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A noninvasive optical approach for assessing chloride extrusion activity of the K–Cl cotransporter KCC2 in neuronal cells

Anastasia Ludwig1,5, Claudio Rivera1,2,3 and Pavel Uvarov4,6*

Abstract

Background: Cation-chloride cotransporters (CCCs) are indispensable for maintaining chloride homeostasis in multiple cell types, but K–Cl cotransporter KCC2 is the only CCC member with an exclusively neuronal expression in mammals. KCC2 is critical for rendering fast hyperpolarizing responses of ionotropic γ-aminobutyric acid and glycine receptors in adult neurons, for neuronal migration in the developing central nervous system, and for the formation and maintenance of small dendritic protrusions—dendritic spines. Deficit in KCC2 expression and/or activity is associated with epilepsy and neuropathic pain, and effective strategies are required to search for novel drugs augmenting KCC2 function.

Results: We revised current methods to develop a noninvasive optical approach for assessing KCC2 transport activity using a previously characterized genetically encoded chloride sensor. Our protocol directly assesses dynamics of KCC2-mediated chloride efflux and allows measuring genuine KCC2 activity with good spatial and temporal resolution. As a proof of concept, we used this approach to compare transport activities of the two known KCC2 splice isoforms, KCC2a and KCC2b, in mouse neuronal Neuro-2a cells.

Conclusions: Our noninvasive optical protocol proved to be efficient for assessment of furosemide-sensitive chloride fluxes. Transport activities of the N-terminal splice isoforms KCC2a and KCC2b obtained by the novel approach matched to those reported previously using standard methods for measuring chloride fluxes.

Keywords: Genetically encoded chloride sensor, Slc12a5 gene, KCC2, Inhibition, GABA

Background

Cation-chloride cotransporters (CCCs) form a protein family comprising nine members, which play an important role in maintaining chloride homeostasis in neuronal, renal, vascular, and other cell types [1]. The only member of the CCC family that shows an exclusively neuronal expression in mammals is the potassium–chloride (K–Cl) cotransporter 2 (KCC2) [2]. KCC2 is indispensable for processes of neuronal migration in developing central nervous system, formation and maintenance of small dendritic protrusions—dendritic spines—in maturing neurons, and for rendering fast hyperpolarizing responses of ionotropic γ-aminobutyric acid (GABA) and glycine receptors in adult neurons [3]. KCC2 deficiency in mature neurons results in elevated levels of intracellular chloride concentration [Cl$^{-}$]i and, as a consequence, in attenuated levels of fast hyperpolarizing GABAergic and glycinergic inhibition [4]. Two protein isoforms KCC2a and KCC2b, which differ only in their most N-terminal parts, are encoded by Slc12a5 gene [5]. Complete genetic ablation of the Slc12a5 gene in mice results in severe motor deficits and absence of respiratory rhythm, causing death immediately after birth [6, 7]. Mice with a specific deletion of the KCC2b isoform express only 5–8% of a basal KCC2 protein level, exhibit frequent generalized seizures, and die 2–3 weeks postnatal [8]. In line with this, three recent studies have associated totally five missenses mutations in the human
KCC2 gene with cases of epileptic seizures [9–11]. The identified KCC2 mutants demonstrate deficit of intrinsic transport activity and/or impaired plasmalemmal expression that considerably reduced chloride extrusion activity. Neuropathic pain is another severe outcome of the impaired GABAergic and glycinegic signaling in central pain pathways that is accompanied by downregulation of KCC2 expression and/or activity [12]. Development of novel drugs augmenting KCC2 activity and/or expression after neuronal trauma has been declared as a plausible way to treat neuropathic pain [13, 14]. Analysis of chemical compounds by high-throughput screening (HTS) requires reliable and robust methods for assessing KCC2 transporter activity in neuronal cell lines.

Both electrophysiological and non-electrophysiological methods have been used so far to analyze activity of the KCC2 protein and its mutant isoforms, though direct measuring of chloride fluxes in neuronal cells by electrophysiological tools is hampered by the electro-neutral nature of the K–Cl cotransport. Yet, several indirect methods allow assessing KCC2 transport activity by exploiting the fact that a reversal potential for inotropic GABA_A (E_{GABA_A}) and glycine (E_{Gly}) receptors depend on [Cl^-]. Thus, E_{GABA_A} in a gramicidin-perforated patch configuration, which does not disturb [Cl^-], provides a close estimation for a steady-state [Cl^-] level [15]. This approach, however, does not necessarily reflect an actual extrusion activity of KCC2, as in the conditions of low cellular chloride conductance even a relatively weak KCC2 activity may significantly decrease [Cl^-]. Another approach is to use a whole-cell patch clamp configuration for measuring somatodendritic E_{GABA_A} gradient in conditions of constant Cl^- loading via somatic patch pipette [16]. One possible caveat of this method is that the endogenous KCC2 activity could be significantly distorted by continuous Cl^- loading, since SPAK and OSR1 kinases, which have been previously shown to regulate KCC2 activity [17–19], are known to be inhibited by high [Cl^-] [20]. One way to avoid this problem is to assess efficiency of neuronal Cl^- extrusion by measuring a recovery time constant (\( \tau \)) of changes in inhibitory post-synaptic potential mediated by GABA_A receptors (IPSP_A) after a brief injection of chloride currents by iontophoresis [21–23]. The short-term character of Cl^- injection minimizes an impact on SPAK/OSR1 signaling cascade, thus allowing a genuine KCC2 extrusion activity to be measured by fitting a curve of IPSP_A amplitude recovery immediately after the cessation of Cl^- injection. This approach has proven to be useful for a pharmacological dissection of different components of chloride conductance in neurons.

Electrophysiological methods described above are labor intensive and time consuming, thus lacking a high efficiency required for HTS applications and for analyzing activity of multiple KCC2 mutants. Moreover, instead of measuring electroneutral KCC2-mediated chloride fluxes directly, these methods provide only indirect correlates of the KCC2 transport activity (steady-state [Cl^-], \( \tau \), somatodendritic E_{GABA_A} gradient). Another strategy to quantify K–Cl cotransport is to directly analyze dynamics of [Cl^-] changes in response to imposed chloride gradients [24]. One of the approaches to follow [Cl^-], dynamics is to measure intracellular accumulation of radioactive chloride (\( ^{36}\text{Cl}^- \)), though a relatively long half-life time of \( ^{36}\text{Cl}^- \) isotope (around 300,000 years) implies a substantial radioactive hazard especially in case of HTS applications [25]. Intracellular chloride concentration can also be measured using chloride-sensitive microelectrodes, but such electrodes are difficult for construction and cannot be used for HTS applications. Chemical assays such as silver chloride gravimetry and atomic absorption spectroscopy have been used for measuring [Cl^-] previously and are still in use [26]. However, both methods are tedious since they require multiple washing steps and separation of cellular and extracellular solutions. One more strategy to overcome problems described above is to quantify K–Cl cotransport by analyzing dynamics of K^+ instead of Cl^- component. Indeed, in case of various potassium channels and transporters, K^+ cation can be substituted for its congeners such as radioactive \( ^{86}\text{Rb}^+ \) [27, 28], nonradioactive \( ^{85}\text{Rb}^+ \) [29, 30], Tl^+ [31–33], and \( \text{NH}_4^+ \) [34], thus fluxes of these cations instead of K^+ can be measured. Although such approaches have been successfully exploited for CCC members in HTS applications in nonneuronal cell lines, their implementation for neuronal cells has so far been limited due to same technical reasons as for the described above Cl-flux methods (poor survival of neuronal cells after multiple washing steps, poor loading and high toxicity of Tl^+ sensitive dyes, a relatively high amount of cells required for an analysis, health hazard issues in case of radioactive \( ^{86}\text{Rb}^+ \) isotope). Moreover, neuronal expression of numerous K^+ channels and transporters impedes an accurate assessment of K^+-fluxes attributed specifically to KCC2. These methods provide low temporal resolution and lack spatial resolution needed for measuring KCC2 activity in small neuronal compartments—axons, dendrites, and dendritic spines.

To overcome limitations inherent to the electrophysiological and K^+ flux-related methods, development of a new class of tools—genetically encoded chloride sensors (GECS)—has been commenced a decade and a half ago [35]. At that time, it was found that fluorescent characteristics of green fluorescent protein (GFP) variants depend on the concentration of halide anions [36]. Since then various GECS have been characterized: YFP-H148Q...
with multiple variants [38] including a membrane-targeted mbYFPQS [39], Clomeleon [40] and its recent modification SuperClomeleon [41], ClopHensor [42] with several modifications [43] including the neuronal variant ClopHensorN [44], as well as Cl-sensor [45] with its glycine receptor (GlyR)-linked variant BioSensor-GlyR [46]. Most of the above-mentioned chloride sensors are fusion proteins comprising a halide-sensitive (YFP, E2GFP) and a halide-insensitive (CFP, DsRed, tdTomato) parts connected by a short linker. Such design allows ratiometric measurements to be done in either FRET or non-FRET mode. A major advantage of GECS is that they make possible noninvasive [Cl\textsuperscript{−}], measurements in neuronal somas and compartments (axons, dendritic spines and shafts). Moreover, many cells can be recorded simultaneously providing a high efficiency required for HTS applications [13]. Furthermore, stable cell lines encoding GECS can be easily propagated in quantities required for screening of large libraries of chemical compounds. Transgenic mice expressing YFP [47], Clomeleon [48], and Cl-sensor [49] have also proved to be useful for studying Cl\textsuperscript{−} dynamics in brain slices and in dissociated neuronal cultures.

A major disadvantage of the YFP-based chloride sensors is their relatively high sensitivity to protons [37, 40, 43, 45, 47, 50–52]. Indeed, inaccuracy in determination of [Cl\textsuperscript{−}], depends on the basal [Cl\textsuperscript{−}], and pH\textsubscript{r} and may reach up to 10 mM [40, 45] at physiologically relevant low [Cl\textsuperscript{−}], [44, 53]. To measure [Cl\textsuperscript{−}], more precisely, independent pH\textsubscript{r} recording using pH-sensitive dyes or genetically-encoded pH sensors is necessary, especially in the cases of significant pH\textsubscript{r} fluctuations induced by neuronal activity [54]. This can be accomplished by using ClopHensor—a chloride sensor that allows simultaneous noninvasive [Cl\textsuperscript{−}], and pH\textsubscript{r} measurements. Even though ClopHensor and its variants [43] provide more accurate [Cl\textsuperscript{−}], measurements [42, 44], usage of these sensors is more technically demanding compared to other GECS for several reasons: (1) three excitation wavelengths instead of two, normally used for other GECS, are needed for acquisition; (2) usage of laser light sources is preferable [42, 44] because two excitation wavelengths for ClopHensor (488-nm pH-dependent E2GFP signal and 458-nm pH-independent E2GFP signal) are closely set; (3) additional photodiode, which measures laser power fluctuations during acquisition, has to be installed into optic paths to correct fluorescence ratios [42, 44]; and (4) a relatively complex data analysis [35].

In the current study, we describe a protocol for noninvasive measuring of KCC2 transport activity in neuronal cells. We have attempted to combine advantages and to avoid known weaknesses of the previously described methods for assaying KCC2 transport activity. Our approach measures KCC2-mediated Cl\textsuperscript{−} fluxes in a robust way using a previously characterized genetically encoded chloride sensor Cl-sensor. In contrast to ClopHensor and its derivatives, usage of Cl-sensor requires neither expensive laser light sources nor a confocal setup [45, 55, 56] and can be accomplished with a conventional fluorescence microscope [57]. Moreover, instead of measuring steady-state [Cl\textsuperscript{−}], levels—indirect correlate of the KCC2 activity, our protocol directly assesses dynamics of KCC2-mediated Cl\textsuperscript{−} fluxes. This new approach can be used for screening chemical compounds augmenting KCC2 activity as well as for assessing a transport activity of KCC2 mutant variants. As a proof of concept, we use this method to compare transport activities of the two previously characterized KCC2 isoforms, KCC2a and KCC2b [5, 58] in neuronal type Neuro-2a cells.

Methods

DNA constructs

Cl-sensor consists of the chloride sensing YFP triple mutant YFP-(H148Q/I152L/V163S) connected by a 20 amino acid linker (GSGSGENLYFAGGGSGGSGS) to the chloride-insensitive CFP [45] (Fig. 1a). Cl-sensor in pGW-CMV expression vector (British Biotechnology, Oxford, UK) and human GlyR α1 subunit expression construct were described previously [55, 59]. Expression constructs for KCC2a and KCC2b splice variants of the neuronal KCC2 cotransporter were characterized previously [5, 58]. pcDNA3.1(−) plasmid (Invitrogen) was used for a mock transfection control.

Cell lines and transfection

Mouse neuroblastoma Neuro-2a cells (CCL-131, American Type Culture Collection, Manassas, VA) were cultured in 35 mm culture dishes in Dulbecco’s Modified Eagle’s Medium (DMEM) supplemented with 10% fetal bovine serum and 100 units/ml penicillin plus 100 μg/ml streptomycin antibiotics mix. For imaging experiments, the cells were plated on 1-cm glass coverslips, transfected the next day with corresponding DNA constructs using Lipofectamine 2000 (Life Technologies) according to manufacturer’s instructions, and used for Cl\textsuperscript{−} imaging 24–48 h after transfection. The transfection mixes differed in the experiments presented in Figs. 2, 3, and 4, but total DNA amount per one reaction (35 mm plate) was always kept 2.15 μg, of which Cl-sensor constituted 0.25 μg. Since it is important to maintain the same amount of total DNA in all transfection reactions, the empty vector pcDNA3.1(−) was added when needed to adjust the total DNA amount to 2.15 μg. So, for the calibration curve experiments (Fig. 2), the cultures were co-transfected with 0.25 μg of Cl-sensor and 1.9 μg of pcDNA3.1(−). In the experiments intended to optimize the loading procedure (Fig. 3), the transfection mix contained 0.25 μg of the Cl-sensor, 0.7 μg of the human GlyR α1 subunit construct, 0.7 μg...
of the KCC2 isoform, and 0.5 µg of the pcDNA3.1(–). In the experiments assessing the chloride extrusion activity of the KCC2a and KCC2b isoforms (Fig. 4a, b), the transfection mix contained 0.25 µg of the Cl-sensor, 0.7 µg of the human GlyR α1 subunit expression construct, 0.7 µg of either of the KCC2 isoforms (Fig. 4a, b), and 0.5 µg of the pcDNA3.1(–). For the corresponding control reaction (Fig. 4c, no KCC2), the transfection mix contained 0.25 µg of the Cl-sensor construct, 0.7 µg of the human GlyR α1 subunit expression construct, and 1.2 µg of pcDNA3.1(–). Our approach is based on simultaneous co-expression of multiple constructs that is a reasonable strategy given previous work [56].

Fluorescence imaging setup

The imaging setup for [Cl]⁻ measurements in neuronal cells transfected with Cl-sensor was based on the inverted Olympus fluorescence microscope (IX71, Olympus, France) and was described previously [57] (Fig. 1b). Briefly, Neuro-2a cells grown on coverslips and expressing Cl-sensor were excited using an X-Cite Series 120Q (Lumen Dynamics Group Inc., Ontario, Canada) light source through the 430(24) nm and 500(20) nm excitation filters (ET430/24× and ET500/20× filters were purchased as a part of the #59217 set, Chroma Technology Corp., Bellows Falls, VT, USA) mounted into the Lambda 10-B Filter wheel (Sutter Instruments Company, Novato, CA).
USA). A transmission neutral density filter (ND 1.3 B–5% Trans, Chroma Technology Corp.) was installed in front of 430(24) nm excitation filter to prevent inactivation of the Cl-sensor by 430 nm light [57]. Olympus LUCPlanFLN 20× objective, NA 0.45 (Olympus, France) was used for taking images of 10–20 cells simultaneously (Fig. 1c). The emitted fluorescence passed through a double bandpass [470(24) + 535(30)] filter (59017 m was purchased as a part of the #59217 set, Chroma Technology Corp., Bellows Falls, VT, USA) and was collected using CoolSNAPHQ Monochrome CCD camera with 0.05 Hz acquisition frequency and 20–50 ms exposure time. Intensities of the collected fluorescent signals corresponding to the excitation wavelengths of 430 nm ($F_{430}$) and 500 nm ($F_{500}$) were corrected for background fluorescence. For that, three ROIs were chosen in nonfluorescent areas for each analyzed optic view. Fluorescent intensities measured for three ROIs were averaged (separately for 430 and 500 nm) to obtain the background values that were subsequently subtracted from $F_{430}$ and $F_{500}$ values obtained for ROIs corresponding to the measured cells from the same optic view. Metamorph software with an option for a multi-dimensional acquisition (MDA) (Roper Scientific SAS, Evry, France) was used to calculate a background-corrected ratio $R = \frac{F_{430}}{F_{500}}$.

**Fluorescence recording protocol**

Coverslips with Neuro-2a cells overexpressing GlyR α1 subunit were fixed inside the imaging chamber, which was mounted onto the inverted Olympus fluorescence microscope. Cells were perfused with an extracellular solution ECS (in mM): 150 NaCl, 2.5 KCl, 2.0 CaCl$_2$, 2.0 MgCl$_2$, 5 HEPES, 10 d-glucose, pH 7.4 using a fast perfusion system described previously [60]. An inner diameter of the quartz perfusion tubes (250 μm) allowed a rapid (within 100 ms) change of solutions in the entire optical field. Bumetanide (10 μM) was added into all solutions to prevent chloride influx mediated by NKCC1 cotransporter. Chloride loading of Neuro-2a cells was accomplished by applying ECS supplemented with 100 μM glycine. Simultaneously with applying glycine, [K$^+$] in the ECS was raised up to 50 mM to increase Cl$^-$ driving force and thus to elevate efficiency of Cl$^-$ loading process. This was done by substituting [Na$^+$] for [K$^+$] in the ECS to keep osmolarity of the final solutions equal 310 mOsm.

All experiments were performed at room temperature (22–24 °C).

**Calibration**

Cl-sensor was calibrated in Neuro-2a cells as described previously [45]. Coverslips were placed in experimental chamber connected to a perfusion system described above. Neuro-2a cells were permeabilized for 5–10 min with 80 mM β-escin (Sigma, St. Louis, USA) added to the extracellular solution (in mM) (150 NaCl, 2.5 KCl, 2.0 CaCl$_2$, 2.0 MgCl$_2$, 5 HEPES, 10 d-glucose, pH 7.4). Calibration solutions containing different [Cl$^-$] (5, 15, 40, 60, 80, 100 μM) were prepared as described previously [45]. Coverslips were placed in experimental chamber connected to a perfusion system described above. Neuro-2a cells were permeabilized for 5–10 min with 80 mM β-escin (Sigma, St. Louis, USA) added to the extracellular solution (in mM) (150 NaCl, 2.5 KCl, 2.0 CaCl$_2$, 2.0 MgCl$_2$, 5 HEPES, 10 d-glucose, pH 7.4). Calibration solutions containing different [Cl$^-$] (5, 15, 40, 60, 80, 100 μM) were prepared as described previously [45].

![Fig. 2](image-url) Calibration of the Cl-sensor in Neuro-2a cells. a Ratio for intensities of fluorescent signals emitted by Neuro-2a cells expressing Cl-sensor after excitation with 430 nm ($F_{430}$) and 500 nm ($F_{500}$) light. Neuro-2a cells were permeabilized with β-escin and incubated in extracellular solutions containing different Cl$^-$ concentrations [Cl$^-$]. An example of stepwise [Cl$^-$] changes is shown. Acquisition interval is 20 s. Mean values and the corresponding SEM are shown for 12 cells recorded in the optic field. b A linear regression for $F_{430}/F_{500}$ changes Δ($F_{430}/F_{500}$) obtained for a broad range of [Cl$^-$] changes (Δ[Cl$^-$]) derived from multiple (n = 8) independent experiments similar to one shown in (a). Each data point shows how the $F_{430}/F_{500}$ ratio changes in response to a change of the extracellular chloride concentration Δ[Cl$^-$]. Error bars represent SEM. Error bars corresponding to −10, +5, and +10 mM data points are too small to be seen on the graph.
50, 100, and 150 mM) were prepared by mixing solutions with a high chloride concentration (150 mM KCl, 10 mM d-glucose, 20 mM HEPES, pH 7.3) and zero chloride concentration (150 mM K-Gluconate, 10 mM d-glucose, 20 mM HEPES, pH 7.3). The calibration solutions were applied into the experimental chamber through a perfusion system, and intensities of fluorescence $F_{430}$ and $F_{500}$ emitted after excitation of Neuro-2a cells with wavelengths 430 and 500 nm were measured. Incubation time in each of the calibration solutions was about 2–3 min to reach steady-state values for $F_{430}$ and $F_{500}$ signals.

**Statistical analysis**

All results are presented as mean ± SEM. The statistical significance was assessed by ANOVA using GraphPad Prizm software (GraphPad Software, CA, USA).

**Results**

**Calibration of the Cl-sensor in cultured Neuro-2a cells**

We aimed to develop a robust optical technique for measuring K–Cl transport activity mediated by electroneutral K–Cl cotransporter KCC2 in neuronal cells. In contrast to other available optical approaches, we intended to assess chloride fluxes through a plasma membrane rather than a steady-state intracellular chloride concentration $[Cl^-]_i$, as the latter one does not necessarily reflect KCC2 activity properly. Since a regular transport mode of KCC2 is known to be chloride extrusion, we developed our protocol to measure chloride efflux similar to the previously published efflux assays [16, 61, 62]. To visualize $[Cl^-]_i$ dynamics in Neuro-2a cells, we used Cl-sensor, a genetically encoded sensor characterized previously [45, 55, 57]. To calibrate Cl-sensor, Neuro-2a cells grown on glass cover-slips and expressing Cl-sensor, were permeabilized with β-escin [55] and incubated sequentially in a series of HEPES-buffered solutions with chloride concentrations $[Cl^-]_o$ varying from 5 to 150 mM. Cells were sequentially illuminated by 500 and 430 nm light, and intensities of the fluorescent signals emitted by the Cl-sensor ($F_{430}$ and $F_{500}$), passed through a double bandpass filter [470(24) + 535(30)], were recorded (Fig. 1). The corresponding ratio $R = F_{430}/F_{500}$ was calculated for each $[Cl^-]_o$ (Fig. 2a). The changes in the extracellular (and in intracellular due to the β-escin permeabilization) chloride concentrations produced the corresponding changes $\Delta R$. Figure 2a represents an example of one of such experiments, where we applied in series the extracellular solutions with different $[Cl^-]_o$ (50, 15, 5, 50, 100, 150, and 5 mM) to the Neuro-2a cells expressing...
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Cl load = High K⁺ ECS + glycine

Δ[Cl], mM

ECS  Cl load  ECS  Cl load  ECS

KCC2a

Cl extrusion, mM/min

Furosemide (+)  Furosemide sensitive

furosemide (-) furosemide (+) furosemide sensitive

Δ[Cl], mM

ECS  Cl load  ECS  Cl load  ECS

KCC2b

Cl extrusion, mM/min

Furosemide (+)  Furosemide sensitive

furosemide (-) furosemide (+) furosemide sensitive

Δ[Cl], mM

ECS  Cl load  ECS  Cl load  ECS

Empty vector

Cl extrusion, mM/min

Furosemide (+)  Furosemide sensitive

furosemide (-) furosemide (+) furosemide sensitive

Cl load = High K⁺ ECS + glycine
Cl-sensor and permeabilized with β-escin. Such graphs allowed us to calculate ∆R values for the corresponding ∆[Cl\(^-\)]\(_o\) changes. Of note, ∆C values can be negative if [Cl\(^-\)]\(_o\) decreases at the subsequent step, or positive in the opposite situation; similarly, ∆R values can also be positive or negative. Experiments similar to the one shown in the Fig. 2a were performed totally with eight 35 mm dishes (in two independent Neuro-2a cultures), and more than 80 Cl-sensor positive cells were recorded. In these experiments the extracellular solutions with varying [Cl\(^-\)]\(_o\) were applied in various orders, and the obtained ∆R values were plotted as a function of ∆[Cl\(^-\)]\(_o\) (Fig. 2b). Nonphysiological ∆[Cl\(^-\)]\(_o\) values (>100 mM) have not been analyzed. The obtained (∆[Cl\(^-\)]\(_o\), ∆R) pairs were fitted by the linear regression with the equation Y = 0.0152X – 0.0834 (r\(^2\) = 0.99). In agreement with Stern–Volmer equation, ∆R demonstrated a linear dependence in the broad range of alterations ∆[Cl\(^-\)]\(_o\) with the slope k = ∆R/∆[Cl\(^-\)]\(_o\) = 0.015 (Fig. 2b), though inconsistency in ∆R determination increased for ∆[Cl\(^-\)]\(_o\) values larger than ± 50 mM. We did not use a nonlinear regression analysis, since the linear regression occurred to be accurate enough for modelling our data in the indicated interval of the chloride changes (<100 mM). In addition, in the case of linear regression, just a single coefficient (k, angle of inclination) is used to transform ∆(F430/F500) into the corresponding changes of the intracellular chloride concentration ∆C.  

Protocol for chloride loading in Neuro-2a cells  
One of the strategies for measuring KCC2 activity is to estimate how efficiently KCC2 copes with the artificially elevated [Cl\(^-\)]\(_i\) level. Chloride loading in such experiments has been successfully accomplished through sharp electrodes [21], patch-clamp glass pipettes [16, 62], and via opening chloride channels of GABA\(_A\) and Gly receptors upon application of the corresponding agonists [45, 55, 57, 63]. The latter approach allows to change [Cl\(^-\)]\(_i\) in multiple cells simultaneously, thus it was a method of choice for our experiments. Neuro-2a cells were transiently transfected with the expression construct for GlyR α1 subunit. When expressed in heterologous system, these subunits can form functional homo-oligomeric chloride channels [64] with high Cl\(^-\) conductance [65]. To enable chloride loading, transfected cells were first pre-incubated in standard ECS for 5 min and then perfused with ECS containing glycine (100 µM) (an arrow in Fig. 3a). ∆R values were recorded every 20 s and transformed into ∆[Cl\(^-\)]\(_i\) using the slope coefficient k obtained during the calibration procedure. Only minor changes ∆[Cl\(^-\)]\(_i\) were detected in Neuro-2a cells expressing α1 GlyR (Fig. 3a). This could be explained by a relatively high basal [Cl\(^-\)]\(_i\) level (about 60 mM) in Neuro-2a cells [66] and, as a consequence, by a relatively weak inward driving force for chloride. To increase the driving force, we shifted the resting membrane potential (RMP) to more depolarized values simultaneously with the application of glycine (Fig. 3b). For this purpose, Na\(^+\) in the extracellular solution was substituted for K\(^+\) to increase concentration of the latter up to 50 mM, while keeping osmolarity constant. Indeed, in high K\(^+\) conditions we observed a fast and efficient chloride loading in Neuro-2a cells (Fig. 3b). Also, in agreement with previous studies [45, 55, 57], we noticed that high K\(^+\) itself can induce Cl\(^-\) loading in Neuro-2a cells even in the absence of glycine application, though a rate of Cl\(^-\) loading was about twice lower (Fig. 3c). This may be explained by unusually high chloride permeability of Neuro-2a cells reported previously [66] or by the KCl-induced depolarization and subsequent acidification of the intracellular compartments. A combination of high K\(^+\) and glycine have been used in all subsequent experiments for efficient Cl\(^-\) loading into Neuro-2a cells.  

Furosemide-sensitive chloride efflux mediated by KCC2a and KCC2b isoforms in Neuro-2a cells  
To measure chloride efflux mediated specifically by KCC2, Neuro-2a cells were loaded with Cl\(^-\) as described above, and kinetics of the subsequent [Cl\(^-\)]\(_i\) recovery to the baseline level was analyzed sequentially in absence and then in presence of furosemide, a known inhibitor of the K–Cl cotransporters [67]. ∆R values were recorded every 20 s and transformed into changes ∆[Cl\(^-\)]\(_i\), using the coefficient k derived from calibration procedure (Fig. 2). After 4 min period of baseline recording in
normal K\(^+\) ECS, cells were loaded with Cl\(^-\) by applying glycine (100 \(\mu\)M) in high K\(^+\) ECS (Fig. 4a–c). To shorten the imaging protocol, Cl\(^-\) loading step was restricted to 10 min, even though in most cases this duration was not enough to reach a plateau level. Cl\(^-\) loading was followed by substitution of the high K\(^+\) ECS solution for the one with normal K\(^+\) and containing no glycine that leaded to the fast [Cl\(^-\)]\(_i\) recovery in cells transfected with KCC2a (Fig. 4a) and KCC2b (Fig. 4b), but not with empty vector (Fig. 4c). A linear regression was used to determine a rate of [Cl\(^-\)]\(_i\) recovery \(\Delta[\text{Cl}^-]/\Delta t\) (red lines in Fig. 4a–c), and the corresponding slopes were calculated (red bars on the right panels of Fig. 4a–c). The steep drop of [Cl\(^-\)]\(_i\) for KCC2a and KCC2b expressing cells can be partly attributed to the KCC2-mediated chloride extrusion and partly to the Cl\(^-\) efflux via chloride channels and other than KCC2 transporters as a result of the change in the driving force for chloride. Bumetanide (10 \(\mu\)M) was added into all solutions to block the activity of Na\(^+\)–K\(^+\)–2Cl\(^-\) cotransporter NKCC1, which is known to be endogenously expressed in Neuro-2a cells [66] and also known to mediate chloride influx. In the second part of the recording, the Cl\(^-\) loading and Cl\(^-\) recovery steps were repeated in the presence of 500 \(\mu\)M furosemide to block the KCC2-specific efflux activity (green lines and bars in Fig. 4a–c). The furosemide-sensitive components of the Cl\(^-\) extrusion for individual Neuro-2a cells (black bars in Fig. 4a–c) were derived by subtracting furosemide(+) components from corresponding furosemide(−) components.

The average chloride efflux characterized by \(\Delta[\text{Cl}^-]/\Delta t\) was 2.9-fold higher in cells transfected with KCC2a and 2.3-fold in cells transfected with KCC2b constructs compared to the mock-transfected cells (grey bars in Fig. 5a). Previous studies reported ~2.7-fold increase for \(^{86}\text{Rb}^+\) uptake in HEK293 cells transiently transfected with either KCC2a or KCC2b isoforms [5], and ~fourfold increase in HEK293 stably transfected with KCC2b isoform [67]. Furosemide (500 \(\mu\)M) attenuated Cl\(^-\) extrusion activity of KCC2a and KCC2b about fourfold, to the level detected in mock-transfected cells in the presence of furosemide (black bars in Fig. 5a). Of note, chloride fluxes in the mock-transfected cells also decreased ~1.9-fold upon furosemide application that is in agreement with \(^{86}\text{Rb}^+\) flux data reported previously for mock-transfected HEK293 cells [5, 67]. Furosemide-sensitive components of \(\Delta[\text{Cl}^-]/\Delta t\) values were considerably higher in Neuro-2a cells transfected with KCC2a (6.3-fold) and KCC2b (5.5-fold) constructs compared to the mock-transfected control, but did not differ significantly from each other (Fig. 5b). These values were close to the ones reported previously for the \(^{86}\text{Rb}^+\) influx in HEK293 cells stably transfected with KCC2b (6.2-fold) [67], as well as for the \(^{86}\text{Rb}^+\) influx in HEK293 cells transiently transfected with KCC2a (~fourfold) and KCC2b (~fourfold) [5, 58].

The above experiments showed that even the relatively short Cl\(^-\) loading step (~10 min) leads to a significant
[Cl$^-\]$ increase that could affect multiple intracellular signaling pathways. Previous studies have reported an important role of the WNK-SPAK signaling pathway in the regulation of the K–Cl cotransport activity mediated by KCC2: the pathway is known to be fully activated after 30–60 min preincubation in low [Cl$^-\]$ [68, 69], resulting in KCC2 inhibition [17, 18, 70, 71]. In contrast, the high [Cl$^-\]$ is known to inhibit SPAK kinase [20], thus leading to the KCC2 activation. Therefore, the Cl$^-\$ loading step could theoretically lead to the inhibition of the WNK-SPAK pathway and, consequently, to the KCC2 activation, thus affecting the KCC2 transport activity. To address this question, we modified our protocol and included two consecutive chloride-loading steps without the furosemide, and then one loading step with the furosemide. Extrusion kinetics after the first and second chloride-loading steps have been compared for both KCC2a and KCC2b isoforms. For both KCC2 isoforms, we revealed a tendency to the increase of the extrusion rate during the second step compared to the first step, but it was not significant (KCC2a: 61 ± 15%, p = 0.055, Student’s t-test, 47 cells in 3 experiments; KCC2b: 54 ± 25%, p = 0.16, Student’s t-test, 71 cell in 3 experiments). It is important to notice that during our standard protocol (with one chloride-loading step without furosemide and one with furosemide) the tendency to the increase of the extrusion rate cannot distort our measurements, as the KCC2 activity at the second step is anyway blocked by the furosemide.

The 10 min chloride loading protocol was used in our study, since it provided the robust [Cl$^-\]$ increase for the reasonably short period. The data presented in Fig. 4 also imply that shorter loading protocols can be used to minimize [Cl$^-\$] changes and subsequent effects on intracellular signaling pathways (e.g. WNK-SPAK). To check whether the chloride extrusion rate depends on the amount of chloride preloaded during the loading step, we compared the short (2-min) and long (10-min) loading protocols. For the KCC2a-transfected cells in the absence of furosemide, an average increase in the [Cl$^-\$] during the 2-min loading step (5.8 ± 0.14 mM, n = 15 cells in N = 4 experiments) was significantly lower (p = 0.0052, Student’s t-test) compared to the 10-min loading protocol (14.1 ± 1.6 mM, n = 57 cells in N = 6 experiments). In the same conditions, the chloride efflux activity after the 2-min loading procedure (7.3 ± 0.24 mM/min, n = 15 cells in N = 4 experiments) was not significantly different (p = 0.19, Student’s t-test) compared to the efflux activity after the 10-min loading step (8.4 ± 1.6 mM/min, n = 57 cells in N = 6 experiments). For the KCC2a-transfected cells in the presence of furosemide, an average increase in the [Cl$^-\$] for the 2-min loading step (6.9 ± 0.37 mM, n = 25 cells in N = 4 experiments) was significantly lower (p = 0.035, Student’s t-test) compared to the 10-min loading (13.4 ± 2.1 mM, n = 51 cell in N = 6 experiments). However, the chloride efflux activity after the 2-min loading procedure (2.6 ± 0.36 mM/min, n = 15 cells in N = 4 experiments) was not significantly different (p = 0.59, Student’s t-test) compared to the efflux activity after the 10-min loading step (2.4 ± 0.6 mM/min, n = 51 cells in N = 6 experiments). Importantly, in both cases (2-min and 10-min loading) we did not observe significant difference for amount of chloride loaded in the presence and absence of furosemide. For the 2-min loading step: (−furo) 5.8 ± 0.14 mM, n = 15 cells in N = 4 experiments versus (+furo) 6.9 ± 0.37 mM, n = 25 cells in N = 4 experiments, p = 0.07, Student’s t-test. For the 10-min loading step: (−furo) 14.1 ± 1.6 mM, n = 57 cells in N = 6 experiments versus (+furo) 13.4 ± 2.1 mM, n = 51 cell in N = 6 experiments, p = 0.8, Student’s t-test. Thus, we conclude that the efflux activity of the KCC2a cotransporter does not depend on the amount of chloride pre-loaded into the cells. In addition, these data show that furosemide does not affect significantly the amount of chloride loaded during the loading step.

**Discussion**

In this study we have described a straightforward technique for assessing efflux activity mediated by K–Cl cotransporter KCC2 in neuronal cells. Our protocol ensures following features: (1) physiologically relevant chloride sensitivity, (2) direct assessment of furosemide-sensitive chloride fluxes instead of a steady-state chloride concentration, (3) measurement of chloride efflux instead of influx, (4) minimal impact of intracellular signaling pathways (e.g. SPAK/OSR1), (5) noninvasiveness, and (6) high throughput. While some of these features individually can be attributed to currently known methods for assessing KCC2 activity, none of the existing methods to our knowledge has so far combined all these features.

As a basis for our technique, we have chosen an optical approach and implemented it by means of the previously characterized Cl-sensor, a triple YFP mutant H148Q/I152L/V163S [45]. Optical approaches in general are known to provide low toxicity, noninvasiveness, and high efficiency. Although expression level of Cl-sensor in our experiments was high enough for stable recordings of chloride fluxes, no signs of cytotoxicity were observed. Morphology of Neuro-2a cells expressing Cl-sensor was normal and did not differ from that of non-transfected cells. Cl-sensor in combination with our imaging setup, which is equipped with 20× objective, allowed us to measure simultaneously the furosemide-sensitive chloride fluxes in about 15 transfected cells during our standard (about 40 min) recording protocol. Similar throughput has been previously observed in...
studies that used Cl-sensor in dissociated cultures [45, 55, 57], brain and retina slices [55], and ex vivo whole-mount DRG samples [49]. More cells can be analyzed by using objectives with lower magnification, but this may require increasing an acquisition time and/or intensity of light sources. Both these factors affect accuracy of Cl− measurements [57], partially because YFP and CFP components of Cl-sensor have differential photobleaching rates [51, 72]. Thus, to prevent photobleaching we kept acquisition time (20–50 ms) and rate (0.05 Hz) minimal. For cells with a very high KCC2 activity, time required for restoration of [Cl−], to the basal level may become too short, thus temporal resolution of the acquisition process may need to be increased. Intrinsic properties of the YFP mutant utilized for Cl-sensor design impose an upper limit for an acquisition rate of about 0.5 Hz [38], thus temporal resolution can be further increased.

Development of GECS has been significantly intensified during last decade, and different chloride sensors have been used for measuring steady-state [Cl−], levels in mammalian cells [35]. However, it has previously been noticed that the steady-state [Cl−] level itself does not necessarily reflect KCC2 extrusion activity properly, as in the conditions of low chloride conductance even a relatively weak KCC2 activity may significantly decrease [Cl−], [16, 62]. Thus, we used Cl-sensor to assess KCC2-mediated chloride efflux by measuring directly Δ[Cl−], in time. This approach required a different calibration protocol for Cl-sensor: while a standard calibration establishes relationship between absolute values of F 430/F 500 ratio and [Cl−], our protocol determines a link between changes ΔR of the F 430/F 500 ratio and changes Δ[Cl−], (Fig. 2b). Changes in [Cl−], were induced by applying calibration solutions with predefined chloride concentrations [Cl−], to Neuro-2a cells permeabilized with β-escin. Importantly, ΔR and Δ[Cl−], were linearly related that is in agreement with previous studies [73], although inconsistency in ΔR determination increased outside physiological range for Δ[Cl−], values larger than ± 50 mM (Fig. 2b). The angle of inclination between the regression line and x-axis allowed us to estimate dissociation constant Kd defined according to the Stern–Volmer equation as R0/Kd, where R0 is F 430/F 500 ratio at zero chloride concentration. Dissociation constant for the Cl-sensor in Neuro-2a cells was found to be ~60 mM that is close to the previously calculated Kd ~ 50 mM for Cl-sensor in cultured neurons [55] as well as for other double and triple YFP mutants analogous to Cl-sensor [38]: H148Q/I152L (~90 mM), H148Q/V150A/I152L (~60 mM), and H148Q/V163S (~60 mM). The calculated Kd ~ 60 mM is close to the value of the basal [Cl−], level in Neuro-2a cells [66], thus making Cl-sensor suitable for assessing chloride fluxes in this model system.

Our protocol includes Cl− loading in Neuro-2a cells with subsequent analysis of how fast KCC2 brings down [Cl−], to the basal level. Previous studies showed that certain GluR subunits form functional homo-oligomeric glycine receptors possessing high Cl− conductance. We used α1 subunit of GlyR to load Neuro-2a cells with Cl− upon application of glycine. Preliminary experiments showed that Cl− loading was relatively weak that could be explained by a relatively high basal [Cl−], in Neuro-2a cells. To make the loading process more efficient, in parallel with glycine application we elevated resting membrane potential by substituting extracellular Na+ for K+ in ECS medium (high K+ medium). The K+-induced depolarization is known to result in [Ca2+]i increase that in turn, via Ca2+-ATPases, could result in acidification up to 0.2 pH units [74]. Since all YFP-based chloride sensors are known to be pH sensitive, the K+-induced depolarization may affect the Cl-sensor recordings. By using Cl-free solutions, the pH-associated Δ[Cl−], values, reported by Cl-sensor, were estimated to be ~5 mM for the 4 min acidification interval [45]. Slightly higher Δ[Cl−], values (~7 mM) have been observed in our system for the same 4 min acidification interval (Fig. 3c). In contrast to the Markova and coauthors, we did not substitute chloride by gluconate in our solutions, thus the slightly increased Δ[Cl−], values could be attributed, for example, to the increased Cl-influx activity of the chloride channels/transporters in the Neuro-2a cells upon the increased driving force induced by KCl application. This approach resulted in a robust Cl− load, increasing intracellular concentration by ~15–20 mM in Neuro-2a cells expressing KCC2 constructs and ~10–15 mM in the mock transfected cells (Fig. 4). Importantly, amplitude of [Cl−], changes during the Cl− loading procedure did not exceed the ±50 mM range of the standard curve (Fig. 2b) that represents a linear interval of dependence between ΔR and Δ[Cl−],. Higher efficiencies of the Cl− loading in KCC2-expressing cells compared to the mock-transfected cells could be explained by the inverted mode of KCC2-mediated transport in the conditions of elevated K+, as it was predicted previously [67]. Even though the inverted mode (influx) is commonly used to assess K− cotransport activity mediated by KCC2 and other members of CCC family, we are not aware of any studies demonstrating that these two approaches are equivalent. Thus, we optimized our approach according to previous studies that measured KCC-mediated chloride efflux [61, 62, 75, 76].

The idea of our approach has been borrowed from another method for assessing the chloride extrusion/intrusion activity—rubidium assay, which is widely used in the chloride-cotransporter field for decades. According to the methodology of the rubidium assay, a
A similar inhibitory effect of furosemide in mock-transfected cells has been reported for HEK293 cells [5, 67, 79] in agreement with data showing endogenous KCC1 and KCC4 expression in HEK293 cells [82]. We found that the furosemide-sensitive chloride efflux was considerably higher in Neuro-2a cells transfected with KCC2a (6.3-fold) and KCC2b (5.5-fold) constructs compared to the mock-transfected control, but did not differ significantly from each other (Fig. 5b). These values are close to the ones reported previously for the 86Rb influx (6.2-fold) in HEK293 cells stably transfected with KCC2b [67] as well as for the 86Rb influx in HEK293 cells transiently transfected with KCC2a (~fourfold) and KCC2b (~fourfold) [5].

Conclusions

In this study we presented a robust and reliable approach for measuring K–Cl transport activity mediated by neuronal cotransporter KCC2. Our noninvasive approach proved to be efficient for measuring physiologically relevant chloride concentrations, allowed direct assessment of furosemide-sensitive chloride fluxes, and in contrast to many existing techniques allowed to assess chloride efflux instead of influx. We used this method to compare transport activities of the N-terminal splice isoforms KCC2a and KCC2b in neuronal cells. Results obtained with our approach matched well to the results acquired previously using standard methods.

Abbreviations

CCC: cation-chloride cotransporter; KCC2: K–Cl cotransporter member 2; GABA: γ-aminobutyric acid; HTS: high-throughput screening; GABA_A: ionotropic γ-aminobutyric acid type A receptors; GlyR: glycine receptors; E_Cl: reversal potential for ionotropic γ-aminobutyric acid type A receptors; E_ClGABA, reversal potential for ionotropic γ-aminobutyric acid type A receptors; SPAK, STE20/SP1-related proline-alanine-rich protein kinase; OSR1: oxidative-stress responsive protein kinase 1; IPSP, inhibitory postsynaptic potential mediated by γ-aminobutyric acid type A receptors; GECS: genetically encoded chloride sensors; GFP: green fluorescent protein; YFP: yellow fluorescent protein; FRET: Förster resonance energy transfer; DMEM: Dulbecco’s Modified Eagle’s Medium; ECS: extracellular solution; NKCC1: sodium (Na_+) potassium (K+) chloride (Cl–) cotransporter member 1.

Authors’ contributions

AL conceived the study, participated in the design of experiments, transfected cell cultures, carried out imaging experiments, was involved in data analysis, and helped in drafting the manuscript. CR participated in the study design, coordinated the experiments, and was involved in drafting the manuscript. PU conceived the study, designed the experiments, assisted in maintaining cell cultures and imaging experiments, processed data, and wrote the manuscript. All authors read and approved the final manuscript.

Author details

1 Neuroscience Center, University of Helsinki, Helsinki, Finland. 2 INSEMM U901, Institut de Neurobiologie de la Méditerranée (INMED), Marseille, France. 3 UMR S901, Aix-Marseille Université, Marseille, France. 4 Department of Anatomy, Faculty of Medicine, University of Helsinki, Helsinki, Finland.
5 Present Address: Ecole Normale Supérieure, Institut de Biologie de l’ENS (IBENS), INSERM U1024, CNRS 8197, Paris, France. 6 Present Address: Department of Biosciences and Neuroscience Center, University of Helsinki, Helsinki, Finland.

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Competing interests

The authors declare that they have no competing interests.

Availability of data and materials

The key data supporting the conclusions of this article are included within the article. The raw data as well as materials used in the study are available from the corresponding author upon reasonable request.

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