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The ATPase activity of saponin-treated rat erythrocytes: regulation by monovalent cations, calcium, ouabain, and furosemide

V.V. Petrunyaka [1], E.A. Panyushkina [1], E.P. Severina [1] and S.N. Orlov [2]

[1] *Institute of Biological Physics, USSR Academy of Sciences, Pushchino, Moscow Region (U.S.S.R.) and* [2] *Department of Physical Chemistry of Biomembranes, School of Biology, M.V. Lomonosov Moscow State University, Moscow (U.S.S.R.)*

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The ATPase activities were studied in rat erythrocytes permeabilized with saponin. The concentrations of calcium and magnesium ions were varied within the range of 0.1–60 μM and 50–370 μM, respectively, by using EGTA-citrate buffer. The maximal activity of Ca^{2+}-ATPase of permeabilized erythrocytes was by one order of magnitude higher, whereas the Ca^{2+}-binding affinity was 1.5–2 times higher than that in erythrocyte ghosts washed an isotonic solution containing EGTA. Addition of the hemolysate restored the kinetic parameters of ghost Ca^{2+}-ATPase practically completely, whereas in the presence of exogenous calmodulin only part of Ca^{2+}-ATPase activity was recovered. Neither calmodulin nor R24571, a highly potent specific inhibitor of calmodulin-dependent reactions, influenced the Ca^{2+}-ATPase activity of permeabilized erythrocytes. At Ca^{2+} concentrations below 0.7 μM, ouabain (0.5–1 mM) activated whereas at higher Ca^{2+} concentrations it inhibited the Ca^{2+}-ATPase activity. Taking this observation into account the Na^+/K^+-ATPase was determined as the difference of between the ATPase activities in the presence of Na^+ and K^+ and in the presence of K^+ alone. At physiological concentration of Mg^{2+} (370 μM), the addition of 0.3–1 μM Ca^{2+} increased Na^+/K^+-ATPase activity by 1.5–3-fold. Higher concentrations of this cation inhibited the enzyme. At low Mg^{2+} concentration (e.g., 50 μM) only Na^+/K^+-ATPase inhibition by Ca^{2+} was seen. It was found that at [NaCl] < 20 mM furosemide was increased ouabain-inhibited component of ATPase in Ca^{2+}-free media. This activating effect of furosemide was enhanced with a diminution of [Na^+] upto 2 mM and did not reach the saturation level unless the 2 mM of drug was used. The activating effect of furosemide on Na^+/K^+-ATPase activity confirmed by experiments in which the ouabain-inhibited component was measured by the $^{86}Rb^+$ influx into intact erythrocytes.

Introduction

The role of Mg^{2+}-dependent adenosine triphosphatases activated by monovalent cations (Na^+/K^+-ATPase) or Ca^{2+} (Ca^{2+}-ATPase) in ion transport has been studied in great detail with regard to mammalian erythrocytes, human and rat red blood cells, in particular [1–3]. In these studies the kinetic parameters of ATPases were determined in unresealed membrane fragments or inside-out vesicles obtained by long-term hypotonic hemolysis. One of the main limitations of this method is the uncontrollable loss of cytoplasmic regulators associated with the inner side of the membrane by Ca^{2+}-dependent interactions. Calmodulin and calnactin provide a typical example of such regulators [4,5].

A limited number of studies of ATPase activity were carried out on right-side-out vesicles which are, more frequently, termed as 'resealed ghosts'. These vesicles are obtained by rapid (1–2 min) hemolysis followed by restoration of the isoosmotic conditions due to addition of aliquots of hypertonic solution. This approach is very helpful in that it permits us to obtain vesicles which preserve the major components of the cytoplasm, on the one hand, and allow for variations in the internal concentration of ATP, Na^+, K^+, Mg^{2+} and Ca^{2+} concentrations within the desired range, on the other. It should be stressed, however, that because of the reversibility of the partial reactions of E_1-E_2 type ATPases their total activities both in right-side-out and inside-out

Correspondence: V.V. Petrunyaka, Institute of Biological Physics, USSR Academy of Sciences, Pushchino, Moscow Region, 142292, U.S.S.R.

vesicles may vary within very broad limits, depending on the values of transmembrane electrochemical gradients of Na^+, K^+ and Ca^{2+} [6]. Thus, to treat erythrocytes with saponin seems to be a very perceptive approach. It is known that at concentrations of 0.002–0.05 per cent saponin interacts with plasma membrane cholesterol by forming pores that are highly permeable for ions, nucleotides and low molecular weight proteins without any effect on the structural organization of membrane proteins which do not interact with cholesterol [7,8]. For example, it has been shown that even 0.2 per cent saponin is unable to change the Ca^{2+}-ATPase activity in membrane fragments of human erythrocytes obtained by hypotonic hemolysis [9]. However, there are only reports concerning the comparative analysis of some kinetic properties of Ca^{2+}-ATPase of saponin-treated human erythrocytes with those of hypotonic membranes [10,11].

The lack of standard conditions with regard to Ca^{2+} and Mg^{2+} concentrations as well as pH, is a considerable obstacle to the analysis of papers concerned with the study of kinetic properties and regulation of transport ATPases. As a rule EGTA is used to vary Ca^{2+} concentration. This EGTA has proven to be a very reliable Ca^{2+} buffer, especially in the pH range of 7.2–7.4 and at [Ca]/[EGTA] < 1 (free calcium concentration < 1 μM). However, it should be stressed that a pH change within the range of 7.1 to 7.3 causes a 2-fold decrease of $[Ca^{2+}]$. Such changes are practically unavoidable in case of resealed vesicles, since even a minor change in the membrane potential, e.g., approx. 10 mV, is sufficient to change the pH_{in} in the given range [12]. Moreover, the kinetic and regulatory properties of Na^+/K^+-ATPase and particularly of Ca^{2+}-ATPase, depend critically on the concentration of free Mg^{2+} [13]. In the majority of papers this parameter is neglected.

The present work deals with the description of kinetic parameters of activity of Na^+/K^+- and Ca^{2+}-ATPases of saponin-treated rat erythrocytes. The kinetic parameters of Ca^{2+}-ATPase were studied with the use of a EGTA-citrate-ATP buffer which allows variation of the Ca^{2+} concentration from 0.01 to 100 μM at 10–500 μM of Mg^{2+}. The same buffer was used to study the effect of ca^{2+} on the activity of Na^+/K^+-ATPase. It was found that ouabain, depending on Ca^{2+} concentration, increases or decreases the Ca^{2+}/ATPase activity by 30–40 per cent. For this very reason to identify th Na^+/K^+-ATPase we compared the enzyme activity in $(Na^+ + K^+)$-containing media and in Na^+-free media. To elucidate the role of endogenous calmodulin in regulation of Ca^{2+}-ATPase, we varied the concentration of the former in suspension of permeabilized erythrocytes or added the compound R24571, a highly potent specific inhibitor of calmodulin-dependent reactions. Besides, taking into account the data on the identification of the furosemide-sensitive Na^+-ATPase in kidney epithelial cells we studied the effect of furosemide on ATPase activity of saponin-treated erythrocytes.

Materials and Methods

The experiment were carried out on male albino rats. Before blood sampling animals were starved with free access to water. The data on the strains of rats used in the study and their age are given in figure captions. Blood was collected from the bifurcation of the abdominal aorta and run into test-tubes coated with heparin (20–40 units per ml of blood). After storage (0–4 °C, 2 h) blood samples were centrifuged for 10 min at 1500 × *g*, plasma and white cells were discarded, and erythrocytes were washed twice under the same conditions with a solution containing 130 mM KCl, 20 mM Tris-HCl and 0.37 mM $MgCl_2$ (pH 7.4, 4 °C). The pellets of packed erythrocytes were stored on the ice for not more than 3–5 h.

Saponin-treated erythrocyte

Hematocrit of erythrocyte suspension adjusted to 10 per cent by using a medium A which contained 130 mM KCl, 8.3 mM $MgCl_2$ ($[Mg^{2+}] = 370$ μM) or 3 mM $MgCl_2$ ($[Mg^{2+}] = 50$ μM), 20 mM Hepes-Tris (pH 7.4 at 37 °C). Saponin (final concentration 0.04%) was added to the suspensions immediately before assay of ATPase activity.

Erythrocyte ghosts

To preserve or to remove endogenous Ca^{2+}-dependent regulators permeabilized erythrocytes were washed with medium A containing 130 mM KCl, 0.37 mM $MgCl_2$, 20 mM Tris-HCl and about 10 μM Ca^{2+} as admixture or the same medium with 2 mM EGTA, respectively. The ghosts were sedimented by centrifugation at 16000 × *g* for 30 min and supernatant was discarded. The precipitate was resuspended in the same medium without EGTA, sedimented as above and resuspended in medium A to reach a volume equal to 10 per cent of the initial volume of paced erythrocytes.

ATPase assay

Permeabilized erythrocyte or suspension of ghosts (100 μl) were mixed with an equal volume of medium B or C and incubated for 10 min at 37 °C. The reaction was stopped by adding 200 μl of ice-cold 10 per cent TCA. The inorganic phosphate content in the protein-free supernatant was measured by Muszbec et al. method [15] with some modifications [16]. Medium B contained the same component as medium A with addition 2 mM ATP (sodium or potassium salt), 2 mM EGTA and $CaCl_2$ (see Table I). In medium C KCl was replaced by NaCl. To obtain desired concentrations of free calcium (Ca^{2+}) two solutions were prepared using media B and

TABLE I

Appropriate concentrations of total calcium (Ca_t^{2+}) and magnesium (Mg_t^{2+}) to produce of predetermined concentrations of Ca^{2+} ions in presence of 50 or 370 μM Mg^{2+} ions, 5 or 10 mM citrate, 1 mM EGTA, 1 mM ATP at pH 7.4, ionic strength 0.16, 37°C.

Ca^{2+} (μM)	Ca_t^{2+} (μM)		
	For 5 mM citrate at 50 μM Mg^{2+} (Mg_t^{2+} = 1660 μM)	For 10 mM citrate	
		at 50 μM Mg^{2+} (Mg_t^{2+} = 2950 μM)	at 370 μM Mg^{2+} (Mg_t^{2+} = 8360 μM)
0.1	632	634	622
0.3	843	849	836
0.5	907	917	900
0.7	939	954	932
1.0	969	990	960
2.0	1020	1062	1005
3.0	1053	1116	1033
5.0	1109	1212	1076
7.0	1158	1303	1115
10.0	1230	1434	1170
60.0	2032	2805	1836

Note: with Mg^{2+} = 50 μM, Mg-ATP = 340 μM; with Mg^{2+} = 370 μM, Mg-ATP = 700 μM.

C: one without $CaCl_2$ and one containing the maximal concentration of $CaCl_2$ (60 μM) (see Table I).

Previously it was shown that under conditions of maximal ATPase activation the release of inorganic phosphate (P_i) was linear up to 30 min incubation. In our experiments the incubation time was 10–20 min. To determine the Ca^{2+}-ATPase activity, the experiments were performed in Na^+-free media, and the rate of P_i accumulation at a given [Ca^{2+}] was compared to that in the absence of Ca^{2+}. The Na^+/K^+-ATPase activity was determined as the difference between the rates of P_i liberation in an ($Na^+ + K^+$)-containing and in an Na^+-free media. To determine the ouabain-inhibited component of the ATPase activity the 0.5–1 mM of this compound was added in the ($Na^+ + K^+$)-containing medium. The Mg^{2+}-ATPase activity was determined by the rate of P_i release in the absence of Na^+ and Ca^{2+}.

Calculation of free calcium and magnesium concentrations

A personal computer program was written using a system of equations described in [17]. The means of stability constants and their temperature coefficients were used from [8,18]. The values of calculated complex stability constants (at pH 7.4, ionic strange 0.16, 37°C) were as follows: $K_{Ca\text{-}EGTA} = 17\,130\,900\ M^{-1}$, $K_{Mg\text{-}EGTA} = 128\ M^{-1}$, $K_{Ca\text{-}ATP} = 7097\ M^{-1}$, $K_{Mg\text{-}ATP} = 15\,706\ M^{-1}$, $K_{Ca\text{-}citrate} = 5785\ M^{-1}$, $K_{Mg\text{-}citrate} = 6950\ M^{-1}$.

To evaluate the activity of the erythrocyte Na^+/K^+-pump the ouabain-inhibited component of the $^{86}Rb^+$ influx was determined by the methods described earlier [19]. The medium C supplemented with 5 mM KCl and 2 μCi $^{86}RbCl$ was used in this study. Intracellular [Ca^{2+}] was varied by adding 10 μM of A23187. To suppress Ca^{2+} activated K^+-channels quinidine (200 μM) was added. The incubation time was limited to 5 min.

Chemicals

The salts were purchased from BDH (U.K.), Serva (F.R.G.) or Soyuzkhimreaktiv (U.S.S.R.). Tris, Hepes, EGTA, ouabain, citric acid, R24571 (calmidazolium), saponin and quinidine were purchased from Serva, ATP (sodium and potassium salts), furosemide and ouabain were from Sigma (U.S.A.). All reagents were analytical grade. $^{86}RbCl$ was from Amersham (U.K.). Bovine brain calmodulin was a kind gift from Dr. E.L. Permyakov (Institute of Biophysics, USSR Academy of Sciences, Pushchino, U.S.S.R.) and purchased from Serva.

Results

Mg^{2+}-ATPase and Ca^{2+}-ATPase

The activity of Mg^{2+}-ATPase in saponin-permeabilized rat erythrocytes at [Mg^{2+}] = 50 and 370 μM was about 12–18 mmol P_i per litre of cells per h (data not presented).

The activity of Ca^{2+}-ATPase increased with an increase in [Ca^{2+}] up to 2–5 μM. Higher concentrations of ca^{2+} inhibited the enzyme (Fig. 1, curve 1). The inhibiting effect of excess Ca^{2+} on Ca^{2+}-ATPase was demonstrated for inside-out vesicles, unresealed membrane fragments and purified enzyme preparations; however, the mechanism of this phenomenon is still obscure [13].

The maximal activity of Ca^{2+}-ATPase of permeabilized erythrocytes varied from 6 to 60 mmol P_i per litre of cells per h (Figs. 1–3). In mongrel albino rats this value calculated for saponin-treated erythrocytes washed with EGTA from endogenous Ca^{2+}-dependent regulators is 6–7 mmol P_i per litre of cells per h (Fig. 1, curve 2), i.e., was close to that obtained earlier for

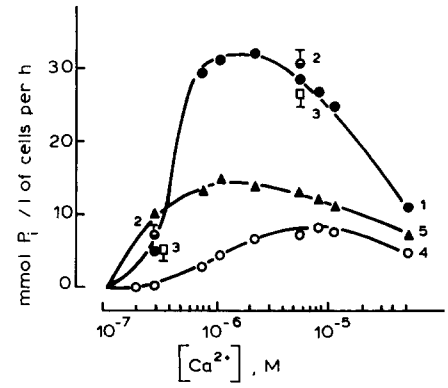

Fig. 1. Ca^{2+}-dependence of Ca^{2+}-ATPase activity in saponin-permeabilized erythrocytes and their ghosts obtained by washing with EGTA (4,5). 1 and 4, control; 2 and 5, +10 μM calmodulin; 3, +10 μM R24571. [Mg^{2+}] = 50 μM. The experiments were performed on male Wistar wag rats aged 4–5 months.

TABLE II

Effects of calmodulin and R24571 on the maximal activity of Ca^{2+}-ATPase

The mean + S.E. (data from four independent measurements). The maximal activity of Ca^{2+}-ATPase in saponin-treated erythrocytes was taken for 100 per cent. * By reason of a great scatter in values in large samples ($n = 15$) the extreme values are given. The experiments were performed on male Wistar vag rats and on Mongrel albino rats aged 4–5 months.

Additions (μM)	Saponin-treated erythrocytes	Ghosts washed at 10 μM Ca^{2+}	Ghosts washed at 2 mM EGTA	Ghosts washed at 2 mM EGTA plus supernatant
Control	100.0 + 7.8	74.7 + 7.0	4.5 – 39.0 *	75.5 + 8.2
R24571 (10 μM)	99.7 + 7.9	–	3.0 – 29.4 *	56.5 + 6.3
Calmodulin (10 μM)	102.3 + 5.4	–	42.0 + 9.2	–

erythrocyte ghosts treated with hypotonic EGTA-containing solutions [20]. Previously it was shown that the affinity to Ca^{2+} and the maximal activity of Ca^{2+}-pump in rat erythrocyte inside-out vesicles washed with EGTA are measured after addition of calmodullin. As can be seen from Fig. 1 (curves 4 and 5), calmodulin added to saponin-perforated erythrocyte ghosts increased the maximal activity and the affinity to Ca^{2+} of Ca^{2+}-ATPase. The calmodulin concentration used in this study (10 μM) was commensurate with its concentration in rat erythrocytes [21]. However, as can be seen from Table II and Fig. 1, exogenous calmodulin failed to induce the complete reconstitution of the system; under study the maximal activity was two times as low as that of saponin-treated erythrocytes prior to the washing in EGTA-containing solution. Unlike calmodulin the hemolysate addition was able to reconstitute the system practically complete. In this case the difference between the maximal activities of Ca^{2+}-ATPase in saponin-treated erythrocytes and their ghosts did not exceed 20–30 per cent.

It is known, that antibiotic R24571 termed as calmidazolium, is the most potent inhibitor of calmodulin-dependent reactions. For example, this compound at concentrations of 5–8 μM fully inhibits the calmodulin-stimulated component of Ca^{2+}-ATPase purified from human erythrocytes [22]. Our previous studies failed to establish any appreciable effect of calmidazolium and endogenous calmodulin on the maximal activity of Ca^{2+}-ATPase of saponin-treated rat erythrocytes [16]. The same results were obtained in the present study. Moreover, as can be seen from Fig. 1 and Table II, the addition of these compounds to permeabilized erythrocytes did not alter the ATPase activity either at low Ca^{2+} concentrations (0.3 μM) or at high ones. This suggest that in intact erythrocytes calmodulin does not control either the maximal activity or the affinity of Ca^{2+}-ATPase to Ca^{2+}.

Note that the data shown in Fig. 1 and Table II were obtained at $[Mg^{2+}] = 50$ μM. It is known that the free Mg^{2+} concentration in human and rat erythrocytes is 200–400 μM [23,24]. We found, however, that an increase in $[Mg^{2+}]$ from 50 to 370 μM did not influence the kinetic parameters of Ca^{2+}-ATPase (Fig. 1, curve 1 and Fig. 2, curve 2). It may be deduced from these data that under these conditions Mg^{2+}-ATPase activity is slightly increased (13 + 0.7 vs. 15 + 2.3 mmol per litre of cells per h).

Effect of ouabain

Most reports testify to the usefulness of ouabain in the determination of Na^+/K^+-ATPase activity. This compound, when used at a concentration of 1 mM, exhibits a remarkable ability to fully inhibit all enzyme isoforms without interfering with the activities of other ATPases, including Ca^{2+}-ATPase. The latter circumstance is especially essential in the study of Ca^{2+} effect on the Na^+/K^+-ATPase activity. To our knowledge the first statement on the absence of ouabain effect on Ca^{2+}-ATPase [25,26] was based on single observations testifying to the lack of effect of relatively low (10^{-5} M) ouabain concentrations on the kinetics of ATP-dependent Ca^{2+} efflux from human erythrocyte right-side-out vesicles [27]. Some recent reports suggest that in unresealed fragments of erythrocyte membranes ouabain activates and/or inhibits Ca^{2+}-ATPase; this effect depends both on ouabain and calmodulin concentrations [28]. In our study ouabain had no effect on the ATPase activity in the absence of Ca^{2+} and Na^+, e.g., Mg^{2+}-dependent ATPases (data not shown). The effect of 1 mM ouabain on the Ca^{2+}-ATPase activity is shown in Fig. 2. It can be seen that with a decrease of Ca^{2+} concentration down to 0.7 μM, ouabain activates Ca^{2+}-ATPase and at $[Ca^{2+}] = 0.2$ μM, increases the enzyme activity. At $[Ca^{2+}] = 2$–5 μM ouabain inhibits

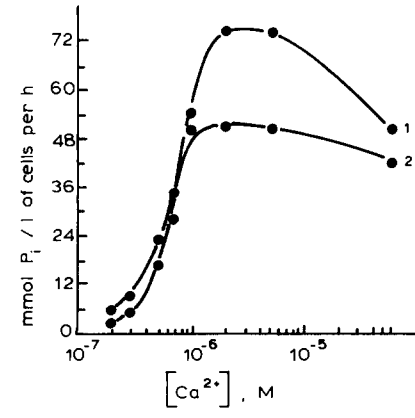

Fig. 2. Ca^{2+}-dependence of Ca^{2+}-ATPase activity in saponin-treated erythrocytes. 1, control; 2, +1 mM ouabain. $[Mg^{2+}] = 370$ μM. The experiments were performed on mongrel albino rats aged 5 months.

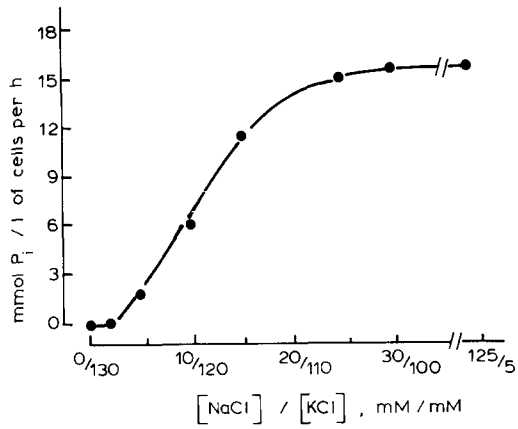

Fig. 3. Dependence of the ouabain-inhibited component of erythrocyte ATPase activity on the Na^+/K^+ ratio. $[Mg^{2+}] = 370$ μM, $[Ca^{2+}] = 0$. The experiments were performed on mongrel albino rats aged 4–5 months.

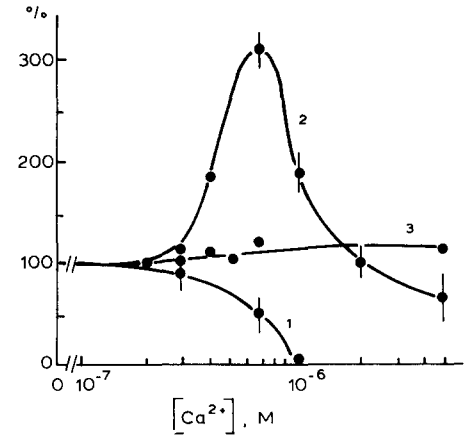

Fig. 4. Ca^{2+}-dependence of Na^+/K^+-ATPase activity in saponin-permeabilized erythrocytes (1,2) and their ghosts washed with EGTA (3). $[Mg^{2+}] = 50$ μM (curve 1) or 370 μM (curves 2 and 3). The experimental curves were obtained as the difference between the ATPase activity in Na^+,K^+-containing media and that in media without Na. The Na^+/K^+-ATPase activity in Ca^{2+}-free media was taken for 100 per cent.

the enzyme by 20–30 per cent. With this in mind, we determined the Na^+/K^+-ATPase activity as a difference between P_i production in $(Na^+ + K^+)$-containing and Na^+-free media.

Na^+/K^+-ATPase

In preliminary experiments we studied the effect of the Na^+/K^+ ratio on the ouabain-sensitive component of Na^+/K^+-ATPase in the absence of Ca^{2+} (1 mM EGTA). As can be seen from Fig. 3, this component is absent at Na^+ concentration below 3 mM. Sodium content in rat erythrocytes varies within the concentration range of 5–15 mmol per litre of cells [29]. Thus, it may be concluded that under condition of ATPase assay (5 per cent hematocrit) the total sodium concentration does not exceed 0.75 mM.

Fig. 4 displays that at 50 μM free Mg^{2+}, the Na^+/K^+-ATPase of permeabilized erythrocytes appeared to be inhibited in parallel with an increase in Ca^{2+} concentration above 0.3–0.4 μM. The half-maximum inhibition of the enzyme activity was seen at Ca^{2+} concentration of 0.7 μM, whereas the complete inhibition occurred at 1 μM Ca^{2+} (curve 1). At Mg^{2+} concentration of 370 μM which corresponded, approximately, to the intracellular concentration of this cation [23,24], the increase in Ca^{2+} concentration in the range of 0.3–0.7 μM caused the activation of Na^+/K^+-ATPase followed by a slight inhibition at $[Ca^{2+}] > 2$ μM (Fig. 4, curve 2). Washing of membranes with EGTA (2 mM) fully eliminated the Ca^{2+} effect on Na^+/K^+-ATPase activity (Fig. 4, curve 3). The data on the effect of the intracellular free calcium concentration on the activity of the Na^+/K^+-pump obtained from the values of the ouabain-sensitive component of $^{86}Rb^+$ influx into intact erythrocytes treated with the $Ca^{2+}(Mg^{2+})$ ionophore A23187 are shown in Fig. 5. It can be seen that at 370 μM Mg^{2+} and at variable Ca^{2+} concentrations (10^{-7}–2.10^{-6} M), the activity of the Na^+/K^+-pump was practically unaffected, showing a 20–30 per cent decrease at $[Ca^{2+}] = 5$ μM.

Effect of furosemide

Furosemide (1 mM) had no effect on the activity of Mg^{2+}-ATPase (date not shown). The effect of furosemide on Ca^{2+}-ATPase is similar to that of ouabain: this compound causes a 2-fold activation of the enzyme at 0.3–0.5 μM Ca^{2+} and its 15–20 per cent inhibition at 2–5 μM Ca^{2+} (Fig. 6). Study of furosemide effect on the activity of Na^+/K^+-ATPase gave most interesting

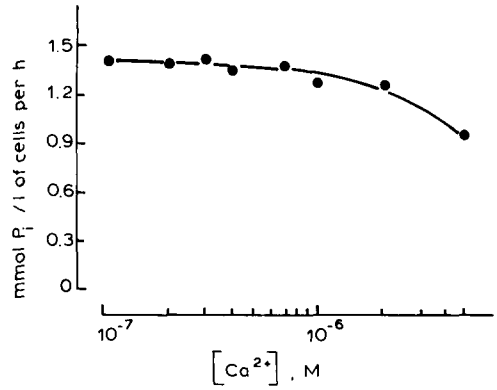

Fig. 5. Ca^{2+}-dependence of the ouabain-inhibited component of $^{86}Rb^+$ influx in rat erythrocytes in presence of ionophore A23187 (10 μM). The incubation medium contained 125 mM NaCl, 5 mM KCl, 8.3 mM $MgCl_2$, 20 mM Hepes-Tris, 5 mM glucose, 10 mM citrate, 622–1836 μM $CaCl_2$ (pH 7.4, 37 °C), 2 μCi/ml of $^{86}Rb^+$ and 200 μM quinidine. In some experiments the incubation medium was supplemented with ouabain (0.2 mM). The suspension hematocrit was 20 per cent. The experiments were performed on Wistar-Kyoto male rats aged 4–5 months.

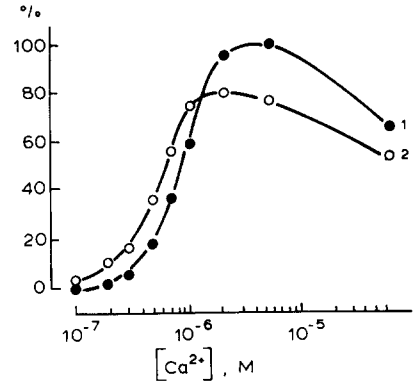

Fig. 6. Ca^{2+}-dependence of Ca^{2+}-ATPase activity in saponin-treated erythrocytes. 1, control; 2, +1 mM furosemide; $[Mg^{2)} = 370\ \mu M$.

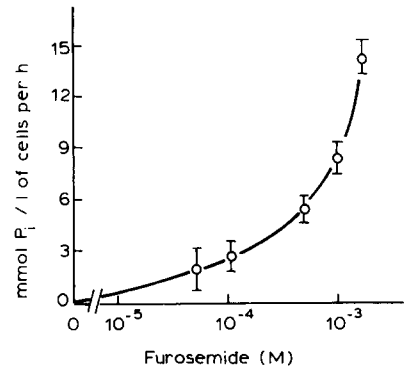

Fig. 8. Dependence of the ouabain-inhibited component of ATPase activity on furosemide concentration. $[Ca^{2+}] = 0$; $[Mg^{2+}] = 370\ \mu M$; $[Na^+] = 2$ mM. The experiments were performed on mongrel albino rats aged 4–5 months.

results. In the absence of Ca^{2+} furosemide slightly and insignificantly increased the Na^+/K^+-ATPase activity under conditions of its maximal activation by sodium ions ($[Na^+] = 30$ mM) (Fig. 7). The activation of Na^+/K^+-ATPase by furosemide enhanced at low NaCl concentration. Thus, at $[Na^+] = 0.5$–0.7 mM, and in the absence of furosemide we failed to observe any inhibition of the enzyme activity by ouabain (Fig. 3, Fig. 7, curve 1). After addition of 1 mM furosemide, the ouabain-inhibited component of ATPase activity reached a level of 8–10 mmol P_i per litre of cells per h (Fig. 7, curve 3), i.e., was only by 50–80 per cent lower than the Na^+/K^+-ATPase activity under sodium saturation in the absence of furosemide (15 mmol P_i per litre of cells per h). Fig. 8 shows the dependence of the ouabain-inhibited ATPase activity at 2 mM Na^+ on furosemide concentration. A significant activation of the enzyme was seen already at 50 μM furosemide but it did not reach the saturation level after its concentration was increased up to 2 mM. An alternative approach to the study of furosemide effects on the activity of Na^+/K^+-ATPase is the determination of the ouabain-sensitive component of $^{86}Rb^+$ influx. The data listed in Table III suggest that the value of this component was by 40–50 per cent lower than that in the presence of 1 mM furosemide. It seems very likely that the 4–5-fold increase of the furosemide-inhibited component of $^{86}Rb^+$ influx revealed in the absence of ouabain is due to the activation of the Na^+/K^+-pump.

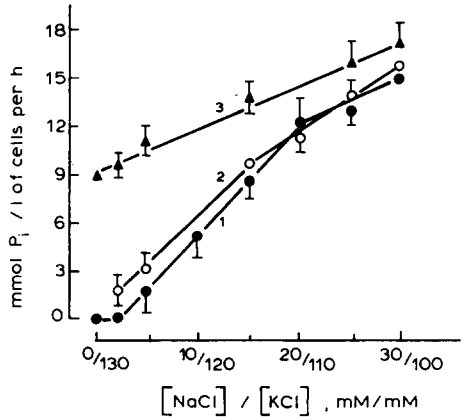

Fig. 7. Dependence of the Na^+/K^+-ATPase activity on the Na^+ and K^+ concentrations ratio. 1, control; 2, +100 μM furosemide; 3, +1 mM furosemide; $[Ca^{2+}] = 0$; $[Mg^{2+}] = 370\ \mu M$. The experiments were performed on mongrel albino rats aged 4 months.

Discussion

The observed dependence of the Ca^{2+}-ATPase activity of saponin-permeabilized erythrocytes on Ca^{2+} concentration (Fig. 1, curve 1) differs essentially not only from that of the ghosts obtained by hypoosmotic hemolysis [20,21], but also from that of permeabilized erythrocyte washed with isoosmotic solutions containing EGTA (Fig. 1, curve 3). These differences affect,

TABLE III

Parameters of K^+ (^{86}RB) influx into the rat erythrocytes

NN	Parameters	mmol per litre cells per h
1	Total flux	2.92+0.09
2	Ouabain-inhibited component	1.43+0.1
3	Furosemide-nonsensitive ouabain-inhibited component	2.0 +0.13
4	Ouabain-nonsensitive furosemide-inhibited component	0.38+0.09
5	Furosemide-inhibited component	0.08+0.06
6	Ouabain + furosemide-nonsensitive component	1.10+0.03
P	$P_{2,3}$	< 0.01
	$P_{4,5}$	< 0.025

Content of incubation medium: 130 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 10 mM glucose, 1 mM NaH_2PO_4, 10 mM Hepes-Tris, 1 μCi $^{86}RbCl$ (pH 7.4, 37°C). Concentrations of ouabain and furosemide 1 mM. The experiments were performed on male Kyoto-Wistar rats aged 4 weeks. For the details of the method see Ref. 20.

primarily, the maximal enzyme activity which is, in both cases, by one order of magnitude lower than that of permeabilized erythrocytes. For this reason, the latter model seems to most adequately reflect the processes occurring in the native cell.

Previous studies with quin2-loaded rat erythrocytes revealed that the rate of $^{45}Ca^{2+}$ influx at $[Ca^{2+}] = 1$ mM was about 40–60 μmol per litre of cells per h [19,30]. Assuming at steady-state condition the 'passive' Ca^{2+} influx and the ATP-dependent Ca^{2+} efflux are equal [2,13,26] and comparing these data with those shown in Fig. 1, it may be concluded that under the basal conditions the Ca^{2+}-ATPase of intact erythrocytes does not exceed 0.1–0.5 per cent of its maximal activity. From the same figure it may be also concluded that at these values of transmembrane calcium fluxes free Ca^{2+} in rat erythrocytes slightly exceeds 10^{-7} M. Our previous studies with Wistar rats showed that this value is 90–130 μM [30]. One should not rule out the possibility that such a comparison is incorrect, because the addition of Ca^{2+} chelators can change the apparent Ca^{2+}-binding affinity of Ca^{2+}-binding and Ca^{2+}-transporting systems of the cell [31].

It is well established that electrical or neurohumoral excitation of cells is associated with an increase in Ca^{2+} concentration from 10^{-7} to 10^{-8}–10^{-5} M. In this case the Ca^{2+}-ATPase activity increases up to 30 mmol P_i per litre of cells per h. Assuming that ATP concentration erythrocytes is about 2 mM, one may presume that in the absence of additional synthesis such an increase in $[Ca^{2+}_{in}]$ leads to rapid (1–2 min) 2-fold decrease of ATP. We do not know such data concerning rat erythrocytes. As for human erythrocytes it was found at $[Ca^{2+}_{out}] = 100$ μM, an addition of A23187 under conditions of monoacetamide-inhibited glycolysis results in 50 per cent loss of ATP during the first 5–7 min of the reaction [32]. It is noteworthy that the maximal activity of Ca^{2+}-ATPase in saponin-treated human erythrocytes is 2–3-times as low as that in rat erythrocytes [33].

Taking into consideration that the Ca^{2+}-ATPase activity in EGTA-treated membranes is restored practically completely after addition of the EGTA extracts (Table II), one may suppose that the enzyme activation is a Ca^{2+}-dependent process. Studies with isolated membrane preparations (ghosts) and purified enzyme revealed that one of such regulators is calmodulin [4,34–36]. However, the results cited herein as well as those obtained in our previous studies [16,30] suggest that in the native cell calmodulin apparently does not take part in the regulation of the ATP-dependent Ca^{2+}-pump activity [37]. It seems likely that a certain role in this process belongs to the protein described by Cunningham et al. [37]. These authors showed that the 20 kDa protein was bound in a Ca^{2+}-dependent fashion to human erythrocyte membranes and underwent phosphorylation by a cAMP-dependent protein kinase, eventually resulting in the lowering of the Ca^{2+}-ATPase activity in Triton-solubilized membrane preparations. Dephosphorylation of the protein activated by 15 μM Ca^{2+} led to a 3-fold increase of the total activity of Ca^{2+}- and Mg^{2+}-ATPases. The role of Ca^{2+} in the regulation of the Na^+/K^+-pump activity in cellular membranes including those of erythrocytes, is not completely understood. The literature data concerning the stimulating effect of Ca^{2+} are rather controversial and far from being complete [5,38–40]. The results obtained by direct measurements of the Na^+/K^+-ATPase activity in the presence of Ca^{2+} also failed to provide any reliable information. The reason for such ambiguity is the inadequacy of experimental conditions because of the impossibility to maintain at a constant level and, in some cases, even to determine with a high degree of accuracy, the Ca^{2+} and Mg^{2+} concentrations [38–40].

The observed dependence of the ouabain-sensitive ATPase activity on Na^+ concentration in saponin-treated erythrocytes in Na^+-free media (Fig. 3) is similar to that of Na^+/K^+-ATPase in native and reconstituted membranes [41]. Low sensitivity to Na^+, i.e., the absence of activity at Na^+ concentrations below 3 mM ($K^+_{0.5} = 15$ mM) allows to inhibition of Na^+/K^+-ATPase by the exhaustion of the Na^+ pool in the reaction medium most specifically. This finding it especially important within the context of the Ca^{2+}-dependent effect of ouabain on Ca^{2+}-ATPase (Fig. 2).

From a comparison of the data shown in Fig. 3 with the values of the ouabain-inhibited rate constants of $^{86}Rb^+$ influx and $^{22}Na^+$ efflux in rat erythrocytes (1–2 mmol per litre of cells per h) [19] we may conclude that under normal conditions the Na^+/K^+-ATPase activity does not exceed 5 per cent of its maximal value.

The revealed inhibiting effect of Ca^{2+} on Na^+/K^+-ATPase at low (50 μM) Mg^{2+} concentrations (Fig. 4) correlates with but does not coincide in sensitivity with the results obtained previously for intact human erythrocytes in the presence of ionophore A23187 [39] as well as for membrane preparations obtained at uncontrollable, although sufficiently high concentrations of contaminant Ca^{2+} [5]. Such a high susceptibility to the inhibiting effect of Ca^{2+}, half-maximal inhibition at 1 μM Ca^{2+} has been found low human erythrocyte Na^+/K^+-ATPase after addition of a purified Ca^{2+}-dependent protein inhibitor, isolated from cytoplasm of erythrocytes or some other cells and termed as calnactin [5]. This result demonstrates the complete integrity of endogenous Ca^{2+}-dependent regulators in saponin-treated erythrocyte membranes as well as the proximity to the native conditions of regulation in the given experimental model.

Qualitatively the activation of Na^+/K^+-ATPase by Ca^{2+} observed in the presence of 370 μM Mg^{2+} (which corresponds to its concentration in the cytosol) is similar to that observed in earlier studies at sufficiently

determined concentrations of Mg^{2+} and Ca^{2+} in rat brain synaptosomal membranes [40] and erythrocyte ghosts [38]. It should be noted, that the quantitatively similar activation (1.5–2.5-fold) of the Na^+/K^+-pump and/or Na^+/K^+-ATPase was found in the case of human erythrocytes by the action of diluted blood serum [42], in fish erythrocytes after the addition of norepinephrine [43,47], in rat hepatocytes after the addition of vasopressin, angiotensin II or norepinephrine [45–48] as well as in lymphocytes under action of mitogenic lectins [48,49]. All the above compounds are known to increase the Ca^{2+} concentration in cell cytosol [45–49]. At the same time, for practically all above examples it was revealed an increase of the rate of the Na^+_{out}/H^+_{in} exchange [50], which suggests that the Na^+/K^+-ATPase activity might be due to the Na^+ elevation in the cytoplasm. Recent studies have demonstrated, however, that the 2–3-fold increase in the activity of Na^+/K^+-ATPase in carp erythrocytes in the presence of beta-agonists is not accompanied by the increase in Na^+_{in} [44]. These data suggest that one of the possible mechanisms of Na^+/K^+-pump activation during cell excitation is the increase in the concentration of internal Ca^{2+}. In this connection it is noteworthy that no activation by biogenic amines of nerve ending Na^+/K^+-ATPase first reported in late 1970s took place when the experiments were conducted in Ca^{2+}-free media [51].

Taking into account the fact that in our study the activation and inhibition by Ca^{2+} of Na^+/K^+-ATPase were completely eliminated after washing of membranes with EGTA, it may be supposed that the washing by the Ca^{2+}-free media removes at least two Na^+/K^+-ATPase regulators, calnactin and a protein activator whose origin is unknown. The lack of enzyme activation at low concentrations of Mg^{2+} suggests that the effect of this activator can be regulated by the protein phosphorylation-dephosphorylation system, in which the enzymes (protein kinases, protein phosphatases and nucleotide cyclases) are activated by rather high concentrations of Mg^{2+} [52,53]. Moreover, Mg^{2+} can modulate the activator binding to the membrane or to the enzyme.

Taking into account the relatively weak inhibiting effect of calnactin and the potent activating effect of the protein activator at high Mg^{2+} concentrations, one can assume that the two Ca^{2+}-dependent regulators compete with each other for the same binding sites in Na^+/K^+-ATPase. Under these conditions the activator displays a higher affinity for Ca^{2+}, and the effect of the inhibitor in the presence of these cations (or Mg^{2+}) appears to be strongly suppressed (Fig. 4).

These findings suggest that erythrocyte membranes contain, besides the well-known Ca^{2+}-dependent inhibitor calnactin, Ca^{2+}-dependent activator of Na^+/K^+-ATPase. Such regulators differ in their affinity for Ca^{2+} and/or Na^+/K^+-ATPase. As a result, the effect of the activator is manifested already at 0.4–0.7 μM Ca^{2+}, whereas its competition with the inhibitor is observed at higher Ca^{2+} concentrations. The decrease in Mg^{2+} concentration eliminates the effect of the activator and increases manyfold that of the inhibitor.

Our attempts to find out whether the Ca^{2+}-dependent effects of ouabain and furosemide on erythrocyte Ca^{2+}-ATPase are due to their interaction with the enzyme or to the structural modification of the membrane, were fairly unsuccessful. Yet, the results of this study clearly demonstrate that the determination of a ouabain-inhibited component in the elucidation of the regulatory role of Ca^{2+} in membranes, characterized by a relatively high activity of Ca^{2+}-ATPase, can hardly be considered as a promising approach.

Another convenient model for Ca^{2+}-dependent regulation of ouabain-inhibited components of K^+ ($^{86}Rb^+$) influx or Na^+ efflux, are native erythrocytes treated with Ca^{2+} ionophores. The first approach used in this study did not reveal the stimulating effect of Ca^{2+} on the Na^+/K^+-pump (Fig. 5). The reasons for the inconsistency of these results with those depicted in Fig. 4 are as follows. First, the regulatory properties of Na^+/K^+-ATPase in membranes, treated with saponin and A23187, differ considerably, Second, the values of $[Ca^{2+}_{in}]$ and $[Ca^{2+}_{out}]$ in intact erythrocytes are also different, presumably due to the relatively low concentration of A23187 and to the shortness of incubation time (5 min). It should be pointed out, however, that the further increase in these parameters is hardly reasonable, since it results in a rapid depletion of the intracellular ATP pool. Third, no equilibrium between extra- and intracellular concentrations of Ca^{2+} could be reached at addition of A23187 at high (300–500 μM) Mg^{2+} to Ca^{2+} (0.1–5 μM) ratios, at a taking into account the known relatively low selectivity of ionophore A23187 ($Mg^{2+} : Ca^{2+} = 1 : 4$) [55] and at differences between the pH_{out} and pH_{in} values [20]. And, finally, unfavorable side effects can result from quinidine used to block the Ca^{2+}-activated K^+-channels which prevent the identification of K^+ fluxes mediated by Na^+/K^+-ATPase. It is well known that quinidine inhibits Na^+/H^+ exchange and influences the systems responsible for Ca^{2+} influx and the protein kinase activity in rat erythrocytes [19]. Therefore, it is quite probable that quinidine may interfere with the regulatory properties of Na^+/K^+-ATPase.

Taking into account the inhibiting effect of furosemide on the ATPase activity, we studied the effects of this compound on transport ATPases [55–59]. The works by Proverbio et al. dating from late 1970's to early 1980's suggest that renal cortex basolateral membranes of guinea pig [55] and rat [56] contain a Mg^{2+}-ATPase component which is stimulated by Na^+ in the absence of K^+. In contrast with Na^+/K^+-ATPase, Na^+-ATPase is not inhibited by ouabain but suppressed by furosemide and ethacrynic acid used at millimolar

concentrations. According to these authors, Na^+-ATPase differs from Na^+/K^+-ATPase by sensitivity to sodium dodecylsulfate, trypsin and Ca^{2+}(Na^+-ATPase could be detected only in the presence of micromolar concentrations of Ca^{2+}) [57,58]. These data and the high utility of furosemide and its derivatives in the detection of systems of facilitated ion transfer in erythrocyte membranes (e.g., $K^+ + Na^+ + 2Cl^-$, $Na^+ + Cl^-$ and $K^+ + Cl^-$ cotransports) [59,60] forced us to examine the effect of this compound on the Na^+/K^+-ATPase activity.

As we know from the literature data all previous experiments designed to investigate the furosemide effect on the activity of plasma membrane ATPases including those cited above [55–58] were conducted at saturating (with respect to Na^+/K^+-ATPase) concentrations of monovalent cations. No significant effect of furosemide on the value of ouabain-inhibited component was found under these conditions. These results are in good agreement with those shown in Fig. 7. At NaCl concentrations higher than 20 mM furosemide has a weak effect on this component. We also failed to establish any significant influence of furosemide on the ATPase activity in the absence of Ca^{2+}, when the entire K^+ pool was replaced by Na^+ (data not shown). It means that Na^+-ATPase is absent in erythrocytes. However, we found that at moderate concentrations of NaCl (< 15 mM) furosemide activates Na^+/K^+-ATPase. As mentioned above, the Na^+_{in} concentration in freshly isolated rat erythrocytes is about 10 mM. It means that the effect of furosemide is due to its interaction with the outer membrane surface, it may be expected that an increase in the ouabain-inhibited component will also be observed during the kinetic analysis of $^{86}Rb^+$ influx; evidence in favour of this hypothesis may be derived from data shown in Table III. It seems likely that the activating effect of furosemide on the ouabain-inhibited component of $^{86}Rb^+$ influx is based on one of following mechanisms: (i) the Na^+/K^+-ATPase operation in the regime of K^+/K^+ exchange which is not accompanied by ATP hydrolysis [61,62]; (ii) the sharp increase in the sensitivity of $3Na + /2K^+$ exchange to $[Na^+]$ and its operation in the regime of K^+-ATPase generating the unidirectional flux of K^+. In the latter case we deal with the inhibition of Na^+/K^+-ATPase by furosemide. The practical solution to this problem demands further verification and experiment. However, the data shown in Figs. 7 and 8 and listed in Table III should be taken into consideration when furosemide and related drugs are being used for therapeutic purposes.

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