Regulatory volume decrease by *Pseudopleuronectes americanus* red blood cells: the nature of the potassium flux pathway

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In response to osmotic swelling, most vertebrate cells regulate volume through a process known as regulatory volume decrease (RVD). In red blood cells (RBCs), including those of the Winter flounder (*Pseudopleuronectes americanus*), RVD is characterized by robust net efflux of K^+, Cl^-, and water [7-9]. K^+ loss during RVD is mediated by K-Cl cotransport (KCC) [13, 14], K^+/H^+ exchange [6] or K^+ conductance (channels) [2, 19], the most common of which is KCC. Furthermore, there are examples where more than one K^+ flux pathway is utilized during RVD [3, 11]. In a cell with a constitutive, robust anion exchanger (AE), all of the above pathways will functionally couple to the AE through changes in $[Cl^-]$ or pH (and therefore $[HCO_3^-]$), resulting in net KCl loss [5]. Based on tests of the relationship of K^+ and Cl^- transport to AE, as well as thermodynamic analyses of K^+ and Cl^- transport, our earlier work indicated that the major flux pathway during RVD by Winter flounder RBCs is KCC [17, 18]. This conclusion was challenged by our recent observation that swelling-induced net K^+ efflux proceeds undiminished in Cl^--depleted cells (5-15 mM), in low Cl^- (< 5 mM) media. The existence of a K^+ flux pathway that is not KCC is suggested by the observation that a substantial part of the K^+ flux during RVD is insensitive to 2 mM furosemide (KCC inhibitor). Our current work is an attempt to further test the hypothesis that RVD is mediated by KCC, in winter flounder RBCs, and to test for the existence of a second K^+ flux pathway during RVD. In addition, we have preliminary data partially characterizing the biochemical control of the major K^+ flux pathway in Winter flounder RBCs.

To identify the flux pathways responsible for flounder RBC volume regulation, we measured net pH, K^+ and Cl^- fluxes using a pH microelectrode, a K^+–electrode, a flame photometer, an ion chromatograph, and a chloridometer, respectively [4]. Data from electrodes were collected using a Power Lab (ADI Instruments, Colorado Springs, CO) data collection system and Chart 4.0.1 (ADI Instruments) analysis software. Net ion fluxes were expressed as millimoles of ion/Kg dry cell solid (dcs). Cell water content was determined gravimetrically on a 5-place analytical balance, and expressed as L H2O/Kg dcs. Flux measurements were performed on cells, in isotonic (360 mOsm), hypertonic (540 mOsm), or hypotonic (180 mOsm) HEPES-buffered Ringer's solution (pH 7.6), where osmolarities were adjusted (± 5 mOsm) by varying the NaCl concentration [7, 8]. The mean serum osmolarity from fish used in these studies was 347 mOsm (ranged 335 to 363, n = 29) measured on a freezing-point osmometer (model 3D3, Advanced Instruments Inc., Norwood, MA). 1 mM ouabain (Na^+/K^+-ATPase inhibitor) was present in all flux media. Intracellular anion replacement was performed by washing cells four times successively in Cl^--free Ringer's solution with either nitrate, sulfamate, or thiocyanate (SCN) as the substituted anion [18]. Where indicated, AE inhibition was achieved by 2 successive 20-minute pre-incubations of RBCs with 10 μM 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid (DIDS).

The coupling of RVD K^+ flux to Cl/HCO_3 exchange: The functional relationship of a K^+ flux pathway to AE can help to reveal the nature of the flux pathway. Briefly, functional coupling between AE and K^+/H^+ exchange will buffer changes in pH secondary to K^+/H^+ exchange, resulting in little or no apparent pH change in a poorly-buffered Ringer's solution (PBR) during RVD. If however, K^+/H^+ exchange is responsible for K^+ loss during RVD, inhibition of AE with DIDS in hypotonic PBR will augment intracellular acidification and extracellular alkalinization. In contrast, if during RVD, AE is

functionally coupled to either KCC or conductive K^+ and Cl^- flux, Cl^- recycling through AE should result in intracellular acidification and extracellular alkalinization, in a hypotonic PBR. In contrast to the situation where AE and K^+/H^+ exchange are coupled, if AE is coupled to either KCC or conductive K^+ and Cl^- flux, inhibition of AE with DIDS will permit net K^+ loss to occur independent of changes in pH.

RBCs, with or without inhibition of AE by DIDS, were suspended in isotonic, hypotonic or hypertonic PBR. Suspensions of RBCs, in PBR (0.5 mM HEPES) at 10% hematocrit, were monitored for DIDS- and volume-dependent changes in pH_o. Similar to what was reported previously, DIDS-pretreated cells in hypertonic PBR produced a decrease in pH_o of approximately 0.68 ± 0.03 pH units in 20 minutes (n = 2); whereas DIDS-free controls showed very little change in the same time period (Table 1). This decrease in pH_o in DIDS-treated cells is attributed to a cell shrinkage-activated Na^+/H^+ exchange (NHE) [16, 18]. The flounder NHE was subsequently identified and has structural and regulatory properties of both mammalian NHE1 and trout ßNHE [16]. In contrast, RBCs in isotonic or hypotonic PBR showed little or no change in pH_o over 20 minutes, with or without DIDS treatment (Table 1). The magnitude of net K^+ efflux during RVD in 20 minutes is similar to the net Na^+ influx seen via Na^+/H^+ exchange in osmotically shrunken cells, yet there is no change in pH_o during RVD. Thus, the K^+ flux pathway cannot be K^+/H^+ exchange. Also, as DIDS is a covalent inhibitor of HCO_3^--dependent transporters, the RVD K^+ flux pathway is not a HCO_3^--dependent K^+ transport. On the other hand, the data were consistent with a Cl^--dependent K^+ flux pathway (KCC or K^+ channels), in that no pH change occurred in the presence or absence of AE. The failure of the media to alkalinize in the presence of AE exchange could be explained by the fact that flounder RBCs are high Cl^- cells and that during RVD there was little or no change in the Cl^- chemical gradient with which to drive Cl/HCO_3 exchange.

Table 1. The unitary change in media pH over 20 minutes of RBCs at 10% hematocrit, in PBR of varied tonicity, with or without DIDS. Values are change in pH units ± SEM.

	Isotonic	Hypertonic	Hypotonic
No DIDS	-0.04 ± 0.01 (n = 4)	-0.12 ± 0.03 (n = 2)	-0.11 ± 0.04 (n = 3)
+ 10 μM DIDS	-0.11 ± 0.01 (n = 3)	-0.68 ± 0.03 (n = 2)	-0.09 ± 0.05 (n = 3)

Is swelling-induced K^+ flux conductive? In general, for robust net K^+ flux to proceed via a conductive pathway (K^+ channels), it must run in parallel with a conductive counter-ion flux of similar magnitude, *i.e.*, charge must be conserved. Assuming that net K^+ loss during RVD is conductive, the only counter-ion flux large enough to support net K^+ flux is that of Cl^-. Previously, using the K^+ ionophore valinomycin (100 nM), we demonstrated that the membrane Cl^- conductance (gCl) is sufficient to support conductive net KCl flux, in resting cells [17, 18]. To further test the notion that gCl is sufficient to support conductive K^+ loss during RVD, we treated RBCs with the Na^+ ionophore SQI-Pr (50 μM) in hypotonic Ringer's solution. A large net Na^+ influx was observed in cells treated with SQI-Pr (124.3 mmoles Na^+ / Kg dcs in 90 minutes, ranged 99.1 to 149.4, n = 2) relative to cells in the absence of SQI-Pr (22.4 mmoles Na^+ / Kg dcs in 90 minutes, ranged 20.3 to 24.5, n = 2). Meanwhile, net K^+ flux during RVD was unaffected by SQI-Pr. When intracellular Cl^- (Cl^-_i) and extracellular Cl^- (Cl^-_o) were replaced with sulfamate, SQI-Pr-mediated (conductive) net Na^+ flux was inhibited in cells in hypotonic media (53% over 90 minutes, n = 1). Similarly, K^+-selective microelectrode recordings in isotonic extracellular media showed that valinomycin-induced (conductive) net K^+ flux was inhibited when intracellular and media Cl^- were replaced with sulfamate (n = 1). Therefore the membrane conductance for sulfamate (gSFM) is less than that of gCl^-. That said, Cl^- replacement with sulfamate had no effect on net K^+ loss during RVD (n = 3). A similar result was obtained by Cl^- replacement with nitrate (n = 1). In contrast, when Cl^-_i and Cl^-_o were replaced with SCN^-, which should have a

higher membrane conductance than Cl^-, SQI-Pr-mediated Na^+ flux was dramatically increased (333 mmoles Na^+ / Kg dcs net influx in 90 minutes, n = 1), confirming the prediction that $gSCN^- > gCl^-$. Interestingly, Cl^- replacement with SCN^- resulted in complete inhibition of swelling-induced K^+ loss during RVD (n = 3). These data demonstrate that net K^+ flux during RVD is not dependent upon anion conductance (gCl^-) and therefore, is not via K^+ conductance. Based upon the anion replacement data, we conclude that K^+ loss by winter flounder RBCs during RVD is mediated by electroneutral K-Anion (K-Cl or K-sulfamate) cotransport (see Table 2).

Table 2. Summary of anions that are transported with K^+ by the RVD pathway in Winter flounder compared to KCC1 from mouse RBCs [1] or from the RBCs of all other mammalian species [13] (+ indicates a transported anion, - indicates a less well- / non-transported anion).

	Flounder RVD K^+ flux	Mouse KCC1 [1]	KCC1 (mammalian RBCs) [13]
Cl^-	+ (n = 3)	+	+
sulfamate	+ (n = 3)	-	-
SCN^-	- (n = 3)	+	-
NO_3^-	+ (n = 1)	+	-

Inhibition of RVD K^+ flux by furosemide or phosphatase inhibitors: 5 mM furosemide, a specific inhibitor of K-Cl cotransport [10, 13, 15], completely inhibited K^+ flux during RVD in Winter flounder RBCs (n = 2). In addition, in many other cells, KCC is inhibited due to net phosphorylation by the serine/threonine phosphatase inhibitors, calyculin A (CLA) and okadaic acid (OKA) [13]. RBCs were pre-incubated in 100 nM CLA or 100 nM OKA for 30 minutes, followed by suspension in hypotonic media. Swelling-induced net K^+ flux was inhibited by 64% in cells treated with calyculin A (n = 1), and by 30% in cells treated with okadaic acid (n = 1), at the 90-minute time point. These data are consistent with the interpretation that K^+ loss during RVD is mediated by KCC.

The effects of protein kinase inhibitors on activation of K^+ flux: In most vertebrate cells, KCC is activated due to net dephosphorylation, by kinase inhibitors, including staurosporine, ML-7, N-ethylmaleimide (NEM), or intracellular Mg^{++} depletion (EDTA + 10 μM A23187) [12, 13]. We treated flounder RBCs with these compounds and observed their effects on net K^+ efflux. Compared to untreated controls (30.6 mmoles / Kg dcs in 90 minutes, ranged 26.1 to 35.0, n = 2), net K^+ efflux was briskly activated by 5 μM staurosporine (149.2 ± 14.8 mmoles / Kg dcs in 90 minutes, n = 3) and to a lesser extent by 50 μM ML-7 (69.5 mmoles / Kg dcs in 90 minutes, ranged 68.2 to 70.8, n = 2). Next, RBCs were Mg^{++} depleted by addition of EDTA and A23187 in a Mg^{++}-free Ringer's solution. Net K^+ efflux strongly activated (135.9 mmoles / Kg dcs in 90 minutes, ranged 101.9 to 169.8, n = 2) in Mg^{++} depleted RBCs, and was attenuated by subsequent addition of 1 mM Mg^{++} to the Mg^{++}-free flux media. K^+ fluxes were also not seen in analogous experiments with Ca^{++}-depletion, in the presence of 1 mM Mg^{++}. Finally, RBCs were treated with the thiol-reactive agent, NEM (2 mM). Yet, counter to what is observed in other cell types, K^+ flux was not stimulated by NEM (n = 2; Table 3).

Table 3. Summary of treatments that activate KCC in flounder RBCs compared to KCC1 in the RBCs of several mammalian species [13] (* [12]) (+ indicates activation, - indicates inhibition/no effect).

	Flounder KCC	KCC1 (mammalian RBCs [13])
Cell Swelling	+ (n = 6)	+
Staurosporine	+ (n = 3)	+
ML-7	+ (n = 2)	+ (*)
Mg depletion	+ (n = 2)	+
NEM	- (n = 2)	+

Although these studies are preliminary, in that our conclusions are based on 1 to 3 experiments of each type discussed, our data nevertheless indicate that swelling-induced net K^+ efflux is mediated by K-Cl cotransport in Winter flounder RBCs. In addition, our data indicate that the flounder KCC has unique kinetic and pharmacological properties: reduced sensitivity to furosemide, insensitivity to NEM, and the capacity to transport alternative anions (sulfamate or NO_3^-) in place of Cl^-. The authors wish to thank John Payne, Jeff Williams and Stine Pedersen for helpful commentary, as well as Shannon Fyrberg, Kate Healy, Girmay Naizghi, Jenn Marchan and Jason Blackshear for their technical support. SF and KH were awarded High School Research Fellowships supported by the Betterment Fund. KH was a Hancock County Scholar. This work was supported by National Institutes of Health (NHLBI) grant #HL21179 (to PMC) and by an American Heart Association fellowship #AHA 0315035Y (to RRR).

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