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Stimulation of a Ouabain-Sensitive Rb^+ Uptake in Human Erthrocytes with an External Electric Field

Engin H. Serpersu and Tian Yow Tsong
Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Summary. (Na, K)ATPase of the red blood cell (RBC) is known to be electrogenic. Activation of this pump hyperpolarizes the RBC membrane by several millivolts. By exposing erythrocytes in an isotonic suspension to an alternating electric field it is possible to modulate transmembrane potential ($\Delta\psi$) of the RBC. We have found that this modulation stimulates uptake of Rb^+, against a chemical concentration gradient, when the applied AC field exceeds 10 V/cm (or an induced $\Delta\psi$ of 6 mV). The voltage-stimulated Rb^+ uptake is completely inhibited by ouabain. Thus, (Na, K)ATPase may be involved. The stimulated Rb^+ uptake is unrelated to the thermal effect by several lines of evidence. First, this uptake is above levels in controlled samples maintained at an identical temperature. Second, this uptake shows an optimum voltage. The maximum stimulation obtained in our experiment (26 amol/RBC·hr) occurs at 20 V/cm, i.e., a $\Delta\psi$ of 12 mV. Above or below this field strength the uptake is reduced. Third, this uptake is AC frequency dependent. It peaks around 1 kHz and diminishes at 1 MHz. The effective range is between 0.1 kHz to 0.1 MHz. A thermal effect would not be frequency dependent. In contrast to the ATP-dependent pumping activity of the (Na, K)ATPase, no stimulated Na^+ efflux is detectable with the AC field. Neither Rb^+ efflux, nor Na^+ influx is stimulated by the AC field. Rb^+ uptake is also stimulated by the AC field in a RBC sample treated with vanadate. The meaning of these observations is discussed.

Key Words electrogenic pump · erythrocytes · (Na, K)ATPase · membrane potential · ion transport · electric field

Introduction

The erythrocyte can maintain large gradients of Na^+ and K^+ ions across a membrane. The resting potential of the cell membrane is equal to the Nernst diffusion potential for the chloride ion (Lassen, 1977; Dunham & Hoffmann, 1980). Na^+ and K^+ ions have several migration pathways through the red cell membrane (Lew & Beauge, 1979; Sarkadi & Tosteson, 1979; Dunham & Hoffmann, 1980; Tosteson, 1981). One of these pathways is mediated by (Na, K)-ATPase. This enzyme catalyzes efflux of $3\,Na^+$ and influx of $2\,K^+$ against their respective chemical concentration gradients (Post & Jolly, 1957; Garrahan & Glynn, 1967). The catalytic cycle of the enzyme is specifically inhibited by ouabain (Schatzmann, 1953), and it consumes one ATP for each $3\,Na^+/2\,K^+$ pumped (Post & Jolly, 1957). Na^+ and K^+ transport through the pump is coupled under normal conditions, and the unequal transport number implies that the pump is electrogenic.

Using a potential-sensitive fluorescent dye, Hoffman, Kaplan and Callahan (1979) have recently measured changes in the membrane potential of RBCs upon activation of the (Na, K)ATPase by the addition of K^+ ion to the external medium (Hoffmann & Laris, 1974; Sims, Waggoner, Wang & Hoffmann, 1974). When ouabain is added to the medium the membrane is depolarized by several millivolts. This indicates that activation of the pump results in hyperpolarization of the cell membrane. However, it is not clear whether the magnitude of the membrane potential change produced by the pump can be accounted for by the net difference in the transport stoichiometry of Na^+ and K^+ ions.

The role of cell membrane potential in ionic transport is not yet fully understood. The application of external electric fields can modulate the membrane potential for further study (Sale & Hamilton, 1968; Riemann, Zimmermann & Pilvat, 1975; Kinosita & Tsong, 1977*a*; Witt, 1979; Schlodder & Witt 1981; Teissie, Knox, Tsong & Wehrle, 1981). Previous work from this laboratory has shown that when erythrocytes are treated with high voltage pulses (2–5 kV/cm) small pores are implanted in the cell membrane, which subsequently cause lysis of the treated cells. The hemolysis can be prevented under certain conditions (e.g., incubation of cells in the presence of high molecular weight compounds such as oligosaccharides), and the membranes can be resealed (Kinosita &

Tsong, 1977*b*). Evidence that the sites of the voltage perforation may include the (Na, K)ATPase has been obtained by studying the effect of ouabain. The high voltage-induced conductance change of a RBC suspension can be partially blocked by ouabain in low ionic strength solutions (Teissie & Tsong, 1980). However, these authors did not observe the same ouabain inhibition in high ionic strength media (i.e., solutions containing 45 mM NaCl or higher). The conductance of red cell suspensions was also changed when the cells were subjected to alternating sinusoidal voltages of a small magnitude (16–32 V/cm) (Teissie & Tsong, 1982). The change in conductance was reduced 20–30% by ouabain, and it was suggested that these ouabain-sensitive, reversible membrane conductance changes resulted from the voltage activation of (Na, K)ATPase. However, a temperature effect could not be unequivocally ruled out, and information about the molecular nature of this effect was lacking.

In this communication, we have measured ionic movements by radioactive tracers and shown that the membrane potential produced by an external electric field can be used to actively transport Rb^+ into the cells in a ouabain-sensitive manner. The voltage-driven Rb^+ uptake is observed with samples either in a high salt medium or in a low salt medium. The uptake is also shown to occur both when the (Na, K)ATPase is inactive at 3 °C and when it is functioning at 20 °C.

Abbreviations Used

amol: atto (10^{-18}) mole
AC: alternating current
ATP: adenosine 5′-triphosphate
ATPase: adenosinetriphosphatase
RBC: red blood cells
Tris: 2-amino-2-(hydroymethyl)-1,2-propanediol
S, NS, OS, and *ONS*: AC stimulated, nonstimulated, ouabain pretreated and stimulated, ouabain pretreated and nonstimulated erythrocyte samples

Materials and Methods

Materials

Ouabain was obtained from Sigma Chemical Co. $^{22}Na^+$ and $^{86}Rb^+$ were from Amersham Corp., and Liquiscint was purchased from Yellow Spring Instruments.

Experimental Set-Up

The chamber for the voltage stimulation and the circuit of the set-up are shown in Fig. 1. The chamber is connected to a Heath Zenith SG-1271 functional generator which can generate an AC field of various wave forms up to 1 MHz. The temperature of the chamber was controlled by circulating water through the two brass blocks which support two platinized platinum electrodes. The steady-state temperature was measured directly by a Teflon-coated thermistor, immersed in the medium, with a Yellow Spring Instruments thermometer during the AC stimulation. The temperature rise due to Joule heating was found to be insignificant, less than 0.2 °C at the maximum stimulating voltage of 20 V/cm.

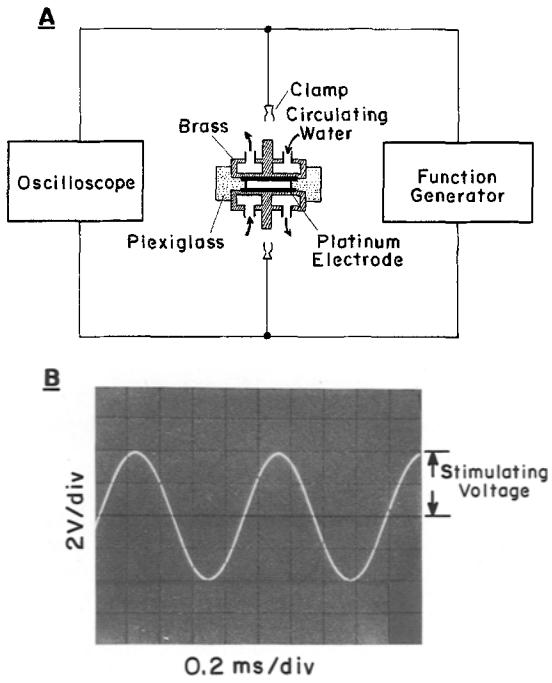

Fig. 1. A schematic of the AC stimulation device. The stimulation chamber is composed of two brass blocks with an internal cavity for cooling water, a plexiglass spacer of 2.5 mm thickness with a 7.5-mm diameter central hole, and a *V*-shaped slit connecting the central hole to the edge. Two flat platinized platinum discs placed between the plexiglass holder and the brass blocks serve as electrodes. The chamber is held by two clamps through which the voltage is applied. The sample is introduced through the *V*-shaped slit, and the voltage with desired frequency is applied with a Heath Zenith SG-1271 function generator. The voltage and frequency of the AC wave are measured by the differential amplifier unit (7A22) of a Tektronix 7704 A oscilloscope. The stimulating voltage reported in this paper is defined as the amplitude of the sine wave as indicated

Ion Uptake Measurements

Freshly drawn human blood was centrifuged, and the red cells were washed three times with five volumes of cold 150 mM NaCl in 10 mM Tris buffer at pH 7.4. The washed cells were usually used on the day of the preparation. In experiments involving preloading of ions, the cells were stored overnight at 4 °C (*see* Table for different loading conditions) and used the next day. The ion uptake experiments were started by incubating the cells for 30 min at room temperature (22±2 °C) in isotonic NaCl, with and without 0.2 mM ouabain. Then, additions were made to give the final ion concentrations shown in the figure legends. After taking 20 µl zerotime aliquots, 150 µl of the suspension was placed between the two platinized platinum electrodes (Fig. 1) at the indicated temperatures. The desired voltage and frequency were then applied to the suspension. Another part of the suspension was incubated at the same

Table. Rb^+ uptake and Na^+ efflux of erythrocytes under various experimental conditions[a] (voltage stimulation was performed at 3 °C with a 20 V/cm, 1 kHz AC field)

Pretreatment		Loaded ion conc. (mM)		Outside ion conc. (mM)[b]		$^{86}Rb^+$ uptake (amol/RBC·hr)			$^{22}Na^+$ efflux (amol/RBC·hr)		
		Na^+	Rb^+	Na^+	Rb^+	*S*	*NS*	*OS*	*S*	*NS*	*OS*
Overnight incubation at 4 °C in	150 mM RbCl ± $^{86}Rb^+$ 15 mM NaCl	ND	27.5	2.5	12.5	44.6±4.2 (3)	29.8±3.7 (3)	28.7±2.4 (3)	ND	ND	ND
	245 mM Sucrose ± $^{22}Na^+$	2	ND	60	2.5	ND	ND	ND	6.6±2.1 (6)	8.6±3.5 (6)	6.1±1.5 (6)
	100 mM NaCl 100 mM	15	ND	2.5	12.5	49.5±2.7 (3)	24.1±0.4 (3)	23.3±0.8 (3)	22.6±5.2 (6)	26.2±1.2 (6)	20.4±1.3 (6)
	Glucose ± $^{22}Na^+$	15	ND	12.5	2.5	ND	ND	ND	20.6±1.7 (6)	21.6±2.5 (6)	20.0±2.0 (6)
	150 mM	22	ND	12.5	12.5	48.1±3.3 (3)	23.6±0.6 (3)	23.2±0.5 (3)	12.7±4.2 (6)	6.1±0.5 (6)	10.3±1.2 (6)
	NaCl	22	ND	12.5	2.5	ND	ND	ND	21.1±1.7 (6)	22.3±1.0 (6)	20.7±3.4 (6)
	± $^{22}Na^+$	22	ND	60	2.5	ND	ND	ND	13.9±1.6 (6)	14.1±1.8 (6)	10.5±0.7 (6)
High-voltage pulsing	With $^{22}Na^+$	5	25	12.5	2.5	ND	ND	ND	15.4±2.7 (6)	14.5±2.6 (6)	12.6±3.1 (6)
	Without $^{22}Na^+$	5	25	2.5	12.5	52.3±6.2 (3)	29.8±3.7 (3)	28.7±2.3 (3)	ND	ND	ND
None		ND	ND	2.5	12.5	48.6±3.9 (4)	23.4±0.4 (4)	23.1±0.8 (4)	ND	ND	ND
		ND	ND	140	10	6.3±0.05 (3)	3.4±0.1 (3)	3.0±0.12 (3)	ND	ND	ND

[a] In all experiments 10 mM Tris buffer at pH 7.4 was used with added ions. Overnight loading was followed by washing the cells three times with isotonic NaCl, and the stimulation experiments were performed as shown in Fig. 2, with the ion concentrations as indicated. Rb^+ efflux from line 1 was 55.9±1.4 (S), 50.7±3.0 (*NS*), 45.1±9.0 (*OS*), (n=12) amol/RBC·hr and Na^+ uptake from row 9 was 3.2±0.8 (*S*), 3.2±0.4 (*NS*), 2.9±0.30 (n=3) amol/RBC·hr.

[b] Appropriate concentration of sucrose was present to make each sample suspension isotonic.

S=stimulated sample; *NS*=nonstimulated sample; *OS*=sample stimulated in the presence of 0.2 mM ouabain. The numbers in parenthesis show the number of determinations. Data are given in mean±SD. 1 amol/RBC·hr roughly equals 1.3 ions/pump-sec, if one assumes the uptake is mediated by the (Na, K)ATPase. ND means not determined.

temperature. At the end of the stimulation, the suspension was taken from the chamber and three 20 µl aliquots (from both the stimulated and the nonstimulated control samples) were withdrawn and washed twice with 500 µl of the same ice-cold medium containing no radioactive tracer. The washed cells were dissolved in 1 N NaOH, bleached with H_2O_2 containing 10% ascorbic acid, then acidified with 1 N HCl and taken into 5 ml Liquiscint and counted in a Packard scintillation counter. The hematocrit value of the cell suspension (stimulated and non-stimulated) was determined for each sample; it was usually 15–20%. Other details of the uptake experiments are explained in the figure legends. In Fig. 2, an outline of the experimental protocol is given for a low ionic strength condition. Other solvent conditions are listed in the Table.

The results are expressed as attomoles/erythrocyte-hour or ions/pumps-sec (1 attomole=10^{-18} moles), by assuming 93 µm^3 cell volume (Kinosita & Tsong, 1979), 200 (Na, K)ATPases per RBC (Ellory & Keynes, 1969; Hoffman, 1969), and also that 50% of the packed cell volume is intracellular space.

Ion Efflux Measurements

The cells were suspended (40% hematocrit) in a medium that contained 10 mM Tris buffer at pH 7.4 and 10–150 mM NaCl with ^{22}Na tracer as shown in the Table or with ^{86}Rb in 150 mM RbCl, 10 mM Tris buffer. The suspensions were stored at 4 °C overnight. The next day, the cells were washed three times with isotonic NaCl solution before adding efflux measurement media, which contained no radioactive tracer but different NaCl concentrations as shown in the Table. Incubation of cells with and without ouabain was followed by the addition of appropriate amounts of RbCl solution. Then voltage was applied and aliquots were taken as described in uptake experiments (Fig. 2). 0.2 ml from the supernatant of the first 0.5-ml wash was taken and was counted for efflux. The cells were then washed once more and the radioactivity remaining in the cells was also determined as a control. No additional counts appeared in the second supernatant. The hematocrit was determined for all samples, as in the uptake experiments.

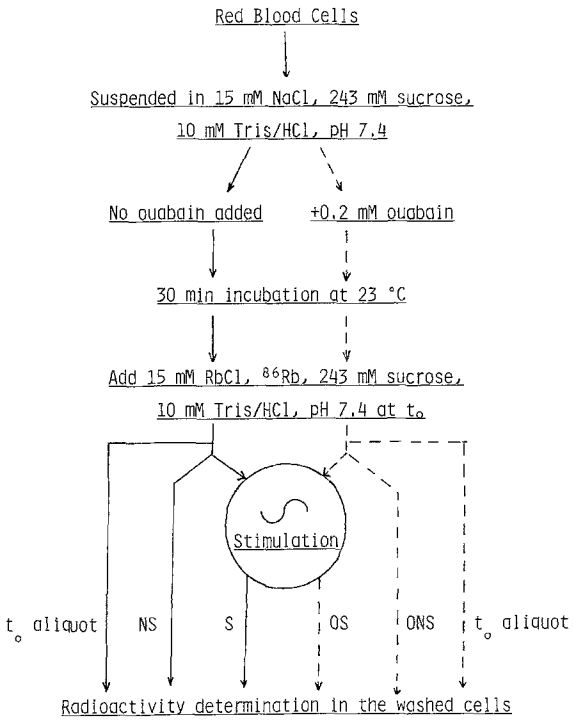

Fig. 2. Procedures for the AC field-stimulation of erythrocytes in a low ionic strength condition. Salt concentration of each experiment is given in figures and in the Table. In ion efflux measurements, 0.2 ml of the first 0.5 ml washing medium was withdrawn for radioactivity counting. *S*: stimulated sample; *NS*: nonstimulated sample; *OS*: sample pretreated with and stimulated in the presence of ouabain; *ONS*: nonstimulated ouabain-containing sample

High Voltage Loading

The red blood cells were suspended at 20% hematocrit in the 10 mM Tris buffer which contained 120 mM RbCl, 15 mM NaCl, 27 mM sucrose and 1 mM $MgCl_2$, either with or without radioactive tracer. The suspension was transferred to the same chamber shown in Fig. 1 which was connected to a Cober 605P high voltage generator, instead of the Heath Zenith function generator (Kinosita & Tsong, 1979). One square wave pulse of indicated duration and voltage (Fig. 7) was applied to each sample. The cells were withdrawn and added to equal volumes of the same pulsing solution which contained radioactive label and 25 mM stachyose, and incubated 120 min at 37 °C in a shaker bath. This treatment resealed the cells (Kinosita & Tsong, 1977*b*). After resealing, the cells were washed 3–4 times with an ice-cold isotonic NaCl solution. The same uptake and efflux procedures described above were then applied to these pulsed and subsequently resealed cells. In all experiments the extent of hemolysis was negligible as measured by leakage of hemoglobin from the treated cells (Kinosita & Tsong, 1977*a*).

Results

Ouabain-Sensitive, AC Field-Stimulated Rb^+ Uptake

When erythrocytes in a low salt isotonic suspension (12.5 mM RbCl, 2.5 mM NaCl, 243 mM sucrose in 10 mM Tris buffer at pH 7.4) were subjected to a stimulation by an AC field, there was an increased uptake of Rb^+ with respect to a nonstimulated sample. Figure 3 shows the result of such an experiment. The AC field was maintained at 16 V/cm and 1 kHz, and the temperature was kept at 3 °C. Passage of AC current at this level did not cause a significant elevation of the suspension temperature (less than 0.1 °C) due to the effective cooling of the chamber design (Fig. 1). 3 °C was chosen in order to minimize the background and maximize the signal to noise ratio. The signal to noise ratio was greatly reduced at higher temperatures (*see* Teissie & Tsong 1982; and also Fig. 6*A* and *B*) because of the increased pumping activity of the (Na, K)ATPase and the increased background permeation of Rb^+ and Na^+ ions in the membrane.

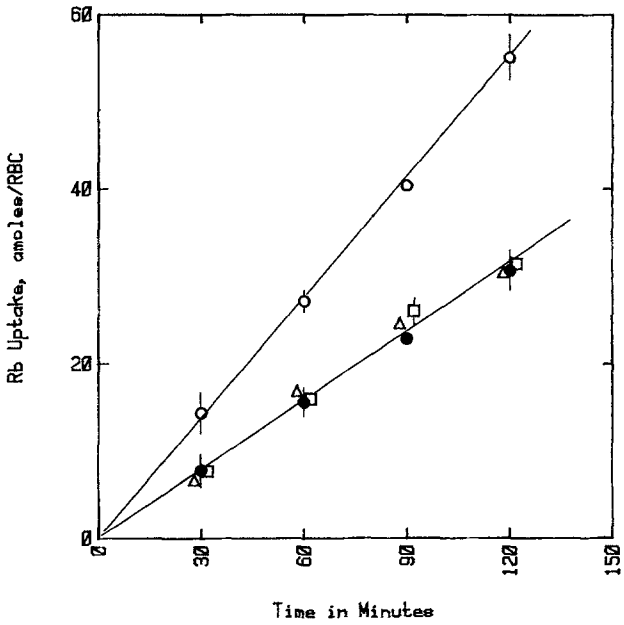

Fig. 3. Time course of Rb^+ uptake. Washed red blood cells were incubated 30 min at room temperature in 10 mM Tris buffer, pH 7.4, containing 15 mM NaCl and 243 mM sucrose with and without 0.2 mM ouabain. At the end of incubation cold RbCl-sucrose solution in 10 mM Tris-HCl, pH 7.4, containing 15–20 cpm per pmol $^{86}Rb^+$ was added to the mixture to give 15–20% hematocrit and the final concentrations of the salts at 12.5 mM RbCl, 2.5 mM NaCl, 243 mM sucrose. 10 µl zero time aliquots were drawn and added to 0.5 ml of cold nonradioactive solution with the same composition as above and washed twice. 150 µl of suspension was introduced into the stimulation chamber and 16 V/cm, 1 kHz AC was applied across the electrodes (o–o), at 3 °C. The rest of the suspension was kept at the same temperature (•–•). At indicated time 10-µl aliquots were withdrawn and washed as mentioned above. Ouabain-containing sample was also treated the same way (*OS*, □; *ONS*, △). Each point represents the average of three separate experiments run in duplicate. A few error bars are shown. They represent the standard deviation from the mean. The slope of the stimulated sample (o) is 28.8 amol/RBC·hr, and of other samples is 15.0 amol/RBC·hr

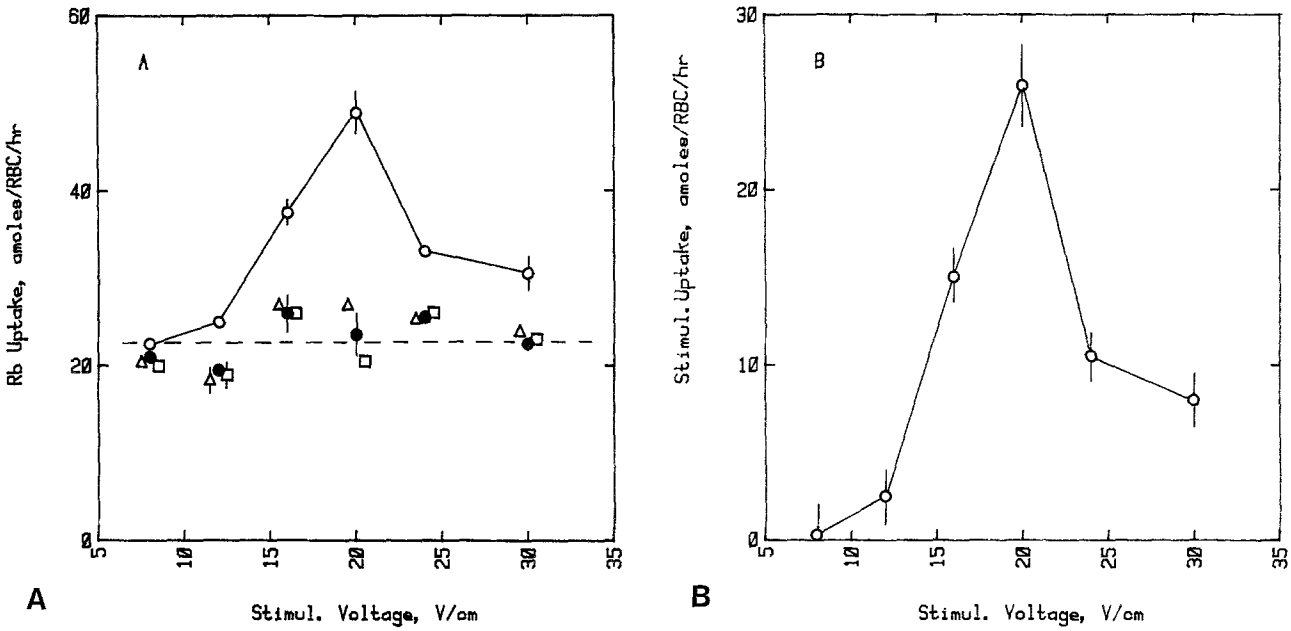

Fig. 4. Voltage dependence of Rb uptake. The red blood cells were treated the same way as in Fig. 3. Samples were treated with various voltages with 1 kHz frequency for 60 min at 3 °C. 20 μl aliquots were drawn both at the beginning and at the end of the stimulation, washed twice with cold nonradioactive medium, and the $^{86}Rb^+$ content of the cells was determined as explained in Materials and Methods. (*A*): Stimulated samples (*S*, ○; *OS*, □), nonstimulated samples (*NS*, ●; *ONS*, △). (*B*): The difference between stimulated and nonstimulated samples. The bars are the standard deviation from the mean (10 determinations for 16 and 20 V/cm, and 6 determinations at other conditions)

As shown in Fig. 3, the AC-stimulated Rb^+ uptake was extremely sensitive to the pretreatment of the red cells with ouabain. In the untreated, stimulated sample (○, *S*) the uptake was 28.8 amol/RBC·hr. The uptake in the ouabain-treated, voltage-stimulated sample (□, *OS*) dropped to 15.0 amol/RBC·hr. At 3 °C, the (Na, K)ATPase was inactive, and the nonstimulated sample (●, *NS*) and the ouabain pretreated, nonstimulated sample (△, *ONS*) gave identical Rb^+ uptake of 15.0 amol/RBC·hr. This value of Rb^+ influx represents an intake through passive permeation and other transport mechanisms. The result shown in Fig. 3 indicates that the voltage-stimulated Rb^+ uptake (13.4 amol/RBC·hr under this condition) can be completely suppressed by ouabain.

Optimum Voltage and Frequency of Stimulation

If the voltage stimulated Rb^+ uptake is directly linked to activity of certain membrane enzyme(s), one would expect to observe optimum values of the voltage and AC frequency of voltage stimulation. This indeed was the case. The dependence of the Rb^+ uptake on the stimulating voltage is shown in Fig. 4. After exposing RBCs to an AC field for 1 hr at 1 kHz, maximum stimulation was found at 20 V/cm. In Fig. 4*A* results of samples with different treatments are given. As expected, the Rb^+ uptake of the ouabain-pretreated, stimulated sample (*OS*, □) was voltage independent, and similar in value to that of the controlled samples (*NS*, ●; *ONS*, △). The dependence on voltage of the ouabain-sensitive, voltage-stimulated Rb^+ uptake is given in Fig. 4*B*. Roughly 24.5 amol/RBC·hr uptake was recorded at 20 V/cm AC field under these conditions.

In Fig. 5 the frequency dependence of the Rb^+ uptake is given when the field was kept constant at 20 V/cm. 1 kHz appeared to be the optimum frequency for the voltage stimulation. The effective range was between 0.1 kHz and 0.1 MHz. Under the optimum condition (20 V/cm, 1 kHz), the stimulated Rb^+ uptake was 24.5 amol/RBC·hr, at 3 °C. This excess uptake more than doubles the background permeation of Rb^+ ion into the RBCs (23.1 amol/RBC·hr).

Voltage Stimulation under Active (Na, K) Pumping

In order to check whether or not a functioning (Na, K) pump could block stimulation by the externally imposed electric field, we have performed similar experiments at 20 °C, both in a high salt and in a low salt media. The result for the low salt condition (12.5 mM RbCl, 2.5 mM NaCl,

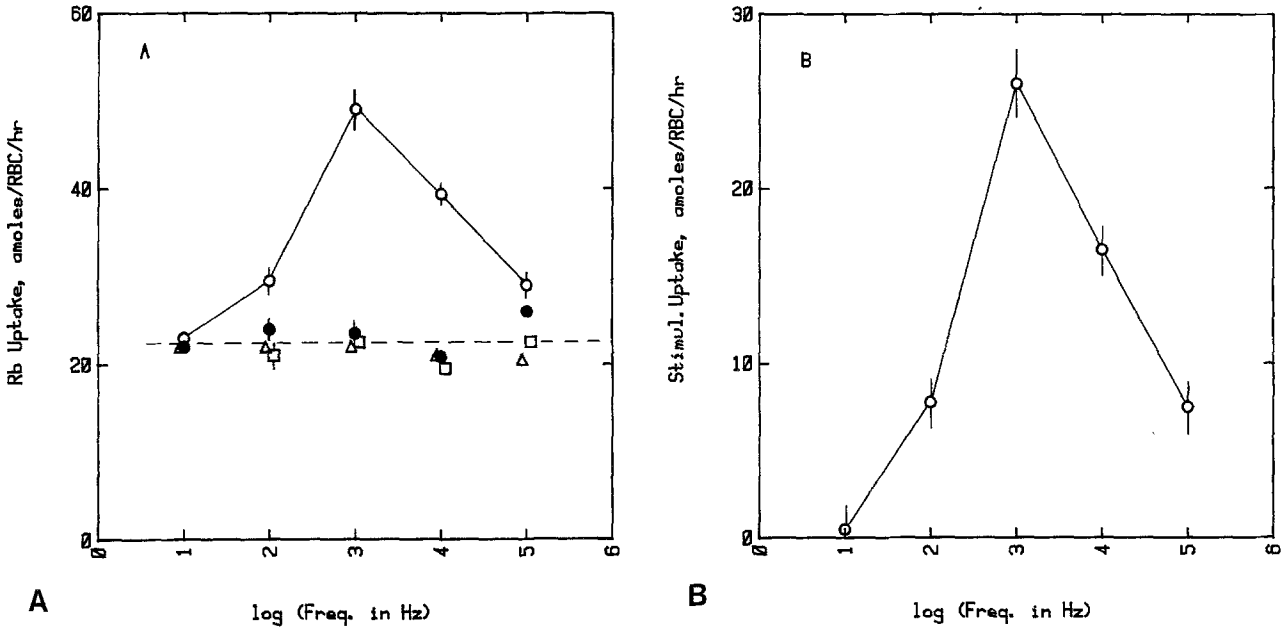

Fig. 5. Frequency dependence of Rb^+ uptake. The procedure was the same as in Fig. 4 except the voltage was 20 V/cm with frequencies shown. (*A*): Stimulated samples (*S*, ○; *OS*, □), and nonstimulated samples (*NS*, ●; *ONS*, △). (*B*): The difference between stimulated and nonstimulated samples. Bars show the standard deviation from the mean (10 determinations at 1 kHz, and 6 at the other conditions)

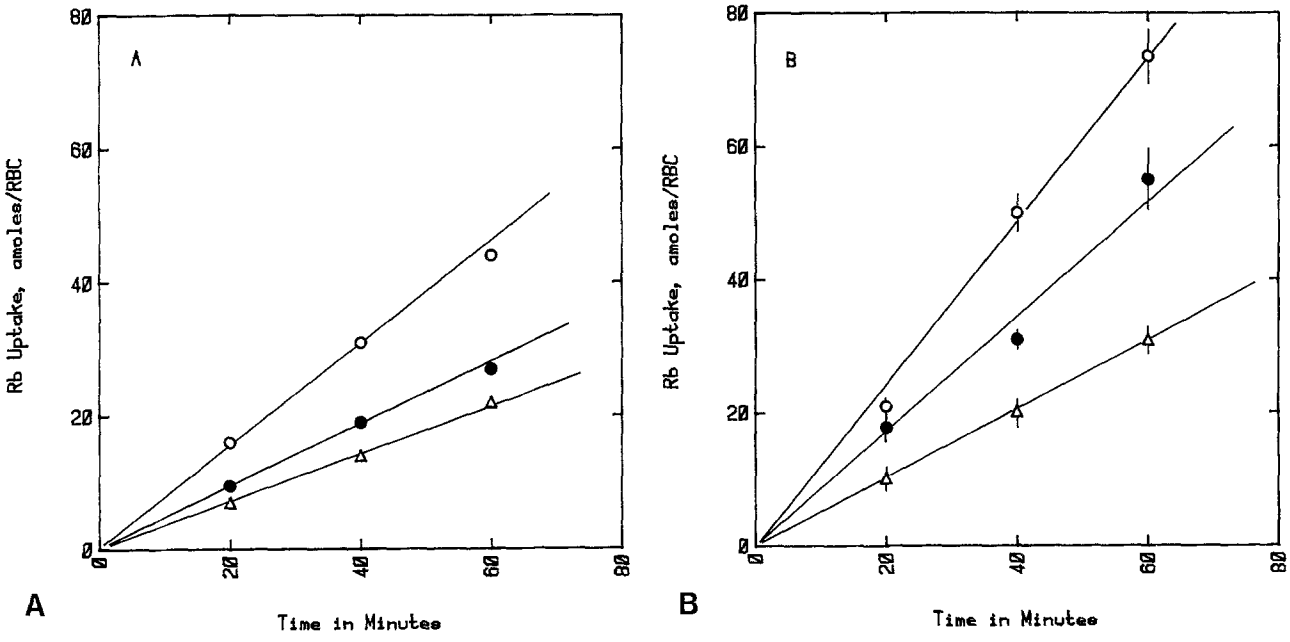

Fig. 6. Stimulation of Rb^+ uptake at 20 °C. (*A*): All steps followed as in Fig. 4, with 20 V/cm, 1 kHz AC field. (○) stimulated sample, (●) nonstimulated sample, (△) ouabain-containing samples. The slopes are 45.0, 27.3, and 21.5 amol/RBC·hr, respectively. (*B*): The cells were preincubated for 30 min at room temperature in isotonic NaCl in 10 mM Tris buffer at pH 7.4 with/without 0.2 mM ouabain. RbCl in 10 mM Tris, pH 7.4, containing 15–20 cpm/pmol $^{86}Rb^+$ were added to the mixture to give the final composition of 140 mM Na^+, 10 mM Rb^+, 15–20% hematocrit. Zero time aliquots were taken and 150 µl of suspension was stimulated at 20 V/cm, 1 kHz at 20 °C. At the indicated time 10 µl aliquots were drawn and washed with cold nonradioactive medium. $^{86}Rb^+$ content was determined as described. Symbols used as in *A*. The slopes are 76.3, 53.3, and 31.2 amol/RBC·hr, respectively, for data in ○, ● and △. *A* presents data from one set of experiments, and *B* presents data from three sets of experiments. Each point is the mean of 3 determinations run in duplicate; bars represent the standard deviation from the mean

243 mM sucrose, and 10 mM Tris at pH 7.4) is shown in Fig. 6*A*. The rate of Rb^+ uptake was 45.0 amol/RBC·hr for the stimulated sample (o, 20 V/cm, 1 kHz), 27.3 amol/RBC·hr for the nonstimulated sample (•) and 21.5 amol/RBC·hr for the ouabain pretreated, nonstimulated sample (Δ). Result for the ouabain pretreated, nonstimulated sample was similar to the *OS* curve (Δ). Thus, the activity of the pump was 5.8 amol/RBC·hr and was increased to 27.3 amol/RBC·hr by the voltage stimulation under these conditions.

The (Na, K) pumping activity is generally higher at the high salt condition (12.5 mM RbCl, 138 mM NaCl, 10 mM Tris at pH 7.4) (Fig. 6*B*). The background uptake was 31.2 amol/RBC·hr (Δ), and when the pump was active this value increased to 53.3 amol/RBC·hr (•). The uptake was further stimulated by the AC field, and 76.3 amol/RBC·hr uptake was recorded with the stimulated sample (o). At high salt concentration, the amount of stimulated Rb^+ transport appeared to increase with increasing temperature (*see* Fig. 6*B* and Table).

No Detectable Voltage-Stimulated Na^+ Movement

No Na^+ influx above controlled samples was detected by the AC stimulation under all the conditions discussed above. To see whether voltage could stimulate Na^+ efflux, it was necessary to load the cells with $^{22}Na^+$. This was accomplished by two different methods.

The first experiment was done by incubating the red blood cells in different concentrations of NaCl solution and 10 mM Tris buffer containing $^{22}Na^+$ overnight at 4 °C. Voltage stimulation was then applied. Table shows that under a variety of conditions, there was no significant $^{22}Na^+$ efflux in the stimulated samples, although the Rb^+ uptake of these cells was effectively stimulated by the AC field. Loading the cells with $^{86}Rb^+$ in isotonic RbCl solution, and then measuring the release of Rb^+ upon stimulation showed no additional release by the AC stimulation (Table).

As an alternative $^{22}Na^+$ loading method, we employed high voltage square-wave pulsing, as was previously used to incorporate larger molecules into the red cells (Kinosita & Tsong, 1977*b*). By using lower field strengths and shorter pulses we were able to incorporate $^{22}Na^+$ into the cells as shown in Fig. 7. The hemolysis at this stage was negligible except for the sample treated with a 40 μsec, 1.8 kV/cm pulse. After resealing, these cells showed no stimulated $^{22}Na^+$ efflux (Table). The reliability of this method was checked by

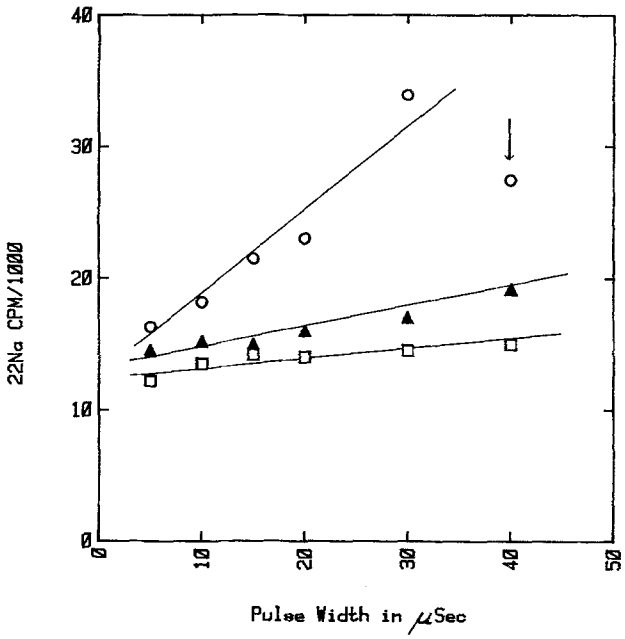

Fig. 7. Loading of $^{22}Na^+$ tracer into red blood cells by the high voltage pulse. Erythrocytes were suspended in 150 μl of 10 mM Tris buffer at pH 7.4, containing 120 mM RbCl, 15 mM NaCl, 1 mM $MgCl_2$, 27 mM sucrose, and $^{22}Na^+$ tracer at room temperature. The hematocrit was 20%. A single square-wave pulse was applied to the suspension at indicated length and voltage. Then the cells were transferred into one volume of resealing solution which contains 21 mM stachyose in addition to the above buffer and incubated for 120 min at 37 °C in a shaker bath. Termination of resealing was followed by three washings with a 140 mM NaCl, 10 mM RbCl, 10 mM Tris buffer, pH 7.4 solution. The incorporated radioactivity was determined as described. Another aliquot of the pulsed cells was transferred into the isotonic solution and kept 4 hr at room temperature. Hemoglobin content of the supernatant was determined at 410 nm against a control which was shocked in H_2O for the same time. Hemolysis was less than 0.5% in all cases except for the point indicated by an arrow. Voltage pulse strength was 1.6 kV/cm (□), 1.7 kV/cm (▲), and 1.8 kV/cm (o)

pulsing another group of cells under exactly the same conditions and ionic compositions without radioactive label. The cells were then resealed and were given to the normal low voltage AC stimulation. The ouabain-sensitive, stimulated Rb^+ uptake was 23.6 amol/RBC·hr in good agreement with the value of 24.5 amol/RBC·hr found with the normal, unpulsed cells. Details of the experiments are given in the Table. The shape of the cells was monitored during AC stimulation by a light microscope connected with a time-lapse video recorder. No detectable shape changes or alignment of the cells were observed. Hematocrit values measured immediately after the AC stimulation also did not indicate volume changes of the cells.

Vanadate inhibits (Na, K) pumping activity of the ATPase by blocking the phosphorylation of the enzyme. A RBC sample was treated with

0.1 mM vanadate for 2 hr, and the low voltage stimulation was performed with this sample in the presence of vanadate. At 3 °C and in a high salt condition (144 mM NaCl, 6 mM RbCl, 1 mM $MgCl_2$), the stimulated sample showed a Rb^+ uptake of 3.51 ± 0.31 amol/RBC·hr compared with 1.23 ± 0.03 amol/RBC·hr for a nonstimulated sample (*see* last row of Table for samples not treated with vanadate). The result indicates that the voltage-stimulated Rb^+ uptake does not depend on a normal functioning of the Na^+ pump.

Discussion

Induced Membrane Potential as the Cause of the Stimulated Rb^+ Uptake

It has been shown that when a cell is exposed to an electric field in suspension, the cell experiences several different effects of the current, e.g., Joule heating of the suspension, electrophoresis of membrane proteins, and the field-induced transmembrane potential (Kinosita & Tsong, 1977*a*). Electrophoresis of membrane proteins can be discounted because of the low magnitude of the electric field and also because of the alternating nature of the applied field. Joule heating has been discussed in great detail in previous publications (*see*, e.g., Kinosita & Tsong, 1977*a*; Teissie & Tsong, 1981). However, the evidence against Joule heating as a cause of the observed Rb^+ uptake is overwhelming in the present experiment. First, direct measurement of the sample temperature with a microthermistor probe (with time constant of 0.2 sec) indicated that the temperature rise due to Joule heating was less than 0.2 °C during all conditions employed. Although nonuniform thermal perturbation could occur at the cell surface, a simple calculation indicated that the maximum effect of local heating (at $\theta = 90°$) should not exceed by 2.3-fold the mean heating, or less than 0.5 °C (unpublished). Controlled samples kept at an identical temperature did not show excess Rb^+ uptake. Secondly, the stimulated Rb^+ uptake exhibited a voltage optimum of 20 V/cm. Below or above this value the efficiency of stimulated uptake was reduced. Joule heating is proportional to the square of the field strength, and the stimulated Rb^+ uptake should increase accordingly when the initial temperature is kept low, at 3 °C. This was not the case. Third, most convincingly, the uptake was dependent on the AC frequency. Joule heating does not depend on the frequency of the AC field. Thus, the observed Rb^+ uptake is most logically attributed to the electric field-induced transmembrane potential.

The magnitude of the field-induced transmembrane potential in a spherical cell is given by

$$\Delta\psi = 1.5 r E \cos\theta$$

where r is the radius of the cell, E the field strength, and θ the angle between the field and the radius to the point of interest on the membrane (Neumann & Rosenheck, 1973; Zimmermann et al., 1974; Kinosita & Tsong, 1977*a*). The relation is valid as long as the conductivity of the membrane is much less than that of the internal and external media (Neumann & Rosenheck, 1973; Kinosita & Tsong, 1979), and this condition is met in our experiment. Using 4.0 μm effective radius for the red cells, we calculated the maximum value of transmembrane potential at the optimum Rb^+ uptake conditions (20 V/cm, 1 kHz) to be 12 mV. The maximum hyperpolarization of the membrane should occur at the loci lying closest to the anode (i.e., $\theta = 180°$). A more elaborate analysis as described by Kinosita and Tsong (1979) did not significantly alter this estimation. Data in Fig. 4 suggest that an induced $\Delta\psi$ of at least 6 mV is required to generate an ouabain-sensitive Rb^+ influx.

High Voltage vs. Low Voltage Induced Ionic Movements

A high electric field (2–3 kV/cm) that generates $\Delta\psi$ of 0.7 to 1 V perforates erythrocyte membranes and renders the membrane freely permeable to Na^+, K^+ ions, and other small sized molecules (Kinosita & Tsong, 1977*a*). The low-amplitude, AC field-stimulated Rb^+ uptake differs from this phenomenon in several respects. (i) The high voltage pores are indiscriminatory to ions or molecules except by size (Kinosita & Tsong, 1977*b*), and movement of these ions or molecules is driven purely by the concentration gradient. The low voltage stimulates Rb^+ or K^+ to move only inward, and there is no stimulated Na^+ movement in either direction. The Rb^+ uptake against the chemical concentration gradient represents an active transport of this ion (*see* next section). (ii) The high voltage-generated pores are irreversible, and are stable at low temperature (Kinosita & Tsong, 1977*b*), but the low voltage effect is completely reversible. RBCs treated with the low AC field behave like freshly prepared cells, and no functional defects have been detected. In fact, after the AC field is removed, the ionic composition of the RBC slowly returns to the initial composition. (iii) The high voltage-generated pores are aqueous

pores and cannot be blocked by specific inhibitors of transport systems or ions once they are open. The low voltage-stimulated Rb^+ uptake is specifically blocked by ouabain.

Voltage and Frequency Optima for AC Stimulation

The (Na, K)ATPase activity and the membrane potential are intimately related (Rapoport, 1970, 1971; Thomas, 1972; Hoffman et al., 1979; Betz et al., 1980). The AC-stimulated Rb^+ uptake increases with an increasing field strength until an optimum value of 20 V/cm ($\Delta\psi$ of 12 mV) is reached. Beyond this value the effect of the AC field diminishes. The range of voltage used here does not cause a leakage of ions, as shown in the controlled samples of Fig. 4*A* (data for *NS, OS* and *ONS* samples). Thus, the diminishing of the stimulation cannot be attributed to a formation of passive permeation pores. One possible explanation is that $\Delta\psi$ exceeding 12 mV alters the gate geometry of an enzyme, which consequently reduces the effectiveness of the Rb^+ pumping activity.

The frequency dependence is most likely associated with kinetic phenomena. The data shown in Fig. 5 indicate a stimulated uptake of 22 Rb^+/pump-sec. Thus, one turnover requires roughly 50 msec (at 3 °C). 1 kHz appears to be too fast to explain this turnover process. It is, however, plausible that 1 kHz could represent an $E \rightleftharpoons E^*$ transition as shown in Fig. 8*B*. Certain conformational changes of protein do occur within this time range (Tsong, 1982).

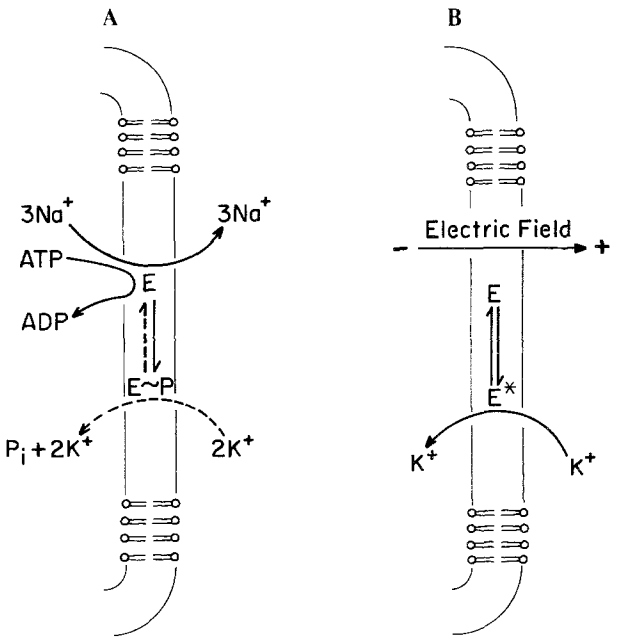

Fig. 8. Mechanisms of the (Na, K)ATPase action. (*A*): The normal, ATP-dependent (Na, K) pumping activity of the enzyme is shown. First, the enzyme is phosphorylated to an $E \sim P$ complex by ATP, and a conformational change of this complex is accompanied by the transport of 3 Na^+ ions out of the RBC. The second step involves the pumping of 2 K^+ ions into the cytoplasm with a concomitant dephosphorylation of the $E \sim P$ complex. This completes one pumping cycle. (*B*): Voltage-stimulated Rb^+ (or K^+) uptake of the RBC is shown. The electric field hyperpolarizes the membrane in one of the two hemispheres of the RBC. This induced electrochemical potential drives the uptake of Rb^+ (or K^+) by the RBC. The electric field may have also induced a conformational change of E to E^*, and the release of this conformational energy is coupled to the pumping of Rb^+. *See* text for details

Mechanisms of AC-Stimulated Rb^+ Uptake

The mechanism of the ATP dependent (Na, K) pumping in the erythrocyte membrane has been described (Lew & Beauge, 1979; Sarkadi & Tosteson, 1979; Dunham & Hoffman, 1980). A simple scheme relevant to the present discussion is given in Fig. 8*A*. In brief, ATP phosphorylates (Na, K)ATPase in the presence of Mg^{2+} and Na^{2+}, and a conformational change of the $E \sim P$ complex is associated with the transport of 3 Na^+ ions out of the RBC. K^+ ion has a high affinity for the phosphorylated enzyme. Transport of 2 K^+ ions into the cell is facilitated by the electrochemical potential, and is accompanied by dephosphorylation of the ATPase. The unequal transport of ions in opposite directions means that the enzyme is electrogenic, and the membrane is hyperpolarized. In the experiment described here, the AC field can polarize the membrane. And, at the hemisphere where the membrane is hyperpolarized, this induced membrane potential can provide energy required for the inward movement of Rb^+, or K^+ ion (Fig. 8*B*). Two observations suggest that the enzyme is a rectifier to Rb^+ or K^+ ion. First, since the opposite hemisphere of the half RBC shown in Fig. 8*B* is expected to be hypopolarized to an equal magnitude, the detection of a net Rb^+ influx means that no Rb^+ efflux can be stimulated by a positive membrane potential. Second, Rb^+ efflux is not induced by AC stimulation of RBC preloaded with Rb^+ (Table).

Although the above interpretation seems to explain the electric origin of Rb^+ influx, a simple energetic consideration indicates that this is not sufficient to account for the observed effect. The free energy of Rb^+ uptake can be expressed by

$$\Delta G = 2.3RT \log \frac{[Rb^+]_{in}}{[Rb^+]_{out}} + F\Delta\psi$$

in which R, T, F, $\Delta\psi$ are, respectively, the gas constant, temperature in degrees Kelvin, Faraday constant, and the membrane potential. At 3 °C, and expressing ΔG in kcal/mol, the relationship has the form

$$\Delta G = 1.26 \log \frac{[Rb^+]_{in}}{[Rb^+]_{out}} + 23.0\Delta\psi.$$

Two situations are examined. First, one assumes that Rb^+ is equivalent to K^+ for the (Na, K)ATPase. In the experiment shown in Fig. 3, ΔG of Rb^+ uptake is roughly 1.0 kcal/mol. A positive ΔG rules out the Nernst potential as a sole source of Rb^+ uptake. In the second situation, Rb^+ is considered to be an independent chemical species. Data in the first row of the Table gives 0.16 kcal/mol for ΔG of Rb^+ uptake, again ruling out the Nernst potential as a sole source of the observed effect. These considerations lead us to suggest that an enzyme conformational change must have also occurred during the AC stimulation (Fig. 8*B*). Further experiments are needed to clarify this point.

One important question remaining to be answered is the role of ATP in the AC-stimulated Rb^+ uptake. At 3 °C no (Na, K) pumping activity is detected; thus, it is unlikely that ATP consumption occurs at this temperature. Yet, Rb^+ uptake is stimulated by the AC field. This does not exclude the possible role of the nucleotide in the Rb^+ influx process. ATP-depleted cells (obtained by incubating RBCs overnight at 37 °C in an isotonic saline containing NaF) lose the ability to uptake Rb^+ by the AC stimulation. However, the ATP-depleted cells are found to be in a deteriorated state, and no conclusion can be drawn by using such cells. It will be necessary to extend this type of experiment to inside-out ghosts.

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