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Furosemide-sensitive Na^+-K^+ cotransport and cellular metabolism in human erythrocytes

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Metabolic depletion of human red cells with 2-deoxy-D-glucose in the presence of EGTA decreased ATP to about 4% of the initial value and increased total ouabain- and furosemide-resistant Na^+ and K^+ effluxes by 20% and 100%, respectively, and furosemide-sensitive Na^+ and K^+ effluxes by 100% and 60%, respectively. When ATP was restored, all the components of Na^+ and K^+ fluxes measured returned to baseline levels suggesting a metabolic dependence.

In 1971, one of us reported that a passive, ouabain-resistant Na^+-dependent Rb^+ influx ($^{86}Rb^+$, used as K^+ analogue) in human red cells was inhibited following treatment with the sulfhydryl reagent iodoacetic acid [1]. As iodoacetic acid irreversibly alkylates glycerylaldehyde phosphate dehydrogenase and hence inhibits ATP synthesis, it was inferred that the Na^+-dependent component of Rb^+ influx (now generally recognized as furosemide-sensitive Na^+-K^+ cotransport [2,4–7]) was dependent on cellular metabolism, most probably on ATP which, however, was not determined [1]. Furthermore, the choice of the chemical used for metabolic depletion prohibited an investigation of the reversibility of the metabolic effects on Na^+-dependent Rb^+ influx. We now present evidence for reversible metabolic dependence of Na^+-dependent K^+ transport by using the non-metabolizable glucose analogue 2-deoxy-D-glucose to deplete cellular ATP, a treatment allowing its subsequent restoration by incubation in the presence of metabolizable substrates. We determined ouabain-resistant zero-trans effluxes of Na^+ and K^+ in the presence and absence of furosemide from cells which were cation loaded [6] to insure V_{max} conditions.

Heparinized blood obtained from two healthy donors was centrifuged for 10 min at 1750 × *g* and plasma and buffy coat were eliminated. Aliquots of packed cells were used to determine cell water and cations, and $[ATP]_c$ [8]. The remaining pellet was suspended at a hematocrit of 5% in a solution containing (mM): 120 NaCl, 30 KCl, 1 $MgCl_2$, 1 EGTA, 2.5 sodium phosphate (pH 7.2) and 0.02 PCMBS to permeabilize the membranes and load the cells with saturating Na^+ and K^+ concentrations [6]. After 20 h at 4°C, cells were divided into two aliquots and pelleted. One pellet was resuspended in sealing solutions to restore $[ATP]_c$ and the low cation permeability [6]. After 1 h at 37°C, cells were washed in a Na^+-, K^+-free washing solution containing (mM): 75 $MgCl_2$, 95 sucrose, 10 Tris/Mops (pH 7.4 at 4°C), 0.1 ouabain, and fluxes were measured in Mg medium containing (mM): 75 $MgCl_2$, 85 sucrose, 10 Tris/Mops (pH

Abbreviations: EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*′-tetraacetic acid; Tris/Mops, tris(hydroxymethyl)aminomethane/4-morpholinepropanesulfonic acid; PCMBS, *p*-chloromercuribenzenesulfonic acid.

7.4 at 37°C), 10 glucose, 0.1 ouabain ± 1 furosemide. The second pellet was resuspended at a hematocrit of 7–8% in a solution containing (mM): 75 NaCl, 75 KCl, 1 $MgCl_2$, 0.1 ouabain, 10 2-deoxy-D-glucose, 4 cysteine (neutralized), ±1 EGTA, and 2.5 sodium phosphate (pH 7.2), and incubated up to 8 h at 37°C with two solution changes. One half aliquot of depleted cells was washed for subsequent flux measurement in the washing solution, the second half was pelleted and resuspended in ATP repletion medium containing (mM): 68 NaCl, 68 KCl, 1 $MgCl_2$, 10 glucose, 5 inosine, 2 adenine, 0.1 ouabain, and 17 sodium phosphate (pH 7.4), ±1 EGTA, and incubated 3 h at 37°C. Cells were then washed for the flux measurement in Mg medium. At the time of completion of each procedure, $[ATP]_c$, cell water and cation content were determined. Zero-trans cation effluxes were measured by two-time-point analysis within the initial velocity range (0 and 40 min) in efflux media and fluxes were calculated in mmol/litre cells as reported elsewhere [6].

Table I shows that cell water and the sum of the cation concentrations were not different between fresh cells and experimental controls which had been PCMBS treated and resealed, although the Na^+/K^+ ratio in the later was 4-fold higher than in fresh cells. When cells were depleted of ATP in the absence or presence of EGTA the water content increased by 6% and 9%, respectively, with regard to the experimental control. This effect may be accounted for by the differences in Na^+ content with respect to the experimental control and by accumulation of 2-deoxy-D-glucose phosphate which cannot be further processed by the glycolytic pathway. Metabolic repletion resulted in the return of cell volume close to that of the control.

Table II shows $[ATP]_c$ and the effect of its alterations on ouabain-resistant zero-trans Na^+,K^+ effluxes. Cellular ATP was slightly higher in fresh cells than in the experimental control but fell to 4% and 6% of the latter's value in cells metabolically depleted in absence and presence of EGTA, respectively. Metabolic repletion restored $[ATP]_c$ to levels close to those of fresh cells. Although in the experimental controls the total ouabain- and furosemide-resistant K^+ fluxes were slightly higher than their Na^+ counterparts, the furosemide-sensitive Na^+ and K^+ effluxes were equal and of the same magnitude reported recently [6]. Upon metabolic depletion in EGTA-free media the total ouabain- and furosemide-resistant Na^+ effluxes increased but not their K^+ counterparts, while both furosemide-sensitive Na^+ and K^+ effluxes fell by some 20%. In the presence of EGTA, however, and with an $[ATP]_c$ statistically not different from that in the absence of EGTA, the furosemide-resistant K^+ efflux doubled leading to a 20% increase of the total K^+ influx and to a drop of the furosemide-sensitive K^+ flux to about 43% of the experimental control. In contrast, the furosemide-sensitive Na^+ efflux was practically abolished, the numbers being not statistically different from zero. Upon repletion of $[ATP]_c$ to 1.1 mmol/litre cells the furosemide-resistant Na^+ and K^+ fluxes fell again to baseline levels, and the furosemide-sensitive Na^+,K^+ fluxes increased to values 10–20% higher than in the experimental control. These data unequivocally establish the reversibility of the effect of metabolic depletion on

TABLE I

WATER CONTENT AND CATION COMPOSITION OF RED BLOOD CELLS

Numbers in parentheses indicate number of experiments. Values are presented as means ± S.E.

Cells	Water content (kg/kg dry cell solids)	Cation composition (mmol/kg water)		
		Na^+	K^+	$Na^+ + K^+$
Fresh cells	1.920 ± 0.009 (6)	14.5 ± 1.3 (8)	115.7 ± 7.0 (8)	130.1 ± 7.7 (8)
Experimental control	1.904 ± 0.013 (6)	47.5 ± 3.1 (8)	88.1 ± 3.5 (8)	131.4 ± 6.2 (8)
Depleted (− EGTA)	2.027 ± 0.038 (4)	57.5 ± 2.0 (4)	81.5 ± 1.9 (4)	139.0 ± 2.1 (4)
Depleted (+ EGTA)	2.069 ± 0.015 (10)	53.5 ± 1.5 (10)	77.2 ± 2.3 (10)	130.7 ± 2.2 (10)
Repleted	1.937 ± 0.011 (4)	52.1 ± 1.5 (6)	78.9 ± 1.8 (6)	130.9 ± 2.6 (6)

TABLE II

EFFECT OF ATP ALTERATIONS ON OUABAIN-RESISTANT Na^+ AND K^+ FLUXES

Numbers in parentheses indicate number of experiments. Values are presented as means ± S.D.

Cells	ATP_c (mmol/litre cells)	Cation fluxes (mmol/litre cells per h)					
		Na^+			K^+		
		Total	Furosemide-resistant	Furosemide-sensitive	Total	Furosemide-resistant	Furosemide sensitive
Fresh	1.19 ±0.09 (6)	–	–	–	–	–	–
Experimental control							
(−EGTA) (2)	0.840	0.85	0.42	0.43	0.95	0.51	0.45
(+EGTA) (8)	0.829 ± 0.090	0.79 ± 0.06	0.39 ± 0.03	0.40 ± 0.04	1.01 ± 0.07	0.56 ± 0.05	0.46 ± 0.02
Depleted							
(−EGTA) (6)	0.035 (2)	1.09 ± 0.07 [b]	0.75 ± 0.08	0.33 ± 0.03	0.91 ± 0.06	0.53 ± 0.08	0.37 ± 0.06
(+EGTA) (6)	0.054 ± 0.006	0.93 ± 0.04	0.85 ± 0.05 [c]	0.08 ± 0.05 [b]	1.23 ± 0.22	1.03 ± 0.21 [a]	0.20 ± 0.06
Repleted							
(−EGTA) (2)	–	0.95	0.53	0.43	1.05	0.56	0.50
(+EGTA) (6)	1.10 ±0.13 (4)	0.98 ± 0.05 [a]	0.50 ± 0.04	0.48 ± 0.03	1.01 ± 0.07	0.48 ± 0.06	0.53 ± 0.04

[a] $p < 0.05$.
[b] $p < 0.01$.
[c] $p < 0.001$.

ouabain-resistant zero-trans Na^+,K^+ fluxes.

These findings suggest a metabolic basis of Na^+-K^+ cotransport in human red cells and thus provide an explanation for the earlier observations made in 1971 [1]. Our work is also in part in line with a report [3] showing that starvation of nystatin treated human red cells in substrate-free solution containing EGTA reduced both Na^+ and K^+ net effluxes by 65%. There is further evidence of reversible metabolic dependence of Na^+-K^+ cotransport in squid giant axon [9], in bird red cells [10] and in MDCK cells [11]. Aside from earlier experiments in dog red cells [12] our work shows for the first time that the effect of metabolic depletion on zero-trans cation fluxes in human red cells was fully reversible upon incubation in metabolizable substrates. At this point it cannot be decided whether ATP itself or other metabolites control passive Na^+ and K^+ fluxes across the red cells membranes. Interestingly, Ca^{2+} has been shown to inhibit Na^+-K^+ cotransport in human red cells [13]. If it is ATP, then the apparent 'affinity' of both Na^+ and K^+ effluxes for ATP must be an order of magnitude higher than that of the *N*-ethylmaleimide stimulated, Na^+-independent K^+ fluxes recently reported to be metabolically dependent for the same cells [14] and for low K^+ sheep red cells [15]. The data of Table II, also show that only in the presence of EGTA was it possible to abolish furosemide-sensitive Na^+ fluxes and reduce K^+ fluxes by 60%. Hence, the apparent 'affinity' of furosemide-sensitive K^+ fluxes for ATP is still higher than that of Na^+ fluxes, and bivalent metal ion chelation may be required. While furosemide-sensitive cation fluxes fell, furosemide-resistant fluxes increased in ATP depleted cells, suggesting perhaps a metabolic basis of their interconvertibility.

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