Swelling activation of transport pathways in erythrocytes: effects of Cl\textsuperscript{−}, ionic strength, and volume changes

HELÈNE GUIZOUARN AND RENÉ MOTAIS
Laboratoire J. Maetz, Département de Biologie Cellulaire et Moléculaire, Commissariat à l'Energie Atomique, and Unité de Recherche Associeè 1855, Centre National de la Recherche Scientifique, 06238 Villefranche-sur-Mer Cedex, France

Guizouarn, Hélène, and René Motais. Swelling activation of transport pathways in erythrocytes: effects of Cl\textsuperscript{−}, ionic strength, and volume changes. Am. J. Physiol. 276 (Cell Physiol. 45): C210–C220, 1999.—If swelling of a cell is induced by a decrease in external medium tonicity, the regulatory response is more complex than if swelling of similar magnitude is due to salt uptake. The present results provide an explanation. In fish erythrocytes, two distinct transport pathways were swelling activated: a channel of broad specificity and a K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter. Each was activated by a specific signal: the channel by a decrease in intracellular ionic strength and the K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter by cell enlargement. A decrease in ionic strength also affected K\textsuperscript{+}-Cl\textsuperscript{−} cotransport activity, but by acting as a negative modulator of the cotransport. Thus cells swollen by salt accumulation respond by activating exclusively the K\textsuperscript{+}-Cl\textsuperscript{−} cotransport, leading to a Cl\textsuperscript{−}-dependent K\textsuperscript{+} loss. By contrast, cells swollen by electrolyte dilution respond by activating both pathways, leading to a reduced loss of electrolytes and a large loss of taurine. Thus two swelling-sensitive pathways, differently regulated, would allow control of the ionic composition of a cell exposed to different volume perturbations.

volume regulation; taurine; volume-activated transports; anion channel; potassium-chloride cotransport

Most cells respond to swelling by activating membrane transport systems that mediate the net loss of osmotically active, cytoplasmic solutes, thereby allowing the cell to undergo a regulatory volume decrease (RVD). Inorganic ions (mainly K\textsuperscript{+} and Cl\textsuperscript{−}) and small organic solutes (amino acids, polyols, and methylamines) are used by cells for such volume control. There is now substantial evidence from a number of mammalian cell types that the volume-regulated efflux of organic solutes may be the taurine, sorbitol, and myo-inositol occurs via a swelling-activated anion channel (2, 18, 44, 45), the volume-sensitive organic osmolyte anion channel, which has not been characterized at the molecular level. For inorganic ions, a number of different swelling-activated K\textsuperscript{+} and Cl\textsuperscript{−} transport mechanisms have been described: a K\textsuperscript{+}-Cl\textsuperscript{−} cotransporter, separate K\textsuperscript{+} and Cl\textsuperscript{−} channels, and a K\textsuperscript{+}/H\textsuperscript{+} antiporter (for review see Refs. 24, 34, and 42).

Fish red blood cells have proved to be a useful model system for the study of aspects of cell volume regulation. Table 1 shows that Na\textsuperscript{+}, K\textsuperscript{+}, Cl\textsuperscript{−}, and taurine account for up to 90% of trout erythrocyte osmolality, with the remaining osmolality due to the presence of greatly or totally impermeant solutes such as ATP, ADP, PO\textsubscript{4}\textsuperscript{3−}, and proteins (mainly Hb). Because cell volume is regulated by the net loss of osmotically active solutes, RVD of trout erythrocytes can only be achieved by the loss of K\textsuperscript{+}, Cl\textsuperscript{−}, and taurine down their electrochemical gradients. However, we previously showed (37) that, depending on how its volume has been altered, the erythrocyte will or will not involve taurine in its volume correction and will choose between different transport systems to jettison K\textsuperscript{+} and Cl\textsuperscript{−}. As illustrated in Fig. 1, when the volume increase is due to a net uptake of salts and obliged water, i.e., resulting in an increase in cell electrolyte concentration, the regulatory response involves only a KCl loss mediated by a strictly Cl\textsuperscript{−}-dependent pathway that displays the characteristics of a K\textsuperscript{+}-Cl\textsuperscript{−} cotransport (4, 37). Such an “isosmotic” swelling can be induced by hormonal stimulation of an Na\textsuperscript{+}/H\textsuperscript{+} exchange or by exposure of the erythrocytes to a solution containing NH\textsubscript{4}Cl. Conversely, when a volume increase of the same magnitude occurs as a result of water entering the cell along its chemical gradient (“hyposmotic” swelling) or dragged by an uncharged solute (e.g., urea), leading to a dilution of cell electrolytes, RVD occurs by a loss of taurine, which accounts for as much as 50% of the total RVD, and by a loss of KCl. The loss of KCl is then mediated by two different systems: a “Cl\textsuperscript{−}-dependent” component and a “Cl\textsuperscript{−}-independent” component, the relative magnitude of the two components varying widely between experiments (20). Furthermore, hyposmotic swelling also activates pathways permeable to diverse cations such as Na\textsuperscript{+}, choline, or tetramethylammonium (TMA) (20, 37). However, although activation of the taurine, K\textsuperscript{+}, and Cl\textsuperscript{−} transport systems will tend to correct volume changes, the simultaneous activations of other pathways may partly counteract RVD [net entry of Na\textsuperscript{+} down its electrochemical gradient (20)] or appear physiologically irrelevant (transport of choline or TMA). In several other fish species, a similar hyposmotic regulatory pattern has been described involving K\textsuperscript{+} (6, 9, 29, 32) and diverse organic solutes (15, 17, 21, 23, 30, 31, 46).

Thus trout erythrocytes possess multiple swelling-sensitive transport pathways. The fact that they adopt different regulatory patterns after isosmotic and hyposmotic swellings of similar magnitude shows that the cells are responding to more than just simple swelling and suggests that cell electrolyte concentration could be involved in regulation of the different pathways. For taurine the pathway activity is related to intracellular electrolyte concentration (37).
in trout erythrocytes. The red blood cells were washed four times in standard saline solution, and the buffy coat was removed by suction. They were then suspended at 20% hematocrit, oxygenated, and incubated overnight at 4°C with 5 mM glucose to ensure that they reached a steady state with respect to ion and water contents before experimentation. We showed previously (5), and it has been confirmed by others (28, 39), that oxy-deoxyhemoglobin transition in fish erythrocytes regulates a Cl⁻–dependent K⁺ transport under isosmotic conditions, with deactivation of the pathway occurring in anoxia or at a very low PO₂. Therefore, experiments at high or variable PO₂ do not allow a clear characterization of the volume-sensitive K⁺ fluxes in these cells. Thus, before experiments the cells were again washed four times in an N₂ atmosphere in the appropriate saline solution, and experiments were performed under an N₂ atmosphere.

Solutions. All experiments were performed in solutions flushed with N₂ and maintained under an O₂- and CO₂-free N₂ atmosphere at 15°C. The basic solution used throughout the experiments contained (in mM) 145 NaCl, 4 KCl, 5 CaCl₂, 1 MgSO₄, and 15 N-2-hydroxyethylpiperazine-N'-3-propanesulfonic acid (pH 7.95, 320 mosmol/kgH₂O). In some experiments, Na⁺ was replaced by 145 mM choline. For Cl⁻–free solutions, Cl⁻ was replaced by nitrate(NO₃⁻) or methylsulfate(MeSO₄). All solutions contained ouabain to give a final concentration of 10⁻⁴ M. Swelling was induced by different procedures (37). 1) Cells were exposed to media of various tonalities; the basic solution (320 mosmol/kgH₂O) was diluted by addition of different volumes of buffered water with 15 mM NaCl substituted for 0–50 mM NaCl ions. This results in a net uptake of electrolytes and osmotic swelling, which is characterized by a slight increase of intracellular ionic strength (µ; see Table 2). Cells respond by releasing K⁺ via a Cl⁻–dependent pathway (K⁺–Cl⁻–cotransport). Hyposmotic swelling is induced by a decrease in extracellular osmolality or by diffusion of an uncharged solute such as urea, generating an osmotic inflow of water, which decreases intracellular ionic strength (see Table 2). Volume-regulatory response is then much more complex, involving taurine loss and K⁺ loss via 2 distinct (Cl⁻–dependent and Cl⁻–independent) pathways. Other pathways are activated, mediating downhill uptake of Na⁺ (which counteracts regulatory volume decrease) and movements of diverse structurally unrelated compounds [e.g., choline, tetramethylammonium (TMA), sorbitol].

The purpose of the present study was to gain a better understanding of the complex situation by analyzing how all the different pathways are stimulated and the nature of their relationship. The study involved modifying trout erythrocyte volume by degrees in various ways and monitoring the solute movements that were induced.

MATERIALS AND METHODS

Materials. Ouabain, DIDS, and N-ethylmaleimide (NEM) were obtained from Sigma Chemical (St. Louis, MO), [methyl-14C]choline, 86Rb⁺, [14C]sorbitol, and 2Na⁺ from Amersham (Little Chalfont, UK), and [14C]taurine from NEN Life Science (Boston, MA). All other chemicals were reagent grade.

Cell preparation. Rainbow trout (Onchorhynchus mykiss) were obtained from a commercial hatchery and kept for at least 1 wk in the laboratory. They were stunned by a sharp blow on the head, and blood was obtained by caudal venipuncture by using heparinized syringes. The red blood

| Table 1. Na⁺, K⁺, Cl⁻, and taurine contents in trout erythrocytes |
|-----------------|------------------|-----------------|
|                | Content, µmol/g dcs | Osmolarity, mosmol/kgH₂O |
| Na⁺            | 12.3 ± 1.5        | 7.1 ± 0.9        |
| K⁺             | 270.9 ± 2.9       | 156.6 ± 1.7      |
| Cl⁻            | 114.9 ± 3.9       | 66.4 ± 2.3       |
| Taurine        | 91.5 ± 3.4        | 52.9 ± 2.0       |

Values are means ± SE; n = 17. Water content was measured as difference between wet and dry cell weight. Amounts of Na⁺, K⁺, Cl⁻, and taurine were measured on extracts from dry cells. Osmolarity of Na⁺, K⁺, Cl⁻, and taurine was calculated from their concentration in cell water. Amounts of Na⁺, K⁺, Cl⁻, and taurine were measured on extracts from dry cells. Osmolality of cell water. Sum of osmolalities calculated in this way (283 mosmol/kgH₂O) represents ~90% of total cell osmolality (320 mosmol/kgH₂O), if it is assumed that cell and external saline osmolalities are identical. dcs, Dry cell solids.

Fig. 1. Isosmotic swelling is induced by a net uptake of electrolytes and osmotically obliged water. Hormonal stimulation of Na⁺/H⁺ exchange or suspension of erythrocytes in an isotonic solution containing NH₄Cl promotes an isosmotic swelling, which is characterized by a slight increase of intracellular electrolyte concentration, i.e., ionic strength (µ; see Table 2). Cells respond by releasing K⁺ via a Cl⁻–dependent pathway (K⁺–Cl⁻–cotransport). Hyposmotic swelling is induced by a decrease in extracellular osmolality (by diffusion of an uncharged solute such as urea), generating an osmotic inflow of water, which decreases intracellular ionic strength (see Table 2). Volume-regulatory response is then much more complex, involving taurine loss and K⁺ loss via 2 distinct (Cl⁻–dependent and Cl⁻–independent) pathways. Other pathways are activated, mediating downhill uptake of Na⁺ (which counteracts regulatory volume decrease) and movements of diverse structurally unrelated compounds [e.g., choline, tetramethylammonium (TMA), sorbitol].
Swelling-activated transports of $K^+$, First, the effect of various degrees of swelling on $K^+$ fluxes was investigated. In Fig. 2A, trout erythrocytes were swollen to various degrees by exposure to isosmotic saline (320 mosmol/kgH$_2$O) containing various amounts of NH$_4^+$ salts in replacement of Na$^+$ salts. Such a swelling, termed isosmotic, results from a net uptake of NH$_4^+$ salts and osmotically obliged water (37). The media contained Cl$^-$ or NO$_3^-$ as anion. Isosmotic swelling of cells in Cl$^-$-containing media was followed by a quite linear increase in the ouabain-insensitive $K^+$ influx as a function of cell volume. By contrast, in NO$_3^-$-containing media there is no discernible influence of cell volume on the $K^+$ flux. Thus trout erythrocytes responded to isosmotic swelling by activating exclusively a Cl$^-$-dependent $K^+$ flux, regardless of the magnitude of the volume increase. In Fig. 2B, trout erythrocytes were swollen to various degrees by exposure to hypotonic media of various osmolalities. In media with and without Cl$^-$ (with NO$_3^-$), hypotonic swelling was followed by an increase in the ouabain-insensitive $K^+$ flux. The fluxes measured in Cl$^-$-containing media were used to measure other unidirectional transports. Uptake measurements refer to values of solutes determined in cells as a function of time. Influx rates were estimated from the amount of radioactivity accumulated within a fixed incubation period that fell within the initial linear phase of the uptake time courses. Radioisotopes were added to the cell suspension just after swelling; the supernatant was kept to measure external radioactivity, and the pellet was weighed wet and dry for cell volume determination. For beta-radioactivity counting, the dry pellet was suspended in 1 ml of distilled water overnight, and radioactivity in the extract was measured by liquid scintillation in a beta counter (Packard). For determination of gamma radioactivity, the pellet was counted dry (Kontron gamma counter). Uptakes are expressed in micromoles per gram of dry cell solids and fluxes in micromoles per gram of dry cell solids per hour. In these experiments the external concentrations of the solutes were generally (in mM) 145 Na$^+$, 145 choline, 5 taurine, 5 sorbitol, and 4 K$^+$.

Ion content and concentration. The dry cells were suspended in 5 ml of distilled water overnight; then 100 µl of 70% (vol/vol) perchloric acid were added to the suspension. After centrifugation at 30,000 g for 10 min the clear supernatant was saved for analysis of cations, Cl$^-$, and amino acids. Ions were measured as previously described (20). A trapping correction of 3.5% was routinely applied to the final calculations. Ion contents were expressed in micromoles per gram of dry cell solids. Ion concentrations in cell water were calculated from ion contents and cell water contents and expressed as millimoles per liter of cell water.

Covalent fixation of DIDS. DIDS was covalently bound to the red cell membrane by incubation in the basic isotonic saline (10% hematocrit with 100 µM DIDS at 15°C for 90 min). Then the reacted DIDS was washed away (1 rinse with 25 vol of saline + 0.5% BSA followed by 2 additional rinses without albumin). Swelling was subsequently induced by suspending the cells in a DIDS-free hypotonic medium.
were substantially greater than those in NO₃-containing media, the difference between the two curves corresponding to the Cl⁻-dependent component of the K⁺ flux (Fig. 3).

Two main observations emerged from these data. The Cl⁻-dependent K⁺ component, considered a K⁺-Cl⁻ cotransporter, was always activated when cell volume increased, but the pattern of activation as a function of cell volume was different depending on the way cells were swollen (Fig. 3). When cell swelling was isosmotically induced, the flux increased considerably and regularly at the first increase in volume. In other words, any deviation from normal volume was immediately “sensed” by cells, which responded by activating the K⁺-Cl⁻ cotransporter, and the greater the volume increase the greater the activation. On the other hand, when swelling was hyposmotically induced, the threshold for activation of the flux, called the set point, was shifted to higher cell volume, and the maximal activity was greatly reduced.

The Cl⁻-independent K⁺ component, which was activated as a function of cell volume under hyposmotic swelling conditions (Fig. 2B), was not activated under isosmotic cell-swelling conditions, irrespective of the degree of swelling (Fig. 2A). In other words, activation of the Cl⁻-independent K⁺ component is not simply triggered by the cell volume increase but is dependent on changes in the intracellular concentration of salts or impermeant compounds. Because the cells were swollen to the same extent in isosmotic and hyposmotic solutions, any changes in the concentration of cytoplasm-impermeant compound were the same with both types of treatment; conversely, the concentration of intracellular electrolytes decreased in the hyposmotically swollen cells and increased in the isosmotically swollen cells (Table 2), indicating that intracellular electrolyte concentration could play a role in the activation process of the Cl⁻-independent K⁺ flux. To investigate this possibility further, two distinct experimental protocols were employed. We compared the activation of the Cl⁻-independent K⁺ flux when dilution of electrolytes results from cell volume increase (Fig. 4A) and when dilution of electrolytes occurs at a constant swollen cell volume (Fig. 4B).

Erythrocytes exposed to hypotonic media of different tonicities show a simultaneous increase in cell volume and decrease in intracellular solute concentration. A direct relationship exists between the two parameters.
an entry of water into the cell causes, e.g., a 10% increase in cell volume (expressed as cell water content per gram of dry cell solids) and a 10% dilution of intracellular electrolytes. Figure 4A depicts a typical experiment. Cell swelling was obtained by suspending trout erythrocytes in hypotonic Cl\(^{-}\)-free, NO\(_3\)-containing media of different tonicities, and the resulting Cl\(^{-}\)-independent K\(^{+}\) flux was measured. Cell volume and Cl\(^{-}\)-independent K\(^{+}\) flux are plotted as a function of electrolyte dilution in Fig. 4A. An electrolyte dilution of <10% had no discernible influence on K\(^{+}\) flux. From five similar experiments the set point for activation was visually estimated and corresponded to an ~10% salt dilution. As shown in Fig. 4A, above the set point the flux increased rapidly and in a linear manner.

To induce electrolyte dilution at a constant cell volume, the following protocol was adopted. Trout erythrocytes were swollen to the same extent (40% volume, the following protocol was adopted. Trout erythrocytes were swollen to the same extent (40% increase in cell water relative to control erythrocytes), activation of the Cl\(^{-}\)-independent K\(^{+}\) component is dependent on a reduction of the intracellular electrolyte concentration; despite a large cell volume increase, no discernible activation was measured when electrolyte concentrations remained equivalent to (dilution factor = 1) or greater (dilution factor = 0.9) than that of control unswollen erythrocytes. Activation started when electrolytes were diluted and increased progressively as a function of electrolyte dilution. The set point for activation, visually estimated from four similar experiments, corresponded to an ~10% dilution (dilution factor = 1.1). Thus, when the cell volume was increased to a constant value, activation was only dependent on the reduction of electrolyte concentration. A comparison of Fig. 4, A and B,

**Fig. 4.** Swelling-activated Cl\(^{-}\)-independent K\(^{+}\) flux (●) as a function of intracellular electrolyte dilution. To measure Cl\(^{-}\)-independent fluxes, NO\(_3\) was used as a Cl\(^{-}\) substitute. A: hypotonic swelling (electrolyte dilution varying as cell volume). Cells, previously incubated in Cl\(^{-}\)-free, NO\(_3\)-containing saline (320 mosmol/kgH\(_2\)O) in which 90 mM NaNO\(_3\) was replaced by urea, NH\(_4\)NO\(_3\), or urea-NH\(_4\)NO\(_3\) mixtures (see MATERIALS AND METHODS).
shows that the electrolyte set point for activation of the Cl\(^-\)-independent K\(^+\) flux was very similar in the two experiments. Because the concentration of cytoplasm-impermeant compounds, such as proteins, is strictly related to volume change, i.e., progressively decreased in Fig. 4A and maintained constant in Fig. 4B, the set point appears insensitive to changes in the concentration of impermeant compounds.

Other swelling-dependent transport systems. In response to swelling, trout erythrocytes activate several membrane pathways other than Cl\(^-\)-dependent and Cl\(^-\)-independent K\(^+\) transport systems; the transport of compounds as structurally diverse as amino acids (taurine) and inorganic (Na\(^+\)) and organic (choline) cations is induced in a similar fashion (20). An organic uncharged solute, sorbitol, can also be transported (see Fig. 7A). It must be pointed out that activation of these pathways allows a large amount of compounds to be transported. For example, when hyposmotically swollen in a Cl\(^-\)-free, NO\(_3\)-containing saline, K\(^+\) loss per hour was 33.81 ± 4.45 µmol/g dry cell solids and taurine loss was 37.97 ± 3.55 µmol/g dry cell solids, whereas Na\(^+\) uptake was 29.10 ± 1.59 µmol/g dry cell solids (5 experiments). In other words, the net Na\(^+\) uptake practically counterbalanced the volume-regulatory K\(^+\) loss mediated by the Cl\(^-\)-independent component; therefore, RVD in a Cl\(^-\)-free medium results exclusively from taurine loss. It is interesting to note that permeability of choline is also very large, even greater than that of Na\(^+\) (20).

However, as described above for the Cl\(^-\)-independent K\(^+\) transport, these pathways are activated when cells are hypotonically swollen but remain inactivated when cells are isosmotically swollen (37). We have shown previously (37) with cells swollen at a constant volume that activation of the taurine pathway is dependent on a decrease in intracellular electrolyte concentration. Experiments were then designed to compare activation of the taurine pathway when dilution of electrolytes occurs at a constant cell volume or results from cell volume increase. Similarly, experiments were performed to analyze the putative role of electrolyte dilution on the volume-sensitive pathways mediating the transport of inorganic (Na\(^+\)) and organic (choline) cations. These results, along with the data reported above for the Cl\(^-\)-independent K\(^+\) pathway, are shown in Fig. 5.

From the analysis of Fig. 5, several observations can be made.

1) For all compounds, i.e., Na\(^+\), choline, taurine, and K\(^+\) (as the Cl\(^-\)-independent component), activation of fluxes and electrolyte concentration were inversely related when cell volume was changing (Fig. 5A) or kept constant (Fig. 5B). In other words, at a constant cell volume, activation of all fluxes was strictly dependent on a reduction of cell electrolyte concentration.

2) The dependence of fluxes on electrolyte concentration was identical for all compounds, suggesting that the different pathways are in some way regulated in a coordinated fashion or that all solutes move via a common pathway.

3) The set points for activation of all solute fluxes were similar (~10% dilution of electrolytes) when electrolyte concentration varied with changes in cell volume (Fig. 5A) or at a constant swollen cell volume (Fig. 5B). In other words, the set points were insensitive to the concentration of impermeant compounds.

In skate hepatocytes it has been shown that activation of the taurine pathway is dependent on intracellular...
lar Cl\textsuperscript{−} concentration (25). The experiments described above were performed in Cl\textsuperscript{−}-containing media, except for those involved in the measurement of the Cl\textsuperscript{−}-independent K\textsuperscript{+} fluxes, which were performed in Cl\textsuperscript{−}-free, NO\textsubscript{3}\textsuperscript{−}-containing media. To test whether a decrease in Cl\textsuperscript{−} concentration, rather than dilution of electrolytes, is responsible, in trout erythrocytes, for activation of all these swelling-sensitive pathways, the following experiments were performed. First, isosmotic swelling was induced by suspending red blood cells in isosmotic media containing NH\textsubscript{4}Cl or NH\textsubscript{4}NO\textsubscript{3} exchanged for the same amount of Na\textsuperscript{+} salt, a condition in which swelling occurs without dilution of electrolytes (Table 2). As indicated above, swelling in NH\textsubscript{4}Cl did not induce activation of the taurine pathway. Figure 6A shows that total replacement of Cl\textsuperscript{−} by NO\textsubscript{3}\textsuperscript{−} also did not induce any activation of the taurine flux. In a second series of experiments, a similar swelling (40% increase in cell water) was induced by suspending erythrocytes in hypotonic, NaCl- or NaNO\textsubscript{3}-containing media, a condition in which swelling is accompanied by a decrease in electrolyte concentration. Figure 6B shows that taurine flux was similar in NO\textsubscript{3}\textsuperscript{−} and Cl\textsuperscript{−} media, i.e., when intracellular Cl\textsuperscript{−} has been totally replaced or only 40% diluted. Replacement of Cl\textsuperscript{−} by MeSO\textsubscript{4} gave similar results (not shown). Thus activation of the taurine pathway appears dependent on the concentration of intracellular electrolytes and not on the concentration of intracellular Cl\textsuperscript{−}. Because Na\textsuperscript{+} and choline fluxes were unaffected by the replacement of Cl\textsuperscript{−} with MeSO\textsubscript{4} (not shown), activation of these solute fluxes, like that of taurine, is dependent on a reduction of cell electrolyte content.

In conclusion, the volume-sensitive pathways for taurine, Na\textsuperscript{+}, choline, and (Cl\textsuperscript{−}-independent) K\textsuperscript{+} were activated by a reduction in electrolyte concentration, and their patterns of activation, including the set points, appeared identical.

All these pathways have been shown previously to be inhibited to a similar extent by a series of anion transport inhibitors [e.g., furosemide, niflumic acid, DIDS, and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB)], and in cases when the dose dependencies were measured, these were also found to be similar (6, 19, 20). Moreover, in trout erythrocytes (19), but not in eel erythrocytes (35), DIDS, when covalently bound to the membrane, inhibited all these pathways, including the Cl\textsuperscript{−}-independent, but not the Cl\textsuperscript{−}-dependent, K\textsuperscript{+} pathway. Figure 7A shows that covalently bound DIDS similarly inhibited the volume-induced transport of sorbitol, one of the other organic solutes transported in response to swelling. Recently, Bursell and Kirk (6) found that NEM (2 mM) was effective as an inhibitor of the swelling-activated taurine transport in eel erythrocytes. As illustrated in Fig. 7B, NEM (1 mM) applied to trout erythrocytes inhibited not only the volume-dependent taurine transport but also Na\textsuperscript{+}, choline, sorbitol, and Cl\textsuperscript{−}-independent K\textsuperscript{+} transports in a similar manner. Thus the pharmacological characteristics of all these pathways appear strikingly similar.

**DISCUSSION**

Trout erythrocytes possess multiple swelling-sensitive transport pathways that, for swelling of similar magnitudes, can be activated to different degrees according to the manner in which the volume change has been brought about. Depending on whether cell electrolyte concentration has been increased or decreased during swelling, the red blood cell will activate only one regulatory pathway (a KCl cotransport) or numerous additional pathways, implicating cell electrolyte concentration in the control of volume-sensitive pathways.

To try to understand this complex regulatory mechanism, we have to identify, for each transport system,
the specific intracellular signal(s) involved in its activation.

K⁺-Cl⁻ cotransporter. A Cl⁻-dependent K⁺ pathway with the characteristics of a K⁺-Cl⁻ cotransporter (4, 6, 37) is activated in response to isosmotic and hyposmotic swelling (Fig. 3). To define Cl⁻-dependent and Cl⁻-independent K⁺ pathways, NO₃⁻ has been used as a Cl⁻ substitute, similar to studies on sheep, human (10, 33, 34), and other fish (6, 32) red blood cells. However, in mouse (1) and trout red blood cells exposed to extreme prelytic cell volume (3), NO₃⁻ has been shown to be a less efficient substitute for Cl⁻ than MeSO₄ or sulamate. However, under the experimental conditions used in this study (maximum 40% increase in cell water content), NO₃⁻ is a reasonably good substitute, since the K⁺ fluxes measured in NO₃⁻-containing media were similar to or only very slightly higher than those in media containing MeSO₄ or sulamate (not shown).

Figure 3 shows that activation of the K⁺-Cl⁻ cotransport as a function of cell volume was different in isosmotically and hyposmotically swollen cells. Isosmotic swelling induced a strong KCl activation as soon as a slight increase in volume occurred, and the KCl activity continued to increase linearly as a function of cell volume. Hyposmotic swelling also induced KCl activation, but under these conditions the efficiency of the K⁺-Cl⁻ cotransport was clearly reduced: 1) the sensitivity to small changes in cell volume was much lower, suggesting that the set point is shifted to higher cell volumes; and 2) the volume dependence of the K⁺ flux was damped, displaying a rather sigmoidal relationship. Hyposmotic swelling promotes an increase in cell volume and a dilution of impermeant compounds, as does isosmotic swelling, but hyposmotic swelling also promotes a dilution of intracellular electrolytes (i.e., a decrease in ionic strength). Thus the volume increase and/or the decrease in impermeant compound concentration appears to be the primary activator of the K⁺-Cl⁻ cotransport, with ionic strength acting as a modulator of the activated cotransporter; K⁺-Cl⁻ cotransport is more efficient at higher than at lower ionic strengths, this parameter acting partly by altering the set point.

Studies on dog red cell ghosts have shown that dilution of cytoplasmic proteins, regardless of cell volume, activated K⁺-Cl⁻ cotransport (8), suggesting that in trout erythrocytes dilution of cytosolic impermeant compounds could be the parameter controlling activation of the K⁺-Cl⁻ cotransport. Moreover, at any particular dog red cell volume, decreases in ionic strength diminished K⁺-Cl⁻ cotransport activity and shifted the set point of the cotransporter to higher cell volume (41). The authors interpreted these results as implying that interaction of cell electrolytes with some intracellular macromolecules induced changes in the activity of a putative regulatory protein (41), such as perhaps the inhibitory, volume-sensitive kinase (27). Our data in Fig. 3 show that the set point for activation of the K⁺-Cl⁻ cotransport was shifted to a higher cell volume when swelling was hypotonically induced (i.e., when ionic strength was decreased); this is in agreement with the results obtained in dog red blood cells. However, ionic strength not only altered the set point for activation but also decreased the maximal activity, indicating a more complex effect of ionic strength than that suggested for dog red blood cells.

Pathways induced by hyposmotic swelling. Swelling of trout erythrocytes, when accompanied by a dilution of intracellular electrolytes (hyposmotic swelling), simultaneously activates a K⁺-Cl⁻ cotransport and the transport of structurally unrelated compounds such as K⁺ (as a Cl⁻-independent component), taurine, Na⁺, choline, and sorbitol. The data in Fig. 5B clearly demonstrate that the dilution of intracellular electrolytes is the factor that tightly controls activation of all...
these pathways, as we have previously shown for taurine (37); when erythrocytes were kept swollen at a constant volume (40%), no activation occurred as long as the concentration of intracellular electrolytes remained higher than or equivalent to that of control, unswollen cells. Some activation was discernible when the intracellular electrolytes were diluted by ~10%. On greater dilution, the flux increased rapidly and linearly. It is conceivable, as in skate hepatocytes (25), that intracellular Cl\(^-\) or K\(^+\) concentration, rather than the electrolyte concentration as a whole, exerts such a control. However, total replacement of Cl\(^-\) by NO\(_3\) or MeSO\(_4\) did not alter the patterns of activation. Moreover, we previously showed (37) that the taurine pathway remained inactive when swelling was induced by catecholamine stimulation of Na\(^+\)/H\(^+\) exchange or exposure to NH\(_4\) salts, indicating that the nature of cations accumulated in the cell (Na\(^+\) in the presence of ouabain, K\(^+\) in its absence, or NH\(_4\)\(^+\)) also did not control pathway activation. Thus it is the concentration of intracellular electrolytes (i.e., ionic strength), and not their nature, that is the factor controlling activation. Moreover, a comparison of Fig. 5, A and B, supplies additional and important information: fluxes of all solutes are controlled by the changes in cell electrolyte concentration (i.e., ionic strength) but are independent of the degree of cell volume increase. For example, at 1.3 electrolyte dilution, fluxes represent 30–35% of maximal value when cell enlargement is 12% (Fig. 5A) or 40% (Fig. 5B). This is a similar feature for each point on the curves. In particular, activation occurs at the same degree of electrolyte dilution when the cell volume is only slightly enlarged (2%, Fig. 5A) or greatly enlarged (40%, Fig. 5B). Thus activation of all these transport pathways is triggered by the decrease in ionic strength and is independent of the degree of cell volume increase. It means that activation is characterized by an "ionic strength set point" and not by a "volume set point."

Studying the regulation of the volume-sensitive taurine channel in C\(_6\) glioma cells, Emma et al. (12) reached a different conclusion: activation of the channel would be controlled by a volume set point that is modulated by changes in intracellular electrolyte concentration. In these experiments, acclimatization of C\(_6\) cells to hypertonic media allowed an increase in intracellular electrolyte level. Cell swelling was then induced by reducing bath osmolality (i.e., hypotonic swelling). It was found that "at high intracellular concentration a larger degree of cell swelling is needed to activate a given amount of organic osmolyte efflux compared with cells that have normal or below normal inorganic ion levels," leading to the conclusion that intracellular electrolyte level modifies the volume set point. This interpretation is satisfying, if it is assumed that activation is triggered first by a volume set point. However, if we assume that activation is primarily triggered by an ionic strength threshold, as shown in trout red blood cells, the results will be identical: at high intracellular electrolyte concentration a larger degree of hypotonically induced swelling (i.e., a larger amount of water diluting electrolytes) is needed to reach the critical ionic strength threshold. Thus only a direct measurement of intracellular electrolyte concentration at each level of cell swelling would confirm that the taurine channel is differently activated in C\(_6\) glioma cells and in trout red blood cells. Nevertheless, it remains possible that the nature of the taurine pathway may be different in the two cell types.

The present results showing that several pathways induced by hypotonic swelling are similarly activated by ionic strength raise two main questions: 1) What is the relationship between these pathways? 2) How does ionic strength affect them?

The fact that fluxes of solutes so structurally diverse as K\(^+\) (as Cl\(^-\)-independent component), Na\(^+\), choline, taurine, and sorbitol are similarly controlled by ionic strength suggests that their movements are in some way regulated in a coordinated fashion. This linkage was first proposed (20) when, having shown that hypotonic swelling activated several transports of structurally unrelated compounds, we noted 1) that all the fluxes were inhibited by DIDS and other inhibitors of the band 3 anion exchanger (e.g., niflumate and furosemide) and 2) that the IC\(_{50}\) for inhibition of cations and taurine was the same. We suggested that the DIDS-sensitive band 3 anion exchanger would control the activity of multiple transport systems. Later, a series of studies performed by various investigators on erythrocytes from other fish species (flounder, skate, and eel) confirmed that hypotonic swelling led to an increase in the membrane transport of a wide range of solutes (with the addition of new compounds such as betaine, polyols, and nucleosides) and that these fluxes were similarly inhibited by several blockers of the anion exchanger (including NPPB). None of the inhibitors is highly specific, and it is possible that their identical effects on the different volume-activated transport pathways are coincidental. However, certain kinetic properties of these transports were studied and were found to be similar. It was then suggested (22, 23, 31, 46) that all these solutes share a common pathway with the characteristics of an anion channel, displaying considerable similarity to channels mediating the volume-regulatory efflux of organic osmolytes from mammalian cells (44). The recent results of Kirk and collaborators (6, 30, 35), obtained with eel erythrocytes, further support this hypothesis of a single swelling-activated, DIDS- and NPPB-sensitive channel of broad specificity. Likewise, it has been observed, using the patch-clamp technique, that in isotonic conditions trout red blood cells possess a significant DIDS-sensitive Cl\(^-\) conductance that is reversibly stimulated by hypotonic cell swelling (11). An additional argument in favor of the single-pathway hypothesis is provided by the results obtained on Xenopus oocytes expressing red cell band 3 anion exchangers (AE1). When expressed in oocytes, the trout AE1 can function as an anion channel mediating the movements of taurine (13) and uncharged solutes such as sorbitol (14a), whereas the highly homologous isoform from a mature mammalian erythrocyte, a cell that has lost the capacity to regulate its volume, fails to function as an anion channel and to transport taurine and sorbitol. Thus these data strongly
support the view of a direct implication of trout band 3 in the formation of an anion-selective channel permeable to structurally unrelated compounds (19, 36). The hypothesis of a single route for all solutes raises the question of how cations might permeate such channels. Electrophysiological studies of swelling-activated, taurine-permeable anion channels indicate a low conductivity to monovalent cations in cultured mammalian cells (26) but a relatively high cation permeability in skate hepatocytes (25). The “background Cl− channel” of hippocampal neurons is also an anion-selective channel that has a significant permeability to monovalent cations, and Franciolini and Nonner (16) proposed a permeation model in which a cation interacts with an anion to move across the membrane. A similar proposal has been made by Bursell and Kirk (6) for the swelling-induced transport of cations in fish erythrocytes.

The mechanism by which cell ionic strength can activate the putative channel remains to be defined. When trout band 3 was expressed in oocytes, the resulting anion current was a sigmoidal function of the level of band 3 expression, consistent with the view that the conductance pathway was formed by, or required, multimeric arrangements of the protein (13). In skate erythrocytes, osmotic swelling resulted in an increase in the relative proportion of band 3 dimers and tetramers in the membrane, leading to the proposal that band 3 aggregation may be involved in osmolyte channel formation/activation (38). Thus it can be envisioned that a change in intracellular ionic strength, by altering cytoskeletal architecture and/or interaction between band 3 and the cytoskeleton, allows rearrangement of band 3 in the membrane. Alternatively, or perhaps additionally, changes in ionic strength could modify the activity of a protein involved in channel regulation, since, according to Parker (40), decreasing cell electrolytes alters the thermodynamic activity of cytoplasmic proteins via electrolyte-macromolecule interaction.

Why two RVD mechanisms in a single cell? The question arises concerning the reasons why a single cell possesses two different regulatory volume decrease transport pathways specifically activated by distinct stimuli: one that mediates selectively a loss of electrolytes (KCl) and is turned on by the volume increase and another that is turned on by the decrease in cell electrolyte concentration and mediates essentially a loss of taurine (the loss of electrolytes occurring via this pathway as Cl− independent K+ being practically counterbalanced by an entry of electrolytes such as NaCl). Clearly, when swelling results from an uptake of electrolytes, the best way for a cell to undergo volume regulation is to activate the pathway that selectively mediates a loss of electrolytes. Conversely, when swelling results from an entry of water, which dilutes cell electrolytes, the best way is then to activate the taurine pathway by using organic osmolytes to recover volume and by preventing an additional decrease of electrolyte level.

In this context, it must be pointed out that, under physiological conditions, trout erythrocytes can be isotonically or hypotonically swollen. When a trout is exposed to deep hypoxia, catecholamines are released that stimulate erythrocyte Na+/H+ exchangers, leading to an accumulation of electrolytes (NaCl) in erythrocytes and cell swelling (4, 14). This sequence of events, by increasing O2 content of erythrocytes at low PO2 (7), allows the fish to survive in deep hypoxia. A return to normoxic conditions, which is accompanied by deactivation of Na+/H+ exchanges, is followed by a recovery of electrolyte content and cell volume (14). The most efficient way to recover volume and normal electrolyte content is then to specifically extrude salts in excess by the simultaneous functioning of the K+/Cl− cotransporter and the Na+/K+ pump and to repress the taurine efflux pathway. However, trout, a quite euryhaline fish, can also be exposed to various salinities, leading to hyposmotic swelling of red blood cells and a decrease in the intracellular electrolyte concentration. Then it makes sense that the cell uses organic osmolytes such as taurine to undergo volume regulation and simultaneously reduce the loss of electrolytes occurring via the K+/Cl− cotransporter (which is always activated by cell swelling). It also makes sense that the signal that turns on the taurine channel and damps the K+/Cl− cotransport is the dilution of cell electrolytes.

Thus when cells are physiologically subjected to isotonic and hypotonic swelling conditions, the presence of two pathways appears beneficial, since they play a complementary role in the simultaneous regulation of cell volume and cell electrolyte content. Although most studies of RVD are performed on cells hypotonically swollen, swelling of many epithelial cell types is physiologically achieved by a net salt uptake (isotonic swelling). Then it is likely that cell types other than trout erythrocytes possess two RVD mechanisms. This possibility is supported by the recent findings obtained in a mammalian cell line (12). In these cells the swelling-induced channel mediating taurine efflux was inhibited when the intracellular electrolyte concentration was initially elevated. Despite this inhibition, however, cells were still able to undergo an RVD, suggesting involvement of another regulatory pathway. Moreover, as proposed by Strange and collaborators (12, 25, 43), this inhibition of the taurine channel by an elevated electrolyte content would have a physiological significance, i.e., to avoid a loss of organic solutes that could cause electrolytes to rise to toxic levels.

Address for reprint requests: R. Motais, CEA (DBCM) and CNRS (URA 1855), BP 68, 06230 Villefranche-sur-Mer Cedex, France.

Received 12 March 1998; accepted in final form 29 September 1998.

REFERENCES

4. Borgese, F., F. Garcia-Romeu, and R. Motais. Control of cell volume and ion transport by β-adrenergic catecholamines in...
C220 SWELLING ACTIVATION OF TRANSPORT PATHWAYS


Downloaded from http://ajpcell.physiology.org/ by [49x745] on September 30, 2017