Cation-Anion-Coupled Cotransport in an Immortalized Neuronal Cell Line (C6 Glioma)

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of forskolin action on the Na/K pump. In a final myocyte, examined using low-[Cl\textsuperscript{-}] (sulfamate) Tyrode’s to diminish such contaminating current, forskolin reversibly enhanced strophantidin-sensitive current at all voltages. So it appears that PKA, stimulated by forskolin, can increase Na/K pump current, but that caution must be exercised in equating cardiotonic steroid-sensitive current with that generated by the Na/K pump. (Supported by NIH grant HL36783.)

### 57. NH\textsubscript{4}\textsuperscript{+} Inhibits K-Cl Cotransport in Low K Sheep Red Blood Cells

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K-Cl cotransport (COT) is mediated by several isoforms of KCC proteins whose cDNAs have been recently cloned. We have earlier demonstrated that, in red blood cells (RBCs), K-Cl COT carries equally K\textsuperscript{+} and Rb\textsuperscript{+}, as well as Cl\textsuperscript{-} and Br\textsuperscript{-}. To further characterize the selectivity of the K\textsuperscript{+} site, we explored whether NH\textsubscript{4}\textsuperscript{+} competes with K\textsuperscript{+} or Rb\textsuperscript{+} and is transported by K-Cl COT in low K (LK) sheep (S) RBCs before and after treatment with N\textsuperscript{-}ethylmaleimide (NEM), a known stimulator of K-Cl COT (Lauf and Theg. 1980. Biochem. Biophys. Res. Commun. 92:1422). To minimize CO\textsubscript{2}-driven Cl/HCO\textsubscript{3} exchange facilitating intracellular NH\textsubscript{4}Cl formation, and hence osmotic hemolysis, LK SRBCs were pretreated with the band-3 anion transport inhibitor DIDS in either Cl\textsuperscript{-} or SO\textsubscript{4}\textsuperscript{2-} media. Both ouabain-resistant Rb\textsuperscript{+} influx and K\textsuperscript{+} efflux were measured in either NH\textsubscript{4}Cl or (NH\textsubscript{4})\textsubscript{2}SO\textsubscript{4}, and Cl\textsuperscript{-}-dependent Rb/K fluxes (K-Cl COT) were calculated from the difference of the values in the two anions. NH\textsubscript{4}\textsuperscript{+} (increased by replacing Na\textsuperscript{+}) reduced basal and NEM-stimulated Cl\textsuperscript{-}-dependent Rb influx by mixed-type inhibition as both \( V_{max} \) decreased and \( K_i \) increased with rising NH\textsubscript{4}\textsuperscript{+} concentrations. The \( K_i \) for NH\textsubscript{4}\textsuperscript{+}, estimated from Rb influx measurements, was \( \sim 50 \text{ mM} \) and found to be three times larger than the \( K_i \) for Rb\textsuperscript{+} in these experiments, whereas the apparent \( K_i \) for NH\textsubscript{4}\textsuperscript{+}-inhibited Cl\textsuperscript{-}-dependent K\textsuperscript{+} efflux was 45 mM. Experiments taking advantage of an external acid pH-induced conversion of NH\textsubscript{4} to NH\textsubscript{3}, were inconclusive: NEM per se modified the pH response of K-Cl COT. However, no reports on the presence of glial K-Cl COT are available yet. The present work was designed to study Na-K-2Cl COT and K-Cl COT in C6 glioma cells. Cell cultures were grown to confluence under 5% CO\textsubscript{2} supplemented air atmosphere in 12-well plates using F12K essential medium containing 15% horse serum, 2.5% fetal bovine serum, and penicillin/streptomycin. Influx of Rb (a K congener) was measured under initial velocity conditions at 6 min and 37°C in Na media containing (mM): 10 RbCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, buffered to pH 7.4 with 20 HEPES/TRIS. Rb influx (nmol/mg protein \( \times \text{min} \) min) was 16.0 in the absence of inhibitors, 14.3 with 1.0 mM ouabain, 7.9 with ouabain and 0.005 mM bumetanide, and 4.3 with the additional presence of 2 mM furosemide. Thus, the Na/K pump represents 10% of the total Rb influx, whereas the Na-K-2Cl COT and K-Cl COT represent 40 and 22%, respectively. Na-K-2Cl COT was inhibited in a dose-dependent manner by bumetanide, whereas 100% inhibition of K-Cl COT occurred at 2 mM furosemide. At its maximum effective concentration, NEM stimulated the Na/K pump and K-Cl COT by 2- and 10-fold, respectively, and inhibited Na-K-2Cl COT by 100%. Cell K contents remained relatively unchanged, but fell by \( \sim 20\% \) after NEM treatment, suggesting opening of a K channel, reported by us in other cells. Using a polyclonal anti-rabbit KCC1 antibody (generously supplied by Dr. Eric Delpire), K-Cl COT in C6 glioma cells was immunohistochemically demonstrated by a strong, punctuate immunofluorescent labeling throughout the cytoplasm and cell membrane. Our results on cultured C6 glioma cells corroborate the immunological findings of Plotkin et al. (1997. Am. J. Physiol. Cell Physiol. 272:C175–C183) and the RT-PCR results of Payne et al. (1996. J. Biol. Chem. 271:16245–16252) showing the KCC1 isoform in astrocytes. Western blots using the same anti-rabbit KCC1 antibody revealed a single strong band of \( \sim 120 \text{ kDa} \), presumably the cytosolic, unglycosylated KCl cotransporter. These studies have therefore established: (a) the functional presence of the Na/K pump, and both Na-K-2Cl and K-Cl COT; (b) the immunohistochemical presence in C6 glioma cells of a KCC isoform of K-Cl COT; and (c) inhibition of Na-K-2Cl COT and simultaneous stimulation of K-Cl COT by NEM, possibly implicating a shared regulatory pathway. Studies in progress are aimed at the Cl dependence and the critical NEM concentration inversely affecting Na-K-2Cl and K-Cl COT. (Supported in part by NIH, AHA, and a WSU SOM alpha grant.)

### 58. Cation–Anion-coupled Cotransport in an Immortalized Neuronal Cell Line (C6 Glioma)

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Ion gradients across the cell membrane are vitally important for proper cell-to-cell communication and general cell homeostasis and are maintained by both primary (ATP-dependent) and secondary active transport mechanisms. Among the latter, Na-K-2Cl cotransport (COT) is present in primary cultures of rat astrocytes (Tas et al. 1987. Biochem. Biophys. Acta. 903:411–416) and implicated in the K spatial buffering capacity of glial cells. However, no reports on the presence of glial K-Cl COT are available yet. The present work was designed to study Na-K-2Cl COT and K-Cl COT in C6 glioma cells. Cell cultures were grown to confluence under 5% CO\textsubscript{2} supplemented air atmosphere in 12-well plates using F12K essential medium containing 15% horse serum, 2.5% fetal bovine serum, and penicillin/streptomycin. Influx of Rb (a K congener) was measured under initial velocity conditions at 6 min and 37°C in Na media containing (mM): 10 RbCl, 2 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, buffered to pH 7.4 with 20 HEPES/TRIS. Rb influx (nmol/mg protein \( \times \text{min} \) min) was 16.0 in the absence of inhibitors, 14.3 with 1.0 mM ouabain, 7.9 with ouabain and 0.005 mM bumetanide, and 4.3 with the additional presence of 2 mM furosemide. Thus, the Na/K pump represents 10% of the total Rb influx, whereas the Na-K-2Cl COT and K-Cl COT represent 40 and 22%, respectively. Na-K-2Cl COT was inhibited in a dose-dependent manner by bumetanide, whereas 100% inhibition of K-Cl COT occurred at 2 mM furosemide. At its maximum effective concentration, NEM stimulated the Na/K pump and K-Cl COT by 2- and 10-fold, respectively, and inhibited Na-K-2Cl COT by 100%. Cell K contents remained relatively unchanged, but fell by \( \sim 20\% \) after NEM treatment, suggesting opening of a K channel, reported by us in other cells. Using a polyclonal anti-rabbit KCC1 antibody (generously supplied by Dr. Eric Delpire), K-Cl COT in C6 glioma cells was immunohistochemically demonstrated by a strong, punctuate immunofluorescent labeling throughout the cytoplasm and cell membrane. Our results on cultured C6 glioma cells corroborate the immunological findings of Plotkin et al. (1997. Am. J. Physiol. Cell Physiol. 272:C175–C183) and the RT-PCR results of Payne et al. (1996. J. Biol. Chem. 271:16245–16252) showing the KCC1 isoform in astrocytes. Western blots using the same anti-rabbit KCC1 antibody revealed a single strong band of \( \sim 120 \text{ kDa} \), presumably the cytosolic, unglycosylated KCl cotransporter. These studies have therefore established: (a) the functional presence of the Na/K pump, and both Na-K-2Cl and K-Cl COT; (b) the immunohistochemical presence in C6 glioma cells of a KCC isoform of K-Cl COT; and (c) inhibition of Na-K-2Cl COT and simultaneous stimulation of K-Cl COT by NEM, possibly implicating a shared regulatory pathway. Studies in progress are aimed at the Cl dependence and the critical NEM concentration inversely affecting Na-K-2Cl and K-Cl COT. (Supported in part by NIH, AHA, and a WSU SOM alpha grant.)