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The Na^+/K^+-ATPase reaction of human erythrocytes is not near equilibrium. A ^{31}P-NMR study

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We have addressed the question of whether the Na^+/K^+-ATPase in the human erythrocyte is in a state of near-equilibrium by varying the extracellular ratio of Na^+ and K^+ and following the cytosolic phosphorylation potential by ^{31}P-NMR and by combined enzymatic colorimetric measurements. There was no correlation at room temperature between the extracellular Na^+/K^+ ratio and the cytosolic phosphorylation potential measured either by NMR or alternative methods. The cytosolic phosphorylation potential measured by NMR was 4100 ± 1300 (S.E.) M^{-1} at an extracellular K^+ concentration of 5.9 mM (Na^+/K^+ ratio of 24.3) and 2800 ± 700 (S.E.) M^{-1} at 75 mM extracellular K^+ (Na^+/K^+ ratio of 0.99). The chemically determined phosphorylation potential was 6400 ± 1200 (S.E.) and 5000 ± 700 (S.E.) M^{-1} at 5.9 and 75 mM extracellular K^+, respectively. Omission of Ca^{2+} from the buffer solutions did not affect the results. A consistent finding in this study was that the NMR-determined value of ATP was about 10–20% lower than the value determined enzymatically on perchloric acid extracts. The inorganic phosphate (P_i) was fully NMR visible.

Introduction

It has recently been proposed that the plasma membrane Na^+/K^+-ATPase is in a state of near-equilibrium [1] although the combined reaction of the Na^+/K^+-ATPase

$$ATP + 3\,Na_i^+ + 2K_o^+ \rightleftarrows ADP + P_i + 3\,Na_o^+ + 2\,K_i^+ \quad (1)$$

with the known concentrations of reactants is out of equilibrium by -3 to -4 kcal/mol [1,2]. A feature shared by Na^+/K^+-ATPases from various tissues but in contrast to other membrane ATPases [1–3]. Assuming that the stoichometry in Eqn. 1 reflects the state in the intact cell, there are no a priori reasons why the cytosolic phosphorylation potential should not drive the reaction to near-equilibrium. The ATP-generating capability in relation to the rate of ion pumping is very high [1].

We have addressed the problem of whether the Na^+/K^+-ATPase reaction in the human erythrocyte is near equilibrium by varying the extracellular Na^+/K^+ ratio and simultaneously measuring the cytosolic phosphorylation potential by ^{31}P-NMR spectrocopy and by enzymatic methods. Part of this work was presented at the seventh Annual Meeting of The Society of Magnetic Resonance in Medicine, San Francisco, August 20–26, 1988.

Materials and Methods

Erythrocytes

Erythrocytes were obtained from venous blood from healthy donors. The erythrocytes were isolated essentially as previously described [4] except that the standard buffer solution used in this study was a modified Krebs-Ringer solution of the following composition: 118.5 mM NaCl, 4.75 mM KCl, 1.8 mM $CaCl_2$, 1.16 mM KH_2PO_4, 1.18 mM $MgSO_4$, 5 mM glucose and 24.9 mM $NaHCO_4$. This buffer solution yielded total extracellular Na^+ and K^+ concentrations of 143.4 mM and 5.9 mM, respectively, well within the physiological ranges [5]. The buffer solution was bubbled with a gas mixture of 5% CO_2/95% O_2 before use, ensuring pH 7.35 and full oxygenation of the erythrocytes. For NMR

Abbreviations: 2,3-DPG, 2,3-diphosphoglycerate; MDP, methylene diphosphonic acid; PCA, perchloric acid.

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analysis the isolated erythrocytes were washed and resuspended in appropriately modified buffer solutions where the K^+ concentration was varied from 5.9 mM to 75 mM. Isoosmolarity was maintained by decreasing the Na^+ concentration correspondingly.

Since high extracellular levels of K^+ may induce depolarisation with concomitant changes in Ca^{2+} permeability and intracellular Ca^{2+} release in liver [6], control solutions without Ca^{2+} at K^+ levels of 5.9 mM and 75 mM were also tested. The hematocrit was determined by a standard technique [4]. Extracellular Na^+ and K^+ were measured potentiometrically by Kodak Ectaderm 400, precalibrated with calibration solutions at the appropriate levels of K^+ and Na^+.

NMR measurements

The erythrocytes were transferred to a standard 10 mm NMR tube and ^{31}P-NMR spectroscopy was performed at 101.3 MHz in a Bruker AM 250 instrument and at 25°C. 90° pulses and a repetition time of 4.6 s were used. This ensured complete relaxation of ATP and ADP ($T_1 = 1.2$–1.3 s) (Petersen, A., et al., unpublished results) while the P_i peak was partially saturated and corrected by a factor of 1.54. This correction factor was obtained by alternately accumulating spectra with a repetition time of 4.6 s and 20 s on the same sample of erythrocytes. The relaxation time, T_1 of the P_i peak was determined to 4.22 s and with the repetition time used a saturation factor of 1.66 may be calculated, only 8% of the experimentally determined value of 1.54. All spectra were accumulated within 20 min. 125 mM methylenediphosphonic acid in 50 mM Tris at pH 9 served as an external reference 16.9 ppm relative to 85% H_3PO_4) [4]. The area of the MDP reference corresponded to 1.56 ± 0.10 mM (S.E.) ($n = 4$), compared to a sample of P_i at 9.81 mM.

For the NMR-derived quantifications of the peak areas, the computer routine of the instrument was used. In the case of the P_i peak, the area was estimated by manual triangulation because of its proximity to the peak of 2-phosphorus of 2,3-diphosphoglycerate, see Fig. 1. This procedure was verified by using authentic samples of P_i and 2,3-diphosphoglycerate at physiological pH levels. It was concluded that the measured P_i peak was intracellular, since in two experiments in which the erythrocytes were washed three times in phosphate-free buffer solutions and subsequently concentrated to a hematocrit of 66% for NMR measurements, the chemical shift of the intracellular P_i peak was found to be the same as compared to experiments with phosphate in the buffer solution. Due to the transmembrane pH difference (approx. 0.2 pH units) the extracellular P_i peak is shifted downfield towards the broad peak for 2-phosphorus of 2,3-DPG [4]. ADP concentrations were estimated by subtracting the β-ATP peak from the combined peaks of γ-ATP and β-ADP. ATP was measured by the β-ATP peak.

Enzymatic and colorimetric measurements

Before and after each NMR measurement, a sample was extracted with perchloric acid essentially as described previously [7] for standard enzymatic assays of ATP and ADP [8] and colorimetric determination of P_i [9]. The intracellular P_i concentration was estimated by measuring the concentration in both the buffer solution and in the neutralized PCA extract of erythrocytes. Knowing the hematocrit, the P_i concentration is obtained [10].

Results

Fig. 1 shows a ^{31}P-NMR spectrum of erythrocytes (hematocrit of 67%) at 101.3 MHz to illustrate the signal-to-noise ratio obtained in this study. A line-broadening factor of 20 Hz was introduced in the main spectrum, whereas the inset spectrum shows the P_i and γ-ATP region with a line-broadening factor of 2 Hz in order to differentiate the intracellular P_i peak [11]. The assignments of the peaks are based on previous studies [7,11].

Table I summarizes the phosphorylation potentials obtained at various levels of extracellular K^+ (varying the extracellular Na^+/K^+ ratio from 24.3 to 0.99) with and without Ca^{2+}. Phosphorylation potentials obtained by both NMR and enzymatic methods are shown. There is no change of the phosphorylation potential within the Na^+/K^+ ratio range studied, regardless of the levels of

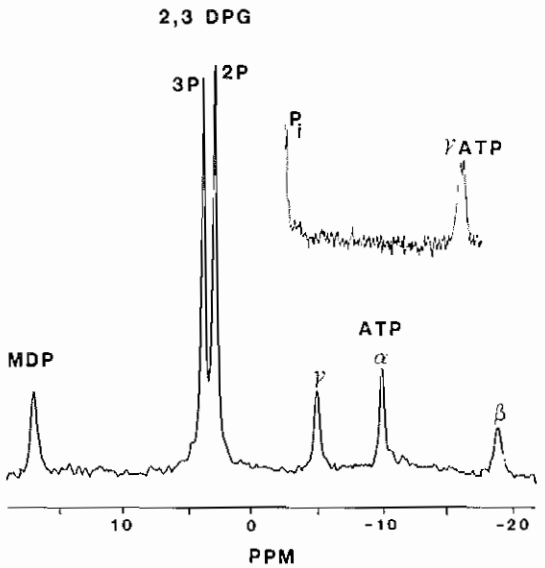

Fig. 1. ^{31}P-NMR spectrum at 101.3 MHz of human erythrocytes suspended in standard Krebs-Ringer buffer solution at a hematocrit of 67%. 240 scans with a repetition time of 4.6 s were accumulated. The inset spectrum shows the P_i peak using a smaller line-broadening factor (2 Hz).

TABLE I

Effect of varying the external K^+ concentration on the phosphorylation potential in human erythrocytes

The erythrocytes were suspended at various external K^+ concentrations as indicated, decreasing the Na^+ level correspondingly. The figures represent the phosphorylation potential defined as (ATP)/(ADP)(P_i), determined both by ^{31}P-NMR and enzymatic methods. At two levels of K^+, Ca^{2+} was omitted as indicated. *n* denotes the number of experiments. Also shown are the intracellular changes in the concentrations of Na^+ and K^+ during the NMR experiment, (−) denoting loss and with one experiment at each level. Results are expressed as mean ± S.E.

K^+ concn. (mmol/l)	(mmol/l RBC)		Phosphorylation potential (M^{-1})	
	ΔNa^+	ΔK^+	by ^{31}P-NMR (*n*)	by enzymatic/colorimetric methods (*n*)
5.9	0	−1.4	4100 ± 1300 (6)	6400 ± 1200 (4)
15	0.4	−7.2	3400 ± 700 (5)	8400 ± 3400 (5)
30	0.2	0.9	3200 ± 1100 (4)	4900 ± 600 (5)
45	−11.9	−4.7	6300 ± 4600 (5)	5200 ± 1600 (4)
75	−4.3	−5.4	2800 ± 700 (5)	5000 ± 700 (5)
5.9 − Ca^{2+}	1.1	−1.4	2000 ± 900 (4)	5500 ± 1200 (5)
75 − Ca^{2+}	−3.6	−2.8	3100 ± 1100 (5)	5600 ± 1800 (5)

Ca^{2+}. The values of phosphorylation potentials at an extracellular K^+ concentration of 5.9 mM (Na^+/K^+ ratio of 24.3) are 4100 M^{-1} by NMR and 6400 M^{-1} by enzymatic methods. These values are in good agreement with a previously published value of 5700 M^{-1} obtained in whole blood [12]. Also shown in Table I are the changes in intracellular levles of Na^+ and K^+. These values were obtained once at each level of Na^+/K^+ ratio by measuring the extracellular concentrations of Na^+ and K^+ before and after the NMR measurements. Knowing the hematocrit, the corresponding changes in the intracellular levels can be calculated. The changes are very small and cannot account for the lack of change in the phosphorylation potential (Table I).

TABLE II

ATP, ADP and P_i concentrations in human erythrocytes

The concentrations are given in mmol/l cell water ± standard error of the mean (S.E.) assuming the water fraction of the erythrocytes to be 0.7. Composite values of the metabolites, regardless of external K^+ concentrations, are given. The NMR-determined values are compared to the enzymatic (ATP, ADP) and colorimetric (P_i) determinations performed on perchloric acid extracts of the same erythrocytes on which the NMR determinations were performed. *n* denotes the number of observations.

	NMR (mmol/l cell water)		Enzymatic/colorimetric (mmol/l cell water)	
	n		*n*	
ATP	34	1.73 ± 0.06 [a]	33	2.23 ± 0.05
ADP	34	0.60 ± 0.05 [b]	33	0.32 ± 0.01
P_i	34	1.61 ± 0.13	33	1.60 ± 0.14

[a] $P < 0.001$ and [b] $P < 0.001$ by a two-tailed *t*-test as compared to the enzymatically determined values.

As shown in Table II the NMR-determined phosphorylation potentials are generally lower than the figures based on combined enzymatic and colorimetric measurements. This reflects a 20% lower NMR-determined ATP value as compared to the enzymatic determination, and a 90% higher NMR-determined ADP value. There is, however, excellent agreement between the NMR and colorimetrically determined P_i values.

Based on a calibration curve obtained from oxygenated hemolysates and using the chemical shift of the 3-phosphorus peak of 2,3-diphosphoglycerate as the pH probe, the intracellular pH was found not to be significantly affected by varying the Na^+/K^+ ratio. The range was found to be 7.14 ± 0.06 to 7.20 ± 0.03 (S.D.), $n = 5$. These results are in agreement with previously published results in a comparable system [4].

Discussion

Two main results came out of this study of human erythrocytes. First, we were unable to observe any change in the cytosolic phosphorylation potential upon a decrease, of up to 25-fold, of the extracellular Na^+/K^+ ratio, corresponding to a 1160-fold change of the apparent equilibrium constant of Eqn. 1 (Table I). We therefore conclude that the Na^+/K^+-ATPase is not in a near-equilibrium state, in contrast to the recent suggestion by Veech [1]. Second, the ^{31}P-NMR visibility of ATP in the erythrocyte is about 80%, and significantly smaller than the full visibility expected (Table II).

Non-equilibrium in the Na^+/K^+-ATPase

The ability of the erythrocyte Na^+/K^+-ATPase to mediate net synthesis of ATP is well known [13]. If the reaction catalyzed by the plasma membrane Na^+/K^+-ATPase was in fact maintained in thermodynamic equilibrium, one would expect a decrease of the cytosolic phosphorylation potential upon decreasing the extracellular Na^+/K^+ ratio (see Eqn. 1). As Table I shows, there is no change of the cytosolic phosphorylation potential as measured by ^{31}P-NMR or by chemical methods. This, in fact, corroborates earlier studies in which it has been shown that the rate of ATP breakdown in erythrocytes suspended in glucose-free media was unrelated to the external Na^+/K^+ ratio [14]. Since K^+ depolarization can cause changes in the permeability and intracellular release of Ca^{2+} in other tissues [6,15], buffer solutions without Ca^{2+} were also tested at K^+ concentrations of 5.9 mM (standard) and 75 mM, corresponding to Na^+/K^+ ratios of 24.3 and 0.99, respectively. As Table I shows, the omission of Ca^{2+} from these buffer solutions did not have any effect on the cytosolic phosphorylation potential. Our data therefore do not support the hypothesis [1] that the Na^+/K^+-

ATPase in human erythrocytes is in a state of near-equilibrium.

The key assumption behind this conclusion is, of course, that the perturbations of the extracellular ratio of Na^+/K^+ with preserved osmotic concentration does not result in significant changes of the intracellular Na^+ and K^+ during the short interval of the NMR measurement after the perturbation, i.e., 20 min. As shown in Table I, the changes in the calculated levels of intracellular Na^+ and K^+ are very small. In order to detect changes in the phosphorylated compounds, a significant part of the normal ATP turnover in the erythrocyte should be utilized by the $Na^/K^+$-ATPase. This requirement is fulfilled as 20% of the ATP production in the erythrocyte is used by the $Na^/K^+$-ATPase [16]. Control experiments with longer incubation times (not shown) in the buffer with 75 mM K^+ showed that the phosphorylation potential was preserved at 4200 M^{-1} after 2 h, whereas near-equilibrium in the $Na^/K^+$-ATPase should lead to an approx. 1100-fold decrease in the phosphorylation potential, see Eqn. 1.

There are several possible reasons for this apparent non-equilibrium state [2]. (i) The stoichiometry in Eqn. 1 may not apply under physiological conditions, rendering the thermodynamic calculations invalid [2,17]. The $Na^/K^+$-ATPase is capable of different transport modes including Na^+/Na^+ and K^+/K^+ exchange [18]. Although these processes do not involve hydrolysis of ATP they do indicate that the $Na^/K^+$-ATPase does not discriminate perfectly between K^+ and Na^+ ions [2,18]. The rationale of this study should, however, be independent of the exact stoiciometry of the $Na^/K^+$-ATPase. (ii) Another possible explanation for the apparent non-equilibrium state in the $Na^/K^+$-ATPase is that one or several of the reactants and products in Eqn. 1 are compartmentalized rendering the thermodynamic calculations, assuming a homogenous cytosolic pool, invalid. It has earlier been shown that the $Na^/K^+$-ATPase in the human erythrocyte utilizes a compartmentalized form of ATP [19,20]. Calculations based on the number of ATP molecules per $Na^/K^+$-ATPase and the known number of $Na^/K^+$-ATPases in the human erythrocyte, indicate that only about 1 in 10000 ATP molecules is involved in the compartmentalized form [19]. This may also explain why a study utilizing the radioactive tracer ^{32}P failed to show significant compartmentation of ATP [21] in accordance with calculations and measurements using near-equilibrium reactions in vitro and in vivo [12]. The fraction of compartmentalized ATP is also too small to explain the decreased visibility of ATP as detected by ^{31}P-NMR. At present, experimental data do not allow a safe conclusion concerning the effect of compartmentation on the thermodynamic calculations.

^{31}P-NMR visibility of ATP

This is, to our knowledge, the first study in which absolute NMR quantification of ATP, ADP and P_i has been attempted in the human erythrocyte. A consistent finding was that the NMR-measured values of ATP were 20% lower and values of ADP were 90% higher than the enzymatic measurements. Furthermore, these results were independent of the extracellular Na^+/K^+ ratio. In contrast to this, there was agreement between the NMR-measured value of P_i and the value obtained by a colorimetric method (Table II). In absolute terms the difference in ATP meausrements by NMR and the enzymatic method is 0.50 mmol/l, with NMR yielding a lower value. While the difference in ADP is 0.28 mmol/l, with NMR giving a higher value (Table II). The NMR determination of ADP as the difference between the (γ-ATP + β-ADP) and β-ATP should be considered less reliable than the measurement of ATP using the β-ATP peak. In this context, it is interesting to note that the enzymatically measured ADP concentration in human erythrocytes agrees with calculations based on near-equilibrium reactions, whereas in other tissues the measured ADP is too high, and is interpreted as intracellular compartmentation/binding of ADP [12]. Since it is very difficult to think of a reason why NMR-determined ADP should give higher values than enzymatic determinations, the results may indicate a systematic underestimation of the β-ATP peak by the amount of 0.28 mmol/l. Another possible explanation might be that the combined γ-ATP and β-ADP peaks contain as yet unidentified peak(s) that are not contained in the β-ATP peak, i.e., nucleoside diphosphates. In ^{31}P-NMR spectra of perchloric acid extracts of erythrocytes there are several smaller peaks in the γ-ATP and β-ADP region not yet identified (unpublished results). The lower ATP as measured by NMR may be related to binding of ATP to hemoglobin, enzymes or membrane. The values for ATP, ADP and P_i as determined by chemical methods are in good agreement with a previous study [12].

In conclusion, we have shown that the membrane $Na^/K^+$-ATPase in the human erythrocyte is unlikely to be in a state of near-equilibrium and that the ^{31}P-NMR visibility of ATP is less than 100%.

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