ClC-2 Channels Regulate Neuronal Excitability, Not Intracellular Chloride Levels

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Synaptic inhibition by GABA_A receptors requires a transmembrane chloride gradient. Hyperpolarization or shunting results from outward current produced by chloride flowing down this gradient, into the cell. Chloride influx necessarily depletes the chloride gradient. Therefore, mechanisms that replenish the gradient (by reducing intracellular chloride concentration, $[Cl^-]_i$) are crucial for maintaining the efficacy of GABA_A receptor-mediated inhibition. ClC-2 is an inward-rectifying chloride channel that is thought to help extrude chloride because inward rectification should, in principle, allow ClC-2 to act as a one-way chloride exit valve. But chloride efflux via ClC-2 nevertheless requires an appropriate driving force. Using computer modeling, we reproduced voltage-clamp experiments showing chloride efflux via ClC-2, but testing the same model under physiological conditions revealed that ClC-2 normally leaks chloride into the cell. The discrepancy is explained by the driving force conditions that exist under artificial versus physiological conditions, and by the fact that ClC-2 rectification is neither complete nor instantaneous. Thus, contrary to previous assertions that ClC-2 helps maintain synaptic inhibition by lowering $[Cl^-]_i$, our simulations show that ClC-2 mediates chloride influx, thus producing outward current and directly reducing excitability. To test how ClC-2 functions in real neurons, we used dynamic clamp to insert virtual ClC-2 channels into rat CA1 pyramidal cells with and without native ClC-2 channels blocked. Experiments confirmed that ClC-2 reduces spiking independently of inhibitory synaptic transmission. Our results highlight the importance of considering driving force when inferring how a channel functions under physiological conditions.

Introduction

Fast synaptic inhibition is mediated predominantly by GABA_A receptors (GABA_ARs) that cause hyperpolarization or prevent (i.e., shunt) depolarization by producing outward current via Cl^- influx. This requires an appropriate driving force, i.e., E_{Cl} must be more negative than V . Whenever Cl^- influx occurs, the transmembrane Cl^- gradient is depleted. The Cl^- gradient must therefore be continually replenished for synaptic inhibition to remain effective. Strong Cl^- influx through GABA_AR can transiently overwhelm Cl^- extrusion mechanisms (Thompson and Gahwiler, 1989; Staley et al., 1995; Staley and Proctor, 1999), and pathological reduction of Cl^- extrusion capacity can cause depolarizing shifts in baseline E_{Cl} (for review, see De Koninck, 2007; Kahle et al., 2008). Understanding the strength and robustness of synaptic inhibition requires that we understand how the transmembrane Cl^- gradient is maintained.

Maintaining low $[Cl^-]_i$ in neurons of the adult CNS is typically attributed to the K^+ - Cl^- cotransporter KCC2, which harnesses the transmembrane K^+ gradient to extrude Cl^- via an electroneutral cotransport process (for review, see Payne et al.,

2003; Blaesse et al., 2009). Other cotransporters and exchangers may contribute to maintaining low $[Cl^-]_i$, but ion channels are typically ill suited for this purpose because, when open, they let ions run down their gradients. Despite this, the voltage- and chloride-sensitive channel ClC-2 has been proposed to help regulate $[Cl^-]_i$ because inward rectification should, in principal, cause it to act as one-way Cl^- exit valve (Staley, 1994; Földy et al., 2010; Rinke et al., 2010; Smart, 2010). Chloride efflux via ClC-2 nevertheless requires an appropriate driving force; specifically, E_{Cl} must be less negative than (i.e., depolarized relative to) V . Those conditions exist when V is manipulated directly by voltage-clamp and high- Cl^- pipette solutions and/or KCC2 blockade render E_{Cl} unnaturally depolarized, but tend not to occur under physiological conditions. Driving force normally promotes Cl^- influx, which means an imperfect exit valve could leak Cl^- into the cell. Indeed, experiments have suggested that ClC-2 reduces excitability by contributing to leak conductance (Madison et al., 1986; Rinke et al., 2010). Whether ClC-2 regulates $[Cl^-]_i$ under physiological conditions, and if not, what its true physiological role is, are important yet unresolved issues.

To resolve these issues, we built a computational model that tracks V and $[Cl^-]_i$ using ClC-2 parameters characterized in CA1 pyramidal cells (Staley, 1994). After validating our model by comparison against past voltage-clamp experiments, we tested it under physiological conditions (i.e., in current clamp, without pipette dialysis, with KCC2 intact). Contrary to voltage-clamp simulations, current-clamp simulations showed that ClC-2 usually leaks Cl^- into the cell, thus producing outward current that directly reduces excitability. We confirmed these results experimentally by using dynamic clamp to insert virtual ClC-2

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current into CA1 pyramidal cells. Our results argue that ClC-2 reduces excitability directly rather than by regulating $[\text{Cl}^-]_i$ for the benefit of synaptic inhibition.

Materials and Methods

Computer simulations. Simulations were conducted using a single-compartment Morris–Lecar model (Prescott et al., 2008) to which adaptation, ClC-2, and synaptic conductances were added.

$$C \, dV/dt = -\bar{g}_{\text{Na}}m_{\infty}(V)(V - E_{\text{Na}}) - \bar{g}_{\text{K}}w(V)(V - E_{\text{K}}) - g_{\text{leak}}(V - E_{\text{leak}}) \\ - \bar{g}_{\text{adapt}}z(V - E_{\text{K}}) - g_{\text{exc}}(t)(V - E_{\text{exc}}) - g_{\text{inh}}(t)(V - E_{\text{GABA}}) \\ - \bar{g}_{\text{ClC2}}p(V - E_{\text{Cl}}) \quad (1)$$

$$dw/dt = \phi_w \frac{w_{\infty}(V) - w}{\tau_w(V)} \quad (2)$$

$$dz/dt = \frac{z_{\infty}(V) - z}{\tau_z} \quad (3)$$

$$dp/dt = \frac{p_{\infty}(V) - p}{\tau_p} \quad (4)$$

$$m_{\infty}(V) = 0.5 \left[1 + \tanh\left(\frac{V - \beta_m}{\gamma_m}\right) \right] \quad (5)$$

$$w_{\infty}(V) = 0.5 \left[1 + \tanh\left(\frac{V - \beta_w}{\gamma_w}\right) \right] \quad (6)$$

$$\tau_w(V) = 1/\cosh\left(\frac{V - \beta_w}{2 \cdot \gamma_w}\right) \quad (7)$$

$$z_{\infty}(V) = \frac{1}{1 + \exp\left(\frac{-\beta_z - V}{\gamma_z}\right)} \quad (8)$$

$$p_{\infty}(V) = \frac{1}{1 + \exp\left(\frac{E_{\text{Cl}} - V_{\text{half}} - V}{V_{\text{slope}}}\right)} \quad (9)$$

where V is voltage and m , w , z , and p are gating variables controlling activation of $\bar{g}_{\text{Na}}$, $\bar{g}_{\text{K}}$, $\bar{g}_{\text{adapt}}$, and $\bar{g}_{\text{ClC2}}$, respectively; m changes instantaneously with V , whereas w , z , and p evolve according to time constants τ_w , τ_z and τ_p . Fast Na^+ conductance $\bar{g}_{\text{Na}} = 20 \text{ mS/cm}^2$, delayed-rectifier K^+ conductance $\bar{g}_{\text{K}} = 20 \text{ mS/cm}^2$, and nonspecific leak conductance $g_{\text{leak}} = 1 \text{ mS/cm}^2$. Adaptation was included only in simulations in Figure 3, *B* and *C*, where $\bar{g}_{\text{adapt}} = 3 \text{ mS/cm}^2$. Excitatory and inhibitory synaptic conductances g_{exc} and g_{inh} and ClC-2 conductance $\bar{g}_{\text{ClC2}}$ are reported in Results. Other parameters were $C = 2 \text{ } \mu\text{F/cm}^2$, $\beta_m = -1.2 \text{ mV}$, $\gamma_m = 18 \text{ mV}$, $\beta_w = -9 \text{ mV}$, $\gamma_w = 10 \text{ mV}$, $\phi_w = 0.25$, $\beta_z = 0 \text{ mV}$, $\gamma_z = 5 \text{ mV}$, and $\tau_z = 100 \text{ ms}$. Activation parameters for ClC-2 were based on Staley (1994): $V_{\text{half}} = 15 \text{ mV}$, $V_{\text{slope}} = -14 \text{ mV}$, and $\tau_p = 300 \text{ ms}$ ($=\tau_{\text{ClC2}}$). Reversal potentials were $E_{\text{leak}} = -70 \text{ mV}$, $E_{\text{Na}} = +45 \text{ mV}$, $E_{\text{K}} = -90 \text{ mV}$, and $E_{\text{exc}} = 0 \text{ mV}$.

GABA reversal potential E_{GABA} must account for Cl^- and HCO_3^- flux through GABA_A channels and was calculated by the Goldman–Hodgkin–Katz equation:

$$E_{\text{GABA}} = 10^3 \frac{RT}{F} \ln \left(\frac{4[\text{Cl}^-]_i + [\text{HCO}_3^-]_i}{4[\text{Cl}^-]_o + [\text{HCO}_3^-]_o} \right), \quad (10)$$

where $R = 8.3 \text{ J} \cdot \text{mol}^{-1} \cdot \text{K}^{-1}$, $T = 310 \text{ K}$ (37°C), $F = 96,485 \text{ C} \cdot \text{mol}^{-1}$, and i and o designate intracellular and extracellular concentrations. The 4:1 relative permeability to Cl^- and HCO_3^- is specified by the factor 4. Intracel-

lular and extracellular $[\text{HCO}_3^-]$ were assumed to remain constant at 11.8 and 25 mM, respectively, since intracellular HCO_3^- is replenished by conversion of CO_2 ; E_{HCO_3} therefore remains constant at -20 mV (as determined by the Nernst equation). Extracellular $[\text{Cl}^-]$ remained constant at 120 mM, whereas $[\text{Cl}^-]_i$ was continuously updated according to

$$d[\text{Cl}^-]_i/dt = \text{SAV} \frac{g_{\text{inh}}x(V - E_{\text{Cl}}) + g_{\text{KCC2}}(E_{\text{K}} - E_{\text{Cl}}) + g_{\text{ClC2}}(V - E_{\text{Cl}})}{F} \\ + \frac{[\text{Cl}^-]_{\text{pip}} - [\text{Cl}^-]_i}{\tau_{\text{pip}}}, \quad (11)$$

where the first term accounts for transmembrane Cl^- flux and the second term (included only in voltage-clamp simulations) accounts for intracellular dialysis from the recording pipette; $\tau_{\text{pip}} = 1 \text{ s}$ and $[\text{Cl}^-]_{\text{pip}} = 40 \text{ mM}$ unless otherwise specified. Surface area to volume ratio is accounted for by $\text{SAV} = 10^{-4} \text{ s/r}$, where $s = 3$ and $r = 6.3 \text{ } \mu\text{m}$ for a spherical soma (surface area = $5 \times 10^{-6} \text{ cm}^2$) and $s = 2$ and $r = 1 \text{ } \mu\text{m}$ for a cylindrical dendrite. For the somatic surface area, $\bar{g}_{\text{ClC2}} = 1 \text{ mS/cm}^2$ gives a total ClC-2 conductance of 5 nS, consistent with Staley (1994). The fraction of GABA_A current attributable to Cl^- flux (as opposed to HCO_3^- flux) is calculated as $x = (E_{\text{HCO}_3} - E_{\text{GABA}})/(E_{\text{HCO}_3} - E_{\text{Cl}})$. Chloride reversal potential E_{Cl} was calculated from the Nernst equation. When KCC2 was included, g_{KCC2} was set conservatively to 0.7 mS/cm^2 (Doyon et al., 2011). Because cotransport is electroneutral (i.e., Cl^- efflux is balanced by K^+ efflux), the term $g_{\text{KCC2}}(E_{\text{K}} - E_{\text{Cl}})$ is included in Equation 11 but is absent from Equation 1. $[\text{Cl}^-]_i$ was assumed to be spatially homogeneous based on effects of intracellular diffusion (Doyon et al., 2011).

Unless otherwise indicated, g_{exc} and g_{inh} were turned on or off but otherwise remained constant. In some simulations, synaptic bombardment was simulated by varying g_{exc} and g_{inh} according to Ornstein–Uhlenbeck processes (Uhlenbeck and Ornstein, 1930; Prescott and De Koninck, 2009):

$$d\zeta = -\frac{\zeta}{\tau} dt + N_{\tau}\xi(0,1)\sqrt{dt}, \quad (12)$$

where $\xi(0,1)$ is a random number with 0 mean and unit variance and N_{τ} is a scaling factor $\sqrt{2/\tau}$ so that $\zeta(t)$ has unit variance. Synaptic conductance $g(t) = \mu + \sigma\zeta(t)$, where μ and σ specify the mean and standard deviation, respectively, of the fluctuating conductance. Parameter values are reported in Results.

Equations were integrated in XPP (Ermentrout, 2002) using the Euler method with a time step of 0.05–0.1 ms. Code will be available in ModelDB.

Electrophysiology. Experimental procedures were approved by the University of Pittsburgh Institutional Animal Care and Use Committee and have been described previously (Prescott et al., 2006). Hippocampal slices were prepared from adult male Sprague Dawley rats. Slices were transferred to a recording chamber perfused with oxygenated (95% O_2 and 5% CO_2) ACSF heated to $31 \pm 1^\circ\text{C}$. CA1 pyramidal neurons were recorded in whole-cell mode with $>70\%$ series resistance compensation using an Axopatch 200B amplifier (Molecular Devices). Membrane potential (after correction for a liquid junction potential of 9 mV) was adjusted to -70 mV through tonic current injection. Intracellular recording solution contained (in mM) 125 KMeSO₄, 5 KCl, 10 HEPES, 2 MgCl₂, 4 ATP (Sigma), 0.4 GTP (Sigma), and 0.1% Lucifer yellow; pH was adjusted to 7.2 with KOH. Using the Ornstein–Uhlenbeck processes described above, virtual synaptic conductances were injected using dynamic clamp (Signal 5, CED). E_{GABA} was fixed at -60 mV , similar to E_{GABA} values in high-conductance state simulations (see Fig. 3C). Neurons were given 10-s-long barrages of virtual synaptic input. Conductance fluctuation amplitude (σ) was fixed at 1 nS and 0.25 nS for g_{exc} and g_{inh} , respectively; the mean conductance (μ) was varied to achieve a range of firing rates. Spikes were counted over the last 9 s of each trial to exclude adaptation during the first second. In interleaved trials, virtual ClC-2 conductance was inserted via dynamic clamp. ClC-2 activation parameters were identical to simulations. We tested at least two $\bar{g}_{\text{ClC2}}$ values per cell, and three levels overall. Simulations indicated that native

and virtual CIC-2 channels have additive effects on excitability (data not shown), but nevertheless, some dynamic clamp experiments were repeated after blockade of native CIC-2 channels by 100 μM zinc (Staley, 1994). To calculate the driving force for CIC-2, E_{Cl} was approximated as -70 mV based on simulation results in Figure 3C. Spike counts from trials with CIC-2 were compared with spike counts from the immediately preceding trial without CIC-2, thereby comparing only responses to equivalent virtual synaptic input and avoiding the confounding influence of any slow drift in excitability. Responses were low-pass filtered at 2 kHz and digitized at 20 kHz using a CED 1401 computer interface.

Results

Model validation based on voltage-clamp simulations

We began by validating our model through comparison with past voltage-clamp experiments. Inward rectification was clearly evident from the CIC-2 I – V curve measured from our model (Fig. 1A); this replicates Figure 1A of Staley (1994). To determine how CIC-2 affects steady-state $[\text{Cl}^-]_i$ assayed by the amplitude of GABA_A R responses, we clamped the model neuron at different V and recorded I_{GABA} in response to 10-ms-long activations of g_{inh} (Fig. 1B, left panel); this replicates Figure 2d of Földy et al. (2010). CIC-2-dependent reduction in inward I_{GABA} reflects the hyperpolarizing shift in E_{Cl} (Fig. 1B, top right panel) caused by CIC-2-mediated inward current (i.e., Cl^- efflux) (Fig. 1B, bottom right panel) over the corresponding voltage range. To be clear, under the simulated conditions with $[\text{Cl}^-]_{\text{pip}} = 40$ mM, $[\text{Cl}^-]_i$ is high (i.e., E_{Cl} is above V_m), which means Cl^- exits the cell via GABA_A R when those channels are open; less Cl^- exits through GABA_A R if some has already leaked out via CIC-2. To determine how CIC-2 affects the rate of Cl^- extrusion, we loaded the model neuron with Cl^- by clamping V at $+60$ mV with g_{inh} on, then g_{inh} was turned off and V_{clamp} was reset to -90 mV. CIC-2 expedited Cl^- clearance (Fig. 1C); this replicates Figure 2f of Földy et al. (2010) and Figure 2 of Rinke et al. (2010). To summarize, our model reproduced CIC-2 rectification properties (Staley, 1994) and the voltage-clamp data that are the basis for concluding that CIC-2 contributes to intracellular Cl^- homeostasis (Földy et al., 2010; Rinke et al., 2010).

Regulation of $[\text{Cl}^-]_i$ in mature central neurons is typically ascribed to KCC2 (see Introduction). Chloride extrusion by CIC-2 and KCC2 are not mutually exclusive, but most previous experiments on CIC-2 (Staley, 1994; Földy et al., 2010; Rinke et al., 2010) were con-

Figure 1. Reproduction of voltage-clamp data in model neuron. **A**, CIC-2 current (I_{CIC2}) was measured at the end of 1-s-long V_{clamp} steps from 0 mV. Traces show responses to voltage-clamp protocol; yellow shading highlights outward I_{CIC2} . Inset shows CIC-2 activation. In **A**, $[\text{Cl}^-]_i$ was fixed at 100 mM, giving $E_{\text{Cl}} = -5$ mV. In **B–D**, $[\text{Cl}^-]_i$ was continuously updated according to Equation 11. KCC2 was not included in voltage-clamp simulations except where indicated in **D**. **B**, Impact of CIC-2 on GABA_A -mediated current (I_{GABA}) was measured at different V_{clamp} by turning on g_{inh} (1 mS/ cm^2) for 10 ms. Inward I_{GABA} (left panel) was less in the model with CIC-2 because inward I_{CIC2} (bottom right panel) caused a hyperpolarizing shift in E_{Cl} (top right panel) by reducing $[\text{Cl}^-]_i$. **C**, Rate of Cl^- extrusion was evaluated by loading the model neuron with Cl^- by turning on g_{inh} (cyan bar) while $V_{\text{clamp}} = +60$ mV, then monitoring recovery of $[\text{Cl}^-]_i$ after g_{inh} was turned off and V_{clamp} was reset to -90 mV. CIC-2 expedited Cl^- removal. **D**, Simulations like in **C** were repeated with CIC-2 and/or KCC2, and with V_{clamp} stepped to -90 mV or -70 mV after Cl^- loading. Unlike CIC-2, KCC2 mitigated Cl^- accumulation and extruded Cl^- equally well regardless of V_{clamp} . **E**, Summary of conditions under which CIC-2 mediates Cl^- efflux.

ducted with cesium in the pipette, which blocks KCC2 (Blaesse et al., 2009) and might thus overemphasize Cl^- extrusion via CIC-2. Therefore, we compared Cl^- extrusion through different mechanisms and with different V_{clamp} steps (Fig. 1D). Whereas V_{clamp} af-

Figure 2. Chloride flux through CIC-2 under physiological conditions (i.e., current clamp, without dialysis, with KCC2). **A**, CIC-2 produced outward current (i.e., Cl^- influx) at all times except during a brief interval (yellow shading) after stimulation, when Cl^- driving force was inverted. **B**, Chloride accumulation and driving force (measured 100 ms after stimulus offset because of CIC-2 kinetics) for different strength inputs to soma and dendrites. Dendritic simulations were conducted with and without (difference from former shown in red) active Na^+ and K^+ currents. Inset shows that $[Cl^-]_i$ kinetics but not steady-state $[Cl^-]_i$ differ between soma and dendrites. **C**, Effects of differences in $[Cl^-]_i$ kinetics when stimulation is brief. In all cases, Cl^- efflux via CIC-2 was limited to a brief interval after stimulus offset and paled in comparison with CIC-2-mediated Cl^- influx occurring at all other times. **D**, CIC-2 current (gray) and conductance (red) for $\tau_{CIC2} = 300 \text{ ms}$ (top panel) and $\tau_{CIC2} = 0.1 \text{ ms}$, i.e., nominally instantaneous (bottom panel). For $\tau_{CIC2} = 300 \text{ ms}$, CIC-2 was weakly active at resting V and deactivated slowly during depolarization; I_{CIC2} changed rapidly because of fluctuations in driving force during spikes, not because of rapid changes in conductance. Slow changes in activation reflect activation kinetics plus slow trends in average V and E_{Cl} . For $\tau_{CIC2} = 0.1 \text{ ms}$, CIC-2 was still weakly active at resting V but rapidly deactivated during spikes. Insets show enlarged view of first spike. **E**, Summary of factors affecting Cl^- flux at different times relative to stimulation.

affected Cl^- extrusion by CIC-2, it did not affect extrusion by KCC2 since KCC2 driving force (calculated as $E_K - E_{\text{Cl}}$) was unaffected. Unlike CIC-2, KCC2 mitigated total Cl^- accumulation because of the direction of its driving force and its persistent activation during depolarization. These data argue that by harnessing the transmembrane K^+ gradient, cotransporter-mediated Cl^- extrusion occurs via a stronger and more stable driving force than channel-mediated Cl^- extrusion. Nevertheless, these data are still consistent with Cl^- efflux via CIC-2 (Fig. 1E).

Direction of Cl^- flux in current-clamp simulations

Current-clamp simulations in the same model revealed a fundamentally different picture from that described above. After strong Cl^- loading, Cl^- efflux via CIC-2 occurred only during a brief interval after stimulus offset (Fig. 2A). This interval corresponds to when Cl^- driving force was inverted, and was absent for stimuli causing less Cl^- accumulation (Fig. 2B) or if stimulus offset was more gradual (data not shown). In fact, CIC-2 allowed Cl^- influx at rest, during stimulation, and even for much of the time after stimulation when $[\text{Cl}^-]_i$ was elevated above resting levels. This pattern was observed in soma and dendrites; the only notable difference was that $[\text{Cl}^-]_i$ kinetics are faster in dendrites, which allows greater Cl^- accumulation during brief stimulation (Fig. 2C) but can also prevent CIC-2 channels from having enough time to activate before $[\text{Cl}^-]_i$ is reduced via other mechanisms (Fig. 2B; see also below). Note in Figure 2B that $[\text{Cl}^-]_i$ did not exceed ~ 20 mM when KCC2 was intact, even during strong stimulation—this defines the upper limit of physiological $[\text{Cl}^-]_i$. These results highlight the importance of driving force and contradict the prevailing view that inward rectification prevents CIC-2 from mediating Cl^- influx.

To investigate why inward rectification fails to prevent Cl^- influx, we compared normal slow-gated CIC-2 conductance [$\tau_{\text{CIC2}} = 300$ ms based on Staley (1994)] with a hypothetical, instantaneously gated CIC-2 conductance (Fig. 2D). For $\tau_{\text{CIC2}} = 300$ ms, CIC-2 produced substantial outward current before deactivation developed following onset of a depolarizing event. In fact, deactivation was negligible during fast events like action potentials (see Fig. 2D, inset) and EPSPs (see below). For deactivation to occur on this faster time scale, τ_{CIC2} must be reduced by $>10\times$. Furthermore, regardless of gating kinetics, inward rectification was incomplete at rest as evidenced by nonzero resting CIC-2 conductance. Thus, inward rectification of CIC-2 is neither instantaneous nor complete. This, coupled with driving force conducive to Cl^- influx, means that CIC-2 normally produces outward current via Cl^- influx. CIC-2 parameters were equivalent in voltage-clamp and current-clamp simulations, which points to testing conditions as the basis for the difference in direction of Cl^- flux (Fig. 2E).

Figure 3. Effects of CIC-2 on neuronal excitability. **A**, Instantaneous firing rate (=reciprocal of interspike intervals) remained constant in model without CIC-2 but increased in parallel with CIC-2 deactivation in model with CIC-2. This demonstrates that spiking is reduced by CIC-2-mediated outward current. Constant current injection (cyan bar) was applied instead of conductance-based stimulation to avoid changes in E_{Cl} caused by Cl^- flux so that g/g_{max} directly reflected CIC-2 activation kinetics (compare with top panel of Fig. 2D). **B**, To investigate how CIC-2 affects spiking driven by synaptic input, a model neuron was bombarded by excitatory and inhibitory synaptic input; $\tau_{\text{exc}} = 2$ ms, $\tau_{\text{inh}} = 10$ ms, $\mu_{\text{exc}} = \mu_{\text{inh}} = 0.5$ mS/cm^2 , $\sigma_{\text{exc}} = \sigma_{\text{inh}} = 0.1$ mS/cm^2 . Sample trace shows that CIC-2 remains active during EPSPs and spikes, which are rapid relative to $\tau_{\text{CIC2}} = 300$ ms. **C**, Average firing rates, E_{Cl} , and E_{GABA} (which accounts for bicarbonate efflux through GABA_AR) for different combinations of g_{exc} , g_{inh} , and g_{CIC2} ; noise parameters as in **B**. In the high-conductance state, strong inhibition is balanced by strong excitation and causes a large depolarizing shift in E_{Cl} and E_{GABA} ; in the low-conductance state, g_{exc} was reduced to give the same baseline spike rate.

Effects of CIC-2 current on neuronal excitability

We predicted that CIC-2 should directly reduce neuronal spiking if the current is indeed outward. To test this, we compared firing rates in models with and without CIC-2 in response to constant current injection (Fig. 3A) and to synaptic-like input (Fig. 3B, C). Under all conditions (including high- and low-conductance states with different E_{Cl} values), CIC-2 reduced spiking. Firing rate reduction was especially pronounced for spiking driven by synaptic input because EPSPs were sufficiently short that they provoked little if any deactivation of CIC-2 (see Fig. 3B).

To test whether CIC-2 reduces spiking in real neurons, we used dynamic clamp to insert virtual g_{CIC2} into CA1 pyramidal neurons recorded *in vitro* and stimulated with virtual synaptic input. As predicted, adding virtual g_{CIC2} reduced spiking with native CIC-2 channels intact (Fig. 4A) or blocked (Fig. 4B). Furthermore, because CIC-2 and synaptic currents implemented by dynamic clamp are not mediated by transmembrane Cl^- flux and because E_{Cl} and E_{GABA} are held constant, our experiments demonstrate that I_{CIC2} can regulate neuronal excitability directly, rather than acting indirectly through modulation of GABA_AR-mediated inhibition.

Discussion

Despite having rectification properties that suggest they act as Cl^- exit valves, our results show that CIC-2 channels normally

Figure 4. Effects of virtual ClC-2 conductance in CA1 pyramidal neurons. **A**, Neurons were stimulated with virtual synaptic inputs applied by dynamic clamp. With native ClC-2 channels intact, stimulus parameters were varied slightly to achieve a range of firing rates in each cell ($n = 4$). In interleaved trials, virtual ClC-2 conductance (3 different g_{max} values with at least 2 values tested per cell) was inserted by dynamic clamp. Plots show spike rates with versus without virtual ClC-2 based on comparison of consecutive trials. Points below dotted line indicate reduction of firing rate by ClC-2. Observed distribution was significantly different from expected; $\chi^2 = 26.0$, $p < 0.001$. Inset shows mean ($\pm$ SEM) reduction in firing rate. Paired t test, $p = 0.088$ for 2.5 nS, $p = 0.005$ (*) for 5 nS, and $p < 0.001$ (**) for 10 nS. **B**, Equivalent experiments ($n = 6$) repeated with native ClC-2 channels blocked by 100 μ M zinc. $\chi^2 = 27.0$, $p < 0.001$. Paired t test, $p < 0.001$ (**). Mean reduction caused by virtual ClC-2 was not significantly different with versus without native ClC-2; t test, $p = 0.60$.

leak Cl^- into neurons. This occurs because Cl^- driving force is almost always against the valve's preferred direction and because ClC-2 rectification has slow kinetics and is incomplete at rest. Therefore, rather than acting primarily to reduce $[Cl^-]_i$ and replenish the transmembrane Cl^- gradient for the benefit of GABA_AR-mediated synaptic inhibition, our data demonstrate that ClC-2 functions under physiological conditions as a leak conductance that directly reduces neuronal excitability.

A role in regulating neuronal excitability is consistent with data showing that ClC-2 reduces input resistance (Madison et al., 1986; Rinke et al., 2010). However, ClC-2 cannot simultaneously provide a Cl^- efflux pathway and shunt depolarizing input, since the latter requires ClC-2 to produce an outward current via Cl^- influx. Shunting still requires Cl^- -mediated outward current to counterbalance cation-mediated inward current (Price et al., 2009)—it is not true that cations simply leak out open Cl^- channels, contrary to what “shunting” might suggest.

Disambiguating whether ClC-2 affects neuronal excitability directly or by modulating the strength of synaptic inhibition is important. For example, consider pathological conditions associated with Cl^- dysregulation (e.g., by KCC2 downregulation): if ClC-2 regulates $[Cl^-]_i$, increased ClC-2 activity could compensate for reduced KCC2 activity and mitigate disinhibition; in contrast, if ClC-2 regulates excitability directly, ClC-2 activity would exacerbate Cl^- accumulation when KCC2 is reduced. Interestingly, a role for ClC-2 in epilepsy based on human data has been suggested but is contentious (Niemeyer et al., 2010), and ClC-2-deficient mice do not have an obvious seizure phenotype (Bösl et al., 2001; Blanz et al., 2007), although subtle effects have been noted (Cortez et al., 2010). It is also notable that ClC-2 itself is subject to significant modulation (Madison et al., 1986; Staley, 1994).

In conclusion, our results contradict the prevailing view that ClC-2 constitutes a Cl^- efflux pathway. Although our model can reproduce voltage-clamp data that are the basis for ascribing this role to ClC-2, the same model tested under physiological conditions

clearly shows that ClC-2 normally leaks Cl^- into the cell, thus regulating neuronal excitability directly. Reminiscent of issues regarding GABA_AR function (e.g., is GABA_AR input inhibitory or excitatory at different developmental stages, in different disease states, in different cellular compartments?), our results emphasize that driving force must be carefully considered when inferring channel function.

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