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Topical Review

The Na-K-2Cl Cotransport system

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Introduction

For numerous animal cell species a quaternary cotransport system has been demonstrated, which is located in the plasma membrane and transports Na^+, K^+, and Cl^- electroneutrally with a stoichiometry of 1 : 1 : 2. It is inhibitable by furosemide and other loop diuretics. Previously, various other transport systems for these ions, which are inhibitable by the same diuretics, had been postulated, such as K^+-Cl^- cotransport [9, 38], Na^+-K^- cotransport [28, 29, 70], primary active Cl^- pump [14, 15, 17, 80], Na^+-K^+ cotransport [34, 62, 63, 97], and Na^+/Na^+ and K^+/K^+ exchange [10, 68, 69, 89, 90, 91]. It is now believed that these are not distinct systems, but merely partial aspects of the above-mentioned single cotransport system. The concept of the Na^+-K^+-$2Cl^-$ cotransport system evolved along different lines which will be briefly described here.

One line started with the "paradoxical" behavior of Cl^- in Ehrlich cells. This ion, which for many decades had been believed to pass the membrane only passively and without mediation, was not always found to behave accordingly: Its distribution between cell and medium often deviates from the Nernst equation at a given electrical potential. Moreover, a rise of the electrical PD, for instance after stimulation of the electrogenic Na^+/K^+ pump by K^+, does not prevent Cl^- to move into the cell, i.e., against its electrochemical potential gradient [49]. This "paradoxical" Cl^- movement was inhibitable by furosemide and was always accompanied by an extra furosemide-inhibitable K^+ movement of similar magnitude, normally also against the corresponding electrochemical potential gradient [38]. The first hypothesis that comes to mind, that of a furosemide-sensitive inward Cl^- pump, does not explain the energetically uphill K^+ movement. More plausible seemed another hypothesis, that of an active K^+/H^+ antiport, which is the presence of a Cl^-/OH^- or Cl^-/HCO_3^- exchange mechanism would account for the observed effects. This hypothesis also had to be discarded, because no evidence of an active K^+/H^+ exchange nor for the participation of an existing SITS- or DIDS-sensitive Cl^-/OH^- exchange could be found. There remained a third hypothesis, that of an active K^+-Cl^- cotransport. Soon, however, improved techniques and shorter test periods revealed that the furosemide-sensitive Cl^- transport exceeded the accompanying K^+ transport by a factor of about 2 [13] and, moreover, that electroneutrality was maintained by a furosemide-sensitive Na^+ flow. This led to the first postulation of a (quaternary) Na^+-K^+-$2Cl^-$ cotransport [35], which could soon be verified on the ground of thorough kinetic and thermodynamic analyses [36, 40], as will be discussed below. As this cotransport had no effect on the electrical PD of the cell membrane, nor was it affected by an imposed electrical PD, it appeared to be electrically silent, in agreement with the indicated stoichiometry. In this form this cotransport is now widely accepted, though its first presentation was met with great scepticism (discussion in 36).

In another line of research it was found that in erythrocytes the exit of Na^+ falls short of what could be accounted for by the ouabain-sensitive Na^+/K^+ pump. A second Na^+ pump, inhibitable by ethacrynate was, therefore, considered [55]. When it turned out that this process also operates inward directed and that this extra Na^+ entry is always associated with an extra entry of K^+, a Na^+-K^+ cotransport [97] was postulated for these cells, and later also for Ehrlich cells and other cells [33, 79]. In avian erythrocytes evidence was found that this Na^+-K^+ cotransport functions in the regulation of cellular volume [32, 64, 84]: It is activated by os-

Key Words secondary active transport · Na-K-2Cl cotransport · regulation of cell volume · loop diuretics · degree of coupling (q)

motic shrinking of the cell and also by norepinephrine, which, supposedly through a β-adrenergic process via cAMP, participates in volume regulation. Later, it could be shown with human erythrocytes that the Na^+-K^+ cotransport depends on the presence of Cl^-, which cannot be replaced by nitrate [19, 20, 25]. It was assumed that Cl^- is cotransported with the two cations, though direct evidence could not be obtained, because the overlapping anion antiport exceeds that of the Na^+-K^+ cotransport by several orders of magnitude. Only later was this assumption confirmed for avian erythrocytes after specific inhibition of the anion exchange [45]. For various other cells, Cl^- dependence at this process is now shown [2–4, 12, 67, 73].

A third line of research was concerned with the detailed action of loop diuretics in renal tubules. After a second Na^+ pump had been excluded, the active movement of Cl^- was identified in microperfusion studies to be the primary target of these inhibitors [15, 80]. Similar findings were reported for other epithelial tissues such as cornea [17] and the intestine of teleosts [98]. Some discrepancies between prediction and findings could be reconciled by the assumption of a secondary active Cl^- transport, i.e. of a Na^+-Cl^- cotransport [29]. For the salt gland of the shark this system was later expanded to include a second Cl^- ion [87], and the resulting Na^+-$2Cl^-$ cotransport appeared to account for most experimental findings satisfactorily. The requirement of K^+ for this loop diuretic-sensitive transport in Henle's loop and other epithelia was discovered more recently on the basis of microperfusion studies [41, 71] and directly by studies with vesicles of various cells [46, 61]. Hence, it would appear that also in the kidney the quaternary cotransport, first described for the Ehrlich cell, is the mechanism underlying all these observations. It is natural that a Na^+-K^+ cotransport, similar to that postulated for the erythrocytes, has never been considered in transepithelial transport, as it would disagree with the electrical findings.

Coupling in Cotransport

Any cotransport implies that the individual flows of the cotransported species are tightly coupled with each other at a fixed stoichiometry. The most rigorous and quantitative test for coupling available is based on "the degree of coupling" (q), derived from the phenomenological equations of Thermodynamics of Irreversible Processes (TIP). It takes into account that each coupled flow is driven, besides by its own (conjugate) driving force, also by the (nonconjugate) driving force(s) of other flow(s). For a simple two-component system the phenomenological equations are

$$J_a = L_{aa}X_a + L_{ab}X_b \tag{1a}$$

$$J_b = L_{ab}X_a + L_{bb}X_b. \tag{1b}$$

J_a and J_b are the flows of solutes A and B, each of which is linked to its driving force X_a and X_b, through the straight coefficients L_{aa} and L_{bb}, and to that of the other solute through a cross coefficient, L_{ab}.

The coupling becomes more explicit in the "quasi-chemical notation," which presupposes a fixed stoichiometry of coupling overlapped by (uncoupled) leakage flows [47].

$$J_a = (\gamma_a^2 L_r + L_a'')X_a + \gamma_a\gamma_b L_r X_b \tag{2a}$$

$$J_b = \gamma_a\gamma_b L_r X_a + (\gamma_b^2 L_r + L_b'')X_b. \tag{2b}$$

L_r is the coefficient linking the flow of the overall process to the total driving force ($X_a + X_b$). L_a'' and L_b'' are (summarizing) leakage coefficients, and γ_a and γ_b the stoichiometric coefficients.

We see that each flow consists of two components, the coupled flow and the uncoupled leakage flow.

The degree of coupling (q) is defined

$$q = \frac{L_{ab}}{\sqrt{L_{aa} \cdot L_{bb}}} = \frac{\gamma_a\gamma_b L_r}{\sqrt{(\gamma_a^2 L_r + L_{aa}'')(\gamma_b^2 L_r + L_{bb}'')}}. \tag{3}$$

A completely coupled pair of flows should give the maximal q of ± 1, which is usually reduced by leakage flows. To obtain q it is not necessary to know all phenomenological coefficients separately; it suffices to determine the ratios of the cross coefficient over each of the straight coefficients. It should be pointed out that in these ratios the variations of the coefficients, which naturally occur with changing driving forces and which severely limit the application of TIP, largely cancel. Accordingly, as has been demonstrated elsewhere [48], q is a far more reliable parameter than the phenomenological coefficients from which it is derived, and the objections raised against the use of irreversible thermodynamics in general do, therefore, not apply to the degree of coupling q.

The simplest experimental way to determine q is through the "pulse-cross response" method:

$$q^2 = \left(\frac{\partial J_a}{\partial J_b}\right)_{X_a} \cdot \left(\frac{\partial J_b}{\partial J_a}\right)_{X_b}. \tag{4}$$

Each partial differential gives the response of the

one flow to an induced change (pulse) of the other one to which it is supposedly coupled. It is unimportant how the primary changes are induced, but in any case, the conjugate driving force of the responding flow must remain constant during the test. If the two flows are plotted against each other, one obtains straight lines over a rather wide range. From the two corresponding slopes q can be derived. The quasi-chemical notation shows that the two corresponding slopes can also be used to determine the upper and the lower limit of the stoichiometric coefficients.

$$\left(\frac{\partial J_a}{\partial J_b}\right)_{X_a} \leq (\gamma_a/\gamma_b) \leq \left(\frac{\partial J_b}{\partial J_a}\right)^{-1}_{X_b}. \quad (5)$$

For the Na^+-K^+-$2Cl^-$ cotransport this procedure has to be extended to all three ion flows concerned, each of which should be coupled directly to the other two. The obscuring effects of the leakage flows can be reduced or even eliminated by specific inhibitors. In the present system advantage could be taken from the specific inhibition by the loop diuretics (furosemide, bumetanide, piretanide). Using only those flux components that are sensitive to these diuretics for the determination of q and γ, one can "filter out" the leakage components, provided that the measuring periods are short enough to avoid changes of the driving forces.

The pulse-cross response test, as described above, was carried out with Ehrlich cells as follows [36, 40]: In cells enriched with Na^+ and depleted of K^+ and suspended in K^+-free buffer the cotransport was initiated by the addition of K^+. One type of experiment was carried out for each of the three ions, Na^+, K^+ and Cl^-. In each case the concentrations of two ion species were kept constant, where that of the third one was varied (Fig. 1). The fluxes of the two unvaried ion species were plotted versus that of the varied species. Thus, it can be shown to which extent the two ion species with constant conjugate driving force respond to the variation of the driving force of the third ion species. Straight lines were obtained with the slopes of almost unity for the two cations and of one half for each cation flow as a function of the Cl^- flow and about two for the Cl^- flow as a function of each of the cation flows.

Using the above slopes to determine q according to Eq. (3) one obtains values very close to unity for all three couplings. It may safely follow that under the experimental conditions no flows other than those of the cotransport are inhibited by furosemide. The stoichiometry was then derived and found to be as expected, namely 1 Na^+ : 1K^+ and 2Cl^- [36, 40]. The above analysis also excludes, at least for Ehrlich cells, that the quaternary cotransport is in reality the sum of two separate binary cotransport systems in parallel, of NaCl and of KCl. Otherwise, the pulse-cross response test would not show any coupling between Na^+ and K^+: in other words, the appropriate degree of coupling, q, should not significantly differ from zero. In the present system it is just this coupling which has been most thoroughly investigated by various authors, for various cell types. Hence, the coupling

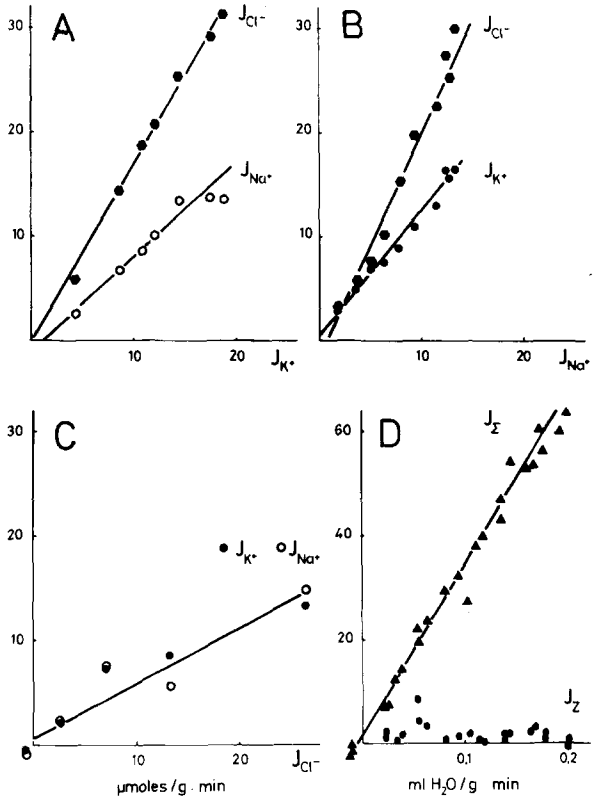

Fig. 1. Pulse-cross response experiment to determine for furosemide-sensitive ion transport the degree of coupling and stoichiometric ratios. Na^+-rich, K^+-depleted Ehrlich cells were incubated for 5 min at 37°C in Krebs-Ringer phosphate buffer with varied Na^+, K^+, and Cl^- content. Furosemide-sensitive fluxes were initiated by addition of K^+ to a K^+-free incubation medium. (For experimental details *see* the original publication.) (*A–C*) The furosemide-sensitive component of uptake of the constant (cross-responding) ions is plotted versus that of the pulsed one. (A) Na^+ and Cl^- constant, K^+ varied. (B) K^+ and Cl^- constant, Na^+ varied. (C) Na^+ and K^+ constant, Cl^- varied. The slopes of the lines are not significantly different from the following values: cation/cation, 1; Cl^-/cation, 2; and cation/Cl^-, 0.5. (*D*) Pooled experimental data from *A–C*. Uptake of osmotic active solutes ($Na^+ + K^+ + Cl^-$) and electrical charge ($Na^+ + K^+ - Cl^-$). The slope of the line for osmotic active solutes versus associated water movement gives an osmolarity of the transported mixture not significantly different from isotonicity. Transfer of electrical charge is not significantly different from zero. (From Ref. 40, reproduced with permission of Elsevier Science Publishers)

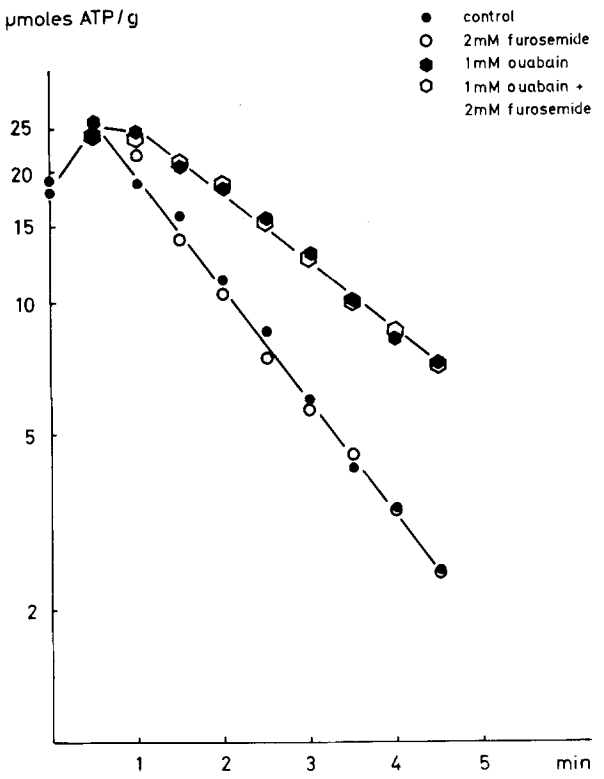

Fig. 2. Coupling of ion transport to ATP hydrolysis. For Na^+-rich, K^+-depleted cells ATP content was determined at different times after activation of transport by addition of K^+ and after blocking of respiratory ATP production by antimycin A. While ATP consumption by ouabain-sensitive pump flux is clearly seen, no influence of furosemide-sensitive cotransport could be observed. (From Ref. 40, reproduced with permission of the publisher)

between K^+ and Na^+ is probably the best established one of the three.

Although the cotransport system has by now been postulated for many other cell types, a rigorous determination of q, based on the pulse-cross response technique described above, so far has not been carried out with any cell other than the Ehrlich cell. It would be reassuring if the q-test was also applied in other systems, for instance to the coupling between Na^+-K^+ cotransport and Cl^- flow in erythrocytes, and to that between Na^+-$2Cl^-$ cotransport and K^+ flow in the loop of Henle. But even though the final proof of the Na^+-K^+-$2Cl^-$ cotransport in most cases may still be missing, the evidence appears very strong that this cotransport is present in all classes of vertebrates.

Treatment of erythrocytes with NEM induces KCl cotransport not inhibitable by loop diuretics [65, 66]. It is not decided now whether a new transport system is induced or whether NEM splits off a part of the quaternary transporter, bearing the K^+- and one (the specific) Cl^- binding site, but not that for the other anion (unspecific?), Na^+ and the diuretic.

Specificity of the Na^+-K^+-$2Cl^-$ Cotransport

Inhibitability

The furosemide-sensitive cotransport system is also inhibited by other loop diuretics such as bumetanide and piretanide. These two are even more powerful and more specific than furosemide: their IC_{50} is lower and their side effects on the anion exchange system are smaller. Also ethacrynate inhibits the cotransport system, though not as such, but in the form of its cysteine adduct. It is much less specific, since as a SH-blocker, it affects many other unrelated processes. As one would expect of various loop diuretics, IC_{50} for inhibition of transport in the isolated perfused tubules of Henle's loops is strongly correlated to their diuretic effect [83]. Moreover, as tested with avian erythrocytes [75] and for Ehrlich cells (M. Götz and P. Geck, *in preparation*), the inhibitory effect of most diuretics in the quaternary cotransport system of these (nonepithelial) cells correlates well with their diuretic effect, supporting the view that the target process is similar for epithelial and nonepithelial cells.

The stilbene derivatives DIDS and SITS and related compounds, which specifically inhibit the anion exchange system through covalent binding to protein, are ineffective towards the cotransport system. This fact helps to distinguish the cotransport from other transport systems [45, 52, 59].

Replaceability of Ion Species

None of the three ion species participating in the quaternary cotransport system can fully be replaced by related species. So Na^+ can only partially be replaced by Li^+, i.e., with the loss of cotransport activity, but not at all by K^+, Rb^+, or Cs^+. K^+ can completely be replaced by Rb^+. Cs^+ is less effective than these, as it reduces the overall transport rate, whereas Tl^+ appears to be more effective than all other cations, even than K^+ and Rb^+ [11, 85, 86]: Under otherwise equal conditions its isotope flux through the cotransport is about ten times that of K^+ and Rb^+ (the corresponding factor for the Na^+/K^+ pump or the K^+ channel is about three). More recent investigations have shown that K^+ can be replaced by NH_4^+ [58]. Cl^- can be replaced by Br^- with the loss of half the transport activity, but not, or not completely, replaced by nitrate. As men-

tioned before, this latter observation is helpful to distinguish the cotransport system from the anion antiport system. Recently, however, more detailed analysis has given evidence that only one of the two Cl^--binding sites discriminates between Cl^- and NO_3^-. This appears to follow from the observations that partial substitution of Cl^- by nitrate shifts the stoichiometric coefficient for Cl^- from two to one [26, 58, 74].

Energetics of Cotransport

Since under normal conditions both K^+ and Cl^- are transported against their respective electrochemical potential difference, the question arises whether this transport is primarily or secondarily active. As under those conditions the third species, Na^+, is maintained at an inward-directed electrochemical potential difference, many authors tend to the view that the cotransport is secondarily active. This agrees with the finding that the cotransport, in contrast to the ouabain-sensitive Na^+/K^+ pump, even working at maximum speed, does not cause any appreciable increase in ATP hydrolysis (Fig. 2) nor, under anaerobic conditions, in glycolytic rate [36, 40]. Yet, this cotransport strongly depends on the presence of intracellular ATP, and in the steady state the sum of the electrochemical potentials of all three ion species seems to be somewhat higher inside than outside [36, 40, 54], but this discrepancy vanishes if one takes into account that the ionic activity coefficients are likely to be lower inside than outside by 10–20%, according to the theory of Debye and Hückel. The ATP dependence of transport may be explained by a regulatory role of ATP, for instance, it may be necessary to phosphorylate proteins crucially involved in cotransport.

Diuretic-sensitive transport is reversible; at very low extracellular K^+ (0.2 mM) it operates net outward directed, a behavior used to isolate transport-deficient mutants [34, 56]. This observation is better explained by secondary than by primary active transport.

In summary, though primary transport has not rigorously been excluded yet, secondary active transport appears to be a plausible and well-supported working hypothesis (Fig. 3).

Function of Cotransport in Volume Regulation

Evidence that the Na^+-K^+-$2Cl^-$ cotransport system functions to maintain cellular volume so far stems mostly from studies with avian erythrocytes [32, 62–64, 84] and, more recently, with Ehrlich cells

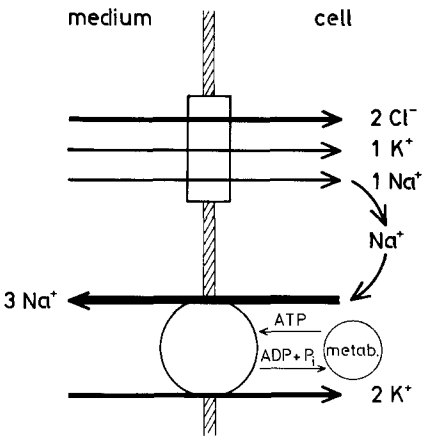

Fig. 3. (Na^+-K^+-$2Cl^-$-cotransport and Na^+/K^+ pump. Furosemide-sensitive cotransport as secondary active transport process. (For details, *see* text)

[37, 39, 52, 54]. With the former it has consistently been observed that osmotic shrinkage of the cells activates the Na^+-K^+ cotransport, which, as mentioned before, is most likely identical with the Na^+-K^+-$2Cl^-$ cotransport. The overall result is a net uptake of KCl into the cell (regulatory volume increase, RVI), as the cotransported Na^+ is immediately replaced by K^+ through the Na^+/K^+ pump. As a consequence, the cellular volume increases again, and as soon as the initial cellular volume is restored the cotransport is inactivated [39]. This behavior is shared by a variety of cell, e.g. rat [23], but not human erythrocyte [1] or frog skin epithelial cells [93], while other cells regulate volume by concerted action of Na^+/H^+- and Cl^-/HCO_3^- exchange mechanisms, e.g., *Amphiuma* red cell [16] and/or human [43], and pig lymphocytes (E. Flaut, *in preparation*). With Ehrlich cells the relationship between Na^+-K^+-$2Cl^-$ cotransport and volume regulation is less obvious. Normal Ehrlich cells shrunken in hypertonic medium fail to restore their original volume [50]. Nonetheless, the Na^+-K^+-$2Cl^-$ cotransport is activated under these conditions, as indicated by an increase in cellular K^+ content and by an up to 20-fold acceleration of the furosemide-sensitive steady-state flux of Rb^+ [37, 39]. Apparently, the driving force under these conditions is too small for the required transport of KCl, probably owing to the high Cl^- content of these cells. This would explain why avian erythrocytes, whose Cl^- content is lower, do normalize their volume in hypertonic medium, and only fail to do so if the extracellular K^+ is as low as 2.5 mM or less. It might also explain why Ehrlich cells, that have been previously adjusted to hypotonic medium, fully normalize their volume through the Na^+-K^+-$2Cl^-$ cotransport if they shrink

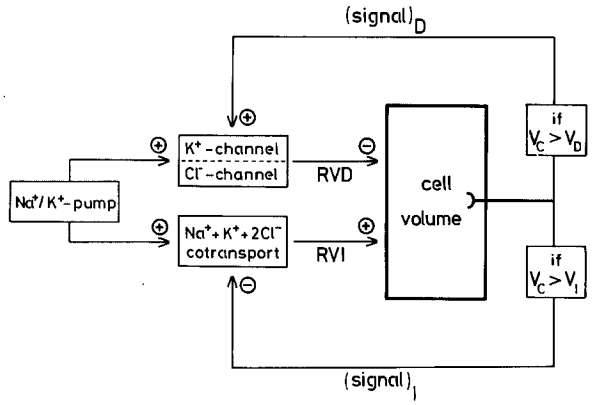

Fig. 4. Feedback control system for cell volume regulation by RVD and RVI. (For explanation, *see* text)

upon resuspension on isotonic medium [52, 54]. It seems that the release of KCl, by which they had overcome the swelling during the hypotonic pretreatment (volume regulatory decrease), favorably altered the driving force of the Na^+-K^+-$2Cl^-$ cotransport. Yet, the lack of driving force for the Na^+-K^+-$2Cl^-$ cotransport system does not fully explain the above-mentioned incompleteness of volume adjustment in hypertonic medium. If, for instance in Ehrlich cells, this driving force is increased by appropriately selected ion concentrations, the volume adjustment is improved, but still remains incomplete (P. Geck, *unpublished observation*). This has also been observed with turkey erythrocytes [92].

Loop diuretic-sensitive ion fluxes are also activated if the cells are shrunken by other than osmotic means: for instance by reducing Na^+, K^+, and/or Cl^- in an otherwise isotonic medium. The cotransport is then activated and can easily be inactivated again by increasing the cell volume, for instance by increasing intracellular content in the osmotic active solutes [39]. It thus appears that the cotransport process is part of a feedback loop to regulate cellular volume by RVI. It is activated when the cellular volume drops below the "set point" value, and is inactivated when this set point is attained or exceeded. Since also the volume regulation after hypotonic shock often seems to remain incomplete, the model hypothesis described below seems to account best for all observations (Fig. 4): Ehrlich cells and many other cell species are capable of regulating their volume in either direction: to increase their volume after shrinkage in hypertonic medium (RVI), on the one hand, and to decrease their volume after swelling in hypotonic medium (RVD), on the other hand. Each of these two processes appears to be effected by a different and separate mechanism: the volume increase (RVI) by the quaternary cotransport system described in this article, and the volume decrease (RVD) by gated K^+ and Cl^- channels [42, 53]. Both are in the end energized by the Na^+/K^+ exchange pump. Each of them appears to have its own "set point," at which it is activated, or inactivated, respectively. Neither of these two set points necessarily coincides with the "normal" cell volume: the set point of the RVD seems to be above this value and that of the RVI below it. In between there appears to be a "blind zone," within which the cellular volume may vary without initiating any of the regulatory mechanisms. As a consequence, the volume regulation may often remain incomplete. For instance, if after hypertonic shock the cellular volume shrinks below the set point, the Na^+-K^+-$2Cl^-$ cotransport system is activated until the volume of the corresponding set point is recovered. Even though at this point the volume is still well below the normal value, and even though the driving force of this cotransport mechanism is still sufficient to operate this process. Similarly, after the cell has swollen in hypotonic shock, shrinkage is effected by opening (gated) K^+- and Cl^- channels, but only until the corresponding set point of the volume is attained, which, however, may be well above the normal volume. Again, the regulation of the volume remains incomplete, although this time in the other direction. This discrepancy between the two set points with the resulting intermediate unregulated "blind" zone may seem disadvantageous. Their main purpose, however, may be to avoid that both mechanisms operate at the same time, as would lead to a dissipation of energy by futile cycles without any regulatory effect.

It is interesting to note that turkey erythrocytes [92] do not appear to possess a RVD mechanism. As a consequence, they can protect their volume from shrinkage, but not from swelling.

Some basic questions still remain:

1. How does the cell sense its volume and compare it with the set points. Several possibilities are thinkable:

a) A certain activator substance of the cotransport, dissolved in the cytoplasm, could be diluted or concentrated, respectively, by the volume change. This appears unlikely, since the cotransport responds already to volume changes by less than 10%, and the inducer concentration changes of the activator may be too small to be effective, unless extremely high cooperatively effects are involved.

b) Cotransport is normally active, upon expansion of the cyto-skeleton following the increase of volume, ligands of the cyto-skeleton might liberate

which inhibit the cotransport system. Attempts to inhibit this process by effectors acting on the cyto-skeleton such as cytochalasine B, colchicine, or vinblastine, even at very high concentrations, were unsuccessful with the Ehrlich cell.

c) Expansion of the cellular membrane could liberate an enzyme, normally hidden in a membrane fold, which catalizes generation of an inhibitor or destruction of an activator of cotransport. Such a hypothesis might explain the high sensitivity of the regulation to even small changes in volume. So far, however, this hypothesis is pure speculation and not supported by any evidence. At least for erythrocytes having a smooth surface, such a hypothesis is very unlikely.

2. The nature of the signal, which is emitted by a change in cellular volume and how it is perceived by the cotransport system, are not known yet. One may think of the involvement of cAMP, or Ca^{++}. But at least in Ehrlich cells neither of these mediators appears to be involved, as will be discussed further below. So far, there is no evidence of any other mediator to be involved either. In contrast, there is good evidence that Ca^{++} plays an important role in RVD.

It may be informative to compare the regulation of Na^+-K^+-$2Cl^-$ cotransport with that of other cellular functions, which also depend on the tonicity of the medium, such as the initiation of protein synthesis [60] and the repair of radiation-induced DNA damages [78], which are both inhibited by a hypertonic medium. The question arises whether the regulating signals are the same in such processes and in cotransport? It is noteworthy that both protein synthesis and cotransport respond to volume changes, independent of how these are induced [39].

Influence of Hormones on the Cotransport

In avian erythrocytes the cotransport system is strongly enhanced if the cellular cAMP level is raised either directly, by the addition of dbcAMP, or indirectly, by catecholamines [6, 32, 45, 63, 70, 75, 84, 92]. The effect is inhibitable by propanolol indicating a β-adrenergic receptor being involved. In duck erythrocytes cAMP raises the "set point" of cellular volume regulation. It appears safe to assume that cAMP does not represent the signal required to turn the transport mechanism on or off, respectively, during the regulation [6, 92], since during activation of the transport process by hypertonic shrinkage cAMP-level is uninfluenced. It seems to be more likely that cAMP-mediated phosphorylation may influence the volume sensor mechanism and/or the set point value.

In Ehrlich cells cAMP does not appear to be involved in the control of cotransport at all. Theophylline in high concentrations inhibits the cotransport, though presumably by lowering the "set point" value of the cellular volume. It is unlikely that this effect involves cAMP, as no change in cAMP could be detected. On the other hand, epinephrine has no additive effect on the cotransport, even though it raises the cAMP level drastically. Other inhibitors of phosphodiesterase, such as rolipram or Ro-20 1724, do not affect the cotransport either, not even in the presence of isoprenaline, in spite of an appreciable rise in cAMP. No change in the cAMP level could be observed in Ehrlich cells if the cellular volume was varied.

After stimulation of quiescent cells by the addition of serum or of growth factors such as EGF, the first noticeable effect is an enhancement of ion transport, in particular, the furosemide-sensitive K^+ influx [8, 21, 72, 77, 81]. This effect is only transient and leads to a net uptake of osmolarity, followed by an increase in cellular volume. Alterations of DNA—and protein synthesis are observable only later, when ion transport has been depressed again and a new steady-state volume of the cell has been established. This phenomenon is similar to that of avian erythrocytes induced by catecholamines. Also here the phenomenon is consistent with the assumption of an altered set point for the RVI.

Both serum and growth factors induce phosphorylation of a protein, presumably through a protein kinase C, since the effect of growth factors can be enhanced by TPA and some other phorbol esters that activate protein kinase C. Those phorbol esters, that do not stimulate the kinase, do not activate the ion transport either.

The stimulation of transepithelial transport in the Henle loop by ADH or its inhibition by prostaglandins probably do not involve the quaternary cotransport system directly, but rather the K^+ channels of the luminal membrane [51]. Similar findings or observations are reported for acetylcholine and cerulein on pancreas cells [88].

Intracellular Ca does not appear to affect the cotransport: its increase through the ionophore A23187 does not prevent inactivation of the cotransport after the "set point" value has been attained, nor does it prevent maximal cotransport activity in shrunken cells. Depletion of cellular Ca does not alter the regulation of cellular volume through the cotransport system. For avian erythrocytes, too, participation of Ca in volume regulation by RVI is excluded, in contrast to volume regulation by RVD, which clearly depends on Ca.

The correlation between loop diuretic-sensitive

cotransport and various pathological states has been extensively studied, but obviously without convincing results. Some of the reported findings are even contradictory, for instance in hereditory hypertension [18, 22, 24, 30, 31, 44, 94–96] the cotransport in human erythrocytes is reported by some authors to be increased, and by others to be decreased or unaltered. The correlation is not much better with other pathological states. Presumably, human erythrocytes are not very suitable for such studies, as they are unable to regulate volume by varying their cotransport rate, in contrast to avian erythrocytes and other nucleated cells.

Chemical Nature of the Transporter

It could be shown in various laboratories that labeled furosemide or bumetanide is specifically bound to isolated membranes of rabbit kidney and of the rectal gland of the shark [27, 46]. This binding is enhanced by K^+ and Na^+ and inhibited by Cl^-.

Using UV irradiation ^3H-bumetanide could be attached covalently and saturably to a membrane protein with the molecular weight of 34 kD [57]. This protein is claimed to be a part of the transport mechanism, though an irreversible and specific inhibition of the Na^+-K^+-$2Cl^-$ transport has not been demonstrated yet. On the other hand, it could be demonstrated with fibroblasts that UV irradiation in the presence of bumetanide irreversibly inhibits this cotransport system. This inhibition is rather specific, provided that the bumetanide concentration is not too high, since otherwise other transport pathways are also affected [7].

In other laboratories it has been found that stimulation of the cotransport by catecholamines, a protein of molecular weight of 200 kD is phosphorylated [5, 82]. Incubation in hypertonic medium, however, does not lead to this phosphorylation, even though the transport is activated. Presumably, the phosphorylated protein is not involved in the transport process directly, but may have a function in the regulatory control system for transport. The same may be true with the 37-kD membrane protein which binds cAMP [6, 76], possibly the protein kinase which phosphorylates the 200-kD protein, since the transport itself is not directly stimulated by cAMP.

References

1. Adraga, N.C., Tosteson, D.C. 1984. *J. Membrane Biol.* **78**:43–52
2. Aiton, J.F., Brown, C.D.A., Ogden, P., Simmons, N.L. 1982. *J. Membrane Biol.* **65**:99–109
3. Aiton, J.F., Chipperfield, A.R., Lamb, J.F., Simmons, N.L. 1981. *J. Physiol. (London)* **310**:208
4. Aiton, J.F., Chipperfield, A.R., Lamb, J.F., Ogden, P., Simmons, N.L. 1981. *Biochim. Biophys. Acta* **646**:389–398
5. Alper, S.L., Beam, K.G., Greengard, P. 1979. *J. Biol. Chem.* **255**:4864–4871
6. Alper, S.L., Palfrey, H.C., De Riemer, S.A., Greengard, P. 1980. *J. Biol. Chem.* **255**:11029–11039
7. Amsler, K., Kinne, R. 1985 (*Abstr.*). *Pfluegers Arch. (Suppl.)* **403**:R94
8. Atlan, H., Snyder, D., Panet, R. 1984. *J. Membrane Biol.* **81**:181–188
9. Aull, F. 1982. *Biochim. Biophys. Acta* **688**:740–746
10. Bakker-Grunwald, T. 1978. *Biochim. Biophys. Acta* **513**:292–295
11. Bakker-Grunwald, T. 1979. *J. Membrane Biol.* **47**:171–183
12. Bakker-Grunwald, T. 1981. *Biochim. Biophys. Acta* **641**:427–431
13. Burckhardt, B.-C. 1979. Ph.D. Thesis, Frankfurt
14. Burg, M.B. 1976. *Kidney Int.* **9**:189–197
15. Burg, M.B., Stoner, L., Cardinal, J., Green, N. 1973. *Am. J. Physiol.* **225**:119–124
16. Cala, P.M. 1980. *J. Gen. Physiol.* **76**:683–708
17. Candia, O. 1973. *Biochim. Biophys. Acta* **298**:1011–1014
18. Canessa, M., Bize, I., Adragna, N., Tosteson, D.C. 1982. *J. Gen. Physiol.* **80**:149–168
19. Chipperfield, A.R. 1980. *Nature (London)* **286**:281–282
20. Chipperfield, A.R. 1981. *J. Physiol. (London)* **312**:435–444
21. Dicker, P., Rozengurth, E. 1978. *Nature (London)* **276**:723–726
22. Duhm, J., Göbel, B.O. 1982. *Hypertension* **4**:468–476
23. Duhm, J., Göbel, B.O., Beck, F.-X. 1983. *Hypertension* **5**:642–652
24. Duhm, J., Göbel, B.O., Lorenz, R., Weber, P.L. 1982. *Hypertension* **4**:477–482
25. Ellory, J.C., Dunham, P.B., Logue, P.J., Stewart, G.W. 1982. *Proc. Natl. Acad. Sci. USA* **77**:1711–1715
26. Epstein, J., Silva, P. 1985. *Proc. Natl. Acad. Sci. USA (in press)*
27. Forbush, B., Palfrey, H.C. 1983. *J. Biol. Chem.* **258**:11787–11792
28. Frizzell, R.A., Dugas, M., Schultz, S.G. 1975. *J. Gen. Physiol.* **65**:769–795
29. Frizzell, R.A., Field, M., Schultz, S.G. 1979. *Am. J. Physiol.* **236**:*F1–F8*
30. Garay, R.P. 1982. *Lancet* **1**:501–502
31. Garay, R. P., Hannaert, P., Dagher, G., Nazaret, C., Maridonneau, I., Meyer, P. 1981. *Clin. Exp. Hypertens.* **3**:851–859
32. Gardiner, J.D., Aurbach, G.D., Spiegel, A.M., Brown, E.M. 1976. *Recent Progr. Horm. Res.* **32**:567–595
33. Gargus, J.J., Miller, L.L., Slayman, C.W., Adelberg, E.A. 1978. *Proc. Natl. Acad. Sci. USA* **75**:5589–5593
34. Gargus, J.J., Slayman, C.W. 1980. *J. Membrane Biol.* **52**:245–256
35. Geck, P. 1979. *Kidney Int.* **17**:405
36. Geck, P., Heinz, E. 1980. *Ann. N.Y. Acad. Sci.* **341**:57–62
37. Geck, P., Heinz, E., Pfeiffer, B. 1981. *Scand. Audiol. (Suppl.)* **14**:25–37
38. Geck, P., Heinz, E., Pietrzyk, C., Pfeiffer, B. 1978. *In:* Cell Membrane Receptors for Drugs and Hormones: A Multidisciplinary Approach. R.W. Straub and L. Bolis, editors. pp. 301–307. Raven, New York

39. Geck, P., Pfeiffer, B. 1985. *Ann. N.Y. Acad. Sci.* **456:**166–182
40. Geck, P., Pietrzyk, C., Burckhardt, B.-C., Pfeiffer, B., Heinz, E. 1980. *Biochim. Biophys. Acta* **600:**432–447
41. Greger, R., Schlatter, S. 1981. *Pfluegers Arch.* **392:**92–94
42. Grinstein, S., Clarke, C.A., Rothstein, A. 1982. *Phil. Trans. R. Soc. London B* **299:**509–518
43. Grinstein, S., Clarke, C.A., Rothstein, A. 1983. *J. Gen. Physiol.* **83:**341–370
44. Gunn, R.B., Silvers, D.N., Rosse, W.F. 1972. *J. Clin. Invest.* **51:**1043–1050
45. Haas, M., Schmidt, W.F., III, McManus, T.J. 1982. *J. Gen. Physiol.* **80:**125–147
46. Hannafin, J., Kinne-Saffran, E., Friedman, D., Kinne, R. 1983. *J. Membrane Biol.* **75:**73–83
47. Heinz, E. 1974. *In:* Current Topics in Membranes and Transport. Vol. 5, pp. 137–159. F. Bronner and A. Kleinzeller, editors. Academic, New York
48. Heinz, E. 1985. *Ann. N.Y. Acad. Sci.* **456:**1–9
49. Heinz, E., Geck, P., Pietrzyk, C., Pfeiffer, B. 1977. *In:* Biochemistry of Membrane Transport. G. Semenza and E. Carafoli, editors. pp. 236–249. FEBS Symp. No. 42. Springer Verlag, Berlin—Heidelberg
50. Hendil, K.B., Hoffmann, E.K. 1974. *J. Cell. Physiol.* **84:**115–126
51. Herbert, S.C., Andreoli, T.E. 1984. *Am. J. Physiol.* **246:***F*745–*F*756
52. Hoffmann, E.K. 1982. *Phil. Trans. R. Soc. London B* **299:**519–535
53. Hoffmann, E.K., Simonsen, L.O., Lambert, I.H.H. 1984. *J. Membrane Biol.* **78:**211–222
54. Hoffmann, E.K., Sjoholm, C., Simonsen, L.O. 1983. *J. Membrane Biol.* **76:**269–280
55. Hoffmann, J.F., Kregenow, F.M. 1966. *Ann. N.Y. Acad. Sci.* **137:**566–576
56. Jayme, D.W., Adelberg, E.A., Slayman, C.W. 1981. *Proc. Natl. Acad. Sci. USA* **78:**1057–1061
57. Jørgensen, P.L., Petersen, J., Rees, W.D. 1984. *Biochim. Biophys. Acta* **775:**105–110
58. Kinne, R., Koenig, B., Hannafin, J., Kinne-Saffran, E., Scott, D.M., Zierold, R. 1985. *Pflueger's Arch* **405, Suppl. 1:**101–105
59. Knauf, P.A., Rothstein, A. 1971. *J. Gen. Physiol.* **58:**190–210
60. Koch, G., Bilello, J.A., Kruppa, J., Koch, F., Oppermann, H. 1980. *Ann. N.Y. Acad. Sci.* **339:**280–306
61. Koenig, B., Ricapito, S., Kinne, R. 1983. *Pfluegers Arch.* **399:**173–179
62. Kregenow, F.M. 1971. *J. Gen. Physiol.* **58:**396–412
63. Kregenow, F.M. 1977. *In:* Osmotic and Volume Regulation. C.B. Jorgensen and E. Skadhage, editors. pp. 379–391. Academic, New York
64. Kregenow, F.M. 1981. *Annu. Rev. Physiol.* **43:**493–505
65. Lauf, P.K. 1983. *J. Membrane Biol.* **73:**246–256
66. Lauf, P.K., Theg, B.E. 1980. *Biochem. Biophys. Res. Commun.* **92:**1422–1428
67. McRoberts, J.A., Erlinger, S., Rindler, M.J., Saier, M.H., Jr. 1982. *J. Biol. Chem.* **257:**2260–2266
68. Mills, B., Tupper, J.T. 1975. *J. Membrane Biol.* **20:**75–97
69. Mills, B., Tupper, J.T. 1976. *J. Cell. Physiol.* **89:**123–132
70. Nellans, H.N., Frizzell, R.A., Schultz, S.G. 1973. *Am. J. Physiol.* **225:**467–475
71. Oberleithner, H., Guggino, W., Giebisch, G. 1983. *Pfluegers Arch.* **396:**27–33
72. Owen, N.E. 1984. *Biochem. Biophys. Res. Commun.* **125:**500–508
73. Owen, N.E., Prastein, M.L. 1985. *J. Biol. Chem.* **260:**1445–1451
74. Palfrey, H.C. 1985. *Physiologist (in press)*
75. Palfrey, H.C., Feit, P.W., Greengard, P. 1980. *Am. J. Physiol.* **238:***C*139–*C*148
76. Palfrey, H.C., Greengard, P. 1981. *Ann. N.Y. Acad. Sci.* **372:**291–307
77. Panet, R. 1984. *Biochim. Biophys. Acta* **813:**141–144
78. Pohlit, W., Heyder, I.R. 1981. *Rad. Res.* **87:**613–634
79. Rindler, M.J., McRoberts, J.A., Saier, M.H., Jr. 1982. *J. Biol. Chem.* **257:**2254–2259
80. Rocha, A.S., Kokko, J.P. 1973. *J. Clin. Invest.* **52:**612–623
81. Rozengurt, E., Mendoza, S. 1980. *Ann. N.Y. Acad. Sci.* **339:**175–190
82. Rudolph, S.A., Greengard, P. 1980. *J. Biol. Chem.* **255:**8534–8540
83. Schlatter, E., Greger, R., Weidtke, C. 1983. *Pfluegers Arch.* **396:**210–217
84. Schmidt, W.F., III, McManus, T.J. 1977. *J. Gen. Physiol.* **70:**59–121
85. Sessler, M., Maul, F.D., Geck, P., Hör, G. 1983. *In:* Nuclear Medicine and Biology. C. Ragnand, editor. pp. 2281–2284. Pergamon, Oxford
86. Sessler, M.J. 1982. M.D. Thesis, Frankfurt
87. Silva, P., Stoff, J.S., Solomon, R.J., Rosa, R., Stevens, A., Epstein, J. 1980. *J. Membrane Biol.* **53:**215–221
88. Singh, J. 1984. *Biochim. Biophys. Acta* **775:**77–85
89. Spaggiare, S., Wallach, M.J., Tupper, J.T. 1976. *J. Cell. Physiol.* **89:**403–416
90. Tupper, J.T. 1975. *Biochim. Biophys. Acta* **394:**586–596
91. Tupper, J.T., Zorgniotti, F., Mills, B. 1977. *J. Cell. Physiol.* **91:**429–440
92. Überschär, S., Bakker-Grunwald, T. 1983. *Biochim. Biophys. Acta* **731:**243–250
93. Ussing, H.H. 1982. *Acta Physiol. Scand.* **114:**363–369
94. Walter, U., Distler, D. 1982. *Hypertension* **4:**205–210
95. Wiley, J.S. 1977. *In:* Membrane Transport in Red Cells. J.C. Ellory, V.L. Lew, editors. pp. 337–361. Academic, New York
96. Wiley, J.S. 1981. *J. Clin. Invest.* **67:**1094–1102
97. Wiley, J.S., Cooper, R.A. 1974. *J. Clin. Invest.* **53:**745–755
98. Zeuthen, T., Ramos, M., Ellory, J.C. 1978. *Nature (London)* **273:**687–689

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