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COMPARISON OF RED CELL AND KIDNEY ($Na^+ + K^+$)-ATPase AT 0°C

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Human red cell and guinea pig kidney ($Na^+ + K^+$)-ATPase were phosphorylated at 0°C. Using concentrations of ATP ranging from 10^{-6} to 10^{-8} M, ATP-dependent regulation of reactivity is observed with red cell but not kidney ($Na^+ + K^+$)-ATPase at 0°C. In particular, with the red cell enzyme only, the following are observed: (i) the ratio of enzyme-bound ATP (E·ATP, measured by the pulse-chase method of Post, R.L., Kume, S., Tobin, T., Orcutt, B. and Sen, A.K. (1969) J. Gen. Physiol. 54, 306s–326s) to steady-state level of total phosphoenzyme (EP) decreases with decrease in ATP concentration and (ii) the apparent turnover of phosphoenzyme (ratio of Na^+-stimulated ATP hydrolysis to level of total EP at steady state) also varies as a function of ATP concentration. In addition, when EP is formed at very low ATP (0.02 μM), and then EDTA is added, rapid disappearance of a fraction of EP occurs, presumably due to ATP resynthesis, only with the red cell enzyme. These differences in behaviour of the red cell and kidney enzymes are explained on the basis of the observed predominance of K^+-insensitive EP in red cell, but K^+-sensitive EP in kidney ($Na^+ + K^+$)-ATPase at 0°C.

Introduction

In an earlier study carried out at 0°C, it was observed that the kinetic behaviour of ($Na^+ + K^+$)-ATPase of human red cells is modulated by ATP in an unexpected manner [1]. Thus, when ^{32}P-labelled phosphoenzyme is formed at very low ATP concentration (0.02 μM) and then EDTA is added, rapid disappearance of the phosphoenzyme intermediate is observed. The initial rapid phase of phosphoenzyme disappearance is, for the most part, not associated with $^{32}P_i$ release and its rate constant, k_d, is several-fold greater than the ratio of Na^+-ATPase (v) to phosphoenzyme intermediate (EP), v/EP, at steady state. This rapid disappearance is a consequence of reversal of phosphorylation and is not observed when higher levels of ATP are used to phosphorylate the enzyme, or when excess non-radioactive ATP (plus Mg^{2+}) are added as a 'chase'. ATP modulation of the equilibrium between an enzyme-ATP complex (E · ATP) and phosphoenzyme product(s) was evidenced also in changes in the ratio E · ATP, (measured by the pulse chase method of Post et al. [2]) to the level of total phosphoenzyme at steady state.

The importance of extending these kinetic studies is their potential in elucidating the fundamental role of ATP binding and release in energy transduction by ($Na^+ + K^+$)-ATPase. However, because of certain limitations of the red cell enzyme (low specific activity, difficulty in measuring phosphoenzyme with ATP concentrations greater than about 10^{-6} M due to non-specific phosphorylations), further kinetic experiments should be carried out with a much more active source of enzyme. With this objective in mind, we have first

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addressed the question whether the previous findings were peculiar to the red cell enzyme and, if so, to elucidate their basis. In this paper, we compare the behaviour of $(Na^+ + K^+)$-ATPase obtained from guinea pig kidney with that of the human red cell.

Methods

Experiments were carried out using $(Na^+ + K^+)$-ATPase from guinea pig kidney cortex prepared according to the method of Post and Sen [3] and from human red blood cell membranes as described previously by Blostein [1]. [γ-^{32}P]ATP was prepared, ^{32}P-labelling of the membrane (EP) and measurements of Na^+-ATPase ($^{32}P_i$ release) were carried out as described previously [1]. All values shown are averages of duplicate or triplicate measurements and for experiments performed three or more times, standard errors are given.

Results

Disappearance of ^{32}P-labelled EP at varying ATP concentration

The phosphoenzyme was formed at 0°C by incubation with [γ-^{32}P]ATP, Mg^{2+} and Na^+. Its disappearance was followed during a subsequent 'chase' with either excess unlabelled ATP (plus Mg^{2+}) or EDTA to stop further phosphorylation. As shown in Table I, labelled phosphoenzyme decayed more rapidly ($k_d = 0.081\ s^{-1}$) after EDTA addition than after MgATP addition ($k_d = 0.027\ s^{-1}$) in red cell, but not kidney enzyme. The latter value is in good agreement with the turnover calculated as the ratio v/EP (0.023 s^{-1}) shown in Table II. These values were estimated from the initial 5-s decrease in EP. Similar experiments done at higher ATP (1 μM) with the red cell enzyme showed that the difference between k_d and v/EP is still evident but to a lesser extent. It was shown previously that the rapid disappearance of EP upon EDTA addition can be attributed to reversal of phosphorylation, presumably EP · ADP $\rightarrow$ E · ATP $\rightarrow$ E + ATP, rather than to release of $^{32}P_i$. It should be noted, of course, that in these and subsequent experiments, EP decay rates calculated on the basis of the initial (5 s) decreases in EP are meaningful only as relative values, for example to compare effects of ATP versus EDTA chases (Table I), or EDTA without and with K^+ (Table IV) since dephosphorylation curves may well be biphasic.

TABLE I

EP DISAPPEARANCE FOLLOWING ATP AND EDTA 'CHASE'

Kidney and red cell membranes were incubated at 0°C, media containing 0.02 μM [γ-^{32}P]ATP, 1.0 mM $MgCl_2$, 25 mM Tris-HCl, and 50 mM NaCl, pH 7.4 at the concentrations of ATP indicated. The volume of the initial incubation medium, including membranes was either 0.1 or 0.2 ml. Reactions were initiated by adding one volume of reaction medium to one volume of membranes in a conical test tube in an ice-water bath at 0°C. Protein concentrations used in this series of experiments ranged from 0.14 to 0.39 mg/ml for the kidney enzyme and 0.22 to 1.92 mg/ml for the red cell enzyme. After 15 s of incubation at 0°C an equal volume of a 'chase' solution consisting of either (i) 15 mM EDTA (Tris form) in 50 mM NaCl, 25 mM Tris-HCl, pH 7.4 or (ii) 20 μM ATP in 50 mM NaCl, 1 mM $MgCl_2$ and 25 mM Tris-HCl, pH 7.4. The reaction was stopped 5 s after the 'chase' and the level of ^{32}P-labelled EP was determined as described in Methods. Control tubes to which no chase was added were included with each experiment. The k_d levels represent the decay rate of phosphoenzyme calculated from the phosphoenzyme level measured 5 s after the addition of the 'chase', compared to the control level. Calculations were based on measurements in presence of Na^+ minus the K^+-baseline level of phosphorylation.

Enzyme source	n	Apparent rate constant for EP disappearance	
		k_d (EDTA chase) (s^{-1})	k_d (ATP chase) (s^{-1})
Kidney	7	0.108 ± 0.01	0.113 ± 0.03
Red cell	7	0.081 ± 0.03	0.027 ± 0.01

Apparent phosphoenzyme turnover measured as ratio of Na^+-ATPase/phosphoenzyme

Measurements of the apparent turnover of phosphoenzyme, the ratio Na^+-ATPase (v) to phosphoenzyme level at steady state, v/EP, were carried out for enzyme from both kidney and red cell at several concentrations of ATP. As shown in Table II, at 0°C and very low ATP concentration (0.02 μM), the kidney phosphoenzyme has a much higher (approx. 5-fold) apparent turnover than the red cell. As the concentration of ATP is increased

TABLE II

APPARENT TURNOVER OF TOTAL PHOSPHOENZYME AS A FUNCTION OF ATP CONCENTRATION AT 0°C

Membranes were incubated in a medium containing varying amounts of [γ-^{32}P]ATP, 0.1 mM $MgCl_2$, 25 mM Tris-HCl and 50 mM NaCl, pH 7.4, at either 0°C or 37°C, as indicated. The reaction volume used for the red cell experiments was 0.2 ml, that used for the kidney enzyme experiments, 0.1 or 0.2 ml. EP and Na^+-ATPase (v) were measured as described in Methods. Calculation of v and EP were based on measurements in presence of Na^+ minus the K^+-baseline activity.

Enzyme source	Temp. (°C)	ATP concn. (μM)	$v/[^{32}P]EP$ (s^{-1})
Kidney	0	0.02	0.087±0.030 (n=14)
		0.20	0,120±0.030 (n=9)
		2.00	0.100±0.040 (n=4)
		20.00	0.110±0.050 (n=3)
	37	2.00	11.49
Red cell	0	0.02	0.017±0.007 (n=5)
		0.10	0.016 [a]
		0.20	0.020
		0.50	0.034 [a]
		2.00	0.068 [a]
	37	2.00	7.39

[a] Data are from the same experiment.

from 0.02 to 2.0 μM, red cell phosphoenzyme turnover tends to increase 3-fold, that of the kidney remains unchanged, even as the ATP concentration is raised to 20 μM. Experiments with the red cell enzyme with concentrations greater than 2 μM were not feasible due to a relatively large baseline activity at the higher ATP levels, i.e. relatively high Na^+-insensitive hydrolysis (K^+-baseline) as well as high 'stable' phosphorylation (K^+-baseline, insensitive to an ATP chase).

The effect of ATP concentration on the 'pulse-chase' level of E · ATP relative to EP at steady state

Using kidney ($Na^+ + K^+$)-ATPase, Post et al. [2] presented evidence indicating formation of an E · ATP complex which precedes EP; neither Mg^{2+} nor Na^+ are required for its formation, but both are required for phosphorylation. In similar 'pulse-chase' experiments, E · ATP was detected in red cell ($Na^+ + K^+$)-ATPase [1].

In the present study, E · ATP was measured in both kidney and red cell systems by incubating at 0°C with concentrations of [γ-^{32}P]ATP ranging from 0.02 to 2.0 μM under non-phosphorylating conditions (1 mM EDTA present; $MgCl_2$ and, in some experiments, Na^+ omitted). After incubation for 15 s, a chase solution containing excess non-radioactive ATP, $MgCl_2$ and NaCl was added and the reaction stopped at intervals thereafter. It is assumed that any radioactively labelled EP formed after the chase reflects the level of E · ATP present before the chase. The subsequent exponential decrease in EP with time, extrapolated to the amount of EP at the time of addition of the chase is then a measure of E · ATP (c.f. Post et al. [2]). The steady-state level of EP was determined under the same conditions, but with Mg^{2+} and Na^+ present initially.

The ratio E · ATP/EP, i.e., 'pulse-chase' EP/'steady-state' EP as a function of ATP concentration is presented in Table III. As shown, E · ATP/EP remains constant in the kidney enzyme (approx. 0.7) but increases with increasing ATP concentration in the red cell enzyme as reported previously [1] and included in Table III for comparison. Similar results were obtained whether or not Na^+ was included in the preincubation with [γ-^{32}P]ATP plus EDTA.

Relative amounts of K^+-sensitive/K^+-insensitive EP

According to studies of Fukushima and Tonomura [4], ADP-insensitive phosphoenzyme precedes an ADP-sensitive form; according to the Albers-Post mechanism, an ADP-sensitive form precedes an ADP-insensitive, K^+-sensitive form [2,5]. Glynn and Karlish [6] pointed out that these observations can be reconciled with the following sequence: $E_1 + ATP \rightleftharpoons E_1 \cdot ATP \rightleftharpoons E_1P \cdot ADP \rightleftharpoons E_1P + ADP$; $E_1P \rightleftharpoons E_2P$ whereby $E_1P \cdot ADP$ represents K^+-insensitive, ADP-insensitive phosphoenzyme, E_1P is ADP-sensitive, K^+-insensitive phosphoenzyme and E_2P is K^+-sensitive phosphoenzyme [6] (subscripts '1' and '2' denote distinct forms of the enzyme, e.g. distinct conformations with respect to sidedness of their cation-facing sites). The possibility that the relative amounts of these (acid-stable) forms at steady state at 0°C differ in red cell and kidney enzyme was tested by examining the relative amounts of K^+-sensitive

TABLE III

RATIO E·ATP/EP AT VARIOUS CONCENTRATIONS OF ATP

These experiments were done as described previously [1]. Briefly in Part A, kidney membranes were first incubated in a medium containing 2 mM EDTA, 25 mM Tris-HCl and [γ-^{32}P]ATP in concentrations of 0.02, 0.20, and 2.0 μM, pH 7.4 at 0°C. 50 mM NaCl was included in some experiments such as the representative red cell enzyme experiment shown. The reaction was started by the addition of 0.075 ml reaction medium to 0.025 ml membranes in a conical test tube. After 15 s of incubation, 0.10 ml of a 'chase' solution containing 0.2 mM ATP (Tris form), 6 mM $MgCl_2$ and either 50 or 100 mM NaCl was added. After the 'chase' the medium contained 0.1 mM ATP, 50 mM NaCl, 3 mM $MgCl_2$, 1 mM EDTA and 12.5 mM Tris-HCl and the final pH was 7.1. Incubation was continued and the reactions were stopped after intervals of 5, 10 and 15 s. The level of phosphoenzyme, representing E·ATP at the point of addition of the chase, was obtained by extrapolating the logarithmic plot of EP versus time after chase, the rate constant for EP disappearance being 0.08 s^{-1} and independent of ATP concentration. This level was compared (ratio E·ATP/EP) to steady-state EP, determined in the same experiment by incubation for 15 s in a medium containing 1 mM $MgCl_2$, 25 mM Tris-HCl, 50 mM NaCl and the corresponding concentration of [γ-^{32}P]ATP, minus the K^+-baseline level. The red cell experiments were performed in an identical manner except that the initial reaction volume was 0.2 ml (0.1 ml membranes, 0.1 ml medium). 0.2 ml of chase solution were added and the reactions were stopped after intervals of 5, 15 and 30 s. For comparison, data from Blostein [1] are included in Part B.

	ATP concn. (μM)	Human red cell			Guinea pig kidney		
		E·ATP [a] (pmol/mg)	EP [a] (pmol/mg)	E·ATP/EP	E·ATP [a] (pmol/mg)	EP [a] (pmol/mg)	E·ATP/EP
A	0.01	–	–	–	–	–	–
							(0.61, $n=2$)
	0.02	0.114	0.577	0.20			
				(0.14±0.07, $n=4$) [b]	7.19	9.36	0.77
							(0.58±0.12, $n=5$) [b]
	0.20	0.501	0.946	0.53			
				(0.36±0.12, $n=6$) [b]	26.59	38.18	0.70
							(0.56±0.17, $n=3$) [b]
	2.00	–	–	–	47.68	65.10	0.73 ($n=1$)
B	Data from Ref. 1.						
	0.02	0.046	0.25	0.18			
	0.05	0.246	1.014	0.24			
	0.20	0.308	0.995	0.31			
	1.00	0.519	0.938	0.55			

[a] Values are from a single representative experiment carried out at the various ATP concentrations shown.

[b] Values in parentheses are mean E·ATP/EP ratios ± standard devations from several ($n \geq 3$) separate experiments.

EP. The membrane preparations were incubated in the presence of 0.02 μM [γ-^{32}P]ATP, 0.1 mM $MgCl_2$ and 50 mM NaCl, pH 7.4 at 0°C for 15 s at which time a 'chase' solution containing either 15 mM EDTA in 50 mM NaCl or 15 mM EDTA in 50 mM NaCl plus 10 mM KCl was added and the reactions stopped 5 s later.

A marked difference between the two enzyme systems with respect to K^+-sensitivity of the phosphoenzyme was observed (Table IV). Based on the 5-s decay in EP following an EDTA chase, it is concluded that K^+ increased the rate constant for disappearance of phosphoenzyme markedly in kidney (4.6-fold) but only slightly in red cell (1.4-fold) ($Na^+ + K^+$)-ATPase (Table IV). Relatively little K^+-sensitive EP was observed also in other studies with red cell ($Na^+ + K^+$)-ATPase carried out at higher ATP concentrations at 0°C [1,7].

In the representative experiment shown in Table IV (Expt. 2), 89% of the total kidney EP disappeared in 5 s in the presence of K^+, but only 37% in absence of K^+. In both instances practically all ($\geq$ 85%) of the decrease in EP appeared as P_i consistent with the conclusion that K^+ accel-

TABLE IV

K^+-SENSITIVITY OF THE PHOSPHOENZYME INTERMEDIATE AT 0°C

Membranes were incubated in media containing 25 mM $MgCl_2$, 0.02 μM [γ-^{32}P]ATP and 50 mM NaCl, pH 7.4. Reactions were initiated by the addition of 1 vol. medium to 1 vol. membranes. After 15 s incubation at 0°C, the reaction was terminated in one set of tubes and to two other sets were added 0.1 ml of a 'chase' solution containing 15 mM EDTA (Tris form) in 50 mM NaCl, 25 mM Tris-HCl, pH 7.4 without (one set) or with 10 mM KCl (another set). The reaction was stopped after a further 5 s of incubation. In the experiment with oligomycin, membranes were pre-incubated with either oligomycin in ethanol (oligomycin present) or ethanol alone as described previously [1] for 20 min at 37°C then 10 min at 0°C. All values represent activity in the presence of Na^+ minus the K^+-baseline activity. n.d., not determined.

Expt. No.	Tissue	K^+ in chase	[^{32}P]EP at steady state (pmol/mg)	[^{32}P]EP 5 s after chase (pmol/mg)	ΔEP (pmol/mg)	ΔP_i (pmol/mg)
1	Red cell	−	0.305	0.185	0.120	n.d.
		+		0.152	0.153	n.d.
2	Kidney [a]	−	11.290	7.093	4.196	3.789
		+		1.299	9.991	8.485
3a	Kidney,	−	25.06	20.96	4.100	-
	oligomycin present	+		19.92	5.140	
3b	Kidney,	−	13.22	9.318	3.901	n.d.
	oligomycin absent	+		0.788	12.43	n.d.

[a] K^+-sensitive EP: 9.991 − 4.196 = 5.795 pmol/mg.
K^+-sensitive P_i: 8.485 − 3.789 = 4.669 pmol/mg.

erates dephosphorylation ($EP + K^+ \rightarrow K \cdot E + P_i$). In contrast to the kidney enzyme, relatively little red cell EP was K^+-sensitive, i.e. 50% disappeared in presence of K^+ and 39% in absence of K^+.

Experiments were also done in which the kidney enzyme was pre-incubated in the presence of oligomycin (30 min at 37°C), since oligomycin is believed to inhibit the E_1P to E_2P conformational transition. As shown in Table IV, oligomycin increased total EP but reduced K^+-sensitive EP to less than 10% of that observed without oligomycin.

Discussion

In this study, we have confirmed and extended earlier observations which suggested that ATP modulates ($Na^+ + K^+$)-ATPase. Thus, at 0°C and as ATP is reduced from about 10^{-6} to 10^{-7} M or less, several kinetic changes are observed: the steady-state ratio of 'pulse chase' $E \cdot ATP$ to EP at steady state is decreased, rapid reversal of a fraction of total EP occurs upon EDTA addition and the apparent turnover of phosphoenzyme is reduced. That this behaviour is observed with red cell but not kidney ($Na^+ + K^+$)-ATPase is most likely a consequence of the finding that the steady state between phosphoenzyme forms at 0°C favors K^+-insensitive EP in the red cell enzyme. Accordingly, in the sequence, $E_1 + ATP \rightleftharpoons E_1 \cdot ATP \rightleftharpoons E_1P \cdot ADP \rightleftharpoons E_1P + ADP$; $E_1P \rightleftharpoons E_2P$, if ATP-dependent modulation occurs prior to the $E_1P \rightleftharpoons E_2P$ step, an effect would not be apparent if the steady state were poised in favor of K^+-sensitive E_2P as in the case of the kidney enzyme at 0°C.

Although ($Na^+ + K^+$)-ATPase from various sources may differ structurally, kinetic differences which are evident as the temperature is reduced may be explained simply on the basis of differences in their lipid environment. Thus, lipids which differ in the temperature-dependency of their physical properties, e.g. lateral compressibility, could have a marked effect on relative rates of ($Na^+ + K^+$)-ATPase reaction steps, particularly those ascribed to conformational changes. Relevant to this possibility is the recent study of Hegyvary et al. [8] who showed that delipidation affected the rate of the phosphoenzyme conformational change, $E_1P \rightleftharpoons E_2P$, shifting the equilibrium toward E_1P.

The idea that ATP might act as a modulator of

the $(Na^+ + K^+)$-ATPase reaction is supported by the reports describing high-affinity and low-affinity ATP sites [9,10]. Several investigators have proposed an alternating-sites, half-of-the sites reactivity mechanism for $(Na^+ + K^+)$-ATPase whereby each catalytic subunit of the (presumably dimeric) enzyme catalyzes the reaction sequence, but with the subunits out of phase [11,13]. Alternatively, low- and high-affinity sites could exist sequentially on one peptide as the enzyme proceeds through different steps in the reaction sequence. In other words, it may be argued that the modulation of kinetics by ATP as evidenced in these experiments with red cell $(Na^+ + K^+)$-ATPase at 0°C is apparent only because the ATP concentration decreases to levels which no longer saturate the site during its lower affinity state. Evidence for a single site which changes its affinity during turnover of $(Na^+ + K^+)$-ATPase has recently been obtained by Moczydlowski and Fortes [14].

Although our results are compatible with two ATP sites, one a modulating site with lower affinity than the other, catalytic site, with the red cell enzyme at 0°C, the K_{ATP} values for both sites are roughly in the micromolar range, that is within the range of affinity of only the 'high-affinity' site described earlier [9,10] but consistent with the two affinities for ATP on the 'Na^+/Na^+' exchange pathway observed by Skou and Esmann [15].

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