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Cation-Chloride Cotransporters in Neuronal Communication

E. Delpire

Two isoforms of the cation-Cl⁻ cotransporter family are expressed in neurons and modulate neurotransmission. NKCC1, a Na⁺-K⁺-2Cl⁻ cotransporter, by raising internal Cl⁻, is responsible for excitatory GABAergic activity in immature brain and in adult sensory neurons. KCC2, a neuronal-specific isoform of the K⁺-Cl⁻ cotransporter, by lowering internal Cl⁻, is critical for inhibitory GABA responses in mature central nervous system neurons.

Brain cation-Cl⁻ cotransporters

To date, seven electroneutral cation-Cl⁻ cotransporters have been described in mammals: a thiazide-sensitive Na⁺-Cl⁻ cotransporter (NCC), two loop diuretic-sensitive Na⁺-K⁺-2Cl⁻ cotransporters (NKCC1 and 2), and four K⁺-Cl⁻ cotransporters (KCC1-4). NCC and NKCC2 are renal-specific cotransporters. The remaining five cotransporters are more widely expressed and are found in the central nervous system. The precise cellular localizations of KCC1, KCC3, and KCC4 have not yet been reported in the brain. NKCC1 has been localized to choroid plexus, oligodendrocytes, and neurons (9), whereas KCC2 expression has been shown to be restricted to neurons (4, 8). These two cation-Cl⁻ cotransporters, functioning in opposite directions (Fig. 1), affect GABAergic and glycinergic neurotransmission through regulation of intracellular Cl⁻ levels.

Development of GABAergic, glycinergic, and glutaminergic neurotransmission

γ-Aminobutyric acid (GABA) and glycine are inhibitory neurotransmitters in the adult nervous system. The inhibitory inputs they generate are essential for proper electric activity in the brain. They balance signals generated by excitatory neurotransmitters (e.g., glutamate) and thus prevent the spread of excitatory activity. GABA and glycine bind to receptors that open Cl⁻ channels (Fig. 1). Depending on the intracellular Cl⁻ concentration, Cl⁻ either enters the cell and hyperpolarizes the plasma membrane or leaves the cell and depolarizes it. The development of GABAergic, glycinergic, and glutaminergic synapses has been best studied in the rat. GABA, which is produced mainly through enzymatic decarboxylation of glutamic acid by glutamic acid decarboxylase (GAD), is found early in rat development. GABAA receptors are also expressed early in development. GABA induces depolarizing, or excitatory, responses during late embryonic and early postnatal life. In contrast, the appearance of glutaminergic synapses is delayed, and glutamate neurotransmission develops progressively during the first postnatal week of life.

It has been proposed that GABA-induced depolarizing responses are important signals for growth of neurites, synaptogenesis, and neuronal plasticity (6). This view is, however, not fully supported by conclusions drawn from mice lacking both isoforms of GAD (GAD65 and GAD67) and completely devoid of GABA. Although these mice die shortly after birth because of a cleft palate and inability to suckle, their brains have normal histology and cytoarchitecture at birth. The brains of these double knockout animals seem to refute the trophic role of GABA during neurogenesis. There remains the possibility, however, that in the absence of GABA, another neurotransmitter compensates for and fulfills its trophic function. Also, the trophic role of GABA might be more significant during postnatal development, when a great number of neuronal connections are being made. Finally, the discrepancy between studies showing the importance of GABA in rat neurogenesis and the normal brain histology observed in double GAD knockout mice might merely reflect differences between species.

For mature neurons that use glycine as an inhibitory neurotransmitter, a similar developmental pattern has been described. Early in development, glycine generates depolarizing (excitatory) responses, but later it produces hyperpolarizing (inhibitory) responses. The change of GABA and glycine responses from depolarizing to hyperpolarizing most likely reflects a change in the driving force for Cl⁻ and a shift in GABA glycine reversal potential during development.

Two recent studies have examined the intracellular Cl⁻ concentrations in neurons during development by using the...
gramicidin-perforated patch technique (2, 7). Both studies have reported a decrease in neuronal Cl– concentration during postnatal development: the first in GABAergic neurons from the neocortex (Fig. 2A), the second in glycinergic neurons from the auditory brain stem (Fig. 2B).

Developmental regulation of NKCC1 and KCC2

Further studies have shown that expression of NKCC1 and KCC2 varies with the developmental switch in GABA responses and with the developmental decrease in intracellular Cl– concentration. Expression of NKCC1 is high at birth and decreases during postnatal development (10). Interesting cell-specific differences exist in the ontogeny of NKCC1. For instance, expression of NKCC1 at birth is high in the cerebellar external granular layer. This layer is composed of progenitor cells that migrate during the first few days of postnatal life. These cells ultimately become granule neurons of the internal granular layer. Expression of NKCC1 in granule neurons then decreases rapidly, reaching very low levels of expression within 1–2 wk. In contrast, neighboring Purkinje neurons express minimal amounts of NKCC1 at birth but display a strong cotransporter signal around postnatal day 14 (10). This timing of NKCC1 appearance coincides with the expression of GAD65, which is maximal around postnatal day 14 and then declines. High expression of GAD65 has been linked to synaptogenesis in Purkinje cells.

In contrast to NKCC1, KCC2 expression is low at birth and increases during postnatal development (1, 4). Both mRNA levels and protein levels increase significantly during the first few weeks of postnatal life. The cotransporter is expressed in both cell body and processes, although the KCC2 signal seems more intense at the nerve termini (4, 15). As previously mentioned, KCC2 is expressed in the cell body and processes of mature central nervous system neurons (4, 15). Because of the large outward K+ gradient, the K+-Cl– cotransporter is capable of driving Cl– against its own electrochemical gradient and setting internal Cl– levels to values below electrochemical potential equilibrium. Using antisense oligonucleotides to KCC2, Rivera and co-workers (11) decreased the level of KCC2 expression in postnatal day 11–13 hippocampal slices and demonstrated a positive shift in GABA/Cl– reversal potential, supporting the view that KCC2 extrudes Cl– in native pyramidal neurons. Thus KCC2 constitutes the long-sought “Cl– pumping mechanism” accounting for hyperpolarizing Cl– influx after brief activation of GABA_A receptors in mature neurons (5, 13).

Can KCC2 participate in both hyperpolarizing and depolarizing GABA responses?

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An interesting feature of GABA neurotransmission, evidenced during high-frequency stimulation, is the reappearance of GABA_A-mediated depolarizing currents. This phenomenon, which occurs at the dendrites but not at cell bodies, is not yet fully understood. It is generally believed that the depolarizing current is mediated by the outward movement of HCO3– ions through the GABA_A receptor/Cl– channel (12). Permeability of the GABA_A receptor to HCO3– is ~1/5 of the permeability to Cl–. The participation of HCO3– in GABA currents is difficult to estimate in the presence of a strong driving force for Cl–. It has been hypothesized, however, that under prolonged stimulation, the Cl– gradient collapses and the HCO3– gradient remains whether or not the cotransporters participate in the regulation of intracellular Cl– in neurons remained to be functionally demonstrated.

NKCC1 accumulates Cl– in sensory neurons

Pharmacological evidence now exists to support the role of NKCC1 in accumulating Cl– in neurons. In contrast to mature central nervous system neurons, peripheral sensory neurons, such as dorsal root ganglion neurons, still exhibit GABA depolarizing currents in the adult. These depolarizing currents are triggered by presynaptic inhibitory axons that establish contact with primary afferent terminals. In this configuration, GABA mediates depolarization of primary afferent terminals, resulting in the blocking of action potential invasion. Thus GABA depolarization initiates presynaptic inhibition, a mechanism that filters the activity of second-order sensory neurons. Using microelectrodes or the gramicidin-perforated patch method, several investigators have demonstrated that amphibian sensory neurons accumulate Cl– well above electrochemical potential equilibrium. The uphill transport of Cl– is mediated by a Na+-K+-2Cl– cotransporter. Evidence supporting this role of the cotransporter are the Na+ and K+ sensitivity of Cl– accumulation and the collapse of the Cl– gradient with exposure to low concentrations of bumetanide, an inhibitor of the Na+-K+-2Cl– cotransporter. Consistent with these functional data is the confirmation of high levels of NKCC1 expression in rat dorsal root ganglion neurons (9).
A role for the cotransporters in epilepsy?

Epilepsy is a very common disorder of the central nervous system that manifests itself as abnormal electric discharges in the brain resulting in time-limited alteration in behavior, motor activity, autonomic function, and/or consciousness. Epileptic seizures are the neurological manifestations of hyperexcitable and hypersynchronized electric activity in the brain. One hypothesis for the hyperexcitability is the possibility of abnormal inhibitory signals in the epileptic brain. On the basis of this premise, various antiepileptic drugs target the GABAergic system, either through enhancement of GABA receptor activity or through inhibition of neurotransmitter reuptake, lengthening the time that the neurotransmitter remains in the synaptic cleft. Recent evidence suggests that counteracting excitatory signals must require a subtle and localized regulation by GABA. Indeed, mice that lack GAD65, one of the GAD isoforms, but that seem to maintain normal levels of GABA in their brains have increased susceptibility to epileptic seizures. Furthermore, although both GAD isoforms have been observed in the same GABAergic neurons, some immunohistochemistry and subcellular fractionation studies localize GAD65 preferentially in the nerve terminals.

Using a hippocampal slice preparation, Hochman and co-workers (3) demonstrated that epileptiform discharges induced by tetanic stimulation of Schaffer collaterals, exposure to 4-aminopyridine (a K⁺ channel blocker) or bicusculine (a GABA_A antagonist), or removal of Mg^{2+} were all inhibited by incubation with millimolar concentrations of furosemide (3). Although at these concentrations the inhibition could be explained by a direct effect of the drug on the GABA_A receptor, the furosemide effect might also indicate the participation of a loop diuretic-sensitive cation-Cl⁻ cotransporter such as KCC2, which is inhibited by loop diuretics like furosemide. Inhibition of KCC2 would likely result in increased intracellular Cl⁻ concentration, leading to reduced hyperpolarizing inhibitory GABA response and thus hyperexcitability.

Additional aspects

Because of their tight relationship to GABAergic neurotransmission, the cotransporters have the capacity to influence many other physiological and pathophysiological processes. First, the cotransporters might be involved in regulating the cyclic firing activity of suprachiasmatic neurons involved in circadian rhythmicity (14). Neurons in this hypothalamic nucleus exhibit GABA depolarizing currents during the day but GABA hyperpolarizing responses at night. Second, the cotransporters might be involved in synchronized thalamocortical activity that occurs during slow wave sleep or anesthesia. Third, the cotransporters might also be involved with the basic mechanisms of alcohol and anesthetic agents through their relationship with the GABA_A receptor. Finally, evidence exists that suggests that neuronal trauma initiates a reversal of GABA currents from hyperpolarizing to depolarizing. These depolarizing GABA currents result in an increase in intracellular Ca^{2+}, reminiscent of GABA function in immature neurons.

Conclusion

Proper electric activity in the brain results from a delicate balance between excitatory and inhibitory inputs at the synapses. Inhibitory neurotransmission involves the activity of ligand-gated Cl⁻ channels. Now and then, these channels elicit excitatory responses that depend on the level of intracellular Cl⁻. Mechanisms involved in Cl⁻ homeostasis therefore play a significant role in neurotransmission. Without

**FIGURE 2.** Decrease in [Cl⁻]i in developing neurons. A: [Cl⁻]i in cortical neurons was estimated from GABA reversal potential measured with the gramicidin-perforated patch method in rat neocortical slices. Cortical neurons originate and migrate from the ventricular zone. [Cl⁻]i in immature neurons of the ventricular zone is high and decreases on migration and differentiation. Redrawn with permission from Owens and co-workers (7). B: glycine/Cl⁻ reversal potentials (E_{glycine}) in developing lateral superior olive neurons were measured in auditory brain stem slices with the gramicidin-perforated patch technique. [Cl⁻]i was calculated using the Nernst equation and an external [Cl⁻] of 133.5 mM. E and P, embryonic and postnatal days, respectively. Modified with permission from Ehrlich et al. (2).
excluding the importance of other Cl\textsuperscript-- transport pathways (the Cl\textsuperscript--/HCO\textsubscript{3}\textsuperscript-- exchanger and some Cl\textsuperscript-- channels such as ClC2), we have discussed in this brief review the evidence implicating NKCC1 and KCC2 in neurotransmission. As the field moves toward new molecular approaches that involve gene knockouts and overexpression, the function of the cation-Cl\textsuperscript-- cotransporters in neurotransmission will be further defined.

NOTE ADDED IN PROOF

We have recently shown that the Na\textsuperscript+-K\textsuperscript+-2Cl\textsuperscript-- cotransporter NKCC1 accumulates Cl\textsuperscript-- in mouse DRG neurons, a mechanism underlying GABA depolarizing responses in these cells. In mice lacking the cotransporter, DRG neurons fail to accumulate Cl\textsuperscript-- and demonstrate abnormal GABA responses. These data, which suggest an absence of depolarization of primary afferent nerve terminals in vivo, are consistent with a sensory perception (nociception) phenotype demonstrated in the NKCC1 knockout mouse (J Neurosci 20: 7531–7538, 2000).

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