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Transient Nature of the Stimulatory" NEM-Effect" on K-Cl Cotransport in KCCL-Transfected HEK293 and Primary Rat Aortic Smooth Muscle Cells

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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

was proposed to inhibit a kinase that by phosphorylation inactivates K-Cl COT (Jennings and Al Rohil. 1999. *J. Gen. Physiol.* 114: 743). Contrary to our work with RBCs, where the "NEM effect" is stable over time, we report here on the transitory nature of this NEM effect in HEK293 cells and primary RASMCs. HEK293 cells either not transfected (normal) or transiently transfected with full-length rbKCC1cDNA (kindly provided by Drs. Gillen and Forbush, Yale University) and RASMCs were treated with NEM concentrations yielding maximum stimulation of endogenous or transfected K-Cl COT. The activity of the system was tested by Rb influx, at various times within 1 h of NEM treatment and removal by washing, in Cl or sulfamate media containing (mM) 0.1 ouabain, 0.01 bumetanide, and 0.01 $GdCl_3$ to inhibit the Na/K pump, Na-K-2Cl COT, and stretch-activated K channels. K-Cl COT is the calculated difference between Rb influx in Cl and sulfamate. Treatment with NEM for 10 min stimulated K-Cl COT by threefold in normal and eightfold in KCC1-transfected cells, and by fivefold in RASMC. The NEM effect was abolished in normal HEK293 cells, and fell by 80% in flKCC1-transfected cells and RASMC after 45 and 10 min, respectively. Full K-Cl COT stimulation was observed in HEK293 cells incubated for 45 min before 10-min NEM treatment and subsequent flux. Furthermore, HEK293 cells first treated with NEM, and then washed and again exposed to NEM, also lost the stimulation of K-Cl COT. Dithiothreitol, a reducing agent, failed to preserve the NEM effect. It is known that the NEM-effect requires the presence of ATP (Lauf. 1983. *Am. J. Physiol. Cell Physiol.* 245:C445). There was no significant difference in the ATP content of control and NEM-treated cells. In contrast, cellular GSH fell by 90%, suggesting a breakdown of the cellular redox potential rather than NEM action through inhibitory thiols as in RBCs (Lauf and Adragna. 1995. *Am. J. Physiol. Cell Physiol.* 269:C1167). We suspect that, in both HEK293 cells and RASMC, NEM induced the initial dephosphorylation responsible for K-Cl COT stimulation, followed by changes in the transport complex resulting in the complete and irreversible dissipation of the stimulatory "NEM effect." (Supported in part by an AHA grant.)

60. Effect of Media pH on the Kinetics of the Sodium/Bicarbonate Cotransporter (NBC) RONA G. GIFFARD and RONALD L. MORGAN, *Department of Anesthesia, Stanford University, Stanford, California*

Regulation of pH in the brain is critical to normal physiological function and in response to pathophysiological disturbances such as cerebral ischemia. NBC is localized to glial cells in the brain. This transporter is DIDS inhibitable, bicarbonate dependent, and electrogenic. When running in an inward direction, NBC alkalinizes the cell. The associated extracellular acidification can dampen neuronal excitability (Rose and Ransom. 1996. *J. Physiol.* 491:291–305). In this study, the effects of acidification on the kinetics of NBC activity of primary cultured astrocytes from mouse cortex were determined. Sodium uptake using $^{22}Na^+$ was used to determine the kinetics of astrocyte NBC.

Astrocyte cultures, prepared from cortices of neonatal Swiss Webster mice, were used after 20–30 d in vitro. Cultures were washed with buffered saline solution containing 5.5 mM glucose (BSS 5.5) at pH 7.4 or 6.8 in the absence of HCO_3^-. After the last wash, the cells were preincubated for 1 h at 37°C in the same buffer. At the end of the preincubation period, the buffer was removed from each well and BSS 5.5 buffer with HCO_3^- was added. This buffer contained 1 mM ouabain, to inhibit the Na^+/K^+ ATPase, 10 μM 5-(*N*-methyl-*N*-isobutyl) amiloride to inhibit Na^+/H^+ exchange and sodium (35–147 mM). The bicarbonate concentrations in the BSS 5.5 buffers at pH 6.8 and 7.4 were 6 and 26 mM, respectively. After adding the BSS 5.5 buffer, 0.2 μCi of $^{22}Na^+$ was added to each well and incubated for 2–6 min. Then the cells were washed with ice-cold phosphate-buffered saline and solubilized with 2% SDS. Aliquots were taken for gamma counting. Initial transport velocities were determined from the time versus sodium uptake plots. The initial velocities and the sodium concentrations were then plotted on a dual reciprocal plot for each pH value.

At a pH value of 7.4, the dual reciprocal plot was a straight line, while at a pH value of 6.8, nonlinear inhibition was found. This type of inhibition indicates that either more than one form of the transporter is present, and the different forms respond differently to acidosis, or that the transporter may associate into a multimeric form or be modified, such as by phosphorylation, in response to the pH. To begin to analyze these different possibilities, the cloned brain NBC (Giffard et al. 2000. *J. Neurosci.* 20:1001–1008) is being expressed in 3T3 cells that do not normally show significant NBC-like activity. This will permit characterization of the behavior of a single species of NBC as a function of pH.

61. Transport Mechanism of the Organic Cation Transporter 2 THOMAS BUDIMAN, ERNST BAMBERG, HERMANN KOEPSELL, and GEORG NAGEL, *Max-Planck-Institut fur Biophysik, D-60596 Frankfurt, Germany*

The organic cation transporter 2 (OCT2) is expressed in kidney and brain, where it serves in homeostasis of exogenous and endogenous amines. We expressed rat OCT2 in *Xenopus laevis* oocytes and studied substrate-induced changes of electrical current with the giant patch-clamp technique. Activation of electric current corresponding to efflux was observed for small organic cations; e.g., tetramethylammonium (TMA), choline, and tetraethylammonium (TEA). In contrast, the bigger cations quinine and tetrabutylammonium (TBA) elicited no change in patch current. However, transport of TMA, choline, and TEA could be inhibited by applying quinine or TBA to the cytoplasmic side. We show that inhibition of organic cation efflux by quinine was competitive with substrates, with an apparent affinity constant of ~1 μM. Transport-mediated electrical current was linearly voltage dependent, with maximal turnover and apparent affinity to substrates both showing voltage dependence. Inclusion of organic cations in the pipette (i.e., the outside) revealed organic cation-induced inward current. Organic-cation–induced currents in both directions were observed when substrates were present on both sides of the membrane. At saturating concentrations of substrates, the resulting conductance was substantially smaller than at transzero conditions. Our results exclude an electroneutral H^+/organic cation$^+$ exchange and suggest the existence of an electroneutral organic cation$^+$/organic cation$^+$ exchange. We propose a model for a carrier-type transport mechanism. (Supported by DFG and MPG.)

62. Pre–Steady State Kinetics of the Neuronal Glutamate Transporter EAAC1 NATALIE WATZKE,* MICHAEL WIESSNER,‡ THOMAS RAUEN,‡ and CHRISTOF GREWER,* **Max-Planck-Institut für Biophysik, Frankfurt, Germany; and* ‡*Max-Planck-Institut für Hirnforschung, Frankfurt, Germany* (Sponsor: Georg Nagel)