Published in final edited form as:

Nat Neurosci. 2009 June ; 12: 725–727. doi:10.1038/nn.2316.

Intracellular zinc inhibits KCC2 transporter activity

Michal Hershfinkel^{1,2}, Karl Kandler^{3,4}, Megan E. Knoch³, Michal Dagan-Rabin^{1,2}, Mandar A. Aras³, Chen Abramovitch-Dahan^{1,2}, Israel Sekler^{2,5}, and Elias Aizenman³

¹Department of Morphology, Ben Gurion University, Beer–Sheva, Israel

²Zlotowski Center for Neuroscience, Ben Gurion University, Beer–Sheva, Israel

³Department of Neurobiology, University of Pittsburgh School of Medicine, USA

⁴Department of Otolaryngology, University of Pittsburgh School of Medicine, USA

⁵Department of Physiology, Ben Gurion University, Beer–Sheva, Israel

Abstract

We report that KCC2 activity, monitored with wide-field fluorescence, is inhibited by intracellular Zn^{2+} , a key component of neuronal injury. Zn^{2+} -mediated KCC2 inhibition produced a depolarizing shift of GABA_A reversal potentials in rat neurons. Moreover, oxygen–glucose deprivation attenuated KCC2 activity in a Zn^{2+} -dependent manner. The link between Zn^{2+} and KCC2 activity provides a novel target for neuroprotection and may be important in activity-dependent regulation of inhibitory synaptic transmission.

Keywords

Zinc; potassium–chloride co–transporter; ischemia

The K^+/Cl^- co-transporter-2 (KCC2) is the major Cl^- outward transporter in neurons, creating a Cl^- equilibrium potential negative to the resting membrane voltage and rendering GABA inhibitory. However, following acute and chronic neuronal injury, KCC2 activity is decreased and GABA becomes excitatory^{1,2,3}, a process that has been tightly linked to neurodegeneration⁴. Another important contributor to neuronal injury and death is an increase of cytosolic Zn^{2+} concentrations ($[Zn^{2+}]_i$)⁵. Although both rise in $[Zn^{2+}]_i$ and decrease in KCC2 have been associated with neuronal injury, whether Zn^{2+} itself can influence KCC2 activity is unknown.

To test whether $[Zn^{2+}]_i$ modulates KCC2 activity we first monitored KCC2-mediated ion transport in HEK 293T cells expressing the co-transporter⁶. NH_4^+ was used as a surrogate ion for K^+ and changes in intracellular pH were monitored using the fluorescent dye BCECF (see Supplemental methods online). KCC2-expressing cells showed a 3-fold faster NH_4^+ -induced acidification rate than cells transfected with empty vector (Fig. 1a,b). Increasing $[Zn^{2+}]_i$ by a 2 min application of Zn^{2+} with the Zn^{2+} ionophore pyrithione (ZnPyr) was immediately followed by a decrease of KCC2-mediated acidification rate, with an IC_{50} of $\sim 50 \mu M$ Zn^{2+} (Fig. 1a–c). Application of Zn^{2+} without pyrithione did not change KCC2 activity (not shown),

Corresponding author: Dr. Michal Hershfinkel, Department of Morphology, Faculty of Health Sciences, Ben–Gurion University, POB 653, Beer–Sheva 84105, Israel. Tel 972–8–6477318, Email: hmichal@gbu.ac.il .

M.E. Knoch present address: Biology Department, Indiana University of Pennsylvania, Indiana, PA 15705, USA.

AUTHOR CONTRIBUTIONS

M.H., M.E.K., M.D.R., M.A.A., C.A.D and E.A. performed, analyzed, and designed the experiments. I.S. and K.K. helped with experimental design and analysis. M.H., K.K., I.S. and E.A. wrote and/or edited the paper.

indicating that KCC2 is inhibited by intracellular Zn^{2+} . Chelating $[\text{Zn}^{2+}]_i$ with N,N,N',N'-tetrakis-(2-pyridylmethyl)-ethylenediamine (TPEN) reversed the effects of ZnPyr (Fig. 1b). Moreover, TPEN alone increased KCC2 activity (Fig. 1a,b), indicating that endogenous levels of $[\text{Zn}^{2+}]_i$ tonically inhibit KCC2.

Next, we determined changes in intracellular chloride $[\text{Cl}^-]_i$ directly with the Cl^- sensitive dye *N*-(ethoxycarbonylmethyl)-6-methoxyquinolinium bromide (MQAE; see Supplemental Methods online). Application of 5 mM KCl reverses KCC2 transport leading to accumulation of intracellular Cl^- (Fig. 1d). Increasing $[\text{Zn}^{2+}]_i$ with ZnPyr blocked the Cl^- influx (Fig. 1d,e), while TPEN enhanced it (Fig. 1d,e), similar to the results observed with NH_4^+ -induced acidification.

We then tested whether KCC2 inhibition by Zn^{2+} is also observed in cortical neurons, which endogenously express the co-transporter. In BCECF-loaded immature neurons (7 days *in vitro*, DIV), expressing low levels of KCC2, NH_4^+ had little effect on intracellular acidification, similar to empty vector-transfected HEK 293T cells (Fig. 1f). In contrast, in mature neurons (>25 DIV), which express high levels of KCC2, NH_4^+ -induced acidification was pronounced. Importantly, in mature neurons ZnPyr also effectively attenuated KCC2 activity (Fig. 1f).

The Zn^{2+} -dependent changes in KCC2 activity should affect the Cl^- gradient and thereby the reversal potential of GABA_A receptor-mediated currents (E_{GABA}). We measured E_{GABA} using the gramicidin perforated patch technique, which leaves intracellular Cl^- undisturbed. A 2 min ZnPyr treatment produced a gradual positive shift in E_{GABA} as, presumably, the intracellular Cl^- concentration decreased with KCC2 inhibition (Fig. 2a). E_{GABA} stabilized within 6–8 minutes after the ZnPyr application to a level ~20 mV positive to control (Fig. 2b,c,d). ZnPyr did not affect E_{GABA} under whole-cell recording conditions with equal extracellular and intracellular chloride (Fig. 2d). The Zn^{2+} -induced shift in E_{GABA} reversed within 30 minutes after removing ZnPyr (Fig. 2d), indicating that neurons can tightly regulate $[\text{Zn}^{2+}]_i$ under non-injurious conditions.

Cerebral ischemia induces acute Zn^{2+} dysregulation in neurons⁷. Therefore, we investigated if KCC2 is inhibited by the sustained increases in neuronal $[\text{Zn}^{2+}]_i$ following oxygen-glucose deprivation in mature neurons (OGD; Fig. 2e). Application of an extracellular Zn^{2+} chelator did not attenuate the $[\text{Zn}^{2+}]_i$ rise following OGD (Fig. 2e), strongly suggesting that it is liberated from intracellular binding sites⁸. Importantly, in ischemic neurons NH_4^+ -induced acidification rates were markedly decreased (Fig. 2f). Moreover, neurons exposed to TPEN during OGD had normal rates of acidification (Fig. 2f), indicating that the rise in $[\text{Zn}^{2+}]_i$ following ischemic insult led to the inhibition of KCC2.

Our results reveal a novel link between $[\text{Zn}^{2+}]_i$ and KCC2 activity. KCC2 inhibition by Zn^{2+} was rapid, suggesting that Zn^{2+} may directly interact with intracellular cysteine or histidine residues of KCC2. Alternatively, Zn^{2+} could contribute to the known regulation of KCC2 activity by phosphorylation⁹ leading to changes in its expression^{9,10}, or by oligomerization¹¹. Zn^{2+} , however, did not induce changes in KCC2 transcription, surface expression, or oligomeric organization (see Supplemental Figs. 1 and 2 online), some of which have been observed in other neuronal injury models^{2,3,4,9,10}. Changes in KCC2 activity without changes in expression have also been reported in a deafness model¹².

The downregulation of KCC2 activity following OGD-induced Zn^{2+} rise may account for acute seizure activity following ischemia¹³. A major factor in neuronal injury following OGD is intracellular release of Zn^{2+} and it was recently shown that it is also critical for spreading depression triggered by OGD¹⁴. Interestingly, a decrease in GABA inhibition has been proposed to be a mechanism contributing to neuronal death following cerebral ischemia⁴. Our results indicate that Zn^{2+} -dependent inhibition of KCC2 may be an important contributor to

neuronal injury. We also suggest that influx of synaptically-released Zn^{2+} or its intracellular release, particularly during periods of intense synaptic activity¹⁵, may dynamically regulate inhibition by modulating KCC2 activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

This work was supported by the US–Israel Binational Science Foundation (BSF2007121, MH & EA), and the US National Institutes of Health (NS043277, EA and DC004199, KK). We thank Karen Hartnett and Mia Jefferson for expert technical assistance.

REFERENCES

1. Woo NS, et al. *Hippocampus* 2002;12:258–268. [PubMed: 12000122]
2. Galeffi F, et al. *J. Neurosci* 2004;24:4478–4488. [PubMed: 15128862]
3. Bonislawski DP, Schwarzbach EP, Cohen AS. *Neurobiol. Dis* 2007;25:163–169. [PubMed: 17045484]
4. Papp E, et al. *Neuroscience* 2009;154:677–689. [PubMed: 18472345]
5. Weiss JH, Sensi SL, Koh JY. *Trends Pharmacol Sci* 2000;21:395–401. [PubMed: 11050320]
6. Lee H, et al. *Eur. J. Neurosci* 2005;21:2593–2599. [PubMed: 15932617]
7. Galasso SL, Dyck RH. *Mol. Med* 2007;13:380–387. [PubMed: 17622314]
8. Aizenman E, et al. *J. Neurochem* 2000;75:1878–1888. [PubMed: 11032877]
9. Wake H, et al. *J. Neurosci* 2007;27:1642–1650. [PubMed: 17301172]
10. Rivera C, et al. *J Neurosci* 2004;24:4683–4691. [PubMed: 15140939]
11. Blaesse P, et al. *J. Neurosci* 2006;26:10407–10419. [PubMed: 17035525]
12. Vale C, Schoorlemmer J, Sanes DH. *J Neurosci* 2003;23:7516–7524. [PubMed: 12930790]
13. Buzsaki G, et al. *Exp. Brain Res* 1989;78:268–278. [PubMed: 2599037]
14. Dietz RM, Weiss JH, Shuttleworth CW. *J. Neurosci* 2008;28:8014–8024. [PubMed: 18685026]
15. Huang YZ, et al. *Neuron* 2008;57:546–558. [PubMed: 18304484]

Figure 1. Increase in $[Zn^{2+}]_i$ inhibits KCC2 activity

(a) KCC2 activity was monitored with the pH-sensitive dye BCECF in HEK 293T cells transfected with a KCC2 or empty vector. Cells were incubated for 2 min with vehicle, Zn^{2+} (100 μM) with pyrithione (5 μM) (ZnPyr), or TPEN (10 μM) and the rate of intracellular pH change following application of NH_4Cl (10 mM) was monitored (see Supplemental Methods online). (b) NH_4^+ -mediated acidification rates (mean $\pm$ SEM) in control and KCC2-expressing HEK 293T cells treated with vehicle ($n=17$), ZnPyr ($n=14$), TPEN ($n=14$), or ZnPyr followed by TPEN ($n=10$); * $p<0.05$, ** $p<0.005$ ANOVA/Tukey, compared to KCC2-expressing NH_4Cl only group. (c) Concentration-inhibition curve of KCC2 activity by Zn^{2+} ($n=11$ for each concentration, mean $\pm$ SEM). (d) KCC2 activity monitored with the Cl^- sensitive dye

MQAE. KCl (10 mM) was applied to KCC2-expressing HEK 293T cells and the rate of Cl^- dependent quenching of the signal was determined. (e) Summary of Cl^- transport rates; ZnPyr, or TPEN, compared to KCl alone (n=6 for each treatment; mean $\pm$ SEM); **p<0.005 ANOVA/Tukey. (f) Endogenous KCC2 activity monitored with BCECF in immature and mature cortical neurons.

Figure 2. Effects of $[Zn^{2+}]_i$ on E_{GABA} , and KCC2 inhibition by OGD in neurons

(a) Representative gramicidin-perforated whole-cell currents ($V_{hold} -50$ mV) in response to $10 \mu M$ GABA before and after a 2 min treatment with ZnPyr ($100 \mu M/5 \mu M$). Scale bars: 300 pA, 1 s. (b) Gramicidin-perforated whole-cell currents before and after inhibition of KCC2 by ZnPyr. Scale bars: 300 pA, 1 s. (c) Current-voltage relationships of peak currents shown in (b). ZnPyr shifted E_{GABA} from -54 to -33 mV. (d) E_{GABA} measured with gramicidin-patch in control neurons ($n=6$), at <1 , and 6–10 min after ZnPyr ($n=10$), and after 30 min rinse ($n=3$). E_{GABA} measured in whole-cell (equal extracellular and intracellular chloride conditions) under control conditions ($n=10$), and after ZnPyr ($n=5$; mean $\pm$ SEM *** $p<0.001$; ANOVA/Tukey) (e) OGD-induced increase in neuronal $[Zn^{2+}]_i$ inhibits KCC2. Neurons were exposed to OGD

for 90 min and loaded with the Zn^{2+} -sensitive dye FluoZin3. A significant Zn^{2+} rise, sensitive to TPEN (20 μM), was monitored at 1 hour following OGD. This increase in cytoplasmic Zn^{2+} , was insensitive to the extracellular Zn^{2+} chelator tricine (1 mM), suggesting the source of the metal was intracellular ($n=7-9$; mean $\pm$ SEM ; * $p<0.05$; ANOVA/Bonferroni). (f) One hour following OGD treatment, neuronal KCC2 activity was monitored with NH_4^+ /BCECF. TPEN treatment during OGD abolished KCC2 inhibition ($n=5-14$; mean $\pm$ SEM ; ** $p<0.005$; ANOVA/Tukey).