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VOLUME-SENSITIVE TRANSPORT SYSTEMS AND VOLUME HOMEOSTASIS IN VERTEBRATE RED BLOOD CELLS

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Summary

Animal cells regulate their volume in the short term by controlling solute movements into and out of the cell. A suite of dissipative transport systems are involved which allow either regulatory volume increase (RVI) or decrease (RVD) responses depending upon the direction of the electrochemical gradients of the solutes. Many of these transporters have been identified at the molecular level and structure–function studies have identified transmembrane transport domains and cytoplasmic regulatory domains. In vertebrate red blood cells, protein phosphorylation appears to be central to the coordinated regulation of transporter activity. Inhibitors of protein phosphatases (PPs) cause inhibition of the K^+/Cl^- cotransporter (a transporter mediating RVD), whilst some inhibitors of protein kinases (PKs) cause activation. A sequence of potential phosphorylation sites appears to constitute a cascade of reactions leading to transporter regulation. PP and PK inhibitors have opposite effects on transporters mediating RVI responses, which is consistent with the coordinated but reciprocal regulation of transporters activated during both RVI and RVD using some common phosphorylation reactions. The transporters are sensitive to other stimuli including, in red blood cells, changes in P_{O_2} and pH. These responses are also sensitive to PK/PP inhibitors and may involve elements of the volume-sensitive transduction pathway.

Key words: protein phosphorylation, K^+/Cl^- cotransport, Na^+/H^+ antiport, volume regulation, oxygenation.

Introduction

Animal cells are surrounded by a thin and flexible plasma membrane which, lacking structural rigidity, is unable to withstand hydrostatic forces. As a result, cells exist in osmotic equilibrium with their solvent environments and behave as near-ideal volume osmometers. However, despite this osmometric behaviour, most cells exist at a characteristic and consistent cell volume despite variations in their osmotic environment and intracellular solute composition. Indeed, work over the past 20 years has established that animal cells possess a very precise and effective control over cell volume under both normal and osmotically disturbed conditions (Hallows and Knauf, 1994; Hoffmann, 1992).

This volume homeostasis, or cell volume regulation, is brought about by controlling the solute content of the cell over a period of a few minutes to a few hours. A regulatory volume decrease (RVD) of cells is brought about by a net loss of cell solute together with osmotically obliged water and *vice versa* for a regulatory volume increase (RVI). The responses may be observed after hypotonic swelling or hypertonic shrinkage of cells as well as following the iso-osmotic movement of solutes into or out of the cell. In some cell types, the regulatory responses may be very rapid (lymphocytes, approximately 2 min) whereas in other cell types they may be slower (trout red blood cells, approximately 40 min). RVD responses have been widely observed, but some cells appear to lack a conventional RVI response. The apparently ubiquitous occurrence of RVD may reflect the potentially catastrophic outcome of uncontrolled cell swelling.

With regard to the mechanisms of cell volume regulation, there are four important features. (1) Cell volume regulation of osmotically disturbed cells occurs in the presence of ouabain, which indicates that the Na^+/K^+ pump is not directly involved, apart from the maintenance of ion gradients. (2) Regulatory solute movements occur through strictly dissipative pathways whose transport activity is altered to achieve the appropriate volume (Sarkadi and Parker, 1991). Uncontrolled solute movements are insignificant in relation to those mediated by transport systems. (3) Solute transport pathways exist in two groups, those that promote solute uptake (the RVI effectors) and those that promote solute loss (RVD effectors). Because the cytoplasm of most animal cells has a high K^+ and a low Na^+ concentration and because volume regulation is dependent upon electrochemical concentration gradients across the plasma membrane, cell shrinkage can only be achieved by increasing membrane permeability to solutes with an outwardly directed electrochemical gradient such as K^+ and the

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organic osmolyte taurine. Regulatory cell swelling can be achieved by increased permeability to solutes with inwardly directed gradients such as Na^+. Thus, in normal low-[K^+] red blood cells, RVD effectors mediate permeability to K^+, Cl^- and amino acids, whereas RVI effectors mediate permeability to Na^+. (4) Because substantial net fluxes of electrolytes are activated during volume regulation, it appears that they are mediated by pathways that are electroneutral. These pathways may be cation/anion cotransporters, exchangers or coupled channels or a mixture of the three. Different cell types appear to utilise different transport pathways to achieve the same ends. Moreover, homologous cell types in different vertebrate phyla may also utilise different transport pathways.

These basic principles have been demonstrated in a wide variety of animal cells, in both suspended cells and in solid tissues, and in many different vertebrate classes and invertebrate phyla. Vertebrate red blood cells have been particularly important in characterising these critical homeostatic responses because they are simple, easy to procure and cell suspensions are relatively homogeneous. However, despite their simplicity, a surprising variety of transport pathways are utilised to control cell volume and these pathways are modulated by other physiological stimuli, such as stress hormones and P_{O_2}.

Current research is directed at three specific goals. First, what are the transport pathways which mediate RVD and RVI and how does their molecular structure relate to their function? Second, what are the functional and evolutionary relationships between RVI and RVD pathways? Third, how are the activities of these transporters regulated in response to altered cell volume? A fourth and critical problem, namely the identity of the volume sensor, remains enigmatic and will not be specifically addressed here.

Identifying the volume regulatory effectors

The transport pathways involved in volume regulation have been characterised by net and unidirectional isotopic flux measurements, by the use of transport inhibitors and salines containing different principal solutes. In trout red blood cells, RVD occurs with the progressive net loss of cellular K^+, Cl^- and amino acids until the reduced cell volume has been attained and net fluxes cease (Garcia-Romeu *et al.* 1991). Part of the swelling-activated K^+ flux was abolished when chloride was replaced by nitrate as principal anion in the saline and a Cl^- concentration gradient can move K^+ against a K^+ concentration gradient, all of which is consistent with a cotransport of K^+ and Cl^- (Brugnara *et al.* 1989). This K^+/Cl cotransporter (KCC) has been identified on the basis of its Cl^--dependence in the red blood cells of many vertebrate species (Table 1), where its activity generally increases in hypotonically swollen cells and decreases in hypertonically shrunken cells. Another component of the swelling-induced K^+ flux in trout red blood cells is observed in a nitrate saline, when the KCC is inactive. This Cl^--independent K^+ pathway (KX) has not been firmly characterised but shows similar pharmacological properties to an organic osmolyte pathway, which carries taurine (Lewis *et al.* 1996). Both taurine- and Cl^--independent K^+ fluxes are increased in hypotonically swollen cells (Bursell and Kirk, 1996; Garcia-Romeu *et al.* 1991) and may be mediated by the same transporter.

In some species, a component of K^+ influx is abolished when saline Na^+ is replaced by an organic cation or when Cl^- is replaced by nitrate. Similarly, Na^+ influx is abolished by removal of saline K^+ or by Cl^- replacment. This is consistent with a Na^+, K^+ and Cl^- cotransport (NKCC) (Haas, 1994), which has been identified in red blood cells of avian species and ferret (Table 1) as well as in Ehrlich ascites cells and epithelial tissues (O'Grady *et al.* 1987). It is strongly activated by hypertonic swelling and inactivated by hypotonic shrinkage, and net flux is thermodynamically determined by the combined concentration gradients for Cl^-, K^+ and Na^+. Net flux measurements have shown a transport Na^+:K^+:Cl^- stoichiometry of 1:1:2 in red blood cells of avian species Haas (1994) and of 2:1:3 in ferret red cells (Hall and Ellory, 1985). In avian species, the red cell NKCC is activated by cyclic AMP and by adrenergic stimulation, though the function of this response is unknown (Palfrey *et al.* 1980).

Finally, in some cell types, the swelling-induced influx of Na^+ is associated with an intracellular alkalisation or extracellular acidification with a Na^+:H^+ stoichiometry of 1:1 (the Na^+/H^+ exchanger, NHE) (Demaurex and Grinstein, 1994). The net H^+ flux induces changes in HCO_3^- concentration, a consequent net exchange of HCO_3^- and Cl^- *via* the anion exhange protein resulting in the net uptake of NaCl with osmotically obliged water. The NHE is powerfully expressed in fish red blood cells (Table 1). In some species, including the Salmonidae, the NHE is activated adrenergically (Fievet and Motais, 1991) whereas in others, including eels, it is shrinkage-activated (Gallardo Romero *et al.* 1996).

The principal transport pathways occurring in vertebrate red blood cells and their respective roles in RVD and RVI are depicted in Fig. 1. Table 1 summarises the known phylogenetic distribution within the vertebrates of the different red cell transporters together with their swelling- and shrinkage-induced sensitivity. Swelling-induced KCCs are widely distributed, whilst the taurine flux pathway and the KX system have been observed widely in lower vertebrates. The RVI effectors are more diverse in that fish and Amphibia express NHE whilst avian species express a NKCC.

Of these transport systems, only the KX system has not been fully characterised at the molecular level. NHE was originally cloned from human lymphocytes by Pouyssegur and colleagues (Sardet *et al.* 1989). This isoform is sensitive to shrinkage, intracellular acidification and growth factors; some progress has recently been made in defining the structural features of a regulatory domain which endow sensitivity to these stimuli (Bianchini *et al.* 1995; Grinstein *et al.* 1994). The trout β-adrenergically activated NHE has also been sequenced (Borgese *et al.* 1992), although this isoform is not particularly volume-sensitive (Gallardo Romero *et al.* 1996). In both cases, the cytoplasmic domain of these NHE isoforms appears to be the main determinant of stimulus-specificity, and chimaeric proteins

Table 1. *Summary of regulatory volume effector transport pathways of vertebrate red cells*

	Regulatory volume decrease					Regulatory volume increase	
	KCC	KX	Tau	Ca^{2+}/Na^{+}	K^{+}/H^{+}	NKCC	NHE
Primates							
Human	?SW	(LIS)	(LIS)			SH?	(Yes)
Carnivores							
Dog	SW			SW			SH
Ferret	SW			SW		Yes	
Bear	SW			SW		No	SH
Rodents							
Rabbit	SW	No					SH
Rat	No RVD?					SH	SH
Mouse	SW						
Guinea pig	SW						SH
Herbivores							
LK sheep	SW	No	(LIS)			No	?No
Horse	SW	No	(SW/LIS)			No	No
Goat	SW						
Cattle	SW		(LIS)				
Avians							
Duck	SW					SH and cAMP	
Turkey	(Yes)	No				SH and cAMP	
Chicken			SW				
Pigeon			SW			cAMP	
Amphibia							
Amphiuma	No				SW	No	SH
Frog	SW					cAMP	SH
Toad			SW			No adrenergic swelling	
Teleosts							
Trout	SW	SW	SW		(Yes)		cAMP
Carp	SW		SW		?		cAMP
Eel	SW	SW	SW				SH
Flounder		SW	SW				
Elasmobranchs							
Skate			SW				
Agnathans							
Lamprey	(NEM)	Yes	No				SH
Hagfish	No RVD response					(Na^{+})*	

KCC, Cl^{-}-dependent K^{+} pathway; KX, Cl^{-}-independent K^{+} pathway; Tau, taurine (or 'osmolyte') pathway; Ca^{2+}/Na^{+}, Ca^{2+}/Na^{+} exchange; NKCC, $Na^{+}/K^{+}/Cl^{-}$ cotransport; NHE, Na^{+}/H^{+} exchange; SW/SH, swelling/shrinkage activated; cAMP, activated by cyclicAMP; LIS, activated by low ionic strength; (), minor pathway; Yes, present but not an RVE; No, probably absent; ?, role or presence/absence uncertain; (NEM), activated by *N*-ethylmaleimide; (Na^{+})*, Na^{+}-dependent but not NKCC; LK sheep, low $[K^{+}]$-containing sheep red cells.

Human: Ellory *et al*. (1982, 1985); Escobales and Canessa (1986); Hall and Ellory (1986); Kaji (1986). Dog: Parker (1983, 1994). Ferret: Ellory *et al.* (1985); Flatman (1983); Hall and Ellory (1995); Milanick (1989, 1992). Bear: Willis *et al.* (1990*a*,*b*). Rabbit: Al-Rohil and Jennings (1989); Brugnara and de Franceschi (1993); Jennings *et al.* (1986). Rat: Duhm and Göbel (1984); Orlov *et al.* (1989); O'Neill (1989). Mouse: Armsby *et al.* (1995); Delpire and Gullans (1994). LK sheep: Dunham and Ellory (1981); Lauf (1981); Lauf *et al.* (1992). Horse: Gibson *et al.* (1993, 1995); Honess *et al.* (1996). Goat and cattle: Ellory *et al.* (1985). Duck: Kregenow (1981); Starke and McManus (1989). Turkey: Palfrey and Greengard (1981); Palfrey and Rao (1983); Pewitt *et al.* (1990). Chicken: Porter and Martin (1992). Pigeon: Shihabi *et al.* (1989). *Amphiuma*: Cala (1980, 1985); Cala and Maldonado (1994). Frog: Gusev *et al.* (1995). Toad: Katz *et al.* (1993); Tufts *et al.* (1987). Trout: Borgese *et al.* (1991); Fievet *et al.* (1993); Garcia-Romeu *et al.* (1991); Guizouarn *et al.* (1993); Motais *et al.* (1992); Nielsen *et al.* (1992). Carp: Jensen (1995); Nikinmaa and Jensen (1992). Eel: Bursell and Kirk (1996); Kirk *et al.* (1992); Lewis *et al.* (1996); Gallardo Romero *et al.* (1996). Flounder: Fincham *et al.* (1987); Fugelli and Thoroed (1986); Thoroed and Fugelli (1994); Thoroed *et al.* (1995). Skate: Goldstein and Brill (1990); Goldstein and Davis (1994); Musch *et al.* (1994). Lamprey: Gusev *et al.* (1992*a*,*b*); Kirk (1991*a*,*b*); Virkki and Nikinmaa (1995). Hagfish: Nikinmaa *et al.* (1993).

of human/trout NHE show sensitivity according to the origin of the cytoplasmic domain (Borgese *et al.* 1994). In the lymphocyte NHE, the putative volume-sensitive site appears to be different from the site(s) postulated to mediate the stimulatory effects of Ca^{2+} and growth factors (Bianchini *et al.* 1995), suggesting that a single transporter can mediate responses to different stimuli.

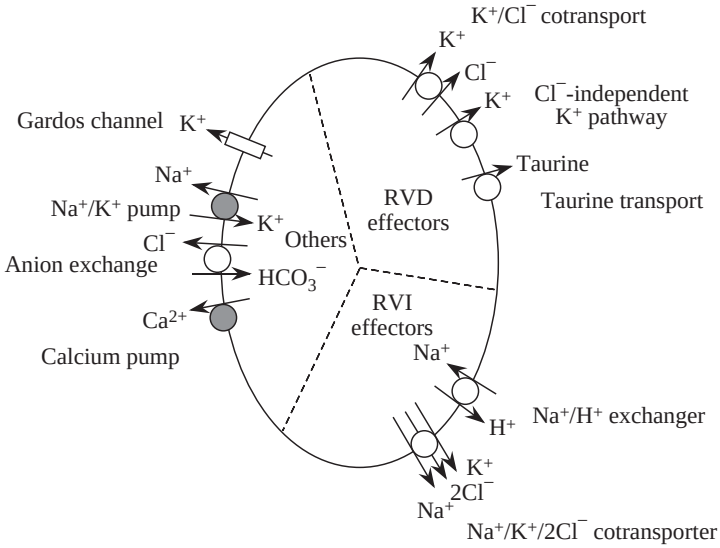

Fig. 1. Schematic diagram illustrating the principal transport pathways across the vertebrate red cell membrane. The arrows indicate the relative net fluxes of solutes given the usual solute distribution of a typical high-[K^+] red cell. RVD, regulatory volume decrease; RVI, regulatory volume increase.

The NKCC has been identified in shark rectal gland by an immunoscreening approach (Xu *et al.* 1994) and in flounder urinary bladder by expression cloning in *Xenopus laevis* (Gamba *et al.* 1994). Other NKCCs have been identified in other tissues and species (Payne and Forbush III, 1994); many more members of this potentially large family of transporters remain to be identified.

The KCC shares some functional characteristics with the NKCC, and the two proteins may be structurally related. Indeed, the KCC from rabbit kidney, rat brain and from human has been cloned by taking advantage of sequence homology with the NKCC (Gillen *et al.* 1996); the KCC1 isoform encodes a 1085-residue polypeptide with 24 % sequence identity with NKCC and a Na^+/Cl^- cotransporter. Secondary structure analyses for both the KCC and the NKCC predict 12 transmembrane domains and a large extracellular loop with potential sites for post-translational modification. Structure–function studies will surely follow to identify the sites involved in cotransporter regulation.

Differential activation of RVD and RVI effectors

Red blood cells placed and equilibrated in an iso-osmotic saline tend to have deactivated transporters. Hypotonic swelling of these cells causes the activation of K^+ flux pathways such as the KCC and the KX systems, whereas hypertonic shrinkage causes the activation of Na^+ flux pathways such as the NHE and the NKCC. Activation of the former group in the usual high-[K^+], low-[Na^+] red blood cells allows the dissipative loss of intracellular K^+ and Cl^- whereas activation of the latter allows the dissipative net gain of extracellular Na^+ and Cl^-.

These net solute movements in high-[K^+] cells are accompanied by osmotically obliged movements of water into or out of the cell. As cell volume is restored, the flux pathways are progressively reduced until they are once again quiescent. Thus, these solute flux pathways show transitions between active and inactive states. Activation of K^+ and Cl^- transport in low-[K^+] red blood cells of some mammals has no effect on cell volume because the concentration gradients for these solutes are abolished. At present, it is unknown whether graded changes observed in unidirectional solute fluxes are due to changes in the transport activity of individual transporters or to changes in the proportion of transporters that have become activated. Jennings proposed a two-step model for transitions between an active and an inactive state, and Dunham has recently proposed a more complex model for sheep red blood cells with a low-activity state as well as the fully deactivated and activated states (Dunham *et al.* 1993). Transitions between slow and intermediate transport rates involve a change in V_{max}, whilst changes from intermediate to high transport rates involve changes in affinity of the transporter for K^+.

A common feature of physiological regulation is the involvement of antagonistically poised effector systems whose activities are coordinated to ensure that only one system can be active at any one time. The distinctive behaviour of the RVI and RVD systems is consistent with this pattern, and we might therefore expect that they are ultimately controlled by a unified mechanism. This concept has been termed ‘reciprocal coordination’ and is evident not only in the differential sensitivity of the two types of effector to cell volume but also, in the red blood cells of some species, in a differential sensitivity to changes in P_{O_2} and to pharmacological manipulation. One good example is the contrasting effects of changes in P_{O_2} upon RVI and RVD systems. The KCC of trout and equine red blood cells is stimulated on transfer of cells from low to high P_{O_2} (Borgese *et al.* 1991; Honess *et al.* 1996; Nielsen *et al.* 1992), whilst transfer of turkey red blood cells from high to low P_{O_2} causes activation of the NKCC. The opposing effects of pharmacological manipulation will be described in subsequent sections.

Control of RVD/RVI effectors by protein phosphorylation

Recently, inhibitors of protein serine/threonine phosphatases

(PPs) have been used to uncover cellular control mechanisms controlling the activity of regulatory volume effectors (RVE). Okadaic acid and calyculin A inhibit the enzymatic activity of purified PPs at nanomolar concentrations and are highly specific in their effects. They also cause inhibition of KCC in rabbit, human and trout red blood cells at the same very low concentrations (Cossins *et al.* 1994; Jennings and Schulz, 1991; Kaji and Tsukitani, 1991). The interpretation of this observation is rather complicated since it depends on these phosphatases acting to dephosphorylate specific phosphorylamino acid residues in proteins which are in the first place phosphorylated by protein kinases (PKs). The kinase and phosphatase therefore catalyse opposing reactions at a specific residue on an as yet unidentified protein involved in the control of cotransporter activity, the degree of phosphorylation reflecting the relative activities of these enzymes. Inhibition by calyculin suggests that the continuing and now unopposed activity of the kinase leads to an inactive cotransporter. By inference, the phosphatase promotes the opposite effect, namely cotransport activation by the dephosphorylation of the critical protein. Thus, transitions between the active and inactive states of the cotransporter involve changes in the relative activity of the enzymes controlling phosphorylation at a specific residue. At this stage, the identity of the phosphorylated protein is unknown, and it is unclear whether one or more proteins or one or more sites on a single protein are involved.

The suggestion of an opposing kinase has led to a search for an inhibitor of protein kinase activity which might also alter cotransport activity. Bize and Dunham (1994) and Cossins *et al.* (1994) have shown in human and trout red blood cells, respectively, that staurosporine causes activation of the KCC. Staurosporine inhibits protein kinase activity with relatively low specificity at micromolar concentrations. However, the KCC activity that was activated by staurosporine was fully inhibited by calyculin, which suggests that the putative kinase activity that caused deactivation of KCC in the presence of calyculin was not the same kinase that was inhibited by staurosporine. The inference was that staurosporine inhibits a kinase that lies upstream to the step catalysed by the calyculin-sensitive phosphatase and that there is more than one phosphorylation step.

The alkylating reagent *N*-ethylmaleimide (NEM) has long been known to cause strong activation of KCC activity in mammalian red blood cells. Kaji and Tsukitani (1991), Jennings and Schulz (1991) and Starke and Jennings (1993) showed that application of a phosphatase inhibitor prior to NEM prevented activation of KCC whereas addition of calyculin after NEM-activation had no effect, which suggested that calyculin and NEM affected the phosphorylation state of the regulatory protein. It was subsequently shown in trout red blood cells that NEM had a slow activating effect and that application of calyculin to partially activated cells caused neither the inactivation of the KCC nor a continuing activation (Cossins *et al.* 1994). Instead, the flux remained constant at the level occurring at the time of calyculin addition. Because the flux was not deactivated by the addition of calyculin, it was suggested that the deactivating kinase was not active and that NEM had caused its inhibition. Thus, the activation caused by NEM alone was due to blockage of the deactivating kinase, allowing a slow, activating phosphatase to continue until all target residues were dephosphorylated and flux was maximal. The apparent blockage of both kinase and phosphatase had prevented transitions between inactive and active cotransporter, achieving a 'clamping' effect, the 'NEM–Cal clamp'. A schematic diagram incorporating these various ideas is presented in Fig. 2. Although this clamp of trout red blood cells dissociates the KCC from control by other stimuli, such as oxygenation, KCC activity is still sensitive to volume perturbation (Cossins *et al.* 1994). This observation suggests that cell volume influences KCC activity by a route that circumvents the clamped phosphorylation site.

More recent studies using inhibitors of tyrosine-directed protein kinase have provided evidence that tyrosine phosphorylation is also involved in controlling the KCC activity. Genistein caused the blockage of KCC in both trout red blood cells (Weaver and Cossins, 1996) and sheep red blood cells (Flatman *et al.* 1995). In trout cells, the tyrosine phosphatase inhibitor vanadate caused a slow and progressive activation of KCC activity. Calyculin A caused blockage of this activity, indicating that the vanadate-sensitive site lay upstream from the calyculin-sensitive site on the transduction pathway controlling the KCC. Genistein also caused blockage of this flux but had no effect upon the activation by either

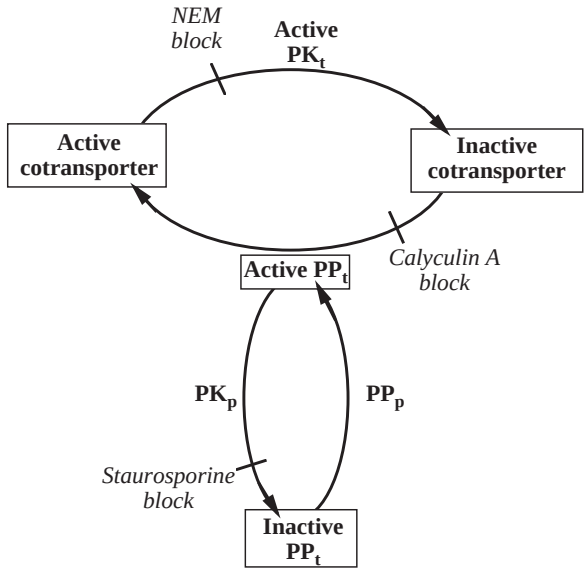

Fig. 2. Schematic diagram of the proposed phosphorylation of an, as yet, unidentified protein which influences the activity of the K^+/Cl^- cotransporter in trout red blood cells. Phosphorylation at this site is controlled by a protein kinase (PK_t) and phosphatase (PP_t). The activity of the PP_t is also controlled by phosphorylation following changes in the relative activity of a second pair of enzymes (PK_p and PP_p). The inhibitors staurosporine, *N*-ethylmaleimide (NEM) and calyculin A are thought to inhibit the indicated steps in this two-step sequence.

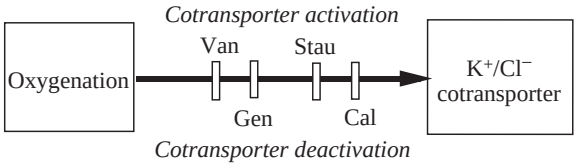

Fig. 3. Schematic diagram of the putative transduction pathway linking the activity of the K^+/Cl^- cotransporter of the trout red cell with oxygenation. The small boxes represent the ordered inhibition of specific steps on the pathway by the indicated reagent. Van, vanadate; Gen, genistein; Stau, staurosporine; Cal, calyculin.

staurosporine or NEM. This genistein-sensitive step lay between the vanadate- and the staurosporine-sensitive steps in what could be a long, multi-step pathway such as that schematically depicted in Fig. 3. In sheep red blood cells, genistein caused blockage of the NEM-activated flux, suggesting that the precise order of inhibitory influences differs among species.

A major problem in studies using inhibitors is their inhibitory specificity for the presumed site of action. Genistein is known to cause inhibition of cellular processes which do not appear to involve tyrosyl phosphorylation (Young *et al.* 1993) and is therefore non-specific. Moreover, inhibition of red cell KCC activity required concentrations well in excess of that required to inhibit purified protein kinases. This disparity might be due to two effects; first, there may be diffusional limitations in gaining access to the intracellular kinase and, second, genistein binds to the ATP-binding site of the kinase and the high intracellular ATP concentrations would compete in binding to the kinase. Thus, some caution should be exercised in attributing the effects of genistein unequivocally to kinase inhibition, and firm evidence will only come from studies of phosphorylation at the protein level. It is worth noting that calyculin does cause KCC inhibition at a very low concentration corresponding to that inhibiting the purified phosphatase. Therefore, its effects in red blood cells can be reasonably attributed to phosphatase inhibition.

Reciprocal control

Coordinating the RVI and RVD effectors requires interaction between the mechanisms controlling their respective activities. Firm evidence for this proposition has come from the opposing effects of PK/PP inhibitors on the RVI and RVD effectors (Cossins, 1991). Whereas the KCC of mammalian and fish red blood cells was inhibited by calyculin, the NKCC in avian red blood cells was strongly activated (Pewitt *et al.* 1990). Calyculin also stimulates the NHE of flounder red blood cells (Y. Weaver, unpublished observations) and NEM causes inhibition. More significantly, we have recently observed a 'clamping' effect of NEM upon the calyculin-activated Na^+ flux in flounder NHE (Y. Weaver and A. R. Cossins, unpublished observations) and the K^+ flux in turkey NKCC; thus, addition of NEM to cells during calyculin activation freezes the activity of the calyculin-activated transporter at a sub-maximal value (Fig. 4), the 'Cal–NEM clamp'.

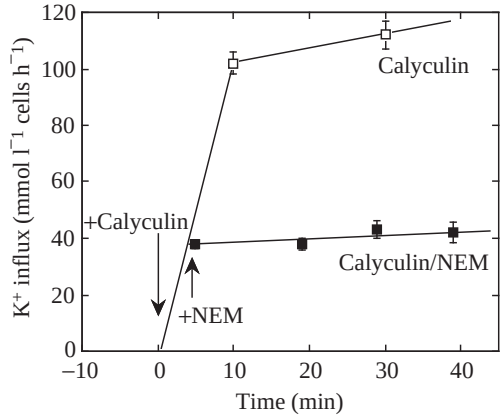

Fig. 4. Evidence for the proposed calyculin–NEM clamp of $Na^+/K^+/2Cl^-$ cotransport in turkey red blood cells. Cells were held under conditions which led to deactivation. Addition of calyculin A (10^{-7} mol l^{-1}) led to increased K^+ influx and the subsequent addition of *N*-ethylmaleimide (NEM, 10^{-3} mol l^{-1}) during activation led to a clamping of activity. Cotransport was neither inactivated nor activated any further (J. S. Gibson, M. Craske and A. R. Cossins, unpublished observation). Values are mean ± S.D. for triplicate measurements on one red cell preparation (for experimental protocol see Cossins *et al.* 1994).

As with the experiments defining the 'NEM–Cal clamp' of the KCC, this 'Cal–NEM clamp' suggests that NEM and calyculin react with enzymes which mediate phosphorylation and dephosphorylation at a single site. However, the opposite orientation of this clamp to that of the NEM–Cal clamp for the KCC suggests that the phosphorylation site in the two systems is the same. This site and the associated PK/PPs appear to be common to the two transduction pathways, the system acting as a switch controlling which of the two effector systems is active under any particular conditions. The PK causes inactivation of an already active KCC but activation of a quiescent NKCC and NHE. Conversely, the PP causes activation of the quiescent KCC and inactivation of the active NKCC and NHE. This mechanism accounts for the activation of one system *or* the other and accounts for the inability to coactivate both systems. However, it does not account for the fact that in volume-static, unstimulated cells the RVD and RVI effectors are both inactive.

In turkey red blood cells, we have found that the NKCC that has been activated by shrinkage is inhibited by the PK inhibitor staurosporine, whereas the calyculin-activated activity is insensitive to staurosporine (N. A. Honess, J. S. Gibson and A. R. Cossins, unpublished observations). These observations suggest that the calyculin-sensitive site, which causes activation, lies downstream of the staurosporine-sensitive step, which causes inhibition. These observations thus agree with the proposed upstream position of staurosporine-sensitive PK in the transduction pathway controlling the KCC in both sheep and trout red blood cells (Bize and Dunham, 1994; Cossins *et*

al. 1994) and are consistent with a conserved order of transduction elements in different vertebrate taxa.

Oxygenation effects on RVEs and the homeostasis of plasma [K^+]

The principal physiological function of the vertebrate red cell is the carriage of oxygen and CO_2, and many molecular and cellular adaptations for optimised function have been described. To this list must be added a new class of cellular responses in which P_{O_2} affects the activity of RVEs and thereby the volume of red blood cells. Nielsen and Lykkeboe (1992) showed that extended incubation of trout red blood cells in a low-P_{O_2} atmosphere led to a progressive reduction of plasma K^+ concentration from 4 to 0.25 mmol l^{-1} over 30–60 min. Restoration of a high P_{O_2} led to a more rapid increase in plasma [K^+] to normal levels. Subsequent work has demonstrated that these changes to extracellular [K^+] are mediated by the KCC, which shows an exquisite sensitivity to P_{O_2} and thereby changes the balance between net K^+ loss *via* the KCC and net K^+ gain by the Na^+/K^+ pump (Borgese *et al.* 1991; Nielsen *et al.* 1992). Cells held at low P_{O_2} have a deactivated KCC. As a result, they accumulate net K^+ *via* the continuing Na^+/K^+ pump together with Cl^- and osmotically obliged water and hence become swollen by approximately 10–15 %. This swelling is approximately threefold greater than that caused by the usual deoxygenation-induced entry of net Cl^- *via* the Cl^-/HCO_3^- exchanger.

Exactly how changes in P_{O_2} alter KCC activity is not well understood. Carbon monoxide also leads to activation of the KCC, which strongly suggests that a protein containing a haem group is involved, the most likely candidate being haemoglobin. Indeed, haemoglobin saturations above 60–70 % are associated with KCC activation, whereas reductions below this threshold cause inactivation. Because calyculin and genistein both reverse oxygenation-induced KCC activation, it is possible that the response is mediated through an extended chain of phosphorylation reactions, as already described.

Equine red blood cells also display an oxygenation-dependent activation of the KCC. The response is also inhibited by calyculin and has a P_{O_2}-sensitivity matching that observed in trout red blood cells (Honess *et al.* 1996). This similarity between these two such disparate species suggests that the phenomenon may be phylogenetically widespread within the vertebrates and may have some broad physiological significance. Indeed, P_{O_2}-sensitive NHEs and NKCCs have been previously observed, although in these cases high P_{O_2} has the opposite effect in reducing (trout NHE) or abolishing activity (carp NHE and turkey NKCC; N. A. Honess and A. R. Cossins, unpublished observations). Thus, oxygenation-linked effects on RVEs are widespread both between different types of transporter and phylogenetically. With respect to the KCC, both trout and horses display high levels of locomotory performance and experience either respiratory hypoxia (as in trout) or exercise-induced tissue hypoxia (as in both horses and trout) in combination with a profound exercise-induced plasma acidosis. The inactivation of KCC at low P_{O_2} may have significant physiological implications, especially during exercise. First, the change in cell volume may affect the rheological properties of the blood, although it is difficult to understand how microcapillary perfusion can be improved by an increase in cell size. Alternatively, the response may lead to the control of plasma K^+ levels in times of great locomotory exertion or hypoxic stress, as observed by Nielsen in tonometered trout blood. Thus, reduction of P_{O_2} below a critical threshold leads to a net sequestration of plasma K^+ by the red cell compartment (Nielsen and Lykkeboe, 1992).

In horse red blood cells, the rate of active K^+ uptake is relatively low. However, KCl loss through the KCC is rapid and this, together with a remarkable increase in haematocrit during exercise, allows the cellular compartment to exert a significant effect upon plasma K^+ levels. Assuming that transitions between active and inactive transporters are rapid, the P_{O_2}-dependence in the systemic blood supply is such that cells within arterial blood should have oxygenation-activated cotransporters, whereas those in the venous blood during exercise should have deactivated cotransporters. Strenuous exercise will affect this situation through a profound plasma acidosis and reductions in arterial and venous P_{O_2}, all of which markedly affect the KCC. Strenuous exercise in horses is also accompanied by a marked hyperkalaemia (Harris and Snow, 1988), the magnitude of which may be influenced by the red cell compartment. Under conditions where the cotransporter is active (i.e. at higher P_{O_2} values), acidosis would produce an increase in net dissipative KCl efflux, with cell shrinkage and an elevation of plasma [K^+]. This situation is reminiscent of sickle cell disease in humans, where an active KCC, coupled with low plasma pH, is thought to contribute to red cell dehydration and consequent rheological effects (Stuart and Ellory, 1988). Conversely, should P_{O_2} be low, inactivation of the KCC, despite the reduction in pH, would result in net active K^+ uptake.

This oxygenation-sensitivity, together with the well-known function of NHEs in controlling intracellular pH, illustrates the way in which RVEs have physiological roles in addition to the regulation of cell volume.

Conclusion

The general problem of how cells show homeostasis of cell volume in the face of aniso-osmotic and iso-osmotic disturbance has been reduced to understanding how a suite of transport systems is controlled. The molecular structure of a least some of these transport systems has been defined, and potential sites for post-translational control have been identified. The processes controlling transporter activity appear to be complex since they endow sensitivity to physiological factors other than cell size (i.e. acidifcation, P_{O_2}, hormones). It is likely that there are a number of steps in a potentially long transduction sequence linking the sensor with the transport system itself. These steps have been pharmacologically mapped using PK or PP inhibitors, and there is some conservation of this sequence among species

and among vertebrate classes. However, the sensor has not been identified in any specific manner and, indeed, there is as yet no consensus on its likely identity.

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