VOLUME REGULATION BY FLOUNDER RED BLOOD CELLS: THE NATURE OF THE ION FLUX PATHWAYS

Robert R. Rigor III, Zhenpeng Zhuang, and Peter M. Cala
Department of Human Physiology, School of Medicine, University of California, Davis CA 95616

Introduction

Volume regulation subsequent to osmotic perturbation is a feature characteristic of most animal cells. Much of what is known about volume regulation is the result of studies of red blood cells (RBCs) (Cossins and Gibson, J Exp Biol 200: 343-352, 1997; Parker, et al, J Gen Physiol 98: 869-80, 1991; Cala, P.M., Mol Physiol 4:33-52, 1983). One of the earliest studies demonstrating vertebrate cell volume regulation was performed at the Mount Desert Island Biological Laboratory using Winter Flounder (*Pseudopleuronectes americanus*) RBCs (Cala, Bull. MDIBL 13: 20-25, 1973; Cala, J Gen Physiol 69: 537-52, 1977). In hypotonic media, flounder RBCs swell and undergo regulatory volume decrease (RVD), characterized by net loss of cellular K^+, Cl^- and water. In hypertonic media, cells shrink and undergo regulatory volume increase (RVI), characterized by net cellular uptake of Na^+, Cl^- and water.

The K^+ efflux pathways most commonly associated with RVD are K^+ and Cl^- conductances, KCl cotransport and K/H exchange. In a cell with a constitutive, robust anion exchanger (AE), the above pathways will functionally couple to the AE through changes in [Cl] or pH (and therefore $[HCO_3^-]$). As a result of functional coupling with the AE, all of the above cation flux pathways will lead to net KCl loss (Cala, P.M., In: *Chloride Channels and Carriers, In Nerve, Muscle and Glial Cells*, New York: Plenum Press, 67-81, 1990). In contrast to flux pathways responsible for RVD, those most often responsible for RVI are Na/H exchange (NHE), Na-K-2Cl cotransport or NaCl cotransport. Again, in a cell with a robust, constitutively active AE, cation-Cl cotransporters will functionally couple to the AE through changes in $[Cl]_i$, while the Na/H exchanger is functionally coupled to the AE through changes in pH, and therefore $[HCO_3^-]$. In all cases, the net result is cell NaCl and water uptake (Cala, op. cit.).

Methods

In attempts to identify the pathways responsible for flounder RBC volume regulation, we measured net cation and Cl^- fluxes and media pH using a combination of flame photometry, a K^+ electrode, a chloridometer, and a pH electrode, respectively (Cala and Hoffmann, Methods Enzymol 173: 330-46, 1989). Net ion fluxes were expressed as millimoles of ion/Kg dry cell solid (dcs). Cell volumes were assessed as the difference between the wet and dry weight of cell pellets, and expressed as L H2O/Kg dcs. Flux measurements were performed on cells, in isotonic (355 mOsm), hypotonic (178 mOsm) or hypertonic (533 mOsm) HEPES-buffered Ringer's solution (pH 7.6), with 1 mM ouabain (Na/K ATPase inhibitor) (Cala, J Gen Physiol 69: 537-52, 1977; Cala, J Exp Zool 199: 339-44, 1977).

Results/Discussion

The coupling of volume regulatory ion fluxes to Cl/HCO_3 exchange: Experiments were performed on cells, with a functional AE or following inhibition by 40 minute preincubation with 10μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS) (Maldonado and Cala, Am J Physiol 267: C1002-C1012., 1994). DIDS was employed together with measurements of net ion flux and intra and/or extracellular pH, since the magnitude of pH change and Cl^- flux in the absence and presence of functional AE can be useful in identification of the cation flux pathway. Briefly, functional coupling between AE and a cation-Cl cotransporter or conductive cation and Cl^- flux, will produce changes in pH, which are eliminated by AE inhibition. In contrast, coupling between AE and alkali metal/H exchangers will buffer changes in pH secondary to cation transport. Therefore, inhibition of AE will accentuate pH changes resulting from net alkali metal/H exchange.

Suspensions of RBCs, in poorly buffered (0.3 mM HEPES) media at 10% hematocrit, were monitored for DIDS and volume-dependent changes in pHo and cell Cl^- content. DIDS pretreated cells undergoing RVI produced a greater decrease in pHo (from 7.56 to 7.37 in 20 minutes) than DIDS free controls (from 7.56 to 7.50 in 20 minutes), supporting the interpretation that NHE is responsible for RVI.

The chloride-dependence of volume regulatory alkali metal fluxes: Since net alkali metal fluxes during RVD and RVI are accompanied by Cl^- flux in the absence of DIDS, it was necessary to determine the basis for Cl^- coupling. In some conditions, intracellular Cl^- (Cl_i^-) was replaced with methanesulfonic acid (MSA). Since MSA/Cl exchange is mediated by AE, intracellular Cl_i^- can be replaced with MSA by suspending cells in Cl^- free, MSA media (Payne, et al., Am J Physiol 259: C819-27, 1990). Because MSA is not transported by cation-Cl cotransporters or the anion conductance, Cl^- replacement with MSA will inhibit conductive cation flux and KCl cotransport (Clemo, et al., Circ Res 84: 157-65, 1999; Kracke and Dunham, Proc Natl Acad Sci USA 87: 8575-9, 1990). Following suspension in isotonic, MSA medium, Cl_i^- was reduced from 120 to 20 mmoles Cl^- / Kg dcs. The cells were treated with DIDS to prevent MSA transport on AE, then suspended in anisotonic Cl^--free media and permitted to volume regulate. The loss of K^+ by osmotically swollen Cl^--depleted cells (29 mmoles / Kg dcs in 2 hours) was nearly identical to that by cells with normal $[Cl^-]_i$ (22 mmoles/Kg dcs in 2 hours). Furthermore, Cl^--depleted, DIDS-treated RBCs exhibited minimal Cl^- loss during RVD, compared to cells with normal $[Cl^-]_i$. That K^+ loss was unaffected by the presence of DIDS and low $[Cl^-]_i$ is counter to expectations of K^+ and Cl^- conductance, and KCl cotransport, yet consistent with K/H exchange -mediated RVD. In contrast, cells suspended in Cl^--free, hypertonic media exhibited no net Cl^- uptake during RVI, yet net Na uptake kinetics and magnitudes were nearly identical to those in media containing Cl^-. These data suggest that net Na^+ flux during RVI is via Na/H exchange.

Identification of volume regulatory flux pathways with specific transport inhibitors: While there are no known inhibitors of K/H exchange, furosemide and bumetanide are commonly used inhibitors of KCl cotransport (Gillen, et al., J Biol Chem 271: 16237-44, 1996; Payne, Am J Physiol 273: C1516-25, 1997). Additionally, amiloride, ethylisopropyl-amiloride (EIPA) and the Hoechst compound HOE694 are all potent inhibitors of the volume regulatory ("housekeeping") Na/H exchanger (isoform NHE1) (Orlowski and Kandasamy, J Biol Chem 271: 19922-19927, 1996). Thus, we initiated studies employing these agents in order to corroborate evidence suggesting that K/H and Na/H exchange are responsible for volume regulation following swelling and shrinkage, respectively. K^+ loss during RVD (69.5 mmoles K^+ / Kg dcs in 2 hours) was minimally inhibited by 1 mM bumetanide (61.5 mmoles K^+ / Kg dcs in 2 hours), and more completely inhibited by 2 mM furosemide (33.7 mmoles K^+ / Kg dcs in 2 hours), suggesting that K^+ loss during RVD is in part due to KCl cotransport. Taken together, these data support the conclusion that more than one K^+ flux pathway contributes to RVD. On the other hand, cells undergoing RVI in hypertonic media showed no reduction in net Na^+ uptake, in the presence of large concentrations either of 1 mM amiloride, 20 μM EIPA or 20 μM HOE694. If RVI is mediated by NHE1, then we must conclude that it is an inhibitor-insensitive NHE1 variant. This conclusion is supported by biochemical/molecular evidence, as discussed below.

Western Blotting and Immunolocalization of the Na/H Exchanger: Immunoblots from Flounder RBC membranes yielded a band of appropriate molecular weight (100 kD) detected by two NHE1-specific antibodies (4E9 monoclonal and XB17 polyclonal), yet was undetectable with an antibody against NHE3 (alpha666) (Maldonado and Cala, op. cit.; McLean, et al., Am J Physiol 276: C1025-C1037, 1999). Furthermore, permeabilized, formaldehyde-fixed whole cells, immunolabeled with XB17 and FITC-conjugated secondary antibody (Zhuang, et al., J Exp Biol 202: 2449-60, 1999), revealed intense fluorescence staining, concentrated in the region of the marginal band, suggesting a

surface-localized population of an NHE1 isoform. This result was not obtained using chicken and Duck RBCs as negative controls. Chicken and duck RBCs, while nucleated, as are those of the flounder, lack volume-regulatory NHE activity (P. Cala, unpublished observations; Lytle, Am J Physiol 274: C1002-10, 1998).

These studies are preliminary, in that our conclusions are based on two or three experiments of each type discussed. Yet, the interpretations are consistent with predicted behaviors of alkali metal/H exchangers and cation-Cl coupled cotransporters, and the consequences of their functional coupling to AE. There are however, some intriguing differences. First, with regard to swelling-induced K^+ loss, our data are consistent with the interpretation that both K/H exchange and KCl cotransport contribute to swelling-induced net K^+ efflux in flounder RBCs. To our knowledge, this is the first indication that a cell may use both K/H exchange and KCl cotransport during RVD. Further investigations are necessary to validate this preliminary conclusion. Our efforts to determine the identity of the Na^+ efflux pathway responsible for RVI are consistent with the interpretation that it is NHE1, yet the flounder NHE is exceptional in that it is insensitive to three commonly employed NHE1 inhibitors. Because of its unique inhibitor insensitivity, the flounder NHE may yield information regarding the nature of NHE inhibition. Work is currently in progress to obtain the complete molecular identity of the flounder RBC NHE. Supported by NIH grant (HL21179), the SCRF and a Schmidt-Nielsen fellowship to PMC.