Sidedness of H+ Action on K-Cl Cotransport in DIDS pH(I)-Clamped Low K Sheep Red Blood Cells with Reduced Mg(I)

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The thiol oxidant diamide (DM) stimulates K-CI cotransport in low K (LK) sheep red blood cells once cellular glutathione (GSH) is oxidized to its dithiol GSSG. This effect is reversed after metabolic or chemical reconstitution of the GSH levels (Lauf. 1988. Journal of Membrane Biology. 101:179) suggesting redox involvement in the control of K-CI cotransport. To further test this hypothesis we took two approaches. We first compared the dose responses of K-CI cotransport activation and GSH oxidation/alkanethiolation by the reversible thiol reagents DM and methylmethane thiosulfonate (MMTS) and of GSH alkylation by the irreversible thiol reagent N-ethylmaleimide (NEM). Second, we studied the effect of elimination of GSH on K-CI cotransport by conjugation to chlorodinitrobenzene (CDNB) via the intracellular GSH transferase prior to exposure to DM, NEM and MMTS. The absolute values of the slopes of K-CI cotransport activation and of GSH oxidation or alkanethiolation were similar but of opposite sign for both DM and MMTS, and different from those for NEM, suggesting a functional relationship between the cellular GSH pool and K-CI cotransport activity. In contrast, the concentration (mmol/liter cells) for 50% activation of K-CI cotransport and 50% reduction of GSH were identical only for MMTS (1.4 vs 1.5) but different for DM (5.3 vs 1.7) and NEM (4.9 vs 2.5) suggesting differences in relative potencies to affect K-CI cotransport and GSH levels. K-CI cotransport increased significantly above base level only after >90% GSH was depleted by CDNB (without reducing APT). The net stimulation by CDNB was less than 20% of that achieved by DM whereas GSH thiols represent 15–20% of the total red cell thiol pool. Treatment with NEM, DM, and MMTS after CDNB failed to activate K-CI cotransport to levels seen with these reagents alone suggesting that all three reagents affected the system via the GSH/GSSG redox system. Based on the dose response curves for NEM-induced K-CI flux activation and GSH alkylation the mechanism by which NEM produces its effect appears to be different. The determinants of the thiol-activated K-CI cotransporter may be either crucial thiol group(s) in the carrier molecule which become unavailable upon CDNB treatment, or oxidized GSH, GSSG, which acts through thiols in the regulatory moiety of the K-CI cotransport complex. (Supported by NIH DK 37,160.)

112. Sidedness of H+ Action on K-CI Cotransport in DIDS pH1-clamped Low K Sheep Red Blood Cells with Reduced Mg, PETER K. LAUF and NORMA C. ADARAGNA, Departments of Physiology and Biophysics, and Pharmacology and Toxicology, Wright State University Medical School, Dayton, Ohio

We have recently proposed a model for proton, H+, and cellular magnesium, Mg, modulation of Cl-dependent K and Rb fluxes through the K-CI cotransporter in volume-clamped low K (LK) sheep red blood cells (Lauf, Erdmann, and Adragna. 1994. American Journal of Physiology. 266:(Cell Physiology. 35):C95). We now have determined the sidedness for the H+ action on K-CI cotransport in these cells with total Mg reduced to 1/20 of control by the divalent cation ionophore A23187 and an external chelator. The HCO3-/Cl exchange inhibitor DIDS (4,4'-disothiocyanatostilbene-2,2'-disulfonic acid) was used in combination with external sucrose to clamp cellular pH, pHc, at values between 6.7 and 8.6, and cell volume close to that of normal cells. Ouabain-insensitive K efflux and Rb influx were then measured in Cl and NO3 media at pHc of 6–9 for each clamped pH, and at various external Rb concentrations, [Rb]o. The difference between the K(Rb) fluxes in Cl and NO3 is the Cl-dependent K(Rb) flux component attributable to K-CI cotransport. Our observations were: (a) DIDS blocked Cl and H+ equilibration with the following pHc, effectiveness: 9.0 = 7.4 > 6.5. (b) Consistent with the reversible DIDS inhibition of K-CI cotransport (Delpire and Lauf. 1992. Journal of Membrane Biology. 126:89), LK cells washed after DIDS treatment exhibited K-CI cotransport activities similar to those of controls; however, DIDS stimulated significantly both K and Rb fluxes in NO3, particularly at pHc 9. (c) An increase in Vmax, of Cl-dependent Rb influx was observed in cells pH-clamped only at pHc = 8.6 when pHc was raised from 6.5 to 9. No major effect on Kmax was seen. (d) The flux reversal points (mM [Rb]o) determined by simultaneous measurements of Cl-dependent K efflux and Rb influx agreed with those estimated from the calculated ratio of the chemical driving forces. Both shifted from ~10 to 40 mM as intracellular Cl increased by about fourfold upon lowering pHc, from 8.6 to 6.7. We conclude that in low Mg, LK sheep red blood cells, with pH, clamped by DIDS, H+ either reduce the
113. Null Mutations of Connexin32 in Patients with X-linked Charcot-Marie-Tooth Disease. DAVID L. PAUL and ROBERTO BRUZZONE, Department of Neurobiology, Harvard Medical School, Boston, Massachusetts (Sponsor, R. A. Frizzell)

Charcot-Marie-Tooth (CMT) disease is a genetically heterogenous disorder characterized by progressive degeneration of peripheral nerves. One form of the demyelinating type is produced by genetic defects on chromosome X. Recently, we have reported that patients with X-linked CMT have single amino acid mutations in the gene encoding connexin32 (Cx32), a protein subunit of the intercellular channels localized at gap junctions (Bergoffen et al. 1993. Science. 262:2039-2042). To understand the role played by Cx32 in the myelinating Schwann cell and in the pathophysiology of CMT, we have compared the functional properties of four mutant Cx32 with those of the wild-type gene. Human Cx32 was PCR-amplified from genomic DNA of CMT patients (family 133: P172 → S; family 243: R142 → W; family 1769: E186 → K; family 51: 175 frameshift) and unaffected controls, subcloned in a transcription vector to produce cRNAs and expressed in Xenopus oocytes. Cells injected with cRNAs coding for either wild-type or mutant Cx32 were manually paired and studied by dual voltage clamp to determine the ability of either mutant to form intercellular channels. While wild-type Cx32 induced the development of large junctional conductances between paired oocytes (averaging 10-20 S), none of the pairs expressing CMT mutants displayed a detectable junctional conductance (< 0.01 S). Functional activity was not rescued by pairing oocytes injected with any of the mutants with cells expressing either the wild-type Cx32 or rat Cx26, another member of the connexin family which functionally interacts with wild-type Cx32 to form intercellular channels. Despite the lack of functional activity, all mutant Cx32 were efficiently expressed by oocytes to the same level of the wild-type, as judged by Western blot analysis. In addition, immunocytochemical analysis suggests that they were also correctly targeted to the sites of membrane apposition between the two cells of a pair. Together, these results show that patients affected by X-linked CMT have null mutations in the Cx32 gene. Because we have localized by immunocytochemistry Cx32 in Schwann cells to the incisures of Schmidt-Lanterman and perinodal regions, we propose that Cx32 may form channels not between cells as in orthodox gap junctions but, rather, between turns of myelin within an individual Schwann cell. Thus, the critical function that may be lost in CMT patients is the exchange of molecules, facilitated by these channels, between the peri-nuclear and peri-axonal regions of Schwann cells. (Supported by NIH grant GM37751.)