Differential Expression of an Inwardly Rectifying Chloride Conductance in Rat Brain Neurons: A Potential Mechanism for Cell-Specific Modulation of Postsynaptic Inhibition

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A voltage-sensitive inwardly rectifying chloride (Cl^-) conductance ($G_{\text{Cl(V)}}$) is present in hippocampal pyramidal but not dentate gyrus neurons and has a significant role in modulation of neuronal inhibition by GABA. $G_{\text{Cl(V)}}$ has the same activation properties as the cloned and expressed Cl^- channel CIC-2. In brain, CIC-2 was detected selectively in neurons, and in hippocampus was detected in the same populations of neurons that demonstrate $G_{\text{Cl(V)}}$. CIC-2 mRNA expression varied widely in different neuronal populations in brain but was greatest in pyramidal and other large neurons and least in interneurons. The observed differential expression of CIC-2 provides a potential molecular basis for the paradoxical excitation produced by GABA_A receptor activation in selected neuronal populations.

[Key words: chloride channels, CIC-2, inhibition, gene expression, CNS, postsynaptic, disinhibition, GABA]

In a previous report, the functional properties of a hyperpolarization activated Cl^- conductance, designated $G_{\text{Cl(V)}}$, was examined in hippocampal neurons (Staley, 1994). This conductance, which had been previously described by others in neurons and non-neuronal cells (Chesnoy-Marchais, 1983; Misgeld et al., 1986; Parker and Miledi, 1988), is shown to be inwardly rectifying with significant conductance only at membrane potentials more negative than the Cl^- transmembrane equilibrium potential (E_{Cl}) and to be of sufficient magnitude and duration to stabilize the relationship between E_{Cl} and resting membrane potential independent of electroneutral Cl^- transport (Staley, 1994). Further, this conductance is present in pyramidal neurons but not neurons of the dentate gyrus (Staley, 1994). These properties suggest that $G_{\text{Cl(V)}}$ could represent a significant cell-specific mechanism for maintaining neuronal Cl^- homeostasis.

Neuronal Cl^- homeostasis determines the direction and amplitude of Cl^- flux gated by GABA_A and glycine receptors. Under most conditions GABA_A and glycine are the principal chemical mediators of neuronal inhibition in the CNS (Misgeld et al., 1986); however, in some neurons GABA_A receptor gated ion flux is paradoxically excitatory (Ben-Ari et al., 1989; Fisman et al.,

1990; Michelson and Wong, 1991; Xie and Smart, 1991, 1993; McIntire et al., 1993; Staley, 1994). For this condition to occur, E_{Cl} must be positive with respect to both the resting membrane potential and the action potential threshold (Staley, 1992; Staley et al., 1992); however, the resting membrane potential must be sufficiently negative that the voltage-sensitive cation channels responsible for generation of action potentials are not inactivated (Zhang and Jackson, 1993). The potential role of $G_{\text{Cl(V)}}$ in determining the effects of GABA_A receptor-mediated Cl^- flux was shown in CA1 pyramidal cells under conditions of neuronal Cl^- loading where GABA_A receptor activation-triggered action potentials only when $G_{\text{Cl(V)}}$ was blocked. Therefore, under normal conditions $G_{\text{Cl(V)}}$ activation clamps E_{Cl} near the resting membrane potential, preventing the development of a driving force for depolarizing Cl^- efflux.

The Cl^- conductance $G_{\text{Cl(V)}}$ that has been observed in hippocampal pyramidal neurons but not dentate gyrus neurons has properties that would be expected for the cloned and functionally expressed Cl^- channel CIC-2. A CIC-2 cDNA, which was cloned from rat brain (Thiemann et al., 1992) and used to generate cRNA for expression in a *Xenopus* oocyte system (Grunder et al., 1992), displayed a voltage dependence similar to $G_{\text{Cl(V)}}$. Both CIC-2 and $G_{\text{Cl(V)}}$ slowly activate at hyperpolarized potentials, deactivate at depolarized potentials, and show no time-dependent inactivation (Thiemann et al., 1992; Staley, 1994). CIC-2 mRNA is a relatively abundant rat brain mRNA, but is widely expressed in other tissues (Thiemann et al., 1992). Since CIC-2 mRNA is widely expressed in non-neuronal tissues and has properties that allow stabilization of cell volume, it has been proposed as a general mechanism for cellular homeostasis (Thiemann et al., 1992). CIC-2 is a member of the supergene family of voltage-gated, Cl^- channels that now includes CIC-0 (Jentsch et al., 1990), CIC-1 (Steinmeyer et al., 1991), CIC-1K (Uchida et al., 1993), and CIC-3 (Kawashi et al., 1994). In addition to the property of voltage-dependent activation, members of this family of genes share a conserved transmembrane domain; however, extensive sequence divergence is present in the amino- and the carboxyl-terminal coding domains (Thiemann et al., 1992; Kawashi et al., 1994). Of the mammalian voltage-gated Cl^- channels, both CIC-2 and CIC-3 have been localized to brain (Thiemann et al., 1992; Kawashi et al., 1994). In this report, we have used *in situ* hybridization methods to determine that the pattern of distribution of CIC-2 is consistent with the pattern of distribution of $G_{\text{Cl(V)}}$ in hippocampus determined by electrophysiologic techniques.

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Figure 1. Detection of CIC-2 mRNA expression in hippocampus with a probe homologous to the 5' coding region. Photomicrographs of coronal sections through an adult rat brain hippocampus taken at the level of the medial habenular nucleus were examined for CIC-2 mRNA expression by *in situ* hybridization. Digoxigenin-labeled cRNA probes homologous to the 5' coding region (bp 63–277) were used. Hybridization was performed with an antisense probe in *A–D* or a sense-strand control probe in *E* and *F*. *A*, A low-power overview with the position of the high-power views

Materials and Methods

CIC-2 probes. A cDNA clone derived from the 5' coding region of CIC-2 was obtained using the reverse transcriptase polymerase chain reaction (RT-PCR) methods (Frohman, 1994) to amplify from polyA-selected RNA prepared from 10⁶ PC-12 cells using a commercially available oligo-dT selection method (Microfast-track, Invitrogen). For amplification of the 5' coding region, a forward primer GGAAGGGA-TGGAGCCTCGAG 42–61 and reverse primer CCCTGGACACTAG-GAACTTGT 277–256, referenced to the published CIC-2 sequence (Thiemann et al., 1992), allowed amplification of a PCR product that was initially cloned into the vector pCRII (Invitrogen, San Diego, CA). A subclone of this species was directionally cloned using an internal restriction site (Pst I; position 63) and a vector-derived EcoRI site ligated into the complementary EcoRI and Pst I sites of Bluescript KS (Stratagene, La Jolla, CA). A downstream coding region probe was obtained by RT-PCR as described above using a forward primer GTA-CCCATGTAGCCCTCAGC 1948–1969 and a reverse primer CCGGAGCTCCTTTAGGGTGAC 2703–2683. The identity of clones was confirmed by sequencing using an Exo[−] Pfu polymerase sequencing kit (Cyclist, Stratagene).

In situ hybridization. Whole rat brain was prepared by perfusion with molecular biology grade HC fixative (Amresco, Solon, OH) followed by immersion fixation overnight at 4°C. The brain was then cut into 0.5 cm slices and immersion fixed for an additional 24 hr in molecular biology grade HC fixative prior to dehydration and paraffin embedding by standard methods. Ten-micrometer sections were cut from paraffin blocks and mounted on pretreated slides (Superfrost plus, Fisher), and stored desiccated at −20°C until use. Immediately prior to use, the tissue sections were cleared of paraffin with xylene washes and rehydrated.

In situ hybridization for detection of CIC-2 mRNA was performed at a final probe concentration of 2 mg/ml with either an antisense- or sense-strand-specific probe transcribed from a plasmid linearized with the appropriate restriction enzyme using either T3 or T7 DNA-dependent RNA polymerase in the presence of a labeling mixture containing digoxigenin-UTP and UTP at a ratio of 0.54 (Boehringer-Mannheim). The conditions of hybridization, RNase treatment and washing were as previously described except that detergent was not included in the hybridization mixture (Smith et al., 1994). Immunodetection of digoxigenin was performed using alkaline phosphatase-conjugated antibodies (Anti-digoxigenin-AP, Fab fragments, Boehringer-Mannheim) at a dilution of 1:5000. Alkaline phosphatase-conjugated antibody binding was detected by incubation of slides at 4°C for 12 hr in 100 mM Tris (pH 9.5), 50 mM MgCl₂, 100 mM NaCl, 1 mM Levamisole, and the chromogens nitroblue tetrazolium (0.34 mg/ml) and 5-bromo-4-chloro-3-indolyl phosphate (0.175 mg/ml), which produce a blue-black precipitate in cells with positive hybridization signal.

Representative sections were photographed with a Nikon Optiphot microscope equipped with Hoffman optics and automated film exposure using T-MAX 100 film. Views of cortex were acquired with an imaging system consisting of a COHU 4900 series CCD camera (Cohu, Inc.) fitted to a either a Nikon Optiphot microscope (Hoffman optics) or a 60 mm Micro-Nikkor macro lens. Digitized images were captured with a Macintosh 840AV computer equipped with a video framegrabber card (LG-3, Scion Corp.) and image analysis software (IMAGE 1.52, National Institutes of Health). Images were then processed using the software package PHOTOSHOP 2.5.1 (Adobe Systems, Inc.) and printed on a Kodak XL7700 photo printer.

Northern blot analysis. Standard methods as described (Sambrook et al., 1989) were used. PolyA-selected RNA was obtained from PC-12 cells using Microfast-track (Invitrogen, San Diego, CA) according to the manufacturers instructions.

Electrophysiology. Whole-cell recordings were obtained at 34°C in rat hippocampal slices as previously described (Staley et al., 1992; Staley, 1994). Where CIC-2 and CIC-3 activation was investigated, Na⁺, K⁺, and Ca²⁺ conductances, HCO₃[−]–Cl[−] exchange, and cation–Cl[−] cotransport were blocked by using artificial cerebrospinal fluid (ACSF)

composed of 109 mM NaCl, 1.25 mM NaH₂PO₄, 10 mM glucose, 20 mM tetraethylammonium chloride, 2.5 mM CsCl, 5 mM 4-aminopyridine, 4 mM MgCl₂, 26 mM *N*-(2-hydroxyethyl)piperazine-*N'*-(2-ethanesulfonic acid) (HEPES), and 0.5 mM furosemide. The pH was adjusted to 7.4 with NaOH, and the ACSF was saturated with 100% O₂. Electrode solutions included 120 mM Cs⁺, 2 mM MgCl₂, 7 mM NaCl, 2 mM QX314, 10 mM HEPES, 1 mM potassium ethylene glycol-bis(β-aminoethyl ether)-*N,N,N',N'*-tetraacetic acid (EGTA), 4 mM potassium adenosine 5'-triphosphate, and 0.3 mM sodium guanosine 5'-triphosphate; pH 7.2. When the resting membrane potential (RMP) and GABA_A synaptic excitation were measured, ACSF was composed of 126 mM NaCl, 2.5 mM KCl, 26 mM HEPES, 2 mM CaCl₂, 2 mM MgCl₂, 1.25 mM NaH₂PO₄, 10 mM glucose, and 0.5 mM furosemide; pH 7.4; K⁺ replaced Cs⁺ in the electrode solution, and QX314 was not used. When the effects of synaptic GABA_A receptor activation were investigated, glutamate receptor antagonists 6,7-dinitroquinoxaline-2,3-dione (DNQX; 10 μM) and kynurenate (1 mM) and the GABA_B antagonist CGP 35348 (100 μM) were added to the ACSF. The stilbene derivative SITS was dissolved in DMSO (final concentration < 0.5%) and applied by bath at a final concentration of 666 μM.

Activation of CIC-2 and CIC-3. CIC-2 currents were measured as the difference between the clamp current immediately after the capacitance transients had decayed (time average, 20–30 msec after the voltage step) and the steady state current. CIC-3 currents were measured using the time-averaged clamp current 20–30 msec after the voltage step. Normalized, leak-subtracted currents were fit to a Boltzmann equation as described previously (Staley, 1994). Because of the overlapping activation potentials for CIC-3 and CIC-2, leak subtraction for CIC-2 exploited the slow activation of this conductance and was based on the clamp current within 50 msec of stepping to the test voltage. Leak subtraction for CIC-3 utilized the ohmic region of the current–voltage relationship between −70 and −30 mV, measured prior to the activation of CIC-2.

Enzymes for modification of RNA and DNA were obtained from Promega (Madison, WI). Deep vent polymerase was from New England Biolabs. All other routine reagents for molecular biology and electrophysiology were from Sigma (St. Louis, Mo.). CGP35348 was a gift of CIBA-GEIGY, (Basel, Switzerland), and QX314 was a gift from Astra (Westborough, MA).

Results

Differential expression of CIC-2 mRNA in hippocampal neurons

CIC-2 cDNA sequence exhibits significant divergence from other cloned voltage-gated Cl[−] channels (CIC-0, CIC-1, CIC-1K and CIC-3) at the amino-terminal coding domain (Thiemann et al., 1992; Kawashi et al., 1994). Based on the published sequence of CIC-2, primers (20-mers) were selected to amplify a 235 bp segment from the amino-terminal coding domain using PC-12 RNA that had been reverse transcribed. The identity of the product was confirmed as described in Materials and Methods by DNA sequence and the ability of labeled probe prepared from this clone to detect a 3.3 kb species on Northern blots of polyA-selected RNA from PC-12 cells as predicted by published results (Thiemann et al., 1992).

We have previously shown that an inwardly rectifying Cl[−] conductance with voltage dependence similar to CIC-2 was expressed in hippocampal pyramidal neurons but not neurons of the dentate gyrus (Staley, 1994). To determine if the expression of CIC-2 mRNA correlated with this conductance, *in situ* hybridization was performed using a 235 bp cRNA probe from the 5' coding region. As shown in Figure 1, an *in situ* hybridization

in *B–D* indicated (*b–d*). *B*, The CA1 region. *C*, The CA3 region. *D*, The CA4 and dentate gyrus. Hybridization with a sense-strand control probe is shown in *E* for the CA3 region and in *F* for CA4 and dentate gyrus. The position of the horizontally elongated interneurons found adjacent to the granule cells is indicated by an arrowhead in *D* and *F*. Hybridization signal (antisense) was detected in the cytoplasm of pyramidal cells but not in the dentate gyrus or in most cell profiles of the molecular layer or subhilar region (*A–D*). No cytoplasmic signal was detected with the sense-strand control probe (*E* and *F*). Scale bars: *A*, 250 μm; *B*, 100 μm for *B–F*.

Figure 2. Activation of CIC-2 and CIC-3 in hippocampal pyramidal neurons. **A**, Voltage-clamp recording using a 125 mM Cl^- electrode solution in a CA3 pyramidal neuron in which cationic conductances were blocked. Initial clamp currents induced by voltage steps from a holding potential

signal was obtained in hippocampal neurons using a cRNA antisense probe (Fig. 1*A–D*), but not a sense-strand control probe (Fig. 1*E,F*). An intense *in situ* hybridization signal was observed in pyramidal cells, which was generally greater in CA3 and CA4 than in CA1 (Fig. 1*B,C*). In contrast, essentially no signal was detected in the dentate gyrus with the exception of occasional scattered cells (Fig. 1*A,D*). GABAergic neurons that are involved in local inhibitory circuits are present throughout the molecular layer surrounding the hippocampus and immediately proximal to the dentate gyrus in the subhilar regions (Ribak et al., 1978; Seress and Ribak, 1983; Michelson and Wong, 1991). CIC-2 mRNA expression was not detected in most cell profiles of the molecular cell layer except for very rare cells. Many cell profiles that have morphologic features of neurons were clearly identified in the molecular layer (Fig. 1*A,B*). Similarly, cells with the horizontally elongated morphology of interneurons were seen proximal to the dentate gyrus that did not express CIC-2 mRNA (Fig. 1*D*). Given the diversity of interneuron classes that have been identified within the hippocampus (Buhl et al., 1994), the presence of occasional interneurons expressing CIC-2 mRNA is not surprising. However, these results indicate that a large portion of interneurons did not express CIC-2 mRNA at detectable levels. In the control sections using the sense-strand probe a faint brown extracellular signal was sometimes been seen in the area of CA3 (Fig. 1*E*). This faint signal was easily distinguishable from specific staining pattern and is the only region of brain that has shown even faint nonspecific signal with the methods used. *In situ* hybridizations were also performed using a probe homologous to the region of CIC-2 from 1948 to 2703 and identical results were obtained (data not shown). These results indicate that in the hippocampus, expression of CIC-2 mRNA was largely restricted to pyramidal neurons.

Electrical expression of an inwardly rectifying Cl⁻ conductance is closely correlated with in situ hybridization signal for CIC-2 mRNA

The previous electrophysiologic study focused on $G_{Cl(V)}$ in the region of CA1 and the dentate gyrus (Staley, 1994). The *in situ* hybridization results indicate that this inwardly rectifying Cl⁻ conductance should be present throughout the pyramidal layer. As shown in Figure 2, an inwardly rectifying Cl⁻ conductance with the activation characteristics of $G_{Cl(V)}$ and CIC-2 was present in all pyramidal cell regions of the hippocampus. Sixteen of 16 pyramidal cells in area CA3 and CA4 demonstrated the $G_{Cl(V)}$ /CIC-2 conductance. CIC-3 is a Cl⁻ channel that is also expressed in the brain neurons, and is heavily expressed in hippocampus (Kawashi et al., 1994). Figure 2*A–C* illustrates that CIC-3 can be functionally differentiated from CIC-2 based on activation at depolarized versus hyperpolarized potentials, the kinetics of activation, and sensitivity to the stilbene derivative SITS. Since

CIC-2 activates at potentials negative to the Cl⁻ reversal potential, activation of CIC-2 can also be demonstrated by depolarization of the RMP in pyramidal cells that are loaded with Cl⁻ via the recording electrode (Fig. 2*D*). When the Cl⁻ reversal potential is set near 0 mV using a 125 mM Cl⁻ electrode solution, activation of CIC-2 results in a gradual depolarization of the RMP due to uncompensated loss of Cl⁻. Activation of CIC-2 drives the RMP toward the Cl⁻ reversal potential, demonstrated by the reduction in amplitude of the spontaneous synaptic potentials due to Cl⁻ flux through the GABA_A receptor-linked ionophore (Fig. 2*D*).

CIC-2 mRNA is differentially expressed in rat brain and spinal cord

Analysis of the distribution of CIC-2 mRNA within the CNS provides for an understanding of one of the cellular mechanisms responsible for neuronal Cl⁻ homeostasis in the CNS. The distribution of CIC-2 mRNA expression detected using the 5'-specific probe is summarized in Table 1 and illustrated for specific regions of the brain as described below in Figures 3–5.

Cerebellum. CIC-2 mRNA expression in cerebellum, as in hippocampus, provides another example of differential expression of CIC-2 in different neuronal populations. In cerebellum, CIC-2 mRNA hybridization was clearly greatest in the Purkinje cells but, in contrast to hippocampus, was expressed in most cells of the molecular layer at moderate levels (Fig. 3*A*). Signal was not detected in the granular cell layer with the exception of scattered cells. Moderate signal was also present in the deep nuclei of cerebellum (Table 1). As shown in Figure 3*B*, no signal was seen with the sense-strand control probe. The pattern of expression within the cerebellum was thus consistent with hippocampus, where the largest class of neurons demonstrated the highest level of expression; however, the expression of CIC-2 mRNA was much greater in cerebellar molecular layer neurons than that detected in the hippocampal molecular layer.

Spinal cord. Motor neurons had the most conspicuous signal (Fig. 3*C*), while sections hybridized with a sense-strand control probe did not demonstrate signal (Fig. 3*D*). CIC-2 mRNA hybridization signal was also detected within central and lateral cervical nuclei and in rare neurons of the posterior horn (Table 1). As in other regions, hybridization was never observed in association with white matter tracks.

Cerebral cortex. CIC-2 mRNA was widely expressed in cortex but varied depending on the region of cortex examined. The coronal image and the corresponding inset in Figure 4 illustrated this variation in expression. In cingulate cortex, CIC-2 mRNA was minimally expressed. In the region of frontoparietal motor cortex expression was largely restricted to layer V and as shown in the inset was minimally expressed in layer IV. In more lateral regions, corresponding to somatosensory cortex, expression was

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of +10 mV demonstrate a linear leak conductance as well as an increased conductance at potentials more positive than -30 mV, consistent with activation of CIC-3. *B*, In the same neuron, perfusion of 666 μ M SITS blocked the CIC-3 conductance. During prolonged voltage steps, an inward current relaxation developed at negative test potentials due to activation of CIC-2. Note the difference in time scales in *A* versus *B* and the much more rapid activation of CIC-3, which is fully activated by the end of the capacitance charging transient. *C*, Current-voltage relationships of CIC-2 (open circles) and CIC-3 (closed circles). Leak-subtracted currents from experiments shown in *A* and *B* were fit to curves based on Boltzmann equations. For CIC-2, $V_{0.1} = -2$, $V_{1/2} = -32$, and the slope factor = 13 mV. For CIC-3, $V_{1/2} = 4$ mV and the slope factor = 14 mV. *D*, Depolarization of the membrane potential of a CA3 pyramidal cell due to Cl⁻ efflux through CIC-2. Current-clamp recording in standard ACSF. Glutamate receptors were blocked, and neuronal Cl⁻ loading was induced by a 125 mM KCl electrode solution. *Bottom inset*, Prior to full activation of CIC-2, Cl⁻ efflux due to spontaneous GABA_A synaptic activity produced bursts of depolarizing membrane potentials (action potentials attenuated by filter and digitization rate). *Top inset*, As CIC-2 was activated, Cl⁻ efflux depolarized the membrane potential toward E_{Cl} , and spontaneous GABA_A synaptic activity no longer depolarized the neuron nor triggered action potentials.

Figure 3. CIC-2 mRNA expression in cerebellum and spinal cord is restricted to specific cell types. Photomicrographs of coronal sections of cerebellum and transverse sections of cervical spinal cord were examined for CIC-2 expression using the 5' coding region probe. *A*, Cerebellum. Strong hybridization signal (antisense) was found primarily within the large Purkinje cells (closed arrow), with occasional labeling of a subpopulation of cells within the granular cell layer (*g*) (open arrow), frequent moderate staining of cell profiles within the molecular cell layer (*m*) (arrowhead), but no signal in the fiber layer (*f*). *B*, Cerebellum control. Section adjacent to *A* hybridized with the sense-strand probe had no detectable signal. The position of Purkinje cells between the granular (*g*) and molecular (*m*) layers is indicated by an arrow. *C*, Anterior horn of spinal cord. Signal was detected with the antisense probe within anterior horn cells (arrows; see Results). *D*, Spinal cord control. Sections hybridized with sense-strand probes show profiles of large motor neurons that contain no signal (arrows). Scale bars, 50 μ m (*A* also for *B*, *C* also for *D*).

widespread but again reduced in layer IV compared to layer V. Ventral to the rhinal fissure, the pyramidal neurons in layer II of primary olfactory cortex expressed the highest levels of CIC-2 mRNA. This pattern of more extensive cortical expression in lateral regions was also observed in coronal sections either an-

terior or posterior to the section shown. As for other regions of brain, no signal was obtained when a sense-strand control probe was used to perform *in situ* hybridizations (data not shown).

Diencephalon. The abundance of CIC-2 mRNA expression varied widely in different regions of the brain. In deep structures

Figure 4. CIC-2 mRNA is differentially expressed in cerebral cortex. Representative hemisection (coronal, 10 μ m) through the anterior diencephalon shows the cortical distribution of CIC-2 hybridization signal. **A**, CIC-2 hybridization signal was least intense from the region of cingulate cortex to frontoparietal primary motor cortex (*boxed area*) where signal was most intense within layer V. Within the frontoparietal somatosensory cortex hybridization signal was more widespread but remained greatest in layer V (*black arrow*). Ventral to the rhinal fissure the primary olfactory cortex consists of three layers and hybridization signal was concentrated within the pyramidal cells of layer II (*white arrow*). **B**, Magnified view of *boxed area* in **A**. The cells within layer V are more intensely labeled than those in layer IV above. No staining was observed in sections stained with the sense-strand control probe (data not shown). Scale bars: **A**, 1 mm; **B**, 50 μ m.

of forebrain signal was present in diagonal band and medial septal nuclei but absent in the lateral septal nucleus (Table 1). Portions of the substantia nigra (pars compacta) and ventral tegmental nuclei expressed large amounts of CIC-2 mRNA (Fig. 5A). Some areas such as caudoputamen, accumbens, and globus

pallidus were devoid of signal with the exception of rare cells (Fig. 5B). Moderate expression was seen throughout the thalamus with the exception of intense signal in the reticulothalamic (Fig. 5C) and the anterodorsal nuclei (Fig. 5D). In epithalamus, the medial habenular nucleus was heavily stained (Fig. 5G). In

the amygdala, marked variation was observed between individual nuclei with the basolateral amygdaloid nucleus demonstrating intense signal while the lateral and medial amygdaloid nuclei contained little signal (Fig. 5I).

Olfactory. Expression of CIC-2 mRNA was restricted in the olfactory bulb to the mitral cell layer (Fig. 5H). As noted in other brain regions, CIC-2 mRNA hybridization signal was lacking in the granular cell layer or in the granular cells that lie in clusters in close proximity to the mitral cells.

Brainstem. In midbrain, minimal staining was detected in the region of the colliculi (Table 1). In contrast, the mesencephalic trigeminal nucleus and locus coeruleus (Fig. 5E,F, respectively) demonstrated intense staining. Staining was also seen in the auditory nuclei, the cuneate/gracile nuclei and in motor nuclei of cranial nerves (Table 1).

Non-neuronal structures. Throughout brain and spinal cord, CIC-2 mRNA expression was confined to regions containing neurons and was not found in white matter, blood vessels or meninges. In most areas, the methods used allowed signal to be localized to the somas of specific neurons. One exception to this pattern of neuron-specific expression was found in the epithelial cell layer of choroid plexus, where significant CIC-2 hybridization signal was present (Fig. 5G). In contrast, CIC-2 mRNA was not expressed at detectable levels in ependymal cells. This suggests that CIC-2 may be involved in Cl^- transport in the choroid plexus.

Discussion

The similarity between the activation properties of the cloned Cl^- channel CIC-2 and the neuronal Cl^- conductance $G_{\text{Cl(V)}}$ (Thiemann et al., 1992; Staley, 1994) and the colocalization of $G_{\text{Cl(V)}}$ to the same neuronal populations that express CIC-2 mRNA, strongly suggest that CIC-2 mediates $G_{\text{Cl(V)}}$. CIC-2 is unique among the channels described to date because of inwardly rectifying characteristics and the osmotic modulation of activation properties, which suggests a fundamental role in cell volume control (Thiemann et al., 1992). However, in the CNS, CIC-2 may have functions in addition to the control of cell volume. This is supported by the pattern of expression of CIC-2 mRNA in the CNS, where expression was limited to specific populations of neurons, undetectable in glia, and marked in the specialized epithelium of choroid plexus. This pattern is in contrast to the ubiquitous pattern that would be expected for a conductance necessary for regulation of cell volume alone.

Neuronal CIC-2 and CIC-3 and regulation of Cl^- conductance

Conductances with properties predicted for the CIC-2 and CIC-3 Cl^- channels can be readily identified in neurons. Both show no significant time-dependent inactivation, and the amplitudes of the whole-cell conductances are approximately the same (Fig. 2C). The channels were distinguished by their activation properties, since CIC-2 was activated at potentials negative to the Cl^- transmembrane equilibrium potential, while CIC-3 was activated at potentials above -30 mV, and we have no evidence that the activation of CIC-3 was affected by the Cl^- equilibrium potential. The block of CIC-3 by stilbene derivatives further distinguishes CIC-3 from CIC-2, which is unaffected by extracellular stilbenes (Fig. 2B; Chesnoy-Marchais, 1983).

CIC-2 and CIC-3 differ further in their pattern of mRNA expression. The restricted pattern of expression of CIC-2 in brain described in this report was in contrast to a more ubiquitous pattern seen with the recently cloned CIC-3 (Kawashi et al., 1994). CIC-3 is a relatively abundant Cl^- channel in brain with outwardly rectifying properties which, like CIC-2, is expressed in neurons but not glia. In contrast to CIC-2, the pattern of CIC-3 expression in neurons is more homogeneous, with signal in both dentate and pyramidal cells of hippocampus, and widespread expression in both olfactory bulb and cerebral cortex (Kawashi et al., 1994).

In patch-clamp studies Cl^- channels are rarely observed in cell-attached mode but are readily seen in detached patches (Blatz, 1991). In whole-cell studies, the amplitude of the Cl^- conductance increases with recording time. These results suggest that Cl^- channels are subject to intracellular regulation. In *Xenopus* oocytes CIC-3 was shown to be inhibited by activators of protein kinase C (PKC) (Kawashi et al., 1994). In hippocampal slice preparations, $G_{\text{Cl(V)}}$ also demonstrates regulation by modulators of second-messenger systems; activators of PKC block $G_{\text{Cl(V)}}$ (Madison et al., 1986) while activation of PKA increases the conductance (Staley, 1994). Therefore, although CIC-3 and CIC-2 have different patterns of distribution, both are subject to regulation by second-messenger systems, suggesting that activation of these channels may be conditional. The specific mechanisms of regulation are unknown for both CIC-2 and CIC-3. It is clear in the case of CIC-2 that the regulatory function resides in the amino-terminal domain, and this function can be maintained even when this domain is placed in a carboxyl-terminal position (Grunder et al., 1992). In CIC-3, a consensus phosphorylation site for PKC is present (Kawashi et al., 1994) but

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Figure 5. CIC-2 mRNA is differentially expressed within subcortical neurons. All photomicrographs were obtained from coronal sections except for H (olfactory bulb), which was obtained from a sagittal section. Hybridization was performed with the 5' region-specific probe. A, Substantia nigra. Strong signal is evident within the pars compacta (arrowhead) and the ventral tegmental area (arrow). Note the relative absence of signal within the pars reticulata (asterisk). B, Caudoputamen was shown penetrated by multiple fiber bundles of the internal capsule (asterisks). CIC-2 hybridization signal was virtually absent except for the rare cell (arrow). C, Reticular thalamic nucleus. Intense signal was detected within this small band (arrow) between the internal capsule/globus pallidus laterally (base of arrow) and the external medullary lamina medially (arrowhead). Also, moderate signal is detected within the ventroposterior thalamic nucleus, medial to the external medullary lamina. D, Anterodorsal thalamic nucleus. Signal was detected within this thalamic nucleus (arrow) lateral to the stria medullaris (asterisk). Moderate signal within the anteroventral and mediodorsal thalamic nuclei was also detected inferiorly. E, Mesencephalic nucleus of the trigeminal nerve. Large, strongly stained, neurons are in evidence, surrounded by unstained neurons and fiber bundles. F, Locus coeruleus. Moderate to strong signal was detected within these medium sized cells. G, Choroid plexus. Intense signal was evident within the choroid epithelium (straight arrow) lying within the third ventricle adjacent to the medial habenular nucleus (asterisk) but signal was absent from nearby ependymal cells (curved arrow). H, Olfactory bulb. CIC-2 hybridization signal was limited to mitral cells (arrow), which lie deep to the olfactory glomeruli (asterisks) and olfactory nerve layer (arrowhead). I, Amygdala. Differential expression was detected between the nuclei of the amygdala. Basolateral/basomedial amygdaloid nuclei and endopiriform nucleus (open and closed arrows, respectively) demonstrated strong signal while signal was absent from the lateral and central amygdaloid nuclei (arrowhead and asterisk, respectively). The corresponding regions demonstrated no cytoplasmic signal when hybridized with a sense-strand control probe (not shown). Scale bars: A, 200 μm for A, C, D, and H; B, 50 μm for B and E–G; I, 250 μm .

Table 1. Distribution of CIC-2 mRNA expression within the rat brain and spinal cord

CNS region	Signal
Olfactory system	
Accessory olfactory bulb	
Mitral cells	+++
Olfactory bulb	
External plexiform layer	—
Glomerular layer	—
Internal granular layer	—
Internal plexiform layer	—
Mitral cell layer	+++
Olfactory nerve layer	—
Olfactory tubercle	—
Primary olfactory cortex	+++
Cortex (see Results)	+ to +++
Basal ganglia	
Accumbens nucleus	—
Caudoputamen	— ^a
Entopeduncular nucleus	— ^a
Globus pallidus	— ^a
Substantia nigra	
Pars compacta	+++
Pars reticulata	— ^a
Ventral tegmental area	+++
Hippocampus	
CA1	++
CA3	+++
CA4	+++
Dentate gyrus	— ^a
Entorhinal cortex	+++
Subiculum	++
Septum	
Diagonal band	+++
Lateral septal nucleus	—
Medial septal nucleus	+++
Amygdala	
Basolateral complex	++
Central nucleus	
Lateral	+
Medial	—
Endopiriform nucleus	++
Lateral nucleus	—
Medial nucleus	
Anterodorsal	—
Anteroventral	+
Epithalamus	
Habenula	
Lateral	+
Medial	+++
Stria medularis	—
Thalamus	
Anterodorsal nucleus	+++
Laterodorsal nucleus	+
Reticulothalamic nucleus	+++
Zona incerta	—
VL	++
VM	++
VPL	++
VPM	++

Table 1. Continued

CNS region	Signal
Hypothalamus	
Arcuate nucleus	—
Dorsal area	++
Dorsomedial area	—
Lateral area	+
Periventricular area	—
Supraoptic nucleus	++
Ventromedial area	+ to ++
Cerebellum	
Cortex	
Molecular layer	++
Purkinje layer	+++
Granular layer	— ^a
Deep nuclei	++
Midbrain/brainstem	
Auditory nuclei	++
Facial nucleus	+++
Gracile/cuneate nuclei	++
Inferior/superior colliculi	—
Locus coeruleus	+++
Mesencephalic trigeminal nucleus	+++
Pontine nuclei	+++
Red nucleus	—
Vestibular nuclei	++
Spinal cord	
Central cervical nucleus	+++
Interneurons (scattered)	+
Lateral cervical/spinal nucleus	+++
Motor neurons (α/γ)	+++
Sensory layers 1–6	— ^a
White matter	—
Non-Neuronal elements	
White matter	—
Choroid epithelium	+++
Ependyma	—
Meninges	—

The intensity of hybridization signal in specific CNS regions was scored on an arbitrary scale from no detectable signal, which corresponded to the intensity detected on control sections (—), to intense labeling (+++).

^a Areas generally not stained but containing the occasional labeled cell.

such sequences are not found in CIC-2 (Thiemann et al., 1992). However, direct regulation by phosphorylation has not been demonstrated for either CIC-2 or CIC-3, and mechanisms of regulation remain an important area of investigation.

Significance of CIC-2 mRNA distribution

The roles of the voltage-sensitive Cl[−] channels and ion-specific inward rectifiers in neuronal physiology are still being determined. One function of inwardly rectifying Cl[−] conductances is stabilization of the relationship between the membrane potential and the Cl[−] equilibrium potential. This prevents paradoxical excitation via GABAergic inhibitory circuits under conditions of Cl[−] loading such as occur during high-frequency synaptic activation (Wong and Watkins, 1982; Thompson and Gahwiler, 1989; Staley, 1994). The finding that CIC-2 mRNA is not expressed in the morphologically identified interneurons of the hippocampal hilar region nor in most of the neuronal profiles of the

molecular layer thus suggests a potential mechanism for the paradoxical excitation of interneurons by activation of GABA_A receptors (Michelson and Wong, 1991). The absence of CIC-2 permits Cl⁻ accumulation in the interneurons to the point that Cl⁻ flux through the GABA_A receptor-associated ionophore is outward, and depolarizes the neuron to the activation voltage of the sodium conductances which underlie the action potential (i.e., Fig. 2D, bottom inset). Therefore, the differential expression of CIC-2 is an important factor in understanding the effects of GABA_A activation.

The most striking feature of the distribution of CIC-2 mRNA was the high level of expression in the largest classes of neurons. As noted, there were several clear exceptions to this pattern. However, the correlation between CIC-2 expression and neuronal size raises the possibility that both CIC-2 and somatic volume are mechanisms of intracellular Cl⁻ homeostasis. High-frequency GABA_A receptor activation leads to intracellular Cl⁻ accumulation (Wong and Watkins, 1982; Thompson and Gahwiler, 1989) and the increase in neuronal Cl⁻ concentration is directly related to the intracellular volume (Qian and Sejnowski, 1990). Therefore, the large somatic volume provides one mechanism to minimize the fluctuation in Cl⁻ concentration. CIC-2 expression is an additional mechanism that prevents shifts in the transmembrane Cl⁻ gradient such that the Cl⁻ reversal potential is positive with respect to the steady-state membrane potential. Such mechanisms might be particularly suited to neurons in which GABA_A receptor activation occurs in high-frequency bursts, which appears to occur in the hippocampal pyramidal cells (Buzsaki et al., 1992). Both of these mechanisms are passive, so that this hypothesis suggests that these neurons would also express high levels of active Cl⁻ transport proteins such as the cation-Cl⁻ cotransporter, as occurs in the choroid plexus (Javaheri, 1991).

The pattern of CIC-2 expression that was observed must also be considered in the context of factors that could modify the functional activity of the channel. CIC-2 function may be regulated by different second-messenger systems in specific neuronal populations. The amount of mRNA may not directly reflect the amount of protein in the cells. Furthermore, the subcellular distribution of CIC-2 will determine the functional effects seen in individual neurons. Specifically, a non-uniform distribution could establish a Cl⁻ gradient within neurons if CIC-2 is present on neuronal processes and soma in different amounts. Therefore, understanding both the regulation and pattern of cell distribution will be required to fully understand the functional significance of the differential expression of CIC-2 in regulating neuronal physiology.

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