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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^-] (sulfamate) Tyrode's to diminish such contaminating current, forskolin reversibly enhanced strophanthidin-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_4^+ Inhibits K-Cl Cotransport in Low K Sheep Red Blood Cells PETER K. LAUF, SUHAIL AHMED, and NORMA C. ADRAGNA, *Departments of Physiology & Biophysics, and Pharmacology & Toxicology, Wright State University, Dayton, Ohio*

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^+ and Rb^+, as well as Cl^- and Br^-. To further characterize the selectivity of the K^+ site, we explored whether NH_4^+ competes with K^+ or Rb^+ and is transported by K-Cl COT in low K (LK) sheep (S) RBCs before and after treatment with *N*-ethylmaleimide (NEM), a known stimulator of K-Cl COT (Lauf and Theg. 1980. *Biochem. Biophys. Res. Commun.* 92:1422). To minimize CO_2-driven Cl/HCO_3 exchange facilitating intracellular NH_4Cl formation, and hence osmotic hemolysis, LK SRBCs were pretreated with the band-3 anion transport inhibitor DIDS in either Cl^- or SO_4^{2-} media. Both ouabain-resistant Rb^+ influx and K^+ efflux were measured in either NH_4Cl or $(NH_4)_2SO_4$, and Cl-dependent Rb/K fluxes (K-Cl COT) were calculated from the difference of the values in the two anions. NH_4^+ (increased by replacing Na^+) reduced basal and NEM-stimulated Cl-dependent Rb influx by mixed-type inhibition as both V_{max} decreased and K_m increased with rising NH_4^+ concentrations. The K_i for NH_4^+, estimated from Rb influx measurements, was ~50 mM and found to be three times larger than the K_m for Rb^+ in these experiments, whereas the apparent K_i for NH_4^+-inhibited Cl-dependent K^+ efflux was 45 mM. Experiments taking advantage of an external acid pH-induced conversion of NH_3 to NH_4^+ were inconclusive: NEM per se modified the pH response of K-Cl COT. To test whether NH_4^+ is actually transported by the K-Cl COT system, Cl-dependent hemolysis was measured in the presence and absence of calyculin and genistein, inhibitors of protein phosphatases and kinases, respectively, and of K-Cl COT. However, no indirect evidence for NH_4^+ transport by the system could be detected with this method. The flux data suggest that in DIDS-treated LK SRBCs, NH_4^+ binds to an external low-affinity site in the transporter and causes allosteric inhibition of K-Cl COT. These results are consistent with the lack of NH_4^+ transport by the hemolysis approach.

58. Cation–Anion-coupled Cotransport in an Immortalized Neuronal Cell Line (C6 Glioma) KENNETH B.E. GAGNON,* NORMA C. ADRAGNA,‡ ROBERT E.W. FYFFE,§‖ and PETER K. LAUF,* **Department of Physiology and Biophysics,* ‡*Department of Pharmacology and Toxicology,* §*Department of Anatomy, and* ‖*Department of Brain Research Center, Wright State University, Dayton, Ohio*

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. *Biochim. 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_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 $CaCl_2$, 1 $MgCl_2$, 10 glucose, buffered to pH 7.4 with 20 HEPES/TRIS. Rb influx (nmol/mg protein × 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 ~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:C173—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 ~120 kD, presumably the cytosolic, unglycosylated K-Cl 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) inactivation 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.)

59. Transient Nature of the Stimulatory "NEM-Effect" on K-Cl Cotransport in KCC1-transfected HEK293 and Primary Rat Aortic Smooth Muscle Cells PETER K. LAUF,* JIN ZHANG, JING ZHANG, and NORMA C. ADRAGNA,‡ **Department of Physiology and Biophysics, and* ‡*Department of Pharmacology and Toxicology, Wright State University, Dayton, Ohio*

K-Cl cotransport (COT) is mediated by several isoforms of KCC proteins whose cDNAs have been recently cloned. The thiol reagent *N*-ethylmaleimide (NEM) (Lauf and Theg. 1980. *Biochem. Biophys. Res. Commun.* 92:1492) has been widely used to demonstrate, by several-fold stimulation, the presence of K-Cl COT in a variety of cells such as red blood cells (RBCs), rat aortic smooth muscle cells (RASMCs), and human embryonic kidney (HEK293) cells transfected with KCC isoforms. In RBCs, NEM