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Proton (H) Modulation of K-Cl Cotransport Through Both Internal and External Sites in DIDS-pH-Clamped Low Magnesium LK Sheep Erythrocytes

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PROTON (H) MODULATION OF K-CL COTRANSPORT THROUGH BOTH INTERNAL AND EXTERNAL SITES IN DIDS-pH-CLAMPED LOW MAGNESIUM LK SHEEP ERYTHROCYTES. ((P.K. Lauf and N.C. Adragna)) Wright State University, School of Medicine, Dayton, OH 45401

The just cloned K-Cl cotransporter (KCC, Gillen et al. JBC 271:16237-44, 1996) is functionally present in low K sheep red blood cells (LK SRBCs) (Lauf et al. Amer. J. Physiol. 263:C917-32, 1992). In a recent thermodynamic study (Lauf & Adragna, J. Gen. Physiol 108:341-350, 1996) we reported that, at constant cell volume, V_{max} of K-Cl influx decreased with external pH (pH_o) in LK SRBCs, with internal Mg (Mg_i) stripped by A23187, and DIDS-clamped at internal pH (pH_i) 8.6, but not at pH_i <7.4, suggesting that H affect KCC at both membrane sides. In controls, H inhibited only inside. We attempted to functionally separate the two H sites. LK SRBCs were titrated to $pH_i = pH_o = \sim 9$, Mg_i stripped by A23187 and EDTA, and subsequently DIDS-pH clamped between $pH_i \sim 8.6$ and <6. Bidirectional K fluxes were measured in Cl and NO_3 at internal K and external Rb concentrations of ~24 and 10 mM, respectively, either at constant pH_o for cells with variable pH_i (8.6 to 6), or at variable pH_o for $pH_i \sim 8.6$ only. Although both K and Rb influxes increased marginally at $pH_i \sim 9$, bidirectional KCC, the calculated K flux differences between the two anions, was insensitive to pH_o. However, lowering pH_i from ~8.6 to 5.8 inhibited both K and Rb fluxes in Cl but also to some extent in NO_3. The shape of the H inhibition curves were different for K and Rb fluxes. Internal protonation caused a distinct hyperbolic inhibition of K-Cl efflux with an IC_{50} for protons at about pH 6.5, whereas Rb-Cl influx fell asymptotically to <1/10 of its activity at pH_i 5.8 not permitting an exact IC_{50} determination. Hence, external and internal H affinities modulating KCC in low Mg_i cells are different with different effects on function. For K-Cl efflux, the IC_{50} for the internal H effect is close to the pK_a for histidine, whereas that for RbCl influx seems to be shifted to higher pH values commensurate with a pK_a shift or a different chemical group. These data are consistent with our three state RAI model for volume-clamped low Mg_i LK SRBCs (Lauf et al., Am. J. Physiol. 266:C95-C103, 1994) in which deprotonation recruits transporters from the R into the active A state and need evaluation in terms of our kinetic asymmetry model (Delpire & Lauf, J. Gen. Physiol. 97:173-193, 1991) and Dunham's ABC activation model (J. Gen. Physiol. 101:733-766, 1993). NIH DK 37,160.

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ION PERMEATION IN GLUTAMATE TRANSPORTERS

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Glutamate transporter-mediated uptake is associated with a Cl^- conductance increase, but unlike the gradients for Na^+, K^+, and H^+, the Cl^- gradient is not measurably coupled to that of glutamate. This conductance is not activated by external binding of the non-transported competitive antagonist kainate. The temperature coefficient (Q_{10}; 20°/10°) for D-aspartate transport mediated by the human subtype EAAT1 was 3.2 ± 0.2, while that of the anion current was 1.0 ± 0.1 (n=5), indicating that the macroscopic anion conductance is independent of the transport rate. The NO_3^- and SCN^- permeabilities of the anion channel (relative to Cl^-) were 11 and 58, respectively. No anomalous mole fraction effect was observed with mixtures of anions. The anion conductance was also activated during reverse transport induced by raising extracellular K^+ (EC_{50} = 35.3 ± 5.7mM; n=5). A macroscopic anion current with a similar permeabilty profile (P_{SCN}/P_{Cl}=61) was observed in excised inside-out patches from oocytes expressing the transporter when excitatory amino acids and Na^+ were applied to the intracellular membrane face with K^+ in the pipette. This current was not observed when K^+ was replaced by choline (n=6). A mutant EAAT3 transporter (E374D) was constructed which mediated K^+-independent obligate amino acid exchange; this mutant exhibited a similar anion conductance. The results suggest that an anion conducting state is associated with a glutamate- and Na^+-occupied state of the transporter rather than a state in the K^+-transporting limb of the cycle.

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REGULATION OF INTRACELLULAR FREE MAGNESIUM IN HELIX ASPERSA NEURONS STUDIED WITH ION-SELECTIVE MICROELECTRODES

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Double-barrelled ion-selective microelectrodes were used to measure transmembrane voltage (Vm) and intracellular free magnesium concentration, $[Mg^{2+}]_i$, in identified neurons (1F,2F and 77F) of the suboesophageal ganglia of *Helix aspersa*. The Mg^{2+} selective barrel contained the neutral ionophore ETH 7025 mixed with PVC, and cyclohexanone (Schaller et al. Pflügers Arch. 423:338, 1993), giving more stable and selective electrodes than previous ones (e.g. ETH 5214). They were calibrated in solutions containing (in mM): KCl, 95; NaCl, 5; Na-Hepes, 5; pH 7.2 and variable concentrations of $MgCl_2$ (0, 0.2, 0.5, 1.0, 2.0, 5.0, 10). The basal $[Mg^{2+}]_i$ was 0.74 ± 0.06 mM (cf. Alvarez-Leefmans et al. J. Physiol. 354: 303, 1984). Replacement of external Na^+ with n-methyl-D-glucamine, produced an increase in $[Mg^{2+}]_i$ from 0.8 ± 0.3 mM up to 1.7 ± 0.4 mM at an initial rate of 0.02 mM/min. Mole by mole replacement of 20 mM external Na^+ with NH_4Cl, produced a transient intracellular alkalinization, followed by an acidification upon removal of NH_4Cl. During the alkalinization, basal $[Mg^{2+}]_i$ decreased from 0.9 mM to 0.1 mM. Upon removal of NH_4Cl, $[Mg^{2+}]_i$ recovered even in the absence of external Mg^{2+}, indicating that the changes in $[Mg^{2+}]_i$ were due to intracellular redistribution. The data suggests that pH_i modulates intracellular Mg^{2+}, probably by affecting cytosolic Mg^{2+} binding or transport. (Supported by NINDS USA Grant NS29227 to FJAL).

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FUNCTIONAL ANALYSIS OF hu MDR 1 PROTEIN EXPRESSED IN YEAST

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A truncated hu MDR 1 cDNA (missing the first 17 amino acid codons of the open reading frame) fused to the Ste 6 promoter of S. cerevisiae (generously provided by Drs. K. Kuchler and J. Thorner [P.N.A.S. 89, 2302 - 2306]) was cloned into the yeast expression vector pVT102. The resulting construct (pMDR1) places both the Ste 6 and alcohol dehydrogenase (ADH) promoters upstream of the hu MDR 1 cDNA. Colonies of pMDR1 stable transformants were found to exhibit high levels of hu MDR 1 protein expression. The strains are resistant to valinomycin, indicating the expressed protein is functional. The expressed protein migrates at about 140 kDa on SDS gels, due to inefficient glycosylation, and we estimate hu MDR 1 protein is about 0.1 % - 0.5 % of the total membrane protein in our highest - expressing clones. Isolated plasma membranes exhibit about a 2 - fold higher rate of ATP hydrolysis in Tris buffer (pH 7.50, 5 mM Mg^{++}) relative to control, and the increased ATPase activity can be further modulated by 10 - 100 μM levels of a variety of hydrophobic compounds. A summary of rates of growth in media of various tonicity and ionic composition, cell volume, intracellular pH, plasma membrane potential, and ion transport for these strains will be presented. Similar data for hu MDR 1 expressing yeast strains created in erg6 and K^+ uptake mutants will also be presented. *This work was performed in the Sackler Laboratory of Membrane Biophysics and was supported by grants from the NIH (GM54516, GM55349) and a Cancer Center Support Grant (NCI-P30-CA-08748). P.D.R. is a Sackler scholar at MSKCC. pV102 was generously provided by the NRC, Canada.*

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ALTERED pHi REGULATION in 3T3 / CFTR CLONES AND THEIR CHEMOTHERAPEUTIC DRUG - SELECTED DERIVATIVES

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Recently (Wei et al., Biophys. J. 69: 883 - 895, 1995) we found that 3T3 cells overexpressing the CFTR exhibit traits of multidrug resistant (MDR) cells. In this work, 3T3 / CFTR clones were selected with either doxorubicin or vincristine to generate series of stable cell lines that exhibit increasing levels of multidrug resistance. Thus, C3D6 (grown in the presence of 600 nM doxorubicin) was selected from C3D4 (grown in 400 nM), which was selected from C3D1 (grown in 100 nM), which was selected from the original 3T3 / CFTR clone C3 (M.J. Stutts *et al., J.B.C. 268,* 20653 - 20658) that was not grown in the presence of drug. A similar series was generated via selection with vincristine. Initial low - level drug selection increases CFTR expression without promoting MDR 1 or MRP expression. Upon continued selection at higher drug concentrations, CFTR mRNA levels decrease while MDR 1 mRNA levels concomitantly increase. At each incremental step of selection intracellular pH (pHi) increases. Cl^-/HCO_3^- exchange (AE) activity is significantly reduced in derivatives overexpressing MDR 1, but not the parental CFTR clones. The set point of Na^+/H^+ exchange (NHE) activity is significantly lower for the non drug - selected 3T3 / CFTR clones, relative to controls, but then increases upon initial drug selection. Overexpression of MDR 1 in the higher level selectants does not appear to further perturb apparent NHE. These data further describe how CFTR and MDR proteins may affect pHi regulation. *This work was performed in the Sackler Laboratory of Membrane Biophysics and was supported by grants from the NIH (GM54516, GM55349) and a Cancer Center Support Grant (NCI-P30-CA-08748). P.D.R. is a Sackler scholar at MSKCC.*

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ALTERED pHi REGULATION IN hu MDR 1 TRANSFECTANTS

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We recently reported on the characteristics of stable LR73 / hu MDR 1 transfectants created without any exposure to chemotherapeutic drugs (M.M.H. *et al., J. Gen. Physiol. 108,* 295 - 313). These clones define the phenotype unequivocally mediated by MDR protein overexpression alone, without any complications associated with exposure to chemotherapeutic drugs. The clones exhibit increased pHi and decreased membrane potential. We have analyzed pHi regulation in detail for these clones, as well as other clones created in Cl^-/HCO_3^- exchanger (AE) - deficient and Na^+/H^+ exchanger (NHE) - deficient backgrounds. Based on Cl^- substitution experiments for clones under constant perfusion with CO_2 / HCO_3^- - buffered media, we conclude that overexpression of hu MDR 1 protein inhibits AE isoform 2 (AE2) function. Conversely, ammonium pulse experiments show that MDR protein overexpression does not perturb NHE function. Cl^- - substitution experiments have been performed under conditions that deplete cellular ATP, in hypotonic and hypertonic media, in the presence of altered Cl^- and HCO_3^- gradients (e.g. altered AE driving force), as well as under Na^+ - free and K^+ - free conditions. In addition, we have performed HCO_3^- - pulse experiments under a variety of conditions. The data describe how MDR protein overexpression perturbs pHi regulation in LR73 cells. *This work was performed in the Sackler Laboratory of Membrane Biophysics and was supported by grants from the NIH (GM54516, GM55349) and a Cancer Center Support Grant (NCI-P30-CA-08748). P.D.R. is a Sackler scholar at MSKCC.*