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BUMETANIDE-SENSITIVE POTASSIUM TRANSPORT AND VOLUME REGULATION IN TURKEY ERYTHROCYTES

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The bumetanide-sensitive ($K^+ + Na^+ + 2Cl^-$)-cotransport system in turkey erythrocytes is activated by either of two treatments: addition of epinephrine or an increase in osmolarity. At elevated (20 mM) K^+ concentration, cotransport activity induced by epinephrine slowly (within 90 min) declines to background level again. This time-dependent inactivation has been linked to bumetanide-sensitive cell swelling. We have compared both the initial rate of cotransport activity and its time dependence after induction by either epinephrine, increased osmolarity or a combination of the two treatments. As a measure of cotransport activity we took the bumetanide-sensitive fraction of $^{86}Rb^+$ influx. Immediately after activation, several kinetic characteristics of this flux (V_{max}; K_m towards K^+; K_i towards bumetanide; pH profile) were identical in cells activated by either treatment. By contrast, cotransport activated by hypertonicity was significantly more resistant towards subsequent inactivation. We show this to be due to the increase in intracellular ion concentrations brought about by hypertonic cell shrinkage. This tended to reverse the driving force for cotransport, and thereby prevented the bumetanide-sensitive swelling associated with inactivation. Our data support the notion that cell volume plays a key role both in the activation and in the time-dependent inactivation of bumetanide-sensitive transport.

Introduction

It has long been known that bird erythrocytes can be induced to take up salt and water by two essentially different experimental treatments: hypertonic shock and the addition of catecholamines (for reviews, see Refs. 1, 2). Subsequent evidence has suggested that both those treatments activate one and the same transport system [3]. This system is separate from the ouabain-sensitive Na^+-pump [4], and inhibited by loop diuretics such as furosemide or bumetanide [5]. It mediates the cotransport of ($1K^+ + 1Na^+ + 2Cl^-$) [6,7].

Little is known about the two activation pathways. On the one hand, catecholamines have been shown to exert their action through the β-adrenergic receptor and through cyclic AMP [3]. Greengard and coworkers [8] have demonstrated the phosphorylation of a membrane protein (molecular weight, 230000) simultaneous with activation of transport. On the other hand, hypertonicity activates the cotransport system by decreasing cellular volume [3]. Neither cyclic AMP [3] nor phosphorylation of a specific membrane protein [8] appear to be involved in the hypertonic activation pathway. However, in view of certain kinetic similarities in the transport activated by either treatment it has been inferred [3] that both activation pathways converge onto a distal rate-limiting step.

A special feature of the epinephrine-activated transport is its 'refractoriness' towards the hormone [9,10] at elevated K^+ concentrations: at 20 mM K^+, after an initial burst of cotransport

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activity following hormonal stimulation, transport rates decline to the basal level again within 1–2 h. This phenomenon has been attributed to the cell swelling that occurs secondary to cotransport activation [10].

The experiments presented here were initiated to further define the relation between the hormonal and hypertonic activation pathways, and the role of cellular volume in the subsequent inactivation of cotransport activity. Our results confirm that the initial kinetics of cotransport activity are independent of the activation pathway. By contrast, the subsequent behaviour of bumetanide-sensitive transport in time was different after the two activating treatments: hypertonic conditions protected against the inactivation normally occurring at elevated K^+ concentrations. We explain this by the effect of osmolarity on the ion gradients, and thereby on the direction of net bumetanide-sensitive salt- and water movements: hypertonicity tended to favour bumetanide-sensitive salt efflux and cell shrinkage, thereby counteracting the inactivation associated with cell swelling.

Methods and Materials

Erythrocytes were obtained from the wing vein of two female turkeys, and washed twice in a saline solution containing (mM): NaCl, 120; KCl, 2.5; $MgCl_2$, 1; Hepes, 20, neutralized with NaOH, 10. pH was 7.4 at 38°C. Cells were diluted to a hematocrit of approx. 20% and incubated for 60–90 min at 38°C in the same medium supplied with 10- mM glucose. After the preincubation K^+ concentration and osmolarity were adjusted as indicated. Osmolarity of the standard saline (plus glucose) was 277 mosM, as determined with a freezing-point osmometer (Vogel OM 801).

For the determination of the initial rate of bumetanide-sensitive K^+ influx, the final cell concentration was approx. 5%. Epinephrine, when present, was added 15 min before $^{86}Rb^+$. The Na^+-pump was inhibited by ouabain (0.2–0.4 mM) 2–5 min before addition of the label. At zero time, $^{86}Rb^+$ (as a tracer for K^+ [1]) was added at 10–50 nCi/ml. After 30 min, duplicate 1-ml samples were spun for 1 min in microfuge tubes containing 0.2 ml silicone oil (Rotitherm H, Roth). After samples of the supernatant were taken, supernatant and most of the silicon oil were aspirated. Pellets were lyzed with distilled water, and protein precipitated with trichloroacetic acid (final concentration, 5%). Radioactivity of cell extracts and supernatants was measured as Cerenkov-radiation in water, in a Packard scintillation counter at a setting corresponding to the ^3H-peak. In this procedure pellet radioactivity contained a component due to the extracellular volume (approx. 15% of the total pellet volume as determined with [^{14}C]sucrose). However, since the extracellular space was not influenced by bumetanide (not shown), and uptake was linear over the first 30 min [6], bumetanide-sensitive K^+ influx was directly calculated from the difference in 30-min uptake in the presence and absence of bumetanide (10–100 μM).

For the experiment of Fig. 3, $^{86}Rb^+$ was added at different time points after activation of bumetanide-sensitive transport. After the cells had been allowed to take up the label for 10 min, duplicate 1-ml samples were spun for 1 min in microfuge tubes (without silicon oil). A sample of the supernatant was taken. To get rid of the extracellular medium, the pellets were washed twice in ice-cold saline (100 mM $MgCl_2$/20 mM Tris-HCl, pH 7.5). From there on, pellets and supernatants were processed as described above.

For the determination of cell water, duplicate samples of 20% cell suspensions were spun for 1 min in preweighed 0.4 ml microfuge tubes. A sample of the supernatant was taken for determination of the extracellular K^+ concentration (by flame photometry). The supernatant and the top layer of the pellet were carefully aspirated. The tubes were weighed, dried overnight at 95°C, and weighed again. Taking the extracellular volume to be 15% of total pellet volume, we calculated cell water (g/g dry wt.) as ((0.85 × wet wt./dry wt.) − 1).

$^{86}Rb^+$ was obtained from Amersham. Bumetanide was the kind gift of Dr. P.W. Feit, Leo, Ballerup, Denmark. All other chemicals were analytical grade.

Expression of results

For the calculation of unidirectional influx, we took $^{86}Rb^+$ to be an ideal tracer for K^+ in these cells [1]. Influx is expressed per ml cells. This

refers to the original hematocrit in standard saline. The results can be converted to a dry wt. base (influx per g dry wt.) by multiplication with a factor 1.74 (see Results).

All experimental points are the average of duplicate determinations. Error bars, where indicated, denote half of the difference between duplicates. For the influx experiments, most duplicates were equal within 10%; for the volume experiments, within 1%.

Results

Initial kinetics of bumetanide-sensitive K^+ influx

In view of the fact that epinephrine and an increase in osmolarity appear to activate one and the same transport system [3,1], we set out to investigate whether there was any interaction between the two activating treatments. As a measure of cotransport activity we took the bumetanide-sensitive fraction of unidirectional $^{86}Rb^+$ influx ($^{86}Rb^+$ serving as a tracer for K^+ in these cells [1]). We determined this influx in media made hypertonic by increasing concentrations of the inert sugar sorbitol, in the absence or presence of a maximally-activating concentration of epinephrine (Fig. 1). In the absence of epinephrine, bumetanide-sensitive transport increased with osmolarity, up to a maximum at about 500 mosM. In the presence of epinephrine, $^{86}Rb^+$ influx at all osmolarities was equal to the influx activated by a saturating increase in osmolarity alone. Qualitatively similar results were found at 2.5 mM and at 20 mM K^+ (Fig. 1). They are in agreement with earlier data on duck red cells [3], and with the notion [3] that the pathways of activation by epinephrine and by hypertonicity converge upon a distal rate-limiting step.

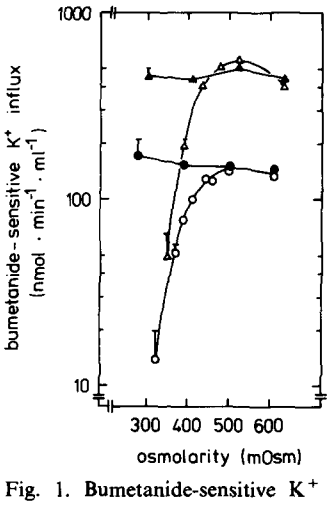

Fig. 1. Bumetanide-sensitive K^+ influx after activation by increasing osmolarity plus or minus epinephrine. The experimental protocol was as described in Methods. Osmolarity was increased by the addition of sorbitol. $^{86}Rb^+$ was added within 15 min after activation. ○, $[K^+]_o = 2.5$ mM, no epinephrine; ●, $[K^+]_o = 2.5$ mM, plus 10 μM epinephrine; △, $[K^+]_o = 20$ mM, no epinephrine; ▲, $[K^+]_o = 20$ mM, plus 10 μM epinephrine.

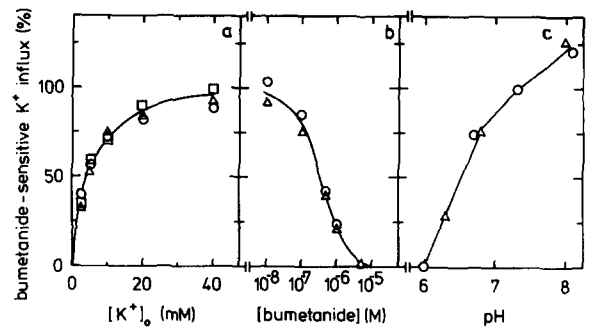

Fig. 2. Bumetanide-sensitive K^+ influx after maximal activation by hypertonicity and/or epinephrine as a function of $[K^+]_o$, bumetanide concentration and pH. For Fig. 2a, $[K^+]_o$ was varied between 2.5 and 40 mM; Cl^--concentration was kept constant by addition of choline chloride (37.5–0 mM). For Figs. 2b and 2c, $[K^+]_o$ was 2.5 mM. For Fig. 2c, the pH was adjusted from its normal value of 7.4 with HCl or NaOH (0.1 M). Bumetanide-sensitive transport was activated by: ○, (a, b): 10 μM epinephrine, (c): 10 μM epinephrine plus 1 mM cyclic AMP; △, 200 mM sorbitol; □, 10 μM epinephrine plus 200 mM sorbitol. 100% values corresponded to (nmol · min^{-1} · ml^{-1}): (a) 1170; (b) 330; (c) 380.

Fig. 2a shows the dependence of bumetanide-sensitive $^{86}Rb^+$ influx on K^+ concentration after activation by either a saturating increase in osmolarity, a saturating concentration of epinephrine, or a combination of those two conditions. It can be seen that, independent of the activating treatment, the apparent K_m for K^+ was approx. 4 mM. Also the apparent K_i towards bumetanide (Fig. 2b; approx. 0.3 μM, see also Ref. 5) and the pH-profile of bumetanide-sensitive $^{86}Rb^+$ influx (Fig. 2c) were identical after either mode of activation.

Time dependence of bumetanide-sensitive transport activity

The data so far have shown that, by several kinetic criteria, the initial rate of $^{86}Rb^+$ influx activated by epinephrine was indistinguishable from that activated by an increase in osmolarity. However, the time-dependent behaviour of $^{86}Rb^+$ influx was different after the two modes of activation. This is illustrated in Fig. 3. After activation by epinephrine at normal osmolarity, transport in the presence of an elevated K^+ concentration (20 mM) declined again in time, as has been found before [9,10]. By contrast, at the same K^+ concentration, transport activated by hypertonic conditions (200 mosM sorbitol) was completely stable (Fig. 3, left half). Transport was also stable when epinephrine was added together with sorbitol (not shown). Thus, the increase in osmolarity protected bumetanide-sensitive transport against 'refractoriness' [9] towards epinephrine. Moreover, the same increase in osmolarity restored bumetanide-sensitive transport activity in 'refractory' cells (Fig. 3, right half). At 2.5 mM K^+, bumetanide-sensitive transport was stable both at normal [1,10] and at elevated osmolarity (not shown). Thus it appeared that extracellular K^+ concentration and medium osmolarity affect the stability of cotransport in opposite ways. We have further investigated this point in the next experiments.

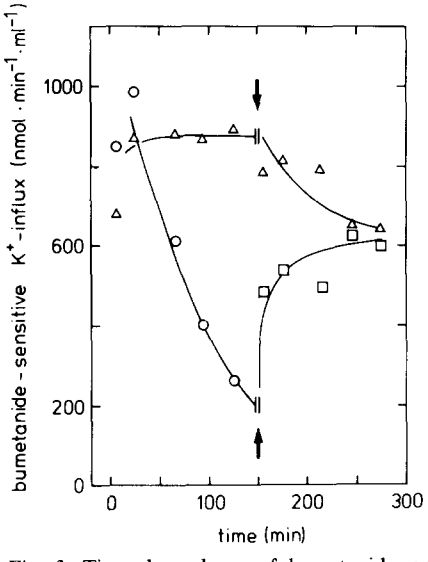

Fig. 3. Time dependence of bumetanide-sensitive K^+ influx after activation by hypertonicity or epinephrine. Influx was followed over 10-min periods (centered around the indicated time points) as described in Methods. Zero time refers to the moment of activation. $[K^+]_o$ was 20 mM. Cells were activated either by 10 μM epinephrine (○) or by 200 mM sorbitol (△). At $t = 150$ min, the osmolarity of the cells originally activated by epinephrine (○) was raised by 200 mosM by the addition of an equal volume of saline containing 400 mM sorbitol (□). At the same time, the cells originally activated by sorbitol (△) were diluted with an equal volume of saline again containing 200 mM sorbitol.

As a measure of the stability of cotransport activity we defined a constant, R, as the ratio of bumetanide-sensitive $^{86}Rb^+$ influx 90 min after activation to that immediately after activation. We determined R at various osmolarities and K^+ concentrations, with and without epinephrine. At each K^+ concentration, R increased with osmolarity (Fig. 4a); conversely, at each osmolarity, R decreased with increasing K^+ concentration (Fig. 4b). In cells that are maximally activated to start with, R theoretically ranges between 0 (total loss of activity after 90 min) and 1 (total retention of activity after 90 min). Experimentally, we found an upper limit for R of 1.1 for cells that were exposed to both epinephrine and increasing hypertonicity (Fig. 4). For cells exposed to hypertonic conditions only, we occasionally obtained values up to $R = 1.7$ (not shown). For the following, we define those sets of conditions for which $R = 0.9$ as 'activity balance points' (we took this value rather than $R = 1$ to allow for any aspecific degen-

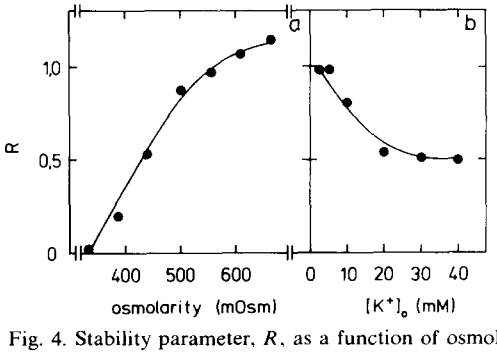

Fig. 4. Stability parameter, R, as a function of osmolarity and $[K^+]_o$. R is defined as the ratio of bumetanide-sensitive K^+ influx between 90 and 110 min after activation ($^{86}Rb^+$ being added at $t = 90$ min) to that measured between 0 and 20 min after activation ($^{86}Rb^+$ being added at zero time). For Expt. 4a, $[K^+]_o$ was 20 mM. For Expt. 4b, osmolarity was 440 mosM; Cl^- concentration was kept constant by the addition of choline chloride (up to 37.5 mM). Epinephrine (10 μM) was present in all incubations.

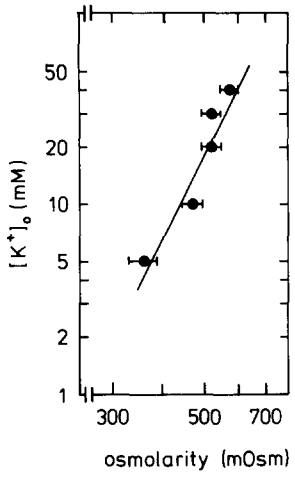

Fig. 5. Activity balance points as a function of $[K^+]_o$ and osmolarity. This figure summarizes the combinations of $[K^+]_o$ and osmolarity for which $R = 0.9$, as determined in a series of experiments similar to those of Fig. 4. For each K^+ concentration, the ends of the bars indicate the osmolarities of the two measured R values bordering the $R = 0.9$ intersection point. Slope of the line, calculated by linear regression, is 4.5.

eration of bumetanide-sensitive transport activity).

Fig. 5 summarizes the activity balance points in a double-logarithmic $[K^+]_o$/osmolarity plane for a series of determinations similar to those of Fig. 4. Two areas can be distinguished: towards the higher left, tansport activities were increasingly unstable; towards the lower right, activities were stable or even increased in time. The line through the balance points separating the two areas has a slope of about 4. This result is not surprising. It indicates that instability of bumetanide-sensitive transport is a consequence of the uptake of four osmotically-active particles per K^+; and thereby confirms [10] that inactivation is a consequence of cell swelling caused by bumetanide-sensitive transport itself. This can be intuitively understood by the following argument. For any point (combination of osmolarity and K^+ concentration) on the line of Fig. 5, the cotransport system is 'balanced'; i.e., the efflux of $(K^+ + Na^+ + 2Cl^-)$ exactly compensates for its influx. Assuming ideal osmotic behaviour of the cells, the immediate effect of a change in osmolarity will be a proportional change in each of the intracellular concentrations of K^+, Na^+ and Cl^-. Under those conditions the cotransport system could in principle be kept 'balanced' (on the line) by a proportional change in extracellular concentration of each of the ions involved. However, in our experimental setup $[Na^+]_o$ and $[Cl^-]_o$ were kept constant. Thus, to keep the system balanced, $[K^+]_o$ has to compensate for the changes in intracellular concentration of those other ions as well. This ultimately is reflected in the fourth-power interrelationship between $[K^+]_o$ and osmolarity found in Fig. 5. A formal derivation is given in the Appendix.

The argument so far implies that the upper-left area of Fig. 5 is associated with cell swelling, the lower-right area with cell shrinkage, and that for the line separating the two areas cell volume remains constant in time. To test this, we performed the experiment of Fig. 6. Cell volume was determined 60 min after activation by epinephrine, in the presence (open symbols) or absence (closed symbols) of bumetanide, at various K^+ concentrations and osmolarities. In the presence of bumetanide cell volume after 60 min varied inversely with osmolarity as expected, and was virtually independent of the K^+ concentration. In the absence of bumetanide, cell volume at all osmolarities showed an upward tilt as a function of K^+ concentration. At each osmolarity, the intersection of the two lines, plus or minus bumetanide, gives that K^+ concentration at which bumetanide-sensitive transport activity did not result in any net

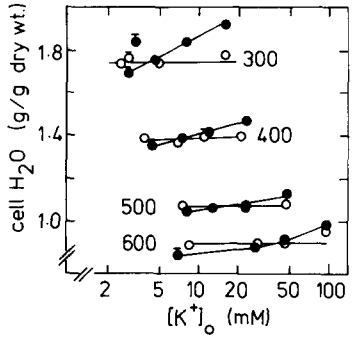

Fig. 6. Bumetanide-sensitive changes in cell water as a function of $[K^+]_o$ at four different osmolarities. Osmolarities were adjusted with sorbitol; the numbers in the figure indicate the final values (in mosM). All samples contained ouabain (0.2 mM) and epinephrine (10 μM). Incubations were started by transferring the suspensions from ice to a 38°C bath. After 60 min, duplicate samples were spun and treated as described in Methods. The K^+ concentrations indicated were those determined after 60 min in the supernatants. ●, no bumetanide; ○, plus 0.1 mM bumetanide.

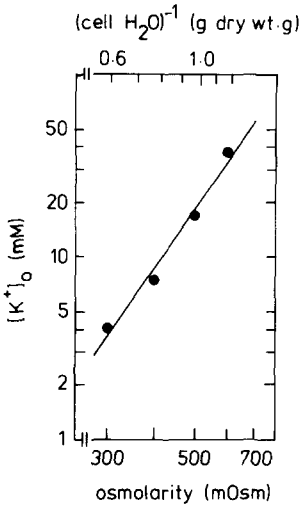

Fig. 7. Volume balance points as a function of a $[K^+]_o$ and osmolarity (or (cell water)$^{-1}$). The intersection points of Fig. 6 for the curves with and without bumetanide were replotted double-logarithmically. Slope of the line, calculated by linear regression, is 3.4.

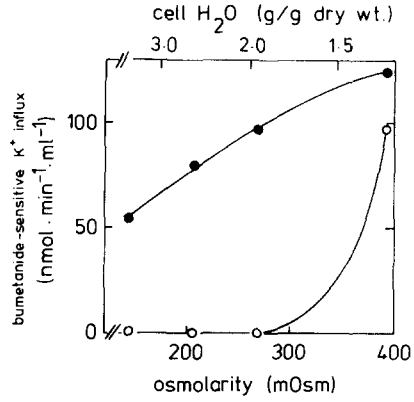

Fig. 8. Bumetanide-sensitive transport in hypotonically-treated cells. For this experiment, the basic saline contained (mM): NaCl, 60; KCl, 2.5; $MgCl_2$, 1; glucose, 10; Tris-HCl, 10, pH 7.4. Osmolarity was increased with sorbitol. The experiment was further performed as that of Fig. 1. ○, control; ●, plus 10 μM epinephrine. The scale for cell water was calculated assuming ideal osmotic behaviour of the cells.

volume change. We have double-logarithmically replotted those 'volume balance points' in Fig. 7. It can be seen that the line connecting them is practically identical to that in Fig. 5.

Figs. 7 and 5 together strongly support the notion that (i) independent of osmolarity, volume changes secondary to the activation of bumetanide-sensitive transport are based on the cotransport of four osmotically-active particles per K^+ ion; i.e., ($1K^+ + 1Na^+ + 2Cl^-$); and (ii) inactivation of cotransport is associated with cell swelling.

However, the relation between cell swelling and inactivation is not straightforward. Fig. 8 shows that in cells that were hypotonically preswollen to volumina by far exceeding those of 'refractory' cells (compare Fig. 6), bumetanide-sensitive transport could still be activated by epinephrine.

Lack of volume-regulatory decrease in turkey erythrocytes

From the experiment of Fig. 8, it appeared that cell swelling by itself did not increase K^+ influx. By contrast, Kregenow [11,2] has reported that duck red cells possess a mechanism to shrink back to their original volume ('volume-regulatory decrease', or VRD) based on an increased K^+ permeability in the swollen state. Apparently, such a mechanism, if present in turkey cells at all, was not very active there. In accordance with this notion we found that the cells reacted as ideal osmometers upon hypotonic shock, and maintained their expanded volume for at least 30 min (immediately after the cells were suspended at 220 mosM, cell water increased from 1.73 ± 0.02 to 2.36 ± 0.02 g/g dry wt.; after 30 min at 38°C, cell volume was 2.34 ± 0.02 g/g dry wt.).

Hypertonic volume control in other cell types is frequently exerted by a Ca^{2+}-dependent K^+ permeability (Gardos effect [12,13]). In human red cells, this permeability can be readily demonstrated after treatment of the cells in a Ca^{2+}-containing medium with the Ca^{2+}-ionophore A23187 [14] or the β-blocker propranolol [13]. Turkey red cells did not respond to either of these treatments (results not shown).

Discussion

Our results confirm that cell volume plays an important role both in the activation and in the time-dependent inactivation of bumetanide-sensitive transport. A model for the activation of bumetanide-sensitive transport in ascites cells has been proposed by Geck et al. [15]. This model states that the activity of the cotransport system is

determined by the discrepancy between the actual cell volume and a predetermined reference volume. We can fit our data on turkey cells into this model by inferring that hypertonicity activates cotransport by decreasing the real cell volume (similar to the situation in ascites cells [15]), whereas epinephrine activates by increasing the reference volume. Although this notion would serve to put the data on turkey cells into a well-defined framework, it leaves several important questions unanswered, notably: (i) what is the nature of the cellular 'memory' involved in the storage of the reference volume? and (ii) what is the nature of the signal indicating the discrepancy between real and reference volume? We have no clue yet as to the answers to these questions. Also the inactivation of cotransport, though clearly linked to cell volume, remains puzzling. In terms of the model by Geck et al. [15], inactivation would simply occur whenever the real cell volume approaches or exceeds the reference volume. Our (and others' [1]) data indicate however that the actual situation is more complex: cotransport could be activated by epinephrine at cell volumes that by far exceed those attained by 'refractory' cells (Figs. 6, 8). Conversely, after hypertonic treatment cotransport was inactivated long before the normal isotonic cell volume had been reached (Figs. 5, 6). This indicates that the time-dependent behaviour of bumetanide-sensitive transport activity is correlated with bumetanide-sensitive volume changes rather than with the absolute cell volume. Thus, in terms of the model of Ref. 15, either another factor, secondary to cell swelling, is involved in inactivation, or the reference volume somehow depends upon the real cell volume.

Nevertheless, the bumetanide-sensitive cotransport system can be, and has been [15,2], considered a volume-controlling device. Strikingly, it is activated only by negative deviations from the normal cell volume. Some cell types also respond to positive deviations of the reference volume with compensatory shrinkage. However, this type of volume control apparently is based on the action of one or more different transport systems. At least in some instances an electrogenic, Ca^{2+}-dependent K^+ permeability is involved [12]. In our turkey red cells such a system was lacking altogether.

Part of the data presented here were discussed at the annual meeting of the Deutsche Gesellschaft für Zellbiologie, München, March 1982 [16].

Appendix

The $(K^+ + Na^+ + 2Cl^-)$-cotransport system is 'balanced' under condition that the sum of the electrochemical gradients of the ions involved is equal to zero:

$$\Delta\tilde{\mu}_{K^+} + \Delta\tilde{\mu}_{Na^+} + 2\Delta\tilde{\mu}_{Cl^-} = 0$$

Since this cotransport is electroneutral, the electrical terms drop out:

$$RT \ln \frac{a^i_{K^+}}{a^o_{K^+}} + RT \ln \frac{a^i_{Na^+}}{a^o_{Na^+}} + 2RT \ln \frac{a^i_{Cl^-}}{a^o_{Cl^-}} = 0$$

Let this relation hold at osmolarity P. Taking the activity coefficients, a, to be equal to 1:

$$[K^+]^P_o = \frac{[K^+]^P_i \cdot [Na^+]^P_i \cdot ([Cl^-]^P_i)^2}{[Na^+]_o \cdot [Cl^-]^2_o}$$

A change in osmolarity to Q, at ideal osmotic behaviour of the cells, will change each of the intracellular ion concentrations, $[K^+]_i$, $[Na^+]_i$, and $[Cl^-]_i$ proportionally, by a factor (Q/P). Then, at constant $[Na^+]_o$ and $[Cl^-]_o$, the extracellular K^+ concentration 'balancing' cotransport at osmolarity Q will be:

$$[K^+]^Q_o = \frac{[K^+]^Q_i \cdot [Na^+]^Q_i \cdot ([Cl^-]^Q_i)^2}{[Na^+]_o \cdot [Cl^-]^2_o}$$

$$= (Q/P)^4 [K^+]^P_o$$

Therefore

$$[K^+]^Q_o / [K^+]^P_o = (Q/P)^4,$$

which is the relationship reflected in Figs. 5 and 7.

Acknowledgements

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gesting the experiment presented in the right half of Fig. 3, and him and Dr. Evert Bakker for critically reading the manuscript.

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