Activation of Electrogenic Rb* Transport of (Na,K)-ATPase by an Electric Field*

Engin H. Serpersu and Tian Yow Tsong‡

From the Department of Physiological Chemistry, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Previous study shows that human erythrocytes when exposed, in an isotonic suspension, to an electric field that generated 6–15 mV of transmembrane potential induced a Rb* uptake that was sensitive to ouabain, a potent inhibitor of (Na,K)-ATPase (Serpersu, E. H., and Tsong, T. Y. (1983) J. Membr. Biol. 74, 191–201). Here we present evidence that this uptake indeed involved the activity of (Na,K)-ATPase. Transport of Rb*, K*, and Na* were carefully monitored during the voltage stimulation. It is shown that the electric field stimulated only the ouabain-sensitive influx of Rb*, and this uptake was against a chemical concentration gradient. The rate of the stimulated Rb* uptake was measured under different intracellular Na* and extracellular Rb* concentrations. The K* for the stimulated Rb* uptake was, respectively, 7 mM for the intracellular Na* and 1.7 mM for the extracellular Rb*, consistent with the values for the red cell (Na,K)-ATPase. Yet, the voltage-sensitive Rb* uptake did not depend on the intracellular ATP level. Neither did the voltage stimulation cause an elevation of ATP concentration in the red blood cells as was observed in mitochondrial and chloroplast ATP synthetase systems under higher electric field conditions. Since only Rb* uptake was stimulated by the voltage, it follows that the Na* and the K* pumping activities of the (Na,K)-ATPase could be decoupled, and the K* pumping activity may derive from the electrogenic component of the enzyme action. In the present case, the applied electric field could polarize the membrane to provide membrane potential required for the electrogenic transport of Rb*. Data also show that vanadate at 180 μM completely inhibited the ATP-dependent Na* and Rb* pumping activities of the enzyme, but only reduced the voltage-stimulated Rb* uptake to 50% level. This represents the first systematic study of the activation of a transport ATPase by an externally applied electric field.

(Na,K)-ATPases1 hydrolyze ATP to transport Na* and K* against their respective chemical concentration gradient across a cell membrane, thus, maintaining the balance of these two ions in many cell and tissue types (1, 2). Under normal conditions, the stoichiometry of the transport in the red blood cell is such that for each ATP consumed, 3Na* are transported out of the cell in exchange of 2K* (3) and this activity is specifically inhibited by ouabain (4). The enzyme is asymmetrically oriented in cell membranes, and its affinity to Na* and K* is very different on each side (5, 6). Depending on cation composition in the two sides, the Na* pump can switch to different modes, e.g. the Na*-Na* exchange mode (7), the K*-K* exchange mode (8), or the uncoupled Na* efflux mode (9), and each mode has a different nucleotide (or P) requirement. Except for the normal 3Na*-2K* exchange mode, all the other modes are nonelectrogenic, i.e. the exchange of ions is electrically neutral. The normal mode of the (Na,K)-ATPase, on the other hand, is electrogenic. This asymmetric pumping of Na* and K* leads to the hyperpolarization of membranes (10, 11). The significance of the membrane polarization, however, remains unclear.

Recent work from many laboratories have shown that it is possible to impose a membrane potential on a cell or organelle by exposing the cell or organelle to an external electric field (12–15). Such applications of an electric field to cell suspensions have been shown to perforate cell membranes, cause changes in membrane conductance (16–18), or activate membrane-bound ATPases (19, 20). This latter effect of the electric field should be useful for the elucidation of the enzyme mechanism and should be of particular interest to biological chemists. Previously, we reported that an AC field in the range of 20 V/cm induced an ouabain-sensitive Rb* uptake by erythrocytes (21). Although the result suggested that (Na,K)-ATPase might be involved, evidence was preliminary and was not compelling. The present study has been undertaken to elucidate kinetic details of the voltage-induced Rb* transport and to provide further evidence that, indeed, the uptake was mediated by the (Na,K)-ATPase. The artificially imposed membrane potential is shown to activate the electrogenic transport activity of the enzyme, and this activation did not alter cellular ATP level. Neither did the voltage stimulation, at the range that activated the K* pump, activate the Na* pump or trigger a synthesis of ATP by the enzyme. The result also suggests that the Na* and the K* pumping activity of the enzyme can be decoupled, i.e. they can function as two independent pumps.

EXPERIMENTAL PROCEDURES

Materials—*2Na* and *86Rb* were obtained from Amersham Corp. Ouabain and luciferin/luciferase were from Sigma. Liquisint was supplied by Yellow Spring Instruments. Other chemicals were of the analytical grade.

Experimental Setup—Details of the experimental setup have been described elsewhere (21). Briefly, the device consists of a cylindrical plexiglas chamber of 150-μl capacity. At the two sides of the chamber

7155
are two platinumized platinum electrodes supported by brass holders. Through each brass holder is a circulating water that maintains the cell suspension in constant temperature. Human red blood cells suspended in various isotonic solutions were placed in the plexiglas chamber and an AC field of 20 V/cm at 1 kHz was then applied to the cell suspension for a designated period of time. After which, the sample was drawn for determination of ionic composition by radioactivity assay.

**Loading of Ions into RBC—**Two procedures were used in the first method, washed red blood cells were resuspended, at 5% hematocrit, in 150 mM KC1, 1 mM MgCl2, 10 mM Tris/HCl buffer at pH 7.4, containing 20 μg/ml of chloramphenicol. pCMBS was added to the suspension in a medium of designated concentration and the suspension was kept at 4°C. The loading solution was changed once after 5-6 h. The next day the cells were collected by centrifugation and washed once with the loading solution minus pCMBS. The cells were then transferred to a recovery medium containing 2 mM adenine, 3 mM inosine, 4 mM cysteine, 10 mM glucose, 1 mM MgCl2, 15 mM KC1, and 10 mM Tris/HCl at pH 7.4 (22), and incubated in a shaker bath at 37°C for 1 h. Afterward, the cells were spun and washed three times with an isotonic KC1 solution, and Na+ content of cells determined. When incorporation of 22Na+ was desired, both the loading and the recovery media contained 22Na+ at 2-4 cpmmol of specific activity. For the ion flux experiments, these cells were washed three times with a medium of designated ion compositions and resuspended in the same medium to 12-17% hematocrit.

In the second method, the cells were incubated for 24 h at 4°C, at 5% hematocrit, in a solution containing 0-150 mM NaCl, 20 mM Tris/HCl, pH 7.4, 20 μg/ml of chloramphenicol, and an appropriate composition of ATP used to maintain the cell integrity. At the end of the incubation period, the cells were then washed and the internal Na+ content was determined by flame photometry, using Li+ as internal standard. These cells were then used for the ion flux experiment. In some cases, leakage of Na+ into the cells during the incubation was determined by using 22Na+ tracer. pCMBS treatment of red cells did not alter the ratio of the ouabain-sensitive Na+ influx to Rb+ influx (the ratios were, respectively, 1.56 and 1.69 for the control and the pCMBS-treated and recovered samples). However, voltage-stimulated Rb+ uptake with pCMBS-treated samples showed a greater variation (50-80%). Thus, we have chosen to alter Na+ content of red cell samples by passive diffusion described above.

**Cation Uptake Measurements—**The experiments followed essentially the procedure described earlier (21). Briefly, cells were suspended, at 12-17% hematocrit, in a standard medium of 140 mM NaCl, 10 mM RbCl with 8-9 tracer, 1 mM MgCl2, 20 mM Tris/HCl, pH 7.4 for experiments measuring the rate of exchange (see tables), with or without 50 μM ouabain. After withdrawing zero time aliquots, 150 μl of each suspension was placed in a stimulation chamber while the rest of the suspensions were kept at the same temperature as the stimulated samples. An AC field at 20 V/cm, 1 kHz was then applied to the ouabain-containing sample and the sample without ouabain for 15 min. These duplicate samples were washed three times with a medium containing no 8-9 tracer. The 8-9 content of samples was then determined as described (21). Also, the hemocrit index of each sample was determined in duplicate.

The specific activity of Rb+ was usually 15-20 cpmmol/pmol. The result of the cation uptake of Rb+ is expressed as attomoles/erythrocyte/h (attomoles/RBC-h), assuming 93 mm3 red cell volume (17) and also that 50% of the cell volume is intracellular space (1 amol/RBC-h = 0.0108 mmol/liter of cells/h). In experiments varying the Rb+ concentration of stimulating medium, Na+ concentration was adjusted so that the sum of two ions was 150 mM and the ATP driven cation uptake was also measured by radioactivity assay to compare with the K0 of the external K+ concentration on the ATP-dependent K+ transport activity. The change in the intracellular concentrations of Na+ and Rb+ during the stimulation was -0.3 ± 0.15 and 0.6 ± 0.20 mM, respectively, as calculated from Na+ and Rb+ tracer measurements at each time point. The Rb+ content of samples was then determined as described above.

**Depletion and Regeneration of ATP in Red Blood Cells—**Red blood cells were suspended, at 5% hematocrit, in a 140 mM NaCl, 10 mM KCl, 1 mM MgCl2, 20 mM Tris/HCl, pH 7.4, solution containing 20 μg/ml of chloramphenicol, and incubated at 26°C for 20 h. Then the cells were further kept at 37°C for 90 min, and then washed once with the medium. ATP content of the cells was determined, and the cells were used for cation uptake experiments immediately. Another portion of the ATP-depleted cells was suspended, at 10% hematocrit, in a recovery medium containing 2 mM adenine, 3 mM inosine, 10 mM glucose, 1 mM MgCl2, 140 mM NaCl, 10 mM KCl, 20 mM Tris/HCl, pH 7.4. ATP content of the cells was then determined and cells were used in the cation uptake experiment. For the determination of ATP concentration, the luciferin/luciferase bioluminescence method was used (24). Fresh luciferin/luciferase mixture was prepared at 20 mg/ml, and 100 μl of this solution was added to 0.1 ml Tris/HCl, pH 7.4, buffer in a cuvette in a LKB Wallace 1250 luminometer. An initial luminescence level was recorded. Luminescence level of aliquots of the perchloric acid extracts of RBC samples appropriately diluted with 0.1 M Tris/HCl, pH 7.4, was measured. The value was then converted to ATP concentration with a calibration curve obtained with ATP standard solutions which also contained the same concentration of perchlorate.

**RESULTS**

**Voltage-induced Rb+ Uptake—**Previous study (21) established that the AC field in the range of 10-30 V/cm, 0.1-100 kHz induced a Rb+ uptake that was inhibited by ouabain. Data in Table I indicate that this uptake was an active transport against the chemical concentration gradient. In experiments 1-4, the cellular concentration of Rb+ was 25-28 mM and the extracellular concentration was 10-12.5 mM. Yet a 20 V/cm, 1 kHz AC field stimulated the uptake of Rb+ (15-25 amol/RBC-h, depending on temperatures) that was completely suppressed by 50 μM of ouabain. Data in Table I also show that the substitution of Rb+ with K+ in the cytoplasm did not alter the result (see experiments 2, 4, and 5). Since this was always the case, in many subsequent experiments the preloading of Rb+ was omitted.

To demonstrate that the voltage-induced Rb+ uptake was not due to a modification of the passive permeability of the membranes by the voltage we have monitored both efflux and influx of Rb+, K+, and Na+, and the result is shown in Table II. Two conditions were used. In experiment 1 (4°C), the cells contained 27.5 mM of Rb+, and the voltage stimulation induced only the ouabain-sensitive uptake, but not the influx of Rb+. Not even the ouabain-insensitive leakage of Rb+ was stimulated by the AC field (compare values for cells unstimulated in the presence of 50 μM ouabain and cells stimulated with an electric field, with the leakage from the same suspension in nonstimulated Na+ movement in either direction was insensitive to the AC stimulation. Results in experiment 2 confirm the above observation. The higher stimulated Rb+ uptake in this case was due to the higher temperature (26°C) in which the experiment was done.

**Voltage-induced Rb+ Transport Dependent on Internal Na+ Concentrations—**The ATP dependent, (Na,K)-ATPase-catalyzed Rb+ transport is known to depend on intracellular Na+ concentration. The rate increases as the internal concentration of Na+ increases, leaving the apparent affinity for Rb+ unaltered (25-27). If the voltage-stimulated Rb+ uptake is mediated by the same enzyme the rate of the uptake should also depend on the internal Na+ concentration. RBC samples with different internal Na+ concentrations were prepared and stimulated by the AC field at 20 V/cm and 1 kHz for 1 h. The Rb+ uptake of these samples was determined by radioactivity assay. Fig. 1A shows the results obtained at 26°C.

The total Rb+ uptake in the absence of voltage stimulation as a function of internal Na+ concentration of the cells is given in the filled circles. When ouabain was present in the medium, the uptake dropped. The difference between the two
Voltage-stimulated Rb uptake in human erythrocytes is an active transport

Rb loading was done either by the passive diffusion method (see "Experimental Procedures") or by using the high voltage loading method (21). After loading, the cells were washed (3 X) with a solution of proper ionic compositions (indicated in the column, Extracellular ion concentration). The extracellular solution was adjusted isotonic with sucrose. After washing, the cells were suspended to a hematocrit of 12-17% with the same solution and Rb uptake was performed. The voltage stimulation was done with an AC field of 20 V/cm at 1 kHz for 60 min. USO, SO, US, and S denote, respectively, unstimulated cells with 50 μM ouabain, stimulated cells with 50 μM ouabain, unstimulated cells, and stimulated cells. Changes in the intracellular ion concentration were less than 0.5 mM for Na, K, and Rb during the Rb uptake experiment. Each data is the mean of 6-27 measurements, and standard deviation is indicated in the parenthesis. (1 amol/RBC-h = 0.0108 mmol/liter of cells/h).

Table I
Voltage-stimulated only Rb uptake

Experimental conditions and symbols used are explained in the legend to Table I. (1 amol/RBC-h = 0.0108 mmol/liter of cells/h). External medium was adjusted to isotonicity by sucrose. USO, SO, US, and S denote, respectively, unstimulated cells with 50 μM ouabain, stimulated cells with 50 μM ouabain, unstimulated cells, and stimulated cells.

Table II

Voltage-stimulated only Rb uptake

Experimental conditions and symbols used are explained in the legend to Table I. (1 amol/RBC-h = 0.0108 mmol/liter of cells/h). External medium was adjusted to isotonicity by sucrose. USO, SO, US, and S denote, respectively, unstimulated cells with 50 μM ouabain, stimulated cells with 50 μM ouabain, unstimulated cells, and stimulated cells.

The above result demonstrates that the presence of Na+ in the cytoplasmic side of the RBC was essential for the (Na,K)-ATPase to respond to the voltage stimulation. Variation in the external Na+ concentration (between 2.5 and 60 mM) did not affect the rate of the voltage-stimulated Rb+ uptake (data
Electrogenic Rb Transport of (Na,K)-ATPase

FIG. 1. Dependence of voltage-induced Rb\(^+\) uptake on cellular Na\(^+\) concentration. Red cells were incubated 24 h at 4 °C in solutions containing 20 mM Tris/HCl, pH 7.4, 1 mM MgCl\(_2\), 20 μg/ml of chloramphenicol, 0–150 mM NaCl, and 0–150 mM KCl (the sum of Na\(^+\) and K\(^+\) was 150 mM). The cells were then spun and resuspended in the standard assay medium (see "Experimental Procedures") at 12–17% hematocrit with 15–20 cpm/pmol of \(^{32}\)Rb\(^+\) specific activity, with and without 50 μM ouabain. 20-μl aliquots from each suspension were withdrawn and washed twice with cold nonradioactive standard medium, then 150 μl from each group was placed in an AC stimulation chamber (21). 20 V/cm at 1 kHz AC field was applied to both chambers for 60 min at 26 °C. The remainder of the two suspensions were also kept at the same temperatures (unstimulated samples). At the end of the stimulation period, 20-μl triplicates from each sample were washed twice as mentioned above. The \(^{32}\)Rb\(^+\) content of the cells was then determined (21). Two other aliquots from each sample were also drawn into capillary tubes and hematocrit index of each sample determined. A, the total Rb\(^+\) uptake in stimulated (○), unstimulated (□), and ouabain-containing stimulated and unstimulated samples (△) is shown. B, the ouabain sensitive part of Rb\(^+\) uptake for the normal (○) and the extra uptake due to voltage stimulation (△) is given. The two data points in the filled circles were obtained with samples (voltage stimulated) in which Na\(^+\) content was depleted with pCMBS method. Each data point represents mean of three separate experiments run in triplicate. Standard deviation from the mean is shown.

FIG. 2. Effect of cellular Na\(^+\) concentration on Rb\(^+\) uptake of red cells. The experiment resembles that shown in Fig. 1 except the temperature was maintained at 6 °C. A, total Rb\(^+\) uptake in voltage-stimulated cells (□), unstimulated cells (○), and ouabain containing stimulated and unstimulated cells (△) is shown. B, the ouabain sensitive part of the normal (○) and the extra uptake due to voltage stimulation (△) is given. The data point in the filled circle was obtained with a stimulated sample in which cellular Na\(^+\) content was depleted with pCMBS method. The data in the open triangle was obtained with the same sample but without voltage stimulation. Each data point represents two separate experiments run in triplicate. The standard deviation from the mean is given.

This is consistent with the notion that the action of Na\(^+\) on this enzyme is membrane side specific (28).

The same experiment was also done at 6 °C. At this temperature the activity of (Na,K)-ATPase was relatively low and the ouabain-sensitive Rb\(^+\) uptake without voltage stimulation was low (Fig. 2, A and B). Voltage stimulation of RBC more than tripled the ouabain-sensitive Rb\(^+\) uptake by the RBC samples. The \(K_m\) values of the (Na,K)-ATPase activity and the voltage-stimulated Rb\(^+\) uptake are again within experimental uncertainty identical, i.e. 8 mM for the internal Na\(^+\) concentration (Fig. 2, A and B).

Dependence of AC-stimulated Rb\(^+\) Transport on External Rb\(^+\) Concentration—Besides the effect of cellular Na\(^+\) concentration, extracellular Rb\(^+\) or K\(^+\) concentrations also affected the ouabain sensitive and the electric field-stimulated Rb\(^+\) uptake. Fig. 3A shows the total Rb\(^+\) uptake in stimulated (open squares), nonstimulated (filled circles), and ouabain-containing samples (open triangles). The ouabain sensitive
Electrogenic Rb Transport of (Na,K)-ATPase

7159

FIG. 3. Effect of external Rb⁺ concentration on the Rb⁺ influx of red blood cells. The Rb⁺ uptake measurements were done as described in the legend to Fig. 1 and under "Experimental Procedures." A, data of total Rb⁺ uptake in voltage stimulated (△), unstimulated (○) samples, and ouabain containing stimulated and unstimulated samples (△) are shown. B, the ouabain sensitive part of the Rb⁺ uptake is shown for the normal (●) and the extra transport due to electric stimulation (■). Each point represents the mean of four separate experiments run in triplicate. Standard deviation is shown.

FIG. 4. Cooperative Rb⁺ uptake on external Rb⁺ concentration. The result of Rb⁺ uptake given in Fig. 3 is replotted in the expanded scale to emphasize the cooperative nature of the Rb⁺ uptake. A, both the plots of the Rb⁺ uptake for the ouabain sensitive part of the normal uptake (○) and the extra uptake due to voltage stimulation (●) show sigmoid dependence on the external Rb⁺ concentration. B, the reciprocal plots indicate that more than one Rb⁺ binding sites were involved in the normal, ouabain-sensitive (○) and the voltage-stimulated (●) Rb⁺ uptake. The maximum rate at 26 °C for the normal (Na,K)-ATPase catalyzed and the voltage-stimulated Rb⁺ uptake was, respectively, 52.6 and 23.5 amol/RBC-h. U means Rb⁺ uptake in amol/RBC-h.

part of Rb⁺ uptake (open circles), i.e. the transport due to the normal (Na,K)-ATPase activity at 26 °C, and the net voltage stimulated part of Rb⁺ uptake (filled squares) are further shown in Fig. 3B. Both curves are similar in shape and they both reach maximum rates around 5 mM extracellular Rb⁺ concentration. Data points at Rb⁺ concentrations below 5 mM are replotted in an expanded scale in Fig. 4A in order to indicate the sigmoid character of the curves. This result suggests that in the process of the voltage-stimulated Rb⁺ transport more than one Rb⁺ binding sites were involved, similar to the Rb⁺ pumping activity of (Na,K)-ATPase (24, 29). In fact, when the data are plotted in the reciprocal form as in Fig. 4B both curves deviate from linearity. In this plot, ouabain-sensitive influx data for unstimulated sample was extracted from the slopes of the time dependence curves with different extracellular Rb⁺ concentrations (data not shown), and the maximum rates obtained were, respectively, 52.6 and 23.5 amol/RBC-h (or 0.57 and 0.25 mmol/liter of cells/h) for the (Na,K)-ATPase-catalyzed and the voltage-stimulated activities. For all the Rb⁺ concentration used, the Rb⁺ uptake by the RBC was linear within the time range studied.

The Hill coefficients obtained from data shown in Figs. 3 and 4 are 1.84 and 1.66 for the ouabain-sensitive, unstimulated Rb⁺ uptake and the voltage-stimulated uptake, respectively. From the same figure, K₅₀ values for extracellular Rb⁺ concentration are extracted to be 0.7 and 1.7 mM, respectively, for the unstimulated and the stimulated Rb⁺ transport.

Effect of Vanadate—The above result clearly indicates that the voltage-stimulated Rb⁺ uptake involved the activity of the (Na,K)-ATPase. Since the voltage stimulation failed to activate Na⁺ efflux (Table II) it would be interesting to examine the effect of ATPase inhibitors on the stimulated Rb⁺ uptake.
Vanadate is a cytoplasmic inhibitor to (Na,K)-ATPase, with a $K_v$ of $10^{-8}$ to $10^{-7}$ M (30, 31). Red blood cell samples were treated with 180 µM vanadate at room temperature for 40 min. In a control experiment, this treatment completely abolished the ouabain-sensitive Na⁺ efflux and Rb⁺ uptake by the RBC. Yet the RBC still exhibited 50% of the voltage-stimulated Rb⁺ uptake activity when treated with an external electric field of 20 V/cm at 1 kHz (Table III). More importantly, the voltage-stimulated Rb⁺ uptake under these conditions was completely suppressed by the presence of 50 µM of ouabain in the external medium (Table III).

**Effect of Intracellular ATP Concentration**—Up to this point all experiments were done with red blood cells of normal ATP level (0.6–1.0 mM). As mentioned, voltage stimulations have been found to trigger synthesis of ATP in mitochondria, chloroplasts, and reconstituted ATP synthetase systems (19, 20). It is, thus, important to check whether the AC stimulation caused an increased ATP consumption, or conversely an elevation of ATP level in the RBC samples. RBC samples were metabolically depleted as described under "Experimental Procedures." The ATP level of these cells dropped from 0.6–1.0 mM to 0.0108 mmol/liter of cells/h). Again, there was no effect on the voltage-stimulated Rb⁺ uptake.

Erythrocytes contain high levels of adenylate kinase and some phosphate hydrolyzing activities that may obscure ATP hydrolysis activity of (Na,K)-ATPase. Since changes in cellular ATP concentration by greater than one order of magnitude did not alter the voltage-induced Rb⁺ uptake, it is unlikely that the voltage treatment stimulated the ATP hydrolysis activity of the (Na,K)-ATPase. A low affinity ATP binding site is believed to regulate conversion of enzyme between the phosphorylated and the dephosphorylated forms (32, 33). The result presented here indicates that this low affinity site was not directly involved in the voltage-stimulated Rb⁺ transport activity.

Another interesting observation was the correlation between the basal level of the (Na,K)-pump and the voltage-stimulated Rb⁺ uptake activity of blood samples from different individuals. The RBC sample from an individual that exhibited a ouabain-sensitive Rb⁺ pumping activity of 79.9 amol/RBC-h compared to the individual of 39.6 amol/RBC-h in Table II also exhibited twice the voltage-stimulated Rb⁺ uptake activity (39.7 amol/RBC-h compared to 22.4 amol/RBC-h). It is not known whether the RBC sample of this individual contained higher enzyme concentration or similar enzyme concentration but with higher specific activity. In any case, the voltage-stimulated, ouabain-sensitive Rb⁺ pumping activity described here increased with increasing basal level of (Na,K)-pump.

**DISCUSSION**

Voltage-stimulated Rb⁺ Uptake Was Due to the Induced

**TABLE III**

Rb uptake of human erythrocytes under various conditions

| No. | Samples                | Temperature | ATP conc | USO | SO | US | S | Ouabain-sensitive basal activity | Ouabain-sensitive stimulated activity | Net stimulated activity |
|-----|------------------------|-------------|----------|-----|----|----|---|----------------------------------|----------------------------------------|------------------------|
| 1   | Fresh RBC              | 6           | 0-1000   | 6.4 | 6.4| 7.0| 9.5| 0.6                             | 3.1                                    | 2.5                    |
|     |                        | 26          |          | (0.3)| (0.3)| (0.1)| (0.4)| (0.3)                          | (0.3)                                 | (0.4)                  |
| 2   | ATP-depleted RBC       | 6           | 16.9     | 8.1 | 7.9| 8.1| 11.7| 0                              | 3.8                                    | 3.6                    |
|     |                        | 26          |          | (1.8)| (0.6)| (0.4)| (0.9)| (0.6)                          | (0.9)                                 | (0.9)                  |
| 3   | ATP-restored RBC       | 26          | 216      | 58.9| 56.5| 92.9| 113.2| 34.0                          | 56.7                                   | 20.3                   |
|     |                        |             | (25)     | (1.9)| (4.5)| (0.5)| (1.5)| (1.9)                          | (4.5)                                 | (1.5)                  |
| 4   | Fresh RBC + 180 µM vanadate | 26 | 50.9     | 52.8| 54.3| 64.9| 3.4 | 21.1                          | 10.6                                   | 10.6                   |
|     |                        |             | (3.6)    | (2.9)| (1.0)| (1.5)| (3.6)| (2.9)                          | (1.5)                                 | (1.5)                  |
| 5   | Fresh RBC with high basal Na⁺-pump activity | 26 | ND       | 81.8| 84.2| 181.7| 201.4| 79.9                          | 117.2                                  | 39.7                   |

Vanadate is a cytoplasmic inhibitor to (Na,K)-ATPase, with a $K_v$ of $10^{-8}$ to $10^{-7}$ M (30, 31). Red blood cell samples were treated with 180 µM vanadate at room temperature for 40 min. In a control experiment, this treatment completely abolished the ouabain-sensitive Na⁺ efflux and Rb⁺ uptake by the RBC. Yet the RBC still exhibited 50% of the voltage-stimulated Rb⁺ uptake activity when treated with an external electric field of 20 V/cm at 1 kHz (Table III). More interestingly, the voltage-stimulated Rb⁺ uptake under these conditions was completely suppressed by the presence of 50 µM of ouabain in the external medium (Table III).

**Effect of Intracellular ATP Concentration**—Up to this point all experiments were done with red blood cells of normal ATP level (0.6–1.0 mM). As mentioned, voltage stimulations have been found to trigger synthesis of ATP in mitochondria, chloroplasts, and reconstituted ATP synthetase systems (19, 20). It is, thus, important to check whether the AC stimulation caused an increased ATP consumption, or conversely an elevation of ATP level in the RBC samples. RBC samples were metabolically depleted as described under "Experimental Procedures." The ATP level of these cells dropped from 0.6-1.0 mM to 0.0108 mmol/liter of cells/h). Again, there was no effect on the voltage-stimulated Rb⁺ uptake.

Erythrocytes contain high levels of adenylate kinase and some phosphate hydrolyzing activities that may obscure ATP hydrolysis activity of (Na,K)-ATPase. Since changes in cellular ATP concentration by greater than one order of magnitude did not alter the voltage-induced Rb⁺ uptake, it is unlikely that the voltage treatment stimulated the ATP hydrolysis activity of the (Na,K)-ATPase. A low affinity ATP binding site is believed to regulate conversion of enzyme between the phosphorylated and the dephosphorylated forms (32, 33). The result presented here indicates that this low affinity site was not directly involved in the voltage-stimulated Rb⁺ transport activity.

Another interesting observation was the correlation between the basal level of the (Na,K)-pump and the voltage-stimulated Rb⁺ uptake activity of blood samples from different individuals. The RBC sample from an individual that exhibited a ouabain-sensitive Rb⁺ pumping activity of 79.9 amol/RBC-h compared to the individual of 39.6 amol/RBC-h in Table II also exhibited twice the voltage-stimulated Rb⁺ uptake activity (39.7 amol/RBC-h compared to 22.4 amol/RBC-h). It is not known whether the RBC sample of this individual contained higher enzyme concentration or similar enzyme concentration but with higher specific activity. In any case, the voltage-stimulated, ouabain-sensitive Rb⁺ pumping activity described here increased with increasing basal level of (Na,K)-pump.
Membrane Potential—It is now recognized that when a cell or an organelle in suspension is exposed to an electric field an electrical potential is generated across the membrane. The magnitude and the sign of the induced membrane potential depend on the position of interest on the membrane surface. For a spherical membrane vesicle, the induced membrane potential, $\Delta \Psi$, is described by,

$$\Delta \Psi = 1.5 \Delta E \cos \theta$$

in which $a$, $E$, and $\theta$ are, respectively, the radius of the vesicle, the electric field strength in volts/cm, and the angle between the field vector and the radial at the point of interest on the membrane surface (13, 15, 20). The maximum potential experienced by a molecule on the membrane surface is $\pm 1.5 \Delta E$ and it occurs at $\theta = 180^\circ$ and $0^\circ$. This is $1.5 \Delta a/d (d$ being the thickness of the membrane) times more intense than that the molecule would experience in solution. For a molecule in the erythrocyte membrane this value is roughly 1000. Our data indicate that maximum stimulation of Rb$^+$ uptake occurred at electric field strength of 20 V/cm, i.e. an induced transmembrane potential of 12 mV (21). Activation of (Na,K)-ATPase is shown to hyperpolarize erythrocyte membranes by this same magnitude (11). The previous study (21) also shows that electric fields below or above 20 V/cm reduced the effectiveness of the stimulation. For field strengths below 35 V/cm no stimulation of Rb$^+$ efflux or movement of Na$^+$ in Na$^+$-K$^+$ transport mode was observed. The constant level of ouabain-insensitive uptake (Tables I and II) makes it less likely that the ouabain binding blocked the electrogenic pump and altered Rb$^+$ uptake indirectly by shifting the membrane potential. These observations indicate that there was no induction of passive permeation pores at this range of voltage. Neither was there any irreversible damage done to the permeation barrier of the red cells as was reported using electric pulses in the kilovolts/cm-range (12–18). Effects of joule heating as the source of the observed Rb$^+$ uptake was unequivocally ruled out by the fact that direct measurements of samples showed negligible temperature elevation (less than 0.5 °C) and that the stimulated uptake was dependent on the frequency of the applied AC field (21). The optimum frequency occurred at 1 kHz when 20 V/cm-electric field was used.

The Voltage-stimulated Rb$^+$ Uptake Involved Activity of (Na,K)-ATPase—Data presented in this article as well as in the previous study (21) support the contention that the voltage-induced Rb$^+$ uptake involved the (Na,K)-ATPase. First, the uptake was against a concentration gradient, indicating that active transport occurred (Table I). Since the uptake was completely inhibited by ouabain, the most likely candidate is (Na,K)-ATPase. Second, the rate of uptake depended on the internal Na$^+$ concentration and the external K$^+$ or Rb$^+$ concentration in the same manner (similar $K_v$ values) as the ATP driven ion translocation through the (Na,K)-ATPase. Third, the Rb$^+$ uptake was consistent with ATP driven Rb$^+$ movement. Since Rb$^+$/K$^+$ efflux and Na$^+$ transport in either direction did not occur, an unrelated activity was unlikely involved (Table II). Finally, erythrocyte samples from an individual with higher basal level of (Na,K)-pump activity also exhibited higher values of AC-stimulated Rb$^+$ uptake.

The stimulated Rb$^+$ influx required the presence of intracellular Na$^+$. The necessity for Na$^+$ ion, without being transported during stimulation may involve the strong inhibitory effect of Na$^+$ on K$^+$-K$^+$ exchange mode of (Na,K)-ATPase (8). Since K$^+$-K$^+$ exchange is not an electrogenic process, intracellular Na$^+$ could hold the enzyme in its electrogenic Na$^+$-K$^+$ transport mode. How did the electric field trigger the K$^+$ pump without simultaneously activating the Na$^+$ pump of the (Na,K)-ATPase? We suggested (21) that an electric field-induced membrane potential could trigger a conformational change of the enzyme, allowing the bound Rb$^+$ (or K$^+$) ions to be translocated into the red cell. We also suggested that since only K$^+$ or Rb$^+$ influx was stimulated by the electric field, the enzyme must, in effect, function as a K$^+$ rectifier. K$^+$ or Rb$^+$ ion could then be driven across the membrane by the membrane potential which either is generated by the efflux of Na ions (11) or, in the present case, induced by the applied electric field. Data in Table III indicate that under identical solvent conditions the rate of the stimulated uptake increased 6–7-fold as the temperature was raised from 6 to 26 °C. The activation energy of the process is approximately 15 kcal/mol. This magnitude of activation process is consistent with a conformational change of a protein. It has been postulated that during the hydrolysis of ATP the catalyzing ions become occluded within the enzyme and are subsequently released after a slow conformational change (32). Indeed, occlusion following a transport of Rb$^+$ and Ca$^{2+}$ has been demonstrated for (Na,K)-ATPase and Ca$^{2+}$-ATPase, respectively (34, 35).

The action of vanadate on the stimulated Rb$^+$ uptake seemed complicated. The vanadate treatment, which inhibited the ouabain-sensitive Rb$^+$ uptake and Na$^+$ efflux in the control cells, did inhibit 50% of the stimulated uptake, which was completely suppressed by ouabain. Vanadate has been proposed as a transitional state analogue for phosphate hydrolysis because of its ability to exist in a stable trigonal bypyramidal structure (36). V-O bond unlike P-O bond forms and breaks rapidly (37). If vanadate forms a complex with an aspartate group (like phosphate), then its stability on the native protein is due to its surroundings rather than to its covalent bond per se (38). Conformational changes within the enzyme upon application of electric field may release vanadate allowing translocation of the already bound Rb$^+$.

All modes of the (Na,K)-ATPase require nucleotides. In spite of the 50% decrease in basal (Na,K)-pump activity when the ATP level dropped from millimolar to 20 μM, no change in the stimulated Rb$^+$ uptake was found (Table II). When the cells were regenerated, they regained 87% of their original basal activity, while stimulated uptake was unchanged (Table II). Determination of intracellular ATP before and after the stimulation did not show significant differences. These results do not directly rule out the requirement for ATP hydrolysis (or synthesis), since, as mentioned, the cells have high levels of adenyate kinase activity. However, uncharged stimulated Rb$^+$ uptake after more than an order of magnitude change in ATP concentration suggests that ATP hydrolysis was not required. Moreover, enzyme turnover that consumed ATP would have stimulated also the Na$^+$ efflux. This clearly was not the case. Since the number of the enzyme was the same in the ATP-depleted and the -undepleted cells, the constant rate of stimulated uptake implies that it was due to the number of enzymes affected by the field instead of their state of activity. However, it is not clear whether nucleotide binding to a high affinity site is required for the observed Rb$^+$ uptake. These findings can be represented by a simple reaction scheme.

```
Membrane potential

$E(Na^+)$, $E(Rb^+)$, $E'(Rb^+)$
```

$E(Na^+)$, $E(Rb^+)$, $E'(Rb^+)$
In this scheme, the membrane potential induces a conformational change of the (Na,K)-ATPase to $E^*$ form. This may be analogous to $E_2$ form of the enzyme in the Albers-Post scheme (39), which has a higher affinity for (Rb$^+$). Then the outside Rb$^+$ combines with this form of the enzyme and is consequently transported inside. The internal Na$^+$ accelerates the analog of (Na,K)-ATPase, which may represent the $E_2$ form of the enzyme in the Albers-Post scheme allowing us to use his luminometer, and to Dr. P. L. Pedersen for helpful discussions and suggestions.

Acknowledgements—We would like to thank Dr. P. L. Pedersen for allowing us to use his luminometer, and Dr. A. Mildvan and B. E. Knox for helpful discussions and suggestions.

REFERENCES

1. Skou, J. C. (1957) Biochim. Biophys. Acta 23, 394-401
2. Post, R. L., Merrit, C. R., Kingsolving, C. R., and Albright, C. D. (1960) J. Biol. Chem. 235, 1796-1802
3. Post, R. L., and Jolly, P. C. (1957) Biochim. Biophys. Acta 25, 118-128
4. Schatzmann, H. J. (1953) Helo. Physiol. Pharmacol. Acta 11, 346-354
5. Cavieres, J. D. (1977) Transport in Red Cells (Ellory, J. C., and Lew, V. L., eds) pp. 1-37, Academic Press, New York
6. Dunham, P. B., and Hoffman, J. F. (1980) Membrane Physiology (Andreoli, T. E., Hoffman, J. F., and Fanestil, D. D., eds) pp. 252-272, Plenum Publishing Corp., New York
7. Glynn, I. M., and Hoffman, J. F. (1971) J. Physiol. (Lond.) 218, 239-256
8. Simons, T. J. B. (1975) J. Physiol. (Lond.) 244, 731-739
9. Glynn, I. M., and Lew, V. L. (1970) J. Physiol. (Lond.) 207, 393-402
10. Thomas, R. C. (1972) Physiol. Rev. 52, 563-594
11. Hoffman, J. F., Kaplan, J. H., and Callahan, T. J. (1979) Fed. Proc. 38, 2440-2441
12. Riemann, F., Zimmermann, U., and Piwat, G. (1975) Biochim. Biophys. Acta 394, 449-462
13. Kinosita, K., and Tsong, T. Y. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 1923-1927
14. Sale, A. J. H., and Hamilton, W. A. (1968) Biochim. Biophys. Acta 163, 37-43
15. Neumann, E., and Rosenheck, K. (1972) J. Membr. Biol. 10, 279-280
16. Kinosita, K., and Tsong, T. Y. (1977) Nature (Lond.) 268, 438-441
17. Kinosita, K., Jr., and Tsong, T. Y. (1979) Biochim. Biophys. Acta 554, 479-497
18. Teissie, J., and Tsong, T. Y. (1980) J. Membr. Biol. 55, 133-140
19. Witt, H. T. (1979) Biochim. Biophys. Acta 505, 355-427
20. Teissie, J., Knox, B. E., Tsong, T. Y., and Wherle, J. (1981) Proc. Natl. Acad. Sci. U. S. A. 78, 7473-7477
21. Serpersu, E. H., and Tsong, T. Y. (1983) J. Membr. Biol. 74, 191-201
22. Garrahan, P. J., and Rega, A. F. (1967) J. Physiol. (Lond.) 193, 459-466
23. Deleted in proof
24. Strehler, B. L. (1968) Methods Biochem. Anal. 16, 99-181
25. Garrahan, P. J., and Rega, A. F. (1967) J. Membr. Biol. 74, 191-201
26. Hoffman, P. G., and Tosteson, D. C. (1971) J. Gen. Physiol. 58, 438-466
27. Baker, P. F., Balustein, M. P., Keynes, R. D., Manil, J., Shaw, T. L., and Steinhardt, R. A. (1969) J. Physiol. (Lond.) 200, 459-496
28. Hoffmann, R. P., and Garrahan, P. J. (1973) J. Physiol. (Lond.) 231, 297-325
29. Hoffman, P. G., and Tosteson, D. C. (1971) J. Gen. Physiol. 58, 438-466
30. Cantley, L. C., Jr., Resh, M. D., and Guidotti, G. (1978) Nature (Lond.) 272, 552-554
31. Garrahan, P. J., and Rega, A. F. (1967) J. Physiol. (Lond.) 218, 239-256
32. Post, R. L., Hegvary, C., and Kume, S. (1972) J. Biol. Chem. 247, 6530-6540
33. Garrahan, P. J., and Rega, A. F. (1967) J. Physiol. (Lond.) 231, 297-325
34. Cantley, L. C., Jr., Resh, M. D., and Guidotti, G. (1978) Nature (Lond.) 280, 510-512
35. Serpersu, E. H., Kirch, U., and Schoner, W. (1982) Eur. J. Biochem. 122, 347-354
36. Linsdell, R. N., Lynn, J. L., Jr., and Lienhard, G. E. (1973) J. Am. Chem. Soc. 95, 8762-8768
37. Kustin, K., and Toppen, D. L. (1973) J. Am. Chem. Soc. 95, 3564-3568
38. Cantley, L. C., Jr., Cantley, L. G., and Josephson, L. (1978) J. Biol. Chem. 253, 7361-7368
39. Dahl, J. L., and Hokin, L. E. (1974) Annu. Rev. Biochem. 43, 327-356