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# N-Ethylmaleimide Treatment of Low K Sheep Red-Blood-Cells at Different Temperatures Reveals Role of Thiols and MG for Activation and inactivation of K-Cl Cotransport

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**M-Pos26**

**N-ETHYLMALIMIDE TREATMENT OF LOW K SHEEP RED BLOOD CELLS AT DIFFERENT TEMPERATURES REVEALS ROLE OF THIOLS AND Mg FOR ACTIVATION AND INACTIVATION OF K-Cl COTRANSPORT.** P. K. Lauf, R. Moezzi and N. C. Adragna. WRIGHT STATE UNIVERSITY SCHOOL OF MEDICINE, Dayton, OH. 45435.

Exposure of low K sheep red blood cells to low concentrations (<1 mM, 5% hematocrit) of N-ethylmaleimide (NEM), activates K-Cl cotransport whereas high NEM concentrations (>2 mM) reverse this effect. At least two types of thiol groups differing in their apparent pK<sub>a</sub> values have been identified. We now report that these two thiols may be further distinguished by NEM treatment at 0°C and 37°C, respectively, followed by analysis of the NEM effect on KCl efflux measured at 37°C in controls and in cells with Mg, reduced by A23187 and EDTA. Results: 1. At 0°C, NEM up to 20 mM solely activated K efflux in Cl, with saturation at <2 min and 1 mM NEM using dithiothreitol to remove NEM. 2. At 37°C, 5 mM NEM reversed KCl flux stimulation to base values, however, with a tenfold larger time constant. 3. Both KCl flux activation and reversal were approximately equal in control and in low Mg<sub>i</sub> cells. Hence Mg<sub>i</sub> per se does not affect thiol alkylation. 4. Similarly, KCl flux, stimulated in low Mg<sub>i</sub> cells in the absence of NEM, was inhibited by 6 mM NEM. Thus both K-Cl cotransport states, one activated by reduction of Mg<sub>i</sub>, and the other by thiol alkylation, which affects regulation by Mg<sub>i</sub>, are similar based on their NEM-inhibition through low affinity thiols. Since ATP is required for both NEM activation and swelling-induced Mg<sub>i</sub> inhibition of K-Cl cotransport, and Mg<sub>i</sub> fails to inhibit NEM-stimulated KCl flux, MgATP may effect the thiol-catalyzed reversal of K-Cl cotransport stimulated by NEM, Mg<sub>i</sub> reduction, and cell swelling, the three principal interventions causing K-Cl cotransport activation. (Supported by NIH 37.160).

**M-Pos28**

**ALZHEIMER'S DISEASE-INDUCED CHANGES IN HUMAN RED CELL MEMBRANE TRANSPORT PROCESSES** (A. Janoshazi, A. Satlin and A. K. Solomon) Biophysical Laboratory, and Dept of Psychiatry, McLean Hospital, Harvard Medical School, Boston MA, 02115.

An altered rate of SO<sub>4</sub><sup>2-</sup> exchange in red cells of patients with Alzheimer's disease (AD) has been reported by Bosman *et al.* (1991). Does this mean that AD red cell band 3 is different from normal? We compared binding kinetics of the fluorescent anion exchange inhibitor, DBDS (4,4'-dibenzamido-2,2'-stilbene disulfonate) in AD red cells with age-matched controls. In normal red cells DBDS binding is saturable, a bimolecular association followed by a monomolecular conformation change which locks bound DBDS to band 3. In four preliminary experiments in AD red cells, the monomolecular conformation change is absent and binding does not saturate in the μmolar concentration range, consistent with an AD-induced conformation change in red cell band 3. Bosman *et al.* also reported one instance of increased binding of the glucose transport inhibitor, cytochalasin B (Cyt B), in AD red cells, suggesting an AD effect on the glucose transport protein (GLUT1). We have previously reported that Cyt B modulates red cell DBDS binding (Janoshazi and Solomon, 1989) indicating that band 3 and GLUT1 are adjacent in the normal cell membrane. Two preliminary experiments show that 2.5 μM Cyt B modulation of DBDS binding kinetics is altered in AD red cells, consistent with an AD-induced conformational change of GLUT1. (Supported by the Council for Tobacco Research and the Alzheimer's Association).

**M-Pos30**

**PHOSPHOLIPID STRUCTURAL SPECIFICITY OF A HUMAN ERYTHROCYTE PHOSPHATIDYL-SERINE-STIMULATED Mg<sup>2+</sup>-ATPASE.** (M. L. Zimmerman, E. Nemerget, and D. L. Daleke) Department of Chemistry, Indiana University, Bloomington, IN 47405.

A candidate aminophospholipid flippase, a lipid-dependent, vanadate-sensitive Mg<sup>2+</sup>-ATPase, has been purified from human erythrocytes. The detergent solubilized enzyme is not active in the absence of added lipid and shows a high degree of specificity for its lipid activator. *sn*-1,2-Diacylphosphatidyl-L-serine (PS) stimulates the enzyme maximally, anionic phospholipids afford partial activation and zwitterionic or neutral lipids do not activate the ATPase. Lipid structural elements required for activation were studied using synthetic PS analogs which were modified in the headgroup, phosphate and glycerol backbone. Activation was reduced by removing the carboxyl group (PE, 28% of PS) or the amine moiety (phosphatidylhydroxypropionate, 28% of PS), or by methyl esterification of the carboxyl group (21% of PS). Phosphatidyl-moserine produce 25% less activity than PS, indicating that the enzyme senses headgroup size. Replacement of the phosphate group with an isosteric, uncharged sulfonate abolished activation of the ATPase, indicating that the phosphate group is another recognition element. ATPase activity was dependent on the stereochemistry of the glycerol backbone, but not the serine headgroup: the D-serine analog of *sn*-1,2-PS was equivalent to the natural L-serine derivative in supporting ATPase activity. However, *sn*-2,3-dilauroyl-phosphatidyl-L-serine and *sn*-2,3-dilauroylphosphatidyl-D-serine afforded only 60% and 32% of the activity in the presence of the *sn*-1,2-isomers. Finally, glycerophosphoserine and phosphoserine, but not the choline analogs or serine alone, significantly inhibited ATPase activation in the presence of PS. These data indicate that the ATPase recognizes several PS structural elements and that each are required for full elaboration of activity. This structural specificity is shared by the aminophospholipid flippase, indicating that these proteins may be identical.

**M-Pos27**

**QUININE AND QUINIDINE INHIBIT K-Cl COTRANSPORT IN LOW K SHEEP ERYTHROCYTES.** N. C. Adragna and P. K. Lauf, WRIGHT STATE UNIVERSITY SCHOOL OF MEDICINE, Ohio, 45401-0927.

Low K (LK) sheep red blood cells (SRBCs) are used as a model to study K-Cl cotransport which plays an important role in cellular dehydration in human erythrocytes homozygous for hemoglobin S. Cinchona bark derivatives such as quinine (Q) and quinidine (QD) are effectively used in the treatment of malaria. In the present study we investigated the effect of Q and QD on K-Cl cotransport in LK SRBCs. Cells with K-Cl cotransport activated by either swelling in hyposmotic media, or by thiol alkylation with N-ethylmaleimide, or by cellular Mg removal through A23187 in the presence of external chelators were exposed to various concentrations of Q and QD and the rate constants of K efflux were determined in Cl and NO<sub>3</sub>. K-Cl cotransport was defined as the Cl-dependent (Cl minus NO<sub>3</sub>) K efflux. K-Cl cotransport stimulated by all three interventions was inhibited by both Q and QD in a dose-dependent manner. Full inhibition of K-Cl cotransport occurred at concentrations of Q > 2 mM, and of QD of 1 mM. The inhibitory effect of Q was manifested in Cl but not in NO<sub>3</sub>, whereas QD also reduced slightly K fluxes in NO<sub>3</sub>. Dixon plots of the 1/K-Cl flux versus the inhibitor concentration revealed curvilinear behavior indicating complex inhibitory kinetics. In contrast to K efflux, Q and QD inhibited KCl influx less effectively in swollen and NEM-treated cells whereas the effect in low Mg cells was more complex. The inhibitory action of these two drugs was reversible and their mechanism of action likely involves diffusion into the cell and inhibition from the cytoplasmic aspect of the membrane. This is the first report of an inhibition by antimalarial drugs of K-Cl cotransport activated by three independent manipulations. (Supported by NIH DK RO1-37160).

**M-Pos29**

**COUPLING OF THE Na FLUX OF THE Na-PO<sub>4</sub> COTRANSPORTER AND THE Na PUMP. EVIDENCE FOR A MEMBRANE POOL OF Na<sup>+</sup>.** (R. B. Gunn and L. Tadayon) Department of Physiology, Emory University School of Medicine, Atlanta, GA 30322.

The ouabain- and disulfonic stilbene-insensitive Na-PO<sub>4</sub> cotransporter in human red cells provides 25% of the <sup>32</sup>P influx in a plasma-like medium, but provides 90% of the labeling of the membrane pool of nucleotides (Shoemaker *et al.*, *J. Gen. Physiol.* 92:449-474, 1988) that are preferential substrates for the ouabain-sensitive Na pump. Experiments were performed to test whether the Na cotransported with the phosphate also had a special relationship to the pump as compared to cytoplasmic Na. Human red cells were loaded with <sup>22</sup>Na by a 60-sec exposure to nigericin (10 μM) in MgCl<sub>2</sub> (110 mM) at 37°C. The ouabain-sensitive <sup>22</sup>Na efflux was measured in 140 mM NaCl ± 1 mM N-methyl-D-glucamine-PO<sub>4</sub>. The external PO<sub>4</sub> appeared to inhibit the pump 13-30%, but when pump function was assayed by <sup>86</sup>Rb influx external PO<sub>4</sub> had no effect. One explanation is that the influx of nonradioactive Na<sup>+</sup> on the Na-PO<sub>4</sub> cotransporter was diluting the specific activity of the pump's substrate pool of Na<sup>+</sup> and decreasing the tracer efflux but not pump turnover or stoichiometry. One predicted consequence of this membrane Na<sup>+</sup> pool is that if the pump is blocked the tracer influx through the Na-PO<sub>4</sub> cotransporter should increase since <sup>22</sup>Na in the pool could no longer be recycled out through the pump. We observed that the influx of <sup>22</sup>Na into red cells (dinitrostilbene-disulfonate-treated to block anion exchange on band 3) in the presence of 1 mM PO<sub>4-out</sub> was increased by ouabain. The coupling of fluxes whereby the product of one enzyme/transporter is not released but used as substrate for another enzyme/transporter is documented in metabolism and the mitochondrial membrane. This relationship for transporters in the plasma membrane of eukaryotes has not been previously described. Supported by USPHS-NIH-HL 28674.

**M-Pos31**

**SENESCENT ERYTHROCYTE REMOVAL** (L. Kantor and H.M. Mizukami) Wayne State University, Detroit, MI 48202. (Spon. by H.M. Mizukami)

An anion transport protein, Band 3 (90-100kda) is involved in the two proposals regarding the senescent rbc's (red blood cells) removal. One by Low *et al.* (1985) suggests that Band 3 molecules cluster due to the possible crosslinking of their n-termini. Such close proximity of antigenic sites leads to bivalent binding of autologous antibodies resulting in rbc removal. Another by Kay (1985) states that the cleavage of Band 3 takes place, forcing a part of it, to the surface of the membrane. Due to the presence of Band 3 fragment on a cell surface, such cell is recognized as immunologically foreign, resulting in autologous antibody binding and eventual removal by macrophages from the circulation. Which proposal is right? Most likely both are.

Our research will elucidate the reason two hypothesis exist - both researchers were looking at the dependent events but at different time periods. We will demonstrate that the initial step is the clustering of Band 3's n-termini as mentioned above, coinciding with the accumulation of oxygen radicals. The radicals lead to oxidation of the clustered Band 3's cysteine residues thus forming disulfide bonds between the Band 3 monomers. This results in conformational changes in Band 3, exposing proteolytic sites, previously hidden in the membrane. The surfacing immunologically different antigenic sites become recognized by autologous IgG, leading to macrophage attachment and eventual removal out of the circulatory system.

We will show densitometrically that Band 3 undergoes decrease in its intact form, meaning degradation is taking place. Further, immunoblots with an antibody to surface portion of Band 3 will demonstrate that Band 3 exists in aggregated forms in middle aged rbc's, whereas old rbc's in addition to aggregation, exhibit breakdown products. Young rbc's display only the intact (90-100kDa) form of Band 3, thus supporting our hypothesis for senescent rbc's removal.