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THE ATP DEPENDENCE OF A OUABAIN-SENSITIVE SODIUM EFFLUX ACTIVATED BY EXTERNAL SODIUM, POTASSIUM AND LITHIUM IN HUMAN RED CELLS

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SUMMARY

The stimulation of ouabain-sensitive Na^+ efflux by external Na^+, K^+ and Li^+ was studied in control and ATP-depleted human red cells. In the presence of 5 mM Na^+, with control and depleted cells, Li^+ stimulated with a lower apparent affinity than K^+, and gave a smaller maximal activation than K^+. The ability of Na^+, K^+ and Li^+ to activate Na^+ efflux was a function of the ATP content of the cells. Relative to K^+ both Na^+ and Li^+ became more effective activators when the ATP was reduced to about one tenth of the control values. At this low ATP concentration Na^+ was absolutely more effective than K^+.

INTRODUCTION

When human red blood cells with normal ATP content are incubated in full sodium media the ouabain-sensitive Na^+ efflux in the presence of external potassium is larger than in K^+-free solutions. However, if the cells have been depleted of their internal energy by incubation with 2-deoxyglucose the ouabain-sensitive efflux of Na^+ is larger in the absence than in the presence of external potassium [1, 2]. This was interpreted to mean that in the energy-depleted cells the dephosphorylation step of the ATPase reaction associated with K^+ entry decreases the availability, at the inner surface of the membrane, of carriers involved in the shuttling of Na^+. A similar relationship between Na^+ efflux and ATP levels was observed in squid giant axons [3] but was related to a hypothetical second ATPase which was apparently inhibited by K^+ [4]. Some properties of the ATPase from broken-membrane preparations are relevant to this behaviour of Na^+ fluxes. In the cases where there is no K^+ in the solution but just Mg^{2+} and Na^+, there is a low level of a Na^+-dependent ATPase activity which is ouabain-sensitive [5, 6]. If the ATP concentration in the mixture is high (about 3 mM), K^+ added to the media increases the overall ATPase activity; but if ATP is in the micromolar range, the addition of K^+ results in an inhibition [4,7]. On the other hand ATP binds tightly to the enzyme in the absence of K^+ [8, 9]. Potassium reduces the affinity of the enzyme for ATP and conversely ATP reduces the affinity of the enzyme for K^+ [10]. The partial inhibition by K^+ at low ATP could

be explained if K^+ not only dephosphorylated the enzyme but also remained attached to it, thus reducing the affinity for ATP. At concentrations at which K^+ and Li^+ produced equal acceleration of dephosphorylation at low ATP, the levels of phosphoenzyme were higher in Li^+ than in K^+ solutions. To account for this Post et al. [11] proposed that the $E \cdot K^+$ complex was more stable than the $E \cdot Li^+$ one, with the consequence that rephosphorylation would be more difficult if K^+ was the dephosphorylating cation.

If the biochemical basis for the relative inhibition of Na^+ efflux by external K^+ at low ATP is that postulated for the ATPase reaction it is conceivable that some evidence could be obtained by comparing the abilities of K^+ and Li^+ to activate Na^+ efflux. In this regard one would expect that as $E \cdot Li^+$ is more unstable than $E \cdot K^+$ (easier to rephosphorylate) the relative ability of Li^+ over K^+ in stimulating a ouabain-sensitive Na^+ efflux would increase as the ATP content of the cells was reduced.

METHODS

Fresh blood samples from healthy donors collected in 10 % (V/V) sodium citrate were used in all cases.

Solutions. All solutions were prepared with deionized water and reagent grade chemicals. The composition of the incubation solutions for efflux measurements was as follows: (a) K^+-free sodium: NaCl, 150 mM; $MgCl_2$, 1 mM; Tris/phosphate, (pH at 37 °C = 7.4) 2.5 mM; (b) K^+-free choline/choline chloride, 150 mM, $MgCl_2$, 1 mM; Tris/phosphate, 2.5 mM. Different sodium concentrations in K^+-free conditions were obtained by mixing appropriate volumes of solutions a and b. (c) Potassium-containing solutions: NaCl, 5 mM; KCl, 0.2–10 mM; choline chloride, 145 mM–[K]; $MgCl_2$ 1 mM; Tris/phosphate, 2.5 mM; (d) lithium containing solutions: NaCl, 5 mM; LiCl, 10–145 mM; choline chloride, 145 mM–[Li]; $MgCl_2$, 1 mM; Tris/phosphate, 2.5 mM. All solutions were calcium-free. Ouabain was obtained from Sigma Co., U.S.A.

ATP depletion. After five washes in K^+-free sodium the cells were incubated for 12 h at room temperature and about 10 % hematocrit in solutions of the following composition: (a) control: NaCl, 148 mM; KCl, 2 mM; $MgCl_2$, 1 mM; Tris/phosphate, 2.5 mM; glucose, 11 mM; (b) depleted: NaCl, 140 mM; KCl, 10 mM; $MgCl_2$, 1 mM; Tris-phosphate, 2.5 mM; 2-deoxyglucose, 3 mM. Both solutions were calcium-free and contained in addition 1 μCi/ml of $^{22}Na^+$, 10 000 units/100 ml penicillin and 0.1 g/100 ml streptomycin. As internal ATP was depleted the ability of the cells to pump out sodium diminished. In preliminary experiments where the electrolyte composition of the solutions was the same in control and depleted cells, the latter ones had higher internal $[Na^+]$ at the end of the incubation. As this had to be avoided in order not to bias the results, the lower pumping capacity of depleted cells was compensated by reducing K^+ and increasing Na^+ in the bathing solutions of the control cells. In this way both groups ended the depletion period with the same Na^+ content (see Results).

Sodium and ATP contents. At the end of the depletion period the cells were washed five times in ice cold K^+-free choline. For sodium analysis about 0.1 ml of cells were lysed in 10 ml of deionized water and then flamed. The cell volume was

obtained from the absorbance of the lysate at 541 nm considering that packed cells give an absorbance of 284. ATP was assayed by the enzymatic method of Sigma, Co. In all cases duplicate samples were taken. All values given in the text are the mean ± S.E.M.

Sodium efflux. The technique for Na^+ efflux is described in detail elsewhere. [12] The rate of Na^+ efflux was determined from the $^{22}Na^+$ lost after 0.5 h incubation at 37 °C. Duplicate or quadruplicate samples, as specified in the legends, were taken. Glucose was present during efflux in control and 2-deoxyglucose, in depleted cells. When present, ouabain was at 10^{-4} M concentration.

RESULTS

Effect of ATP depletion on the ouabain sensitive Na^+ efflux as a function of external Na^+, K^+ and Li^+ concentrations

In a first group of experiments the activation curves of the ouabain-sensitive Na^+-efflux as a function of external Na^+, K^+ and Li^+ concentration was determined. In the experiments which follow the ATP was reduced by an average of 10- to 12-fold in the depleted cells, whereas the sodium content was similar in both groups and within the normal range. The figures are a plot of the ouabain sensitive rate constants of Na^+ efflux, obtained as described in Methods, as a function of the external activating cation concentration. The activation by external Na^+ is shown in Fig. 1. In both controls (950±80 micromol ATP and 7.8±1.0 millimol Na^+/l cells) and depleted cells (82±10 micromol ATP and 8.7±1.2 millimol Na^+/l cells) there was a large reduction in Na^+ efflux when [Na^+] was increased from 0 to 5 mM; this observation has already been reported [13, 14] and is currently taken as an inhibition by external Na^+ of the uncoupled Na^+ efflux through the Na^+ pump. From 5 mM Na^+ up the ouabain-sensitive Na^+ efflux increased but without reaching saturation even at

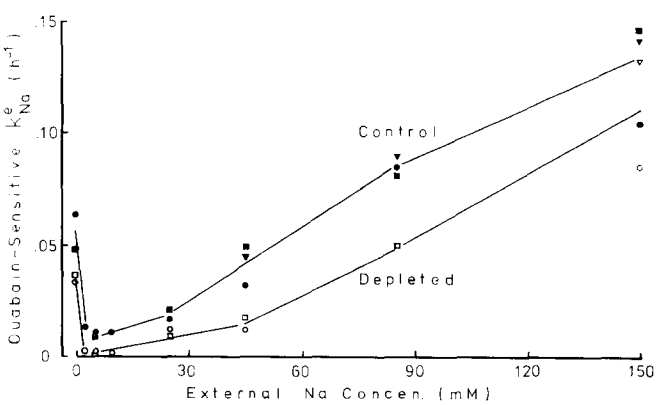

Fig. 1. Activation curve of the ouabain-sensitive Na^+ efflux as a function of the external Na^+ concentration in control and ATP-depleted human red cells. Different symbols correspond to different experiments carried out in duplicate. Control cells (filled symbols) had an initial 950±80 (mean ± S.E.M.) μmol/l cells ATP and 7.8±1.0 mmol/l cells Na^+. In depleted cells (open symbols) the ATP was 82 ±10 μmol/l cells and Na^+ was 8.7±1.2 mmol/l cells. Ouabain was used at 10^{-4} M concentration. For details see Methods.

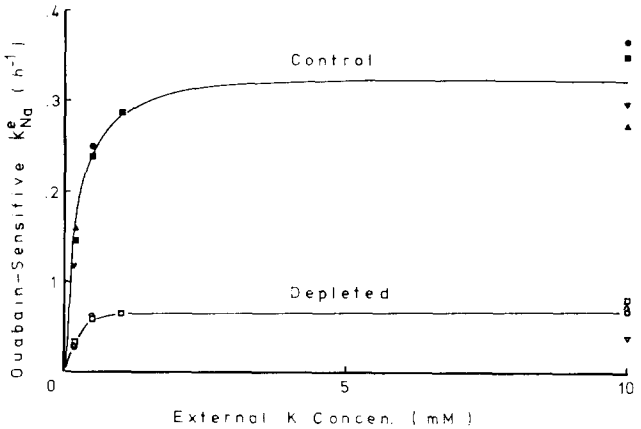

Fig. 2. Activation of a ouabain-sensitive Na^+ efflux by external K^+ in control (filled symbols) and ATP depleted (open symbols) human red cells. The general procedure was as described in Methods. All solutions contained 5 mM Na^+ and the total osmolarity was maintained with choline chloride. Different symbols correspond to different experiments done in duplicate. The initial ATP content was 980 ± 50 μmol/l cells in control and 78 ± 8 μmol/l cells in depleted cells. The Na^+ content was 9.3 ± 1.6 mmol/l cells in control and 9.0 ± 1.3 mmol/l cells in the depleted cells.

150 mM. The Na^+ efflux was always lower in the depleted cells: percentage-wise this reduction seemed more conspicuous at low external Na^+. As the aim of this work was to study the cation-activated Na^+ effluxes through the Na^+ pump, it was desirable to reduce the uncoupled Na^+ efflux through the same pathway to a minimum. In

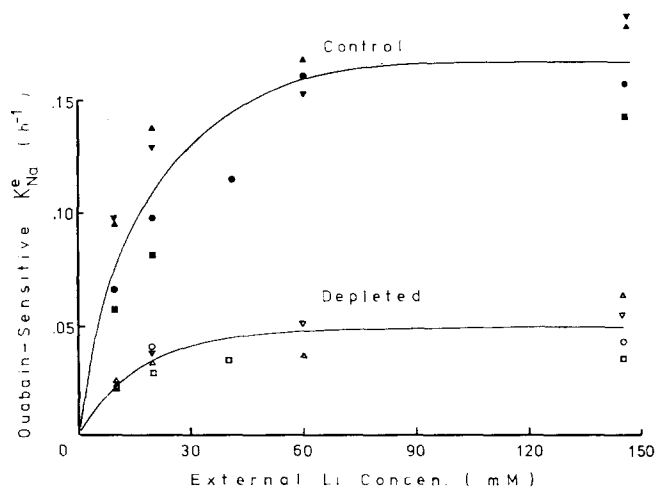

Fig. 3. Activation of a ouabain-sensitive Na^+ efflux by external Li^+ in control (filled symbols) and ATP depleted (open symbols) human red cells. The procedure was as described in Methods. All solutions contained 5 mM Na^+ and the total osmolarity was maintained with choline chloride. Control cells had an ATP content of 950 ± 120 μmol/l cells and an Na^+ content of 9.1 ± 0.9 mmol/l cells. In the depleted cells the initial ATP was 106 ± 14 μmol/l cells and the Na^+ content was 9.8 ± 1.1 mmol/l cells. Different symbols correspond to different experiments carried out in duplicate.

order to accomplish this, and in accordance with the results from above, in the experiments on K^+ and Li^+ activation 5 mM Na^+ was also present in the external media. In this way only the Na^+ efflux coupled to the influx of an external cation would remain. Fig. 2 summarizes the experiments on the K^+-activated Na^+ efflux. The reduction in the Na^+ efflux in the depleted group was dramatic at all K^+ concentrations investigated, with both curves showing an hyperbolic shape. From the reciprocal plot the V values were 0.330 h^{-1} (rate constant) in control and 0.063 h^{-1} in depleted cells. The apparent K_m had the same value in both groups of about 0.25 mM. In other words, a 12-fold reduction in the ATP content largely reduced the K^+-activated ouabain-sensitive Na^+ efflux without appreciable effect on the apparent affinity of the system for external potassium. The ATP content was 980 ± 50 μmol/l cells in the control and 78 ± 8 μmol/l cells in the depleted cells; the Na^+ content, showing no difference, was 9.3 ± 1.6 mmol/l cells and 9.0 ± 1.3 mmol/l cells respectively. The Li^+ activation curves are shown in Fig. 3. In this case the scatter of the data was larger than with potassium; however, the general picture was the same. From the reciprocal plot, which also gave scatter, the V was 0.157 h^{-1} in control and 0.053 h^{-1} in depleted cells. Within the resolution of the method the apparent K_m values were similar with a value of about 13 mM in both instances. At 145 mM, lithium was well above the saturating concentration. The depletion procedure reduced the ATP content from 950 ± 120 μmol/l cells to 106 ± 14 μmol/l cells, whereas the Na^+ content remained at 9.1 ± 0.9 mmol/l cells and 9.8 ± 1.1 mmol/l cells respectively.

Effect of ATP depletion on the maximal ouabain-sensitive Na^+ efflux activated by external Na^+, K^+ and Li^+

So far the data are insufficient to show unambiguously any preferential effect of the ATP depletion on any of the three cation-activated ouabain-sensitive Na^+ effluxes, although they strongly suggest the Na^+-activated is the less affected. One reason for the dispersion of the results is that for each activation curve different samples of blood were used. To avoid this source of uncertainty a further group of experiments were performed using the same blood sample to determine the ouabain-sensitive Na^+ efflux in control and in depleted cells when incubated in full Na^+, 10 mM K^+ and 145 mM Li^+ all at the same time. Quadruplicate samples were taken for each experiment, the results of which are given in Table I. For the control cells the magnitude of the maximal ouabain-sensitive Na^+ efflux followed the sequence $K^+ > Li^+ > Na^+$, with all the differences statistically significant. As K^+ and Li^+ were at saturating concentration the experiments also indicate that Li^+, besides having less affinity for the system, does not give the same V as K^+. This agrees with similar results in skeletal muscle [15]. In the depleted cells the maximal activation of Na^+ efflux followed a different sequence, $Na^+ > K^+ > Li,^+$ again with all differences statistically significant. This means that although all fluxes were reduced by the ATP depletion they were affected in different proportion. Thus, the percentage of the remaining fluxes was 20.3 ± 2.1 in K^+, 29.4 ± 2.5 in Li^+ and 73.1 ± 5.0 in Na^+. Another way of analyzing these data is to normalize them by taking the ratios of the rate constants in Na^+ and Li^+ divided by those obtained in K^+. If there were no effect of ATP depletion these ratios should be the same in control and depleted cells. In fact, the Na^+/K^+ activation ratio was 0.416 ± 0.018 (mean ± S.E.M.) in control cells and 1.505 ± 0.089 in depleted cells. The Li^+/K^+ activating ratio had the value of $0.531 \pm$

TABLE I

THE ABILITY OF EXTERNAL SODIUM, POTASSIUM AND LITHIUM IN PROMOTING A OUABAIN-SENSITIVE SODIUM EFFLUX IN CONTROL AND ATP-DEPLETED HUMAN RED CELLS

Freshly-drawn human red cells were treated as described in Methods. The rate of Na^+ efflux was followed at saturating concentrations of K^+ and Li^+ and at the highest isotonic NaCl Ringer. The K^+ and Li^+ solutions had in addition 5 mM Na. The average initial internal Na^+ was 10.4 ± 1.1 (mean ± S.E.M.) mmol/l cells in control and 10.4 ± 1.9 mmol/l cells in depleted cells. The initial ATP content was 890 ± 75 μmol/l cells in control and 97 ± 15 μmol/l cells in depleted cells. Values in the same row correspond to simultaneous determinations in the same blood sample. Each experiment was carried out in quadruplicate. Values are rate constant for ouabain-sensitive $^{22}Na^+$ efflux, in h^{-1}.

	10 mM K^+, 5 mM Na^+, 135 mM choline			145 mM Li^+, 5 mM Na^+			150 mM Na^+		
	Control	Depleted	$D/C \cdot 100$	Control	Depleted	$D/C \cdot 100$	Control	Depleted	$D/C \cdot 100$
	0.274	0.042	15.2	0.148	0.037	25.0	0.115	0.076	66.5
	0.299	0.077	25.7	0.179	0.054	30.3	0.127	0.108	85.1
	0.376	0.095	25.2	0.178	0.065	36.7	0.139	0.121	86.7
	–	–		0.181	0.038	21.0	0.132	0.098	73.9
	0.322	0.055	17.1	0.174	0.049	28.1	0.153	0.082	53.5
	0.278	0.051	18.4	0.140	0.049	35.2	0.129	0.079	72.8
Mean	0.310	0.064	20.3*	0.167	0.049	29.4*	0.129	0.094	73.1
S.E.M.	0.019	0.010	2.1	0.007	0.004	2.5	0.007	0.007	5.0

* $P < 0.025$; In all other cases $P < 0.001$.

0.021 in control and 0.842±0.055 in depleted cells. In all cases the differences were significant at the $P < 0.001$ level. The values for ATP and Na^+ contents were similar to those obtained in the experiments of the previous section thus, the control group had 980±75 μmol/l cells ATP and 10.4±1.1 mmol/l cells Na^+, whereas in the depleted group the ATP was 97±15 μmol/l cells and Na^+ was 10.4±1.9 mmol/l cells. In addition it is worth emphasising that the depletion procedure had no detectable effect on the ouabain-insensitive Na^+ effluxes. In every case the difference between the ouabain-treated and the non-ouabain-treated cells was statistically significant.

DISCUSSION

The present experiments indicate that the relative ability of external cations to promote a ouabain-sensitive Na^+ efflux in human red cells is modified in ATP depleted better as compared with normal cells. Relative to K^+ both Na^+ and Li^+ became activators when ATP was reduced by a factor of about 10. How much of this is due solely to the ATP reduction and how much to associated changes as ADP and perhaps P_i contents cannot be known with certainty. The activation by Na^+ at low ATP could be considered entirely due to the classical Na : Na exchange [13] as this exchange has been shown to be independent of ATP but to vary fairly linearly with internal ADP [16]. However, the results in Table I would require that a 90 % reduction in ATP was accompanied by a reduction in ADP of only 27 %. This might suggest that in

addition some other mechanism is involved. One possibility as suggested by Glynn and Karlish [17], is a release from inhibition of the uncoupled Na^+ efflux at external Na^+ higher than 5 mM. Another possibility of a K^+-like action of Na^+ through the entire cycle of the pump. In the experiments with K^+ and Li^+ solutions, 5 mM Na^+ was always present; this Na^+ concentration was shown to inhibit to a large extent the uncoupled Na^+ efflux (Fig. 1). The K^+ and Li^+ activation can then be considered a genuine stimulation of the Na^+ pump [5, 15]. The increased effectiveness of Li^+ relative to K^+ as ATP was reduced is compatible with the hypothesis that the dephosphorylating cations on the ATPase reaction remain attached to the enzyme after dephosphorylation, thus reducing its affinity for ATP, and where the $E \cdot K^+$ complex is more stable than the $E \cdot Li^+$ one [11]. Interestingly enough a K^+-like action of Na^+ would also fit in this scheme as it is easy to imagine $E \cdot Na^+$ the most favourable for rephosphorylation. An effect of ATP from the inside, shifting the $E \cdot Na^+ \rightleftharpoons E \cdot K$ equilibrium [18] does not seem useful as an explanation because the cation composition and cell ATP were the same in all incubation solutions. An effect of K^+ coming from the outside, would also agree with observations in resealed ghosts which suggest that the inhibitory effect of K^+ on the ATPase at low ATP is from the outside [17]. External K^+ has been shown to reduce the enzyme ATP affinity [19] and this could be thought of as responsible for the K^+ and Li^+ differences. However against this explanation is the lack of the counterpart effect, that is the reduction in ATP did not seem to modify the apparent affinities of the external site for K^+ or Li^+. Finally, it might be that not all Li^+ goes through the pump by a K^+-like action, but some can replace Na^+ through the $Na^+ : Na^+$ exchange as well, which as such would be less sensitive to ATP depletion. To exclude this alternative ATP consumption and Na^+/ATP ratios under K^+ and Li^+ stimulation would need to be measured.

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It has recently been suggested [20] that the external sites at which K^+ activates translocation and dephosphoryiation (beta sites) are different from those at which it reduces the enzyme-ATP affinity (alpha sites). The interaction of Li^+ with the beta sites also produces dephosphorylation, but in contrast with K^+, the binding of Li^+ to the alpha sites does not change the enzyme-ATP affinity [21]. If this is the case, the K^+ and Li^+ differences reported in this paper could still be a consequence of a process taking place at the external side of the membrane without showing any ATP effect on the apparent affinities for K^+ and Li^+ translocation.

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REFERENCES

1 Garrahan, P. J. and Glynn, I. M. (1967) J. Physiol. 192, 189–216
2 Glynn, I. M., Lew, V. L. and Luthi, U. (1970) J. Physiol., 207, 371–391
3 De Weer, P. (1970) J. Gen. Physiol., 56, 583–620
4 Czerwinski, A., Gitelman, H. T. and Welt, L. G., (1967) Am. J. Physiol. 213, 786–792
5 Skou, J. Ch. (1960) Biochim. Biophys. Acta 42, 6–23
6 Fujita, M., Nagano, K., Mizuno, N., Tashima, Y., Nakao, T. and Nakao, M. (1967) J. Biochem. (Tokyo), 61, 473–477
7 Blostein, R. (1970) J. Biol. Chem. 245, 270–275
8 Nørby, J. G. and Jensen, J. (1971) Biochim. Biophys. Acta 233, 104–116
9 Jensen, J. and Nørby, H. J. (1971) Biochim. Biophys. Acta 233, 395–403
10 Hegyvary, C. and Post, R. L. (1971) J. Biol. Chem. 246, 5234–5240
11 Post, R. L., Hegyvary, C. and Kume, S. (1972) J. Biol. Chem. 247, 6530–6540
12 Beaugé, L. (1975) Biochim. Biophys. Acta, 401, 95–108
13 Garrahan, P. J. and Glynn, I. M. (1967) J. Physiol. 192, 159–174
14 Lew, V. L., Hardy, M. A. and Ellory, J. C. (1973) Biochim. Biophys. Acta 323, 251–266
15 Beaugé, L. (1975) J. Physiol. 246, 397–420
16 Glynn, I. M. and Hoffman, J. F. (1971) J. Physiol. 218, 239–256
17 Glynn, I. M. and Karlish, S. J. D. (1975) J. Physiol. 250, 33–34 P
18 Skou, J. Ch. (1974) Ann. N.Y. Acad. Sci., 242, 168–184
19 Robinson, J. D. (1967) Biochemistry, 6, 3250–3258
20 Robinson, J. D. (1975) Biochim. Biophys. Acta 384, 250–264
21 Robinson, J. D. (1975) Biochim. Biophys. Acta 413, 459–471