Chloride homeostasis differentially affects GABAA receptor- and glycine receptor-mediated effects on spontaneous circuit activity in hippocampal cell culture

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Abstract

The potassium-chloride cotransporter 2 (KCC2)-dependent intracellular chloride level determines whether neurons respond to GABA and/or glycine by depolarization or hyperpolarization. However, still unknown is the role of KCC2-dependent chloride homeostasis in regulating the spontaneous activity of neuronal circuits via GABAA receptor (GABA\textsubscript{A}R) and the glycine receptor (GlyR). In this study, patch-clamp recordings were performed to measure the change of spontaneous neuronal activity in cultured hippocampal neurons. Our results showed that inhibition of KCC2 with furosemide, as well as blockade of GABA\textsubscript{A}R with bicuculline, significantly enhanced circuit activity. Perfusion with bicuculline further enhanced the effects of furosemide on spontaneous circuit activity, while furosemide did not alter the effects of bicuculline. Surprisingly, blockade of GlyR not only induced obvious tonic currents, but also significantly decreased spontaneous synaptic activity. Moreover, inhibition of KCC2 did not change the depressive effect of strychnine on neuronal circuits. Our findings suggest that KCC2-dependent chloride homeostasis is mainly involved in GABA\textsubscript{A}R-mediated synaptic inhibition whereas GlyR-mediated tonic action plays a totally different role in regulating hippocampal circuit activity.

Keywords: KCC2; GABA\textsubscript{A} receptor; Glycine receptor; Tonic current; Spontaneous circuit activity; Hippocampal neuron

Bursting and rhythmic activity are characteristic features of neuronal processing as recognized in electroencephalographic patterns [2,5,20]. In the central nervous system, GABA\textsubscript{A} receptor (GABA\textsubscript{A}R) and glycine receptor (GlyR) are crucial in controlling the activity of neuronal networks; spontaneous circuit activity can change from nonburst to burst by inhibition of these two receptors [4,7]. GABA\textsubscript{A}R-mediated inhibition is accomplished mainly through two pathways: (1) via synaptically released GABA acting on postsynaptic GABA\textsubscript{A}R, which produces synaptic inhibition (also termed phasic inhibition); and (2) via induction of a sustained, background form of inhibition (also called tonic inhibition) by ambient GABA, resulting from persistent activation of extrasynaptic GABA\textsubscript{A}R [16,24]. GlyR-mediated effects function through similar pathways in corresponding neuronal circuits, but only the tonic actions of GlyR are found in the hippocampus [14,17]. Over the past few years, it has become apparent that loss of phasic inhibition prevents the recovery of burst patterns to nonburst activity [9,18]. However, the influence of tonic action on spontaneous circuit activity is still unclear.

Intracellular chloride concentration ([Cl\textsuperscript{−}]) and ligand-gated chloride channels (such as GABA\textsubscript{A}R and GlyR) are critical factors in determining the strength of synaptic inhibition [19,21]. Furthermore, [Cl\textsuperscript{−}], determines the orientation of chloride channel-mediated effects of depolarization or hyperpolarization, which may contribute to shaping circuit activity by regulating the balance between the strength of synaptic inhibition and excitation [9,21]. Recent investigations provided evidence that the K\textsuperscript{+}–Cl\textsuperscript{−} cotransporter 2 (KCC2) plays a dominant role in determining [Cl\textsuperscript{−}], and GABAergic hyperpolarization in mature neurons [11,21], while the Na\textsuperscript{+}–K\textsuperscript{+}–Cl\textsuperscript{−} cotransporter isoform 1 (NKCC1) is a major source of Cl\textsuperscript{−} influx in immature neurons [19]. Furthermore, the loop diuretic, furosemide (100 \textmu M), was used in such studies as a specific pharmacological tool to inhibit KCC2 [19,30]. However, it remains unknown how KCC2-dependent chloride homeostasis regulates the nat-
ural activity of neuronal circuits via GABA\textsubscript{A}R and GlyR in hippocampus.

To mimic the spontaneous activity of brain in vivo and to exclude the potential contribution of HCO\textsubscript{3}\textsuperscript{-}, cultured hippocampal neurons with high levels of spontaneous spiking were perfused with HEPES-buffering solution. This cell culture system provided relatively well-controlled conditions to evaluate the action of phasic and tonic effects on spontaneous circuit activity, in which synaptic GABA\textsubscript{A}R and extrasynaptic GlyR are highly expressed [14,24]. Meanwhile, patch clamp recording was performed to examine the potential roles and differences between phasic and tonic action.

The care of animals used for these experiments followed the guidelines and protocols approved by the Animal Care and Use Committee of the Institute of Neuroscience, Shanghai Institute for Biological Sciences, Chinese Academy of Sciences. Low-density cultures of dissociated embryonic rat hippocampal neurons were prepared as described [30] with some modifications. Hippocampi were removed from E17 to E20 embryonic rats and treated with trypsin for 12–15 min at 37 °C, followed by gentle trituration. The dissociated cells were plated at densities of 25,000 to 60,000 cells/ml on poly-L-lysine coated glass cover slips in 35 mm petri dishes. The plating medium was Dulbecco’s minimum essential medium (DMEM, Gibco) supplemented with 10% fetal calf serum (GIBCO), and 10% Ham’s F12 with glutamine (GIBCO). Twenty-four hours after plating, the culture medium was changed to Neurobasal Media containing 5% B-27 supplement (GIBCO). Cultured neurons were used for patch clamping 12 days after plating.

Somatic whole-cell patch-clamp recordings under voltage-clamp mode were made at room temperature (22–25 °C). The composition of the standard extracellular solution was (in mM): 150 NaCl, 5 KCl, 1 MgCl\textsubscript{2}, 2 CaCl\textsubscript{2}, 10 HEPES, and 10 glucose. The pH was adjusted to 7.4 with tri-hydroxymethyl aminomethane. Patch pipettes were prepared from glass microcapillaries with a micropipette puller (Model P-97, Sutter Instrument Co., USA), and their resistances ranged from 4 to 6 M\textOmega. Composition of the patch pipette solution was (in mM): 125 K-gluconate, 15 KCl, 10 HEPES, 8 NaCl, 4 Mg\textsubscript{2+}-ATP, 0.3 Na\textsubscript{2}GTP, 10 Na\textsubscript{2}-phosphocreatine and 2 EGTA (pH 7.30). Only cells with access resistances between 10 and 20 M\textOmega were accepted for analysis. Membrane potential was held at −70 mV, unless otherwise noted.

In order to test the change of [Cl\textsuperscript{-}], gramicidin-perforated whole cell recordings were made from cultured hippocampal neurons [30]. For experiments, neurons were placed into the recording chamber and constantly perfused with standard extracellular solution using a fast perfusion system (ALA VM8, ALA Scientific Instruments, New York, NY). This system, using separate tubes controlled by electronically operated valves, allowed a complete exchange of external solution surrounding a neuron within 10 ms. The recording pipettes were tip-filled with internal solution of 150 mM KCl and 10 mM HEPES, and back-filled with the solution containing gramicidin (20 μg/ml). These cells were held for at least 30 min until access resistance stabilized. The reversal potential of GABA\textsubscript{A}R currents (\(E_{\text{GABA}}\)) was determined from the current–voltage (I–V) relation obtained by sequentially clamping the membrane at different voltage levels.

Signals were amplified by an Axonpatch-200B amplifier (Axon Instruments, Foster City, CA) and filtered at 5 Hz. Data were acquired and analyzed using Axoscope 9.0 (Axon Instruments). All drugs were purchased from Sigma (St. Louis, MO). Drugs were first dissolved in ion-free water or DMSO, and then diluted to the final concentrations in the standard external solution just prior to use.

Origin (Microcal Software) and Excel (Microsoft, Seattle, WA) were used for data display and analysis. Statistical comparison was carried out using Student’s \(t\) test for comparison between two groups and analysis of variance (ANOVA) for multiple comparison with \(P<0.05\) (*) or 0.01 (**) considered significant. Three or more group comparisons were conducted using ANOVA. “\(N\)” represents the number of neurons studied. All data were expressed as the mean ± S.E.M.

In order to evaluate the modulating effects of KCC2-dependent chloride homeostasis on spontaneous circuit activity, we first used an inhibitor of KCC2 in cultured hippocampal neurons. Consistent with previous investigations [9], perfusion of furosemide (100 μM) to the neuron and its surrounding area significantly increased the amplitude (1.34 ± 0.07 times control, \(n=12, P<0.01\)) and frequency (2.27 ± 0.38 times control, \(n=12, P<0.01\)) of spontaneous synaptic activity (Fig. 1). Furthermore, no tonic currents were recorded after furosemide.

![Fig. 1](image-url)

Fig. 1. Effects of furosemide on spontaneous circuit activity in cultured hippocampal neurons. (A) Furosemide (100 μM) enhanced the frequency and amplitude of spontaneous synaptic inward currents (sPSCs, \(V_h = −70\) mV). (B) Summary of the effects of furosemide on the amplitude of sPSCs. (C) Summary of the effects of furosemide on the frequency of sPSCs.
application. Moreover, we observed that furosemide disrupted the KCC2-dependent intracellular chloride gradient as confirmed by the significant, depolarization-oriented change of equilibrium potential for Cl\(^-\) (Fig. 2; 7.08 ± 0.89 mV, \(n=7\), \(P<0.01\)). Meanwhile, no significant alteration in the slope conductance of GABA-induced chloride current was observed, which indicated that furosemide did not block GABA\(_{\alpha}R\)s (Fig. 2B). These results demonstrate that KCC2-dependent chloride homeostasis plays a critical role in determining spontaneous circuit activity without affecting tonic conductance.

Then, we further investigated the contribution of GABA\(_{\alpha}R\) in regulating circuit activity. Consistently, we observed that 10 \(\mu\)M bicuculline, an antagonist of GABA\(_{\alpha}R\), significantly enhanced spontaneous circuit activity (Fig. 3, sPSCs amplitude increased to 1.24 ± 0.06 times control, \(P<0.05\); sPSCs frequency augmented to 2.45 ± 0.66 times control, \(n=7\), \(P<0.01\)). Furthermore, perfusion with bicuculline also altered the firing pattern of the neuronal circuit. Subsequently, we examined the potential crosstalk between KCC2-dependent chloride homeostasis and GABA\(_{\alpha}R\)-mediated inhibition in regulating spontaneous circuit activity.

Fig. 2. Effects of furosemide on equilibrium potentials of ligand-gated Cl\(^-\) currents. (A) Furosemide shifted the equilibrium potential (\(E_{\text{GABA}}\)) of GABA\(_{\alpha}R\)-mediated currents to a more depolarized level. (B) Current–voltage relationship of 10 \(\mu\)M GABA-induced Cl\(^-\) currents in gramicidin-perforated patch recording before and after perfusion with furosemide. (C) Summary of the shift of \(E_{\text{GABA}}\).

Fig. 3. Crosstalk between KCC2-dependent chloride homeostasis and GABAergic (phasic) inhibition in regulating spontaneous circuit activity. (A) Application of bicuculline (10 \(\mu\)M) induced a significant increase of frequency and amplitude of sPSCs. (B) Summary of the effects of bicuculline on the amplitude of sPSCs. (C) Summary of the effects of bicuculline on the frequency of sPSCs. (D) Representative recordings in the presence of furosemide and bicuculline. (E) Summary of the change of the amplitude of sPSCs after co-perfusion of furosemide and bicuculline. (F) Summary of the change of the frequency of sPSCs after co-perfusion of bicuculline and furosemide. (G) Typical recordings of sPSCs in the presence of bicuculline, and subsequent coapplication of bicuculline and furosemide. (H) Summary of the change of sPSCs amplitude after blockade of GABA\(_{\alpha}R\) and KCC2. (I) Summary of the change of sPSCs frequency after blockade of GABA\(_{\alpha}R\) and KCC2.
circuit activity. Application of bicuculline after furosemide treatment further altered the spontaneous circuit activity (Fig. 3D; sPSCs amplitude decreased to 0.66 ± 0.11 times that of furosemide; sPSCs frequency increased to 2.16 ± 0.58 times that of furosemide, n = 6, P < 0.05). On the other hand, perfusion with furosemide did not change bicuculline-enhanced circuit activity (Fig. 3G; sPSCs amplitude unchanged: 1.09 ± 0.15 times that of bicuculline; sPSCs frequency was also not altered: 1.08 ± 0.13 times that of bicuculline, n = 6, P > 0.05). These results suggest that KCC2-dependent chloride homeostasis mainly functions through GABAAR-mediated inhibition in modulating spontaneous circuit activity.

Surprisingly, we observed that bicuculline depressed rather than enhanced spontaneous circuit activity in a subpopulation of neurons with substantial tonic GABAA currents (data not shown), suggesting that tonic current plays a different role. However, it is difficult to distinguish phasic from tonic effects of GABAAR by pharmacological means. For the sake of clarity, we examined the effects of extrasynaptic GlyR on spontaneous circuit activity. It is well known that GlyR only shows tonic actions, since no glycinergic synaptic transmission is recorded in hippocampal neurons [14,17]. Consistently, perfusion with strychnine not only induced a remarkable tonic current but also significantly depressed spontaneous synaptic activity (Fig. 4; tonic current: 8.51 ± 2.06 pA; sPSCs amplitude decreased to 0.63 ± 0.11 times control; sPSCs frequency was reduced to 0.37 ± 0.12 times control, n = 6, P < 0.01). These results suggest that GlyR-mediated tonic effects play a totally different role in neuronal processing than synaptic GABAAR-mediated phasic inhibition.

Subsequently, we investigated possible crosstalk between KCC2-dependent chloride homeostasis and GlyR-mediated tonic action on circuit activity. Interestingly, we observed that furosemide slightly increased spontaneous circuit activity after blockade of GlyR (Fig. 4E; sPSCs amplitude increased to 1.29 ± 0.07 times that of strychnine; sPSCs frequency augmented to 1.21 ± 0.09 times that of strychnine, n = 4, P < 0.05). On the other hand, strychnine greatly depressed furosemide-enhanced circuit activity with a significant tonic current (Fig. 4H; sPSCs amplitude decreased to 0.44 ± 0.07 times that of furosemide; sPSCs frequency reduced to 0.72 ± 0.03 times that of furosemide, n = 4, P < 0.01). These results indicate that KCC2-dependent chloride homeostasis mainly underlies synaptic GABAAR-mediated phasic inhibition in regulating spontaneous circuit activity, and not GlyR-mediated tonic action.

Firstly, our findings pinpoint that KCC2-dependent chloride homeostasis, GABAAR and GlyR play different roles in regulating spontaneous activity in cultured hippocampal neuronal
circuits. Secondly, we showed that KCC2-dependent chloride homeostasis mainly functions via regulating synaptic (phasic) inhibition in modulating spontaneous circuit activity. Thirdly, we demonstrated that extrasynaptic GlyR-mediated tonic action is independent of the intracellular chloride gradient established by KCC2 in regulating spontaneous circuit activity as evidenced by blockade of GlyR in hippocampal neurons. These results provide new insights into neuronal processing and circuit signal integration.

Changes in KCC2 level (or activity) and KCC2-dependent chloride homeostasis underlie many physiological and pathophysiological conditions [19]. Indeed, several recent investigations provided strong evidence that KCC2 function is regulated by certain patterns of activity, which underlie the plasticity of GABAAR-mediated phasic inhibition in hippocampal neurons [6,29,30]. Consistent with previous reports, we further confirmed that disruption of KCC2-dependent chloride homeostasis significantly enhances spontaneous synaptic activity via synaptic GABAAR-mediated disinhibition of phasic inhibition [9]. These results may be explained by: (1) increased activity itself significantly downregulates the amount of KCC2 [22]. Thus, there are no further alterations of circuit activity after furosemide application; (2) phasic inhibition plays a critical role in setting the temporal window for synaptic integration and synchronizing networks of neurons [24].

Tonic modulation mediated by ligand-gated chloride channels is another important process in neuronal information coding and integration. This occurs in different brain regions and shows neuron-type-specific differences in magnitude and pharmacology [15,23,24]. Previous investigations demonstrated that the processing of rate-coded information is regulated by tonic inhibition (extrasynaptic GABAAR) via synaptic GABAAR-mediated disinhibition of phasic inhibition [9]. These experiments and mathematical operations further showed that the granule cell input–output relationship depends on the level of tonic inhibition and properties of the excitatory input [1,8,28]. However, the effects of tonic action on spontaneous circuit activity are still unclear. Interestingly, we observed that blockade of GlyR significantly decreased the firing frequency of the neuronal circuit while inducing an obvious tonic current. This was somewhat unexpected, as the shunting effect of activation of presynaptic inhibitory channels has been suggested to reduce the amplitude of the presynaptic action potential [3]. In addition, the activation of GlyR, which are expressed in interneurons and pyramidal neurons in the CA1 region of the hippocampus, leads to the shunting of neuronal firing [25]. However, GlyR-induced membrane depolarization activates voltage-gated Ca2+ channels and the subsequent increase in Ca2+ facilitates action potential-evoked neural transmission [10,27,31]. In light of these later findings, a more likely explanation is that extrasynaptic GlyR-mediated tonic action plays a totally different role from synaptic GABAAR-mediated phasic inhibition in neuronal circuits.

It is now clear that the charge carried by the activation of tonic receptors (such as GABAAR) can be three times that produced by phasic inhibition, while the frequency of phasic events is high [15,24]. Could KCC2-dependent chloride homeostasis underlie the physiologically powerful effect of tonic action on spontaneous neuronal activity? This appears unlikely because the activity of hippocampal neuronal circuits was consistently depressed by strychnine, with an obvious tonic current in the process of KCC2 inhibition. Indeed, KCC2 is absent from neuronal axons and [Cl−], is maintained at a relatively higher level in the axon than in other parts of the neuron [26]. Consistently, axonal GlyR-mediated Cl− conductance is excitatory and depolarizing due to an outwardly oriented flux of Cl− [27]. On the other hand, several recent investigations provided strong evidence for cross-inhibition between GABAAR- and GlyR-mediated currents [12,13,32].

Taken together with recent investigations on chloride homeostasis and ligand-gated receptors [9,14,24,26,27], the present results suggest a surprising complexity of signal integration in active neuronal circuits by detecting neuronal circuit activity and the release of neurotransmitters (such as GABA and glycine) via KCC2, GABAARs and GlyRs, which may rapidly shape this activity. Moreover, considering that KCC2-dependent chloride homeostasis variously affects GABAAR- and GlyR-mediated action in regulating spontaneous circuit activity, further investigation is required for more precise analysis of the impact of KCC2 on the regulation of inhibitory transmission and clarification of the mechanisms underlying its contribution to shaping circuit firing patterns.

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References


