

Supplemental Material

Estimate of bumetanide insensitive Cl⁻ fluxes. The simplest explanation for bumetanide insensitive Cl⁻ transport was Cl⁻ diffusion from neighboring areas of dendrite. To test this hypothesis, we calculated the volume of active dendrite based on the dendritic diffusion coefficient for Cl⁻ (Kuner and Augustine, 2000) and the Cl_i depletion / recovery data obtained in the presence of bumetanide. If the volume and geometry of the active dendrite predicted by a diffusion model were reasonable, this would support the idea that the bumetanide-insensitive Cl⁻ transport was due to diffusion. These estimates do not change the accuracy of our results, because the dendritic geometry estimates obtained here were not used in any calculations.

We calculated the volume of the dendrite (*Vol*) based on the charge transfer of the depletion current (*I_{load}*) and the magnitude of the Cl_i recovery (ΔCl_i). *I_{load}* was converted from Coulombs to moles using Faraday's constant, $F = 96,487 \text{ C/mol}$. Cl_i at $t = 0$ ($Cl_{i(t=0)}$) and Cl_i at steady state ($Cl_{i(ss)}$) were estimated from the monoexponential fit of the Cl_i recovery data where steady state was the value at $t = 6$ seconds:

$$Cl_{i(ss)} - Cl_{i(t=0)} = \Delta Cl_i = \frac{I_{load(mol)}}{Vol} \quad (11)$$

Rearranging,

$$Vol = \frac{I_{load(mol)}}{Cl_{i(ss)} - Cl_{i(t=0)}} \quad (12)$$

For our first estimate, we assumed a cubic dendrite, so the length (Len) would be equal to the cube root of the volume. We refined this estimate using a least squared fit algorithm to find Len more precisely. The Cl^- concentration gradient $Grad_{Cl}$ was estimated by

$$Grad_{Cl} = \frac{Cl_i - Cl_{i,ss}}{\frac{1}{2} Len} \quad (13)$$

where $\frac{1}{2} Len$ is the diffusion distance, assuming both ends of the dendritic length are open. The Cl^- flux is then given by Fick's Law of Diffusion:

$$Flux_{Cl} = Grad_{Cl} \times D_{Cl} \times \frac{Vol}{Len} \quad (14)$$

where D_{Cl} is $2 \times 10^{-5} \text{ cm}^2 \times \text{s}^{-1}$, the diffusion coefficient for Cl^- (Kuner and Augustine, 2000). The change in Cl_i as a function of time (t) is therefore

$$\frac{dCl_i}{dt} = \frac{(t_2 - t_1) \times Flux_{Cl_i}}{Vol} \quad (15)$$

The mean predicted geometry of the active area of dendrite was: volume $663 \pm 130 \text{ } \mu\text{m}^3$; length $106 \pm 13 \text{ } \mu\text{m}$; radius $1.4 \pm 0.2 \text{ } \mu\text{m}$ ($n = 5$ cells recorded with $0 \text{ mM Na}_{\text{pipette}}$). This model demonstrates that the rate of Cl_i recovery in the presence of bumetanide was consistent with Cl^- diffusion from regions of the dendrite where $GABA_A$ currents were not activated into the region where $GABA_A$ currents had been activated. These values were not used to calculate any of the Cl^- transport results reported in this manuscript, and therefore do not influence the accuracy of our results.

NKCC1 transport ratio. NKCC1 transport has been shown to have a *unidirectional* $\text{Na}^+:\text{K}^+:\text{Cl}^-$ transport stoichiometry of 1:1:2 (Russell, 2000). Measuring “unidirectional” transport implies that once an ion has been transported across the membrane, it stays on that side of the membrane. In other words, NKCC1-mediated ion self-exchange (where NKCC1 transports one of the ions back across the membrane) and ion transport by other transporters such as Na^+/K^+ -ATPase do not confound stoichiometry measurements. An important caveat is that our transport ratio estimate (1 Na^+ : 4 K^+ : 5 Cl^-) reflects *net* ion transport, rather than unidirectional transport (Russell, 2000) because transport of Na^+ and K^+ by NKCC1 will induce compensatory activity of Na^+/K^+ -ATPase (Figures 5 – 7). To test the hypothesis that NKCC1 is at thermodynamic equilibrium at the steady state Cl_i in other systems, we used the published ion concentrations summarized in Table 3 of Russell (2000) to calculate the predicted Cl_i for NKCC1 at thermodynamic equilibrium in several experimental systems (Equation 8; Russell, 2000). The supplemental figure shows that NKCC1 is at or near thermodynamic equilibrium at the steady state Cl_i in many systems using the widely reported unidirectional $\text{Na}^+:\text{K}^+:\text{Cl}^-$ transport ratio of 1:1:2 (Equation 8; Russell, 2000). In several reports, the 1:1:2 transport ratio predicts steady state Cl_i to be much higher than the observed Cl_i . Interestingly, in each of these cases, a *net* $\text{Na}^+:\text{K}^+:\text{Cl}^-$ transport ratio (1:2:3 or 1:4:5) predicts steady state Cl_i to be at or near the observed Cl_i , indicating NKCC1 transport at thermodynamic equilibrium, as we have shown in neonatal neurons.

Because our estimates reflect net ion transport, it is possible that our estimate of NKCC1 reflects the combined transport activities of NKCC1 and Na^+/K^+ -ATPase. Na^+/K^+ -ATPase moves Na^+ in the opposite direction, but K^+ in the same direction, as NKCC1. This reduces the net transport ratio of Na^+ compared to K^+ and Cl^- , which is what we observed with an estimated $\text{Na}^+:\text{K}^+:\text{Cl}^-$ ratio of 1:4:5 (compared to the canonical 1:1:2 ratio). However, stoichiometry estimates were unchanged from control when we blocked $\alpha 2/\alpha 3$ Na^+/K^+ -ATPase isoforms with dihydroouabain. This suggests that at

least the $\alpha 2/\alpha 3$ isoforms of Na^+/K^+ -ATPase did not significantly contribute to the 1:4:5 NKCC1 stoichiometry estimate.

It is important to note that regardless of the transport ratio used to describe NKCC1-mediated transport, the overall result we observe is consistent with NKCC1-mediated Cl^- transport in the direction that allows the free energy of the combined $\text{Na}^+:\text{K}^+:\text{Cl}^-$ gradient to return to zero following a transient change in Cl_i .

Supplemental Figure. Predicted Cl_i for NKCC1 at thermodynamic equilibrium correlates with observed Cl_i according to unidirectional (1:1:2, $\blacklozenge$) and net (1:2:3, $\circ$; 1:4:5, $\square$) $\text{Na}^+:\text{K}^+:\text{Cl}^-$ transport ratios according to Equation 8. Reported intracellular and extracellular Na^+ , K^+ , and Cl^- concentrations are from Table 3 of Russell (2000). In ascending order of observed Cl_i , the data are from neonatal rat CA1 neurons; heart muscle #1; frog retinal pigment; frog dorsal root ganglion; osteosarcoma; heart muscle #2; bovine aortic endothelium; shark rectal gland; Ehrlich ascites tumor cells; and squid giant axon. The line represents unity, where NKCC1 is predicted to be at thermodynamic equilibrium (no net Cl^- transport) at the steady state Cl_i .

References:

Kuner T, Augustine GJ (2000) A genetically encoded ratiometric indicator for chloride: capturing chloride transients in cultured hippocampal neurons. *Neuron* 27:447-459.

Russell JM (2000) Sodium-Potassium-Chloride Cotransport. *Physiol Rev* 80:211-276.