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Single-File Diffusion through the Ca^{2+}-Activated K^+ Channel of Human Red Cells

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Summary. The ratio between the unidirectional fluxes through the Ca^{2+}-activated K^+-specific ion channel of the human red cell membrane has been determined as a function of the driving force ($V_m - E_K$). Net effluxes and ^{42}K influxes were determined during an initial period of ~90 sec on cells which had been depleted of ATP and loaded with Ca. The cells were suspended in buffer-free salt solutions in the presence of 20 μM of the protonophore CCCP, monitoring in this way changes in membrane potential as changes in extracellular pH. ($V_m - E_K$) was varied at constant E_K by varying the Nernst potential and the conductance of the anion and the conductance of the potassium ion. In another series of experiments E_K was varied by suspending cells in salt solutions with different K^+ concentrations. At high extracellular K^+ concentrations both of the unidirectional fluxes were determined as ^{42}K in- and effluxes in pairs of parallel experiments. Within a range of ($V_m - E_K$) of −6 to 90 mV the ratio between the unidirectional fluxes deviated strongly from the values predicted by Ussing's flux ratio equation. The Ca^{2+}-activated K^+ channel of the human red cell membrane showed single-file diffusion with a flux ratio exponent n of 2.7. The magnitude of n was independent of the driving force ($V_m - E_K$), independent of V_m and independent of the conductance g_K.

Key Words single-file diffusion · Ca^{2+}-activated K^+ channel · human erythrocytes

Introduction

Since Gardos (1958) demonstrated a Ca^{2+}-activated net efflux of potassium ions from human red cells, Ca^{2+}-activated K^+ channels have been detected in a wide variety of cells (*cf.* Latorre & Miller, 1983).

Whereas the Ca^{2+}-activated K^+ conductance pathways found in, e.g., central neurones of *Helix aspersa* (Meech & Standen, 1975) and rabbit skeletal muscle membrane (Latorre et al., 1981) at once were thought of as ion channels, there was a pronounced tendency to interpret the Ca^{2+}-activated K^+ net efflux from human red cells as a mediated flux. This idea arose because the net efflux of K^+ from ATP-depleted cells was found to be a complex function of extracellular K^+ concentration, pH and intracellular Na^+ concentration (*cf.* Hoffman et al., 1980).

To distinguish between a potassium-selective carrier mechanism and a potassium-selective ion channel, Hamill (1981), using the patch-clamp technique, measured currents across the membrane of Ca-loaded human red cells. Currents occurred in discrete steps of unit amplitude and variable duration, with a linear current-voltage relationship indicating a unit conductance of about 18 pS. According to Hille and Schwarz (1978) a unit conductance of this magnitude in itself rules out models based on carrier diffusion mediated conductance.

In recent patch-clamp measurements, Grygorczyk and Schwarz (1983) confirmed a single-channel conductance value of about 20 pS for the Ca^{2+}-activated K^+ channel of the human red cell. In addition, they found that the channel showed inward rectification. Within a range of −100 to 0 mV, no voltage dependence could be detected in the patch-clamp studies of Hamill (1981) and Grygorczyk and Schwarz (1983). Thus the incipient closure of the channels that was found during A23187-induced oscillations in the K^+ conductance of the human red cell membrane cannot be explained as a result of voltage dependence, as previously suggested (Vestergaard-Bogind, 1983).

A large number of K^+-specific ion channels are known at present among which the Ca^{2+}-activated type constitutes one category ranging in single-channel conductance from 20 to 240 pS. For the 200-pS channels, the so-called maxi K^+ channels (Latorre & Miller, 1983), a special model with a short channel containing a single ion has been suggested in order to explain the very high conductance. No deviation from the flux ratio test (Ussing, 1949) is therefore expected in the case of the maxi-channels. In contrast, the delayed rectifier of the squid axon (Hodgkin & Keynes, 1955) and the inward rectifier of frog striated muscle membrane

(Horowicz et al., 1968), K^+-specific ion channels of lower conductance, both show single-file diffusion with a flux ratio exponent of 2.5 and 2, respectively.

Heretofore the flux-ratio test apparently has not been systematically applied to any of the Ca^{2+}-activated K^+ channels. In the present paper it is shown that the channel of the red cell membrane shows single-file diffusion with a flux ratio exponent of ~2.7.

Abbreviations

CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DIDS, 4,4′-diisothiocyanostilbene-2,2′-disulfonic acid; Trizma base, Tris (hydrozymethyl) aminoethane; EGTA, ethylene glycol bis (β-aminoethyl ether)-N,N′-tetraacetic acid.

Materials and Methods

Chemicals

All inorganic salts (pro analysis) were purchased from Merck; Trizma base and CCCP were from Sigma. The ionophore A23187 was from Calbiochem; DIDS was from Pierce Chemical Co.; di-*n*-butylphthalate and sucrose (Aristar) were from BDH. The test combination for determination of hemoglobin was from Boehringer.

Cells

Freshly drawn blood from healthy human donors was heparinized and centrifuged. Plasma and the buffy coat were aspirated, and the cells were washed twice in 5 vol high-K salt solution (90 mM KCl/66 mM NaCl/150 μM $MgCl_2$/50 μM EGTA, pH ~ 7.4). The cells were depleted of ATP and 2,3-diphosphoglycerate as previously described (Vestergaard-Bogind & Stampe, 1984), washed three times in high-K salt solution and either stored on ice as packed cells (A cells) or incubated for 30 min at 37°C in a high-K salt solution (90 mM KCl/66 mM NaCl/25 or 100 μM $CaCl_2$/1 μmol per liter cells of A23187) and then stored on ice as packed cells (B cells). By this preincubation in the presence of calcium and ionophore A23187, cells preloaded with fixed concentrations of calcium were obtained. The cellular calcium content was determined by use of ^{45}Ca in a parallel incubation and the intracellular concentrations of ionized Ca were calculated from the determined ^{45}Ca contents of cells, using the previously found value of 0.19 for α (Vestergaard-Bogind & Stampe, 1984), α being the ratio between the concentration of ionized Ca per liter cell water and total Ca per liter cells (Ferreira & Lew, 1976). The intracellular concentration of ionized Ca was 10 or 20 μM in the two main series of experiments.

In each experimental series the initial cellular contents of water and K were determined on samples from the packed cells stored on ice.

Phthalate Method

Cellular contents of ^{42}K and K^+ and extracellular concentrations of K^+ were determined by the phthalate method of Lew and Brown (1979), modified in order to determine cellular and extracellular concentrations of K^+. In centrifuge tubes 875 μl of a solution (155 mM Trizma acetate, 2 mM EGTA, 3.3 mM $LiNO_3$, pH 7.7) was layered on top of 400 μl di-*n*-butylphthalate (density 1.042 to 1.045 at 20°C) and the tubes were stored on ice.

During an experiment 100-μl samples of the cell suspension (hematocrit ≃ 3.1%) were transferred to the cold phthalate tubes, and 5 sec later the tubes were centrifuged for 30 sec at 18,000 × *g*. The 3.1 μl of cells were now isolated as a pellet under the phthalate layer. Extracellular concentrations of K^+ were determined by flame photometry directly on the diluted top-phases of the phthalate tubes. The rest of the top phase and the phthalate were removed and the cell pellets were processed for scintillation counting of the ^{42}K content and flame photometric determination of the K^+ content.

Membrane Potential

Changes in membrane potential were determined according to the method of Macey et al. (1978). The experiments were carried out with cells suspended in buffer-free solution at a hematocrit of 3.1% in the presence of CCCP, a protonophore which mediates a very fast electrochemical equilibration of protons across the cell membranes. Since the intracellular phase is heavily buffered, a change in membrane potential and accordingly in equilibrium potential of protons enforces a shift in the extracellular pH. At the end of an experiment Triton X-100 was added, resulting in immediate hemolysis of all cells. Since all buffering capacity was confined to the cellular phase, the pH of the hemolysate reflected the original cellular pH value, a value which remained constant during the experiment. Absolute values of membrane potentials were calculated from the differences between the peak values of extracellular pH, and the corresponding cellular pH values, assuming that a ΔpH of 1.00 unit is equal to a membrane potential of −61.0 mV.

pH

pH in the suspensions was measured with a Radiometer G2222C glass electrode with a K4112 calomel electrode as a reference, connected to a Radiometer TTT2 pH meter. The pH measurements were calibrated by means of two Radiometer buffers (7.38 and 9.91) at 37°C.

Experimental Procedure

100 μl of the packed cells (*see above*), depleted of ATP and 2,3-diphosphoglycerate, were transferred to 3000 μl buffer-free salt solution, thermostatted at 37°C and vigorously stirred. When Ca-loaded cells (B cells) were used, the experiments were started by the addition of cells to the salt solution [156 mM (Na^+ + K^+ (with ^{42}K) as Cl^- or NO_3^- salt) and 20 μM CCCP]. When cells with normal Ca content (A cells) were used, the addition of ionophore A23187 (5 μmol per liter cells) to the cells suspended in a salt

solution [154 mM NaCl, 2 mM KCl (with ^{42}K), 25 μM $CaCl_2$ and 20 μM CCCP] initiated the experiments. In this type of experiment a cellular concentration of ionized calcium saturating with respect to activation of the K^+ channels, was obtained within a few seconds.

During the first ~90 sec of each experiment 100-μl samples of suspension were transferred to cold-stored phthalate tubes (*see above*) at intervals of about 7 sec, to determine the influx of ^{42}K into the cells and net efflux of K^+ from the cells. Extracellular pH was recorded to monitor the hyperpolarization of the cells (*see* Fig. 1). At the end of an experiment three samples were taken for determination of the total compartments of ^{42}K, K^+ and hemoglobin (a compartment is here used as an amount per liter of cells). In a sample of extracellular phase the final hemoglobin concentration was measured for calculation of hemolysis during the experiment. The experiments were terminated by addition of Triton X-100 to the suspension and pH decreased within a few seconds to the value of the hemolysate, a value which is equal to the cellular pH (*see* Fig. 1).

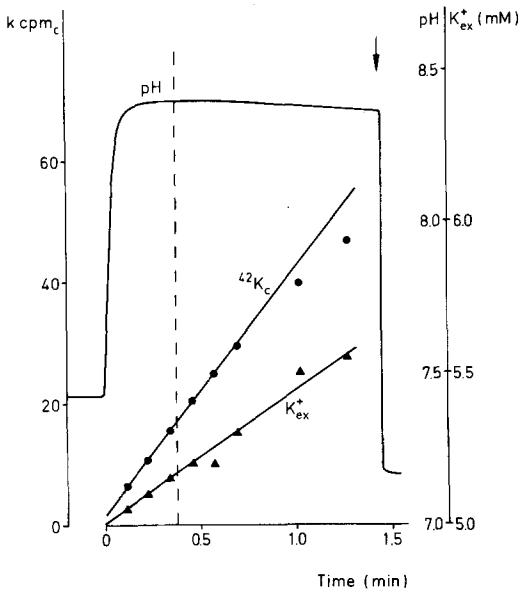

Fig. 1. Results of a typical experiment with ^{42}K influx and K^+ net efflux from ATP-depleted cells preloaded with Ca (10 μM). The abscissa is time in minute and the ordinates are cellular content of ^{42}K in kilo-counts per minute (kcpm$_c$), pH and extracellular concentration of potassium K^+_{ex}. The arrow indicates addition of Triton X-100, which causes immediate hemolysis of all cells so that the pH of the hemolysate becomes equal to the cellular pH. The membrane potential V_m is calculated from the difference between the external pH and the cellular pH. The parameters used to calculate the flux ratio at the time indicated by the dotted vertical line are K^+_{ex}, kcpm$_c$ and V_m together with K^+_{ex} at zero time and the K^+_{ex} and kcpm$_c$ slopes at the dotted line (*see* section on Calculations). 20 μM CCCP was added. pH —; K^+_{ex} ▲; kcpm$_c$ ●

Calculations

Since the cell suspension is a closed system with respect to K^+ and ^{42}K, the total compartments of $K^+(S_T)$ and $^{42}K(P_T)$ are always equal to the sum of the corresponding cellular (S_c and P_c) and extracellular (S_{ex} and P_{ex}) compartments. The net flux J_{net} is equal to the difference between the unidirectional ef- and influxes, J_{ef} and J_{in}. The total compartments are time invariant whereas the cellular and extracellular compartments and the fluxes vary with time. The change with time of the extracellular ^{42}K compartment can be written as:

$$\frac{dP_{ex}}{dt} = -\frac{dP_c}{dt} = J_{ef}\frac{P_c}{S_c} - J_{in}\frac{P_{ex}}{S_{ex}}. \tag{1}$$

Taking into account the above-mentioned relations between compartments and fluxes, Eq. (1) can be solved to give:

$$\frac{J_{ef}}{J_{in}} = \frac{\frac{dP_c}{dt} + \frac{P_{ex}}{S_{ex}}J_{net}}{\frac{dP_c}{dt} + \frac{P_c}{S_c}J_{net}}. \tag{2}$$

Equation (2) is valid under net flux conditions (Fig. 1 shows a net flux experiment) and is composed of elements, which can be determined experimentally, thus allowing calculation of the flux ratio.

In experiments with no or very small net fluxes (*compare* Fig. 2), the initial unidirectional fluxes were simply calculated from the tracer flux and the chemical compartment on the cis side.

Results

In 1949 Ussing derived the flux ratio equation relating the unidirectional fluxes J_{ef} and J_{in} for passive, independent movement of ions across a membrane. In the case of potassium ions the equation would be:

$$\frac{J_{ef}}{J_{in}} = \frac{[K]_c}{[K]_{ex}}\exp\left[\frac{V_m F}{RT}\right] = \exp\left[\frac{(V_m - E_K)F}{RT}\right] \tag{3}$$

where $[K]_c$ and $[K]_{ex}$ are the intra- and extracellular concentrations of K^+, E_K is the Nernst equilibrium potential of K ions, V_m is the membrane potential, and F, R and T have their usual meaning. At least one of the unidirectional fluxes has to be measured as a tracer flux and a unidirectional flux is operationally defined as such.

In their classical work demonstrating single-file diffusion in the delayed rectifier of the squid axon, Hodgkin and Keynes (1955) determined the ratio between the undirectional fluxes in voltage-clamped axon over a wide range of values of the driving force ($V_m - E_K$). They then plotted the ratios be-

Table 1. Flux ratio exponent n determined at different $(V_m - E_K)$ values at a fixed E_K[a]

Nr	E_K (mV)	V_m (mV)	$V_m - E_K$ (mV)	J_{net} (mmol/liter cells · hr)	J_{in}	J_{ef}	$\frac{J_{ef}}{J_{in}}$	n
A1	−101.9	−75.9	26.0	1430	126	1556	12.4	2.58
A2	−101.6	−78.6	23.0	1138	168	1306	7.78	2.38
A3	−100.5	−82.4	18.1	894	219	1113	5.08	2.39
A4	−102.6	−88.4	14.2	800	236	1035	4.40	2.79
A5	−102.0	−96.0	6.0	367	441	808	1.83	2.70
B1	−105.2	−71.9	33.4	950	31.0	981	31.6	2.76
B2	−102.2	−59.7	44.2	1355	20.1	1375	68.3	2.65
B3	−104.0	−49.9	54.1	1543	7.3	1550	213	2.65
B4	−102.9	−38.9	64.0	1731	2.4	1734	710	2.74
B5	−107.7	−16.3	91.4	1694	0.13	1694	12700	2.76
B6*	−107.0	−18.6	88.4	1030	0.29	1031	3520	2.47
C1	−108.3	−73.7	34.6	1091	33.6	1125	33.5	2.71
C2	−108.1	−70.8	37.3	889	17.1	906	53.1	2.84
C3	−108.7	−66.2	42.5	884	7.2	892	124	3.02
C4	−109.8	−53.4	56.4	593	2.3	895	264	2.64
C5	−110.8	−41.8	69.0	450	0.63	450	717	2.55

[a] Results from the V_m series. The columns are from the left: Experimental series and number *Nr*; potassium Nernst equilibrium potential E_K; membrane potential V_m; the driving force $(V_m - E_K)$; potassium net efflux J_{net}; potassium single influx J_{in}; potassium single efflux J_{ef}; the potassium flux ratio J_{ef}/J_{in}, and the flux ratio exponent n. * indicates an experiment where all Cl was substituted by NO_3. Calculations are described in a special section. The three experimental series are: variations in anion conductance obtained by aid of DIDS (A); variations in anion Nernst equilibirum potential by means of different degrees of extracellular sucrose substitution (An^- = 156 to 10 mM) combined with suboptimal cellular concentrations of ionized Ca (B); and variations in potassium conductance achieved by variations in the cellular concentration of ionized Ca (g_K = (10 to 50) $\mu S/cm^2$), (C).

tween the determined unidirectional fluxes on a log-scale versus $(V_m - E_K)$.

In experiments with red blood cells it is impossible to adjust the membrane potential by passing clamping currents through the membranes, and variations in $(V_m - E_K)$ therefore had to be achieved by different means. Opening of the Ca^{2+}-activated K^+ channels of the human red cell membrane results in a cell with a membrane potential V_m totally dominated by the Nernst equilibrium potentials of potassium and chloride ions across the membrane E_K and E_{Cl} and the corresponding membrane conductances g_K and g_{Cl}. According to Hodgkin and Huxley (1952) the current and net flux of potassium ions are given by the equation

$$I_K = J_{net}F = (V_m - E_K)g_K \tag{4}$$

where I_K is the current and J_{net} the net flux of K ions. Equation (4) thus defines the membrane conductance of K ions g_K. In normal human red cells suspended in Cl-Ringer's the anion conductance g_{An} is equal to the chloride conductance g_{Cl}, but in a number of experiments all chloride in the cellular and extracellular phases was replaced by nitrate (NO_3 cells). This results in an unchanged Nernst equilibrium potential ($E_{An} = E_{Cl} = E_{NO_3}$), whereas the conductance of nitrate ions g_{NO_3} is about 2.5 times higher than g_{Cl} (*unpublished observation*). The current and net flux of the anions are given by an equation corresponding to Eq. (4). If net current flow through the membrane is zero, that is $(dV_m/dt) = 0$, which is the case in this system when $I_K = -I_{An}$, then the membrane potential is given by the equation:

$$V_m = \frac{(E_{An} \cdot g_{An}) + (E_K \cdot g_K)}{(g_{An} + g_K)}. \tag{5}$$

Flux ratios were determined at various values of $(V_m - E_K)$ applying three different strategies. In the first approach (V_m series) V_m was varied at a constant E_K and an almost constant extracellular K^+ concentration of ~2 mM. Net efflux of K ions (J_{net}) and net influx of ^{42}K were measured and the flux ratio was calculated from Eq. (2). Different V_m values were obtained by variations in E_{An}, g_{An} and g_K (*compare* Eq. 5). The highest degrees of hyperpolarization (most negative V_m values) were obtained in a series of experiments in which g_{An} was decreased at a normal value of E_{An} (about −12 mV)

Table 2. Flux ratio exponent n determined at different V_m and E_K values[a]

Nr	E_K (mV)	V_m (mV)	$V_m - E_K$ (mV)	J_{net} (mmol/liter cells · hr)	J_{in}	J_{ef}	$\frac{J_{ef}}{J_{in}}$	n
A1	−125.9	−83.5	42.4	1035	12.6	1048	82.9	2.78
A2	−109.9	−79.9	30.0	913	41.7	954	22.9	2.78
A3	−99.0	−75.4	23.6	706	67.1	773	11.5	2.77
A4	−86.8	−70.5	16.3	593	144	736	5.12	2.67
A5	−72.1	−60.2	11.9	517	244	762	3.12	2.56
A6	−55.5	−47.7	7.8	399	328	727	2.22	2.73
A7	−37.1	−33.5	3.6	277	409	686	1.68	3.80
A8*	−106.1	−59.1	47.0	1204	8.6	1213	140	2.81
B1	−12.1	−13.7	−1.2	—	783	835	1.07	—
B2	−12.1	−13.1	−1.0	—	963	895	0.93	—
B3	2.5	1.2	−1.3	—	874	974	1.11	—
B4*	2.1	−4.6	−6.7	−338	643	305	0.47	2.97

[a] Results from the E_K series (A) and the double-tracer series (B). The headings are as in Table 1. E_K variations were obtained by variations in extracellular K^+ concentration (K^+_{ex} = 1 to 33 mM). In the double-tracer series K^+_{ex} was 90 mM in B1 and B2 and 156 mM in B3 and B4. The cells in B2 and B3 were pretreated with DIDS.

and with maximal Ca^{2+} activation of the K^+ channels. In these experiments ATP-depleted cells (A cells) were suspended in a Ringer's solution containing 2 mM K^+ (with ^{42}K), and 25 μM Ca^{2+}. The K^+ channels were opened by a sudden increase in the intracellular concentration of ionized Ca induced by the addition of A23187. Different membrane potentials and net effluxes of KCl were obtained by variation in g_{Cl} by preincubation of the cells for 10 min in the presence of different concentrations of DIDS (0 to 50 μM) (Knauf et al., 1977). The results from these experiments are compiled in Table 1, section A.

In the remaining series of experiments ATP-depleted cells, preloaded with ionized Ca, were used (B cells). A sample of packed cells (with open K^+ channels) was dumped in the appropriate salt solution at zero time and V_m and fluxes were determined for ~90 sec, as described in Materials and Methods. According to Eq. (5) the lowest degree of hyperpolarization should result from a combination of high g_{An} (NO_3 cells), positive E_{An} (sucrose substitution) and low g_K (suboptimal concentrations of cellular calcium). Intermediates between the extreme V_m values of −16 and −96 mV were obtained by combinations of the above-outlined conditions. Thus, in some experiments cells preloaded with suboptimal concentrations of Ca^{2+} were used in order to obtain conditions where low values of g_K were combined with normal values of g_{Cl} (*see* Table 1, section C).

In the second approach, samples of packed cells with open K^+ channels (~10 μM of ionized Ca in the cellular phase) were dumped in Ringer's containing from 1 to 33 mM K^+ (with ^{42}K). In this series of experiments, referred to as the E_K series, V_m as well as E_K varied. As in the V_m series, the net efflux of K ions and the ^{42}K net influx were determined. At higher extracellular concentrations of K^+, the K^+ net effluxes were determined as the decrease in cellular potassium. The result of such an experiment is shown in Fig. 1, while all data from the E_K series are presented in Table 2, section A.

Finally, in the third series, ATP-depleted cells were preloaded with 20 μM of ionized Ca. Part of these cells (with open K^+ channels) were additionally loaded for 10 min in a K^+-equilibrium Ringer's (90 mM K^+) with ^{42}K. Samples of packed ^{42}K-loaded cells were dumped in Ringer's containing 90 or 156 mM K^+, and V_m, possible net flux and ^{42}K efflux were determined. In parallel experiments samples of packed cells (not loaded with ^{42}K) were dumped in Ringer's containing 90 or 156 mM K^+ (with ^{42}K). In this case V_m, possible net flux and ^{42}K influx were determined. The flux ratio was then calculated from the isotope fluxes found in pairs of parallel experiments. These experiments were performed with and without pretreatment with DIDS. In the DIDS-treated cells the net influx of KCl from the 156 mM KCl Ringer's was reduced to a minimum and the depolarization was maximal. In addition the experiments in 156 mM K^+ were performed with NO_3 cells dumped in 156 mM KNO_3, achieving in this way a maximal net influx and a minimal degree of depolarization. The data from these experiments, referred to as the double-tracer series, are presented in Table 2, section B, and the result of a typical experiment is shown in Fig. 2.

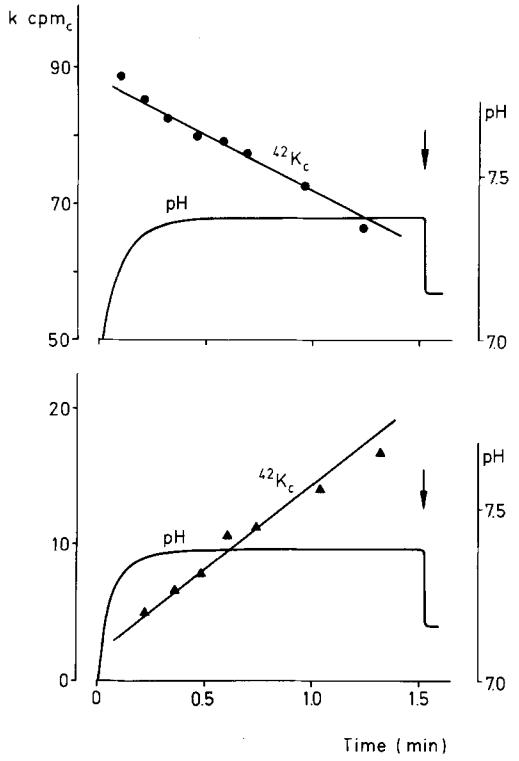

Fig. 2. Results from two experiments with parallel determination of single efflux J_{ef} and single influx J_{in}. The abscissa is time in minutes and the ordinates are cellular contents of ^{42}K in kilocounts per minute (kcpm$_c$) and pH. Membrane potential is calculated as described in Fig. 1. J_{ef} and J_{in} are calculated from the slopes of the kcpm$_c$ curves and the K^+ compartment on the cis side. The upper panel shows J_{ef}, ● and the lower panel shows J_{in}, ▲. 20 μM of CCCP was present. The cells were preloaded with 20 μM Ca^{2+}. — pH

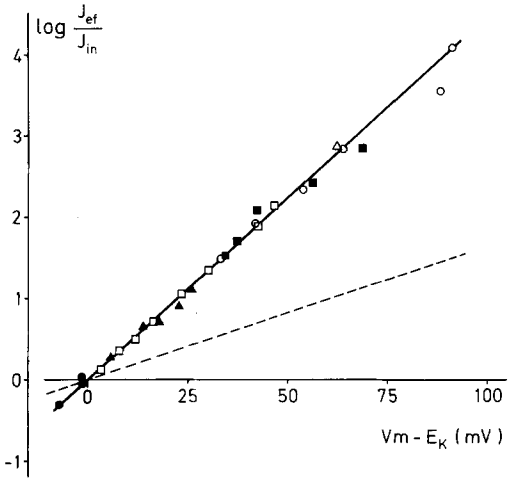

Fig. 3. The logarithm of the potassium flux ratio log J_{ef}/J_{in} as a function of the driving force $(V_m - E_K)$. V_m is the membrane potential and E_K is the Nernst equilibrium potential of K^+. The flux ratio changes one decade every 23 mV, which is equal to a flux ratio exponent $n = 2.7$. The dotted line indicates a value of $n = 1$ corresponding to unrestricted electrodiffusion. All the values from Tables 1 and 2 are shown. Table 1A ▲. Table 1B ○. Table 1C ■. Table 2A □. Table 2B ●. 50 μM quinine present △

In all the above-mentioned experiments the hemolysis of cells after ~70 sec was determined to be less than 1 percent.

Discussion

As seen from Tables 1 and 2 and Fig. 3 flux ratios have been determined within a span of positive $(V_m - E_K)$ values which is larger than that of Hodgkin and Keynes (1955), whereas values in the range where V_m is more negative than E_K are almost lacking. It should be noted that we use a convention of signs which is opposite to that of Hodgkin and Keynes and accordingly use the ratio J_{ef}/J_{in}. In order to obtain flux ratio values within the range where V_m is more negative than E_K the concentration gradients of potassium ions across the red cell membranes have to be reversed and certain technical problems with respect to a sufficiently accurate determination of fluxes have to be solved. Approaches along these lines are, however, in progress.

As expected from Eq. (3), a linear relationship between the logarithm to the ratio between the unidirectional fluxes and the driving force $(V_m - E_K)$ was found (Fig. 3). However, just as Hodgkin and Keynes (1955) found in their study of the delayed rectifier channel, the slope of the line is too steep, with a tenfold change in the flux ratio for a 23-mV change in $(V_m - E_K)$. According to the flux-ratio equation a straight line with a slope corresponding to a tenfold change per 61 mV (at 37°C) should result.

Hodgkin and Keynes (1955) showed that in a long pore containing up to n ions simultaneously, which are constrained to move in single file, the ratio between the unidirectional fluxes as a function of the driving force can be described by the equation:

$$\frac{J_{ef}}{J_{in}} = \exp\left[\frac{n(V_m - E_K)F}{RT}\right]. \quad (6)$$

Here n, the flux ratio exponent, is the average number of ions interacting in the channel under the given experimental conditions. As seen from Tables 1 and 2 the values of the exponent in our experiments do not deviate significantly from that (2.5) reported by Hodgkin and Keynes (1955).

As the absolute value of $(V_m - E_K)$ decreases,

the error in the calculated n values increases sharply so that n cannot be calculated accurately at the smallest values of $(V_m - E_K)$ (*compare* Table 2: A7, B1–3), whereas the spread is hardly visible in the log-scale plot of Fig. 3.

In experiments where $(V_m - E_K)$ values ≥ 90 mV were obtained, the tracer influx, as could be expected, became extremely small. Since the corresponding net effluxes of KCl were very high, the tracer influx had to be determined within ~ 90 sec. We have therefore omitted results from experiments in which the values of the tracer influxes were ≤ 0.1 mmol per liter cells per hr.

Recently, Begenisich and De Weer (1980) have determined potassium flux ratios across the membrane of internal perfused, voltage-clamped squid axons at various V_m and E_K values. They found that between −4 and −38 mV the exponent in the Hodgkin-Keynes equation (Eq. 6) appeared to be a function of the membrane potential. As shown in Fig. 4 no significant voltage dependence of n was detectable within the range of membrane potentials of −16 to −96 mV, at a constant E_K value of ~−105 mV.

It has previously been shown that CCCP-mediated increases in the extracellular pH do not affect the net efflux of K ions from the cells (Stampe & Vestergaard-Bogind, 1985). The fact that n is independent of the membrane potential and therefore independent of the degree of alkalinization of the extracellular phase supports and extends this result.

If, as indicated by the results of Grygorczyk and Schwarz (1983), the Ca^{2+}-activated K^+ channel of the human red cell membrane is an inward rectifier, then it might have been expected that greater similarities with respect to deviation from the flux ratio equation (Eq. 3) would exist between the red cell channel and the inward rectifier of frog striated muscles, than between the red cell channel and the delayed rectifier of squid axons. This is, however, clearly not the case.

Horowicz et al. (1968) found that within a range of $(V_m - E_K)$ values of 30 mV the flux ratio of the inward rectifier of striated frog muscle membrane followed the Hodgkin-Keynes equation (Eq. 6), but with an n value equal to 2. In these experiments the variations in $(V_m - E_K)$ were obtained by varying K^+_{ex} from 100 to 25 mM at a constant membrane potential of about −18 mV, or varying the membrane potential between +20 and −20 mV at a constant E_K value of about −18 mV. In the last-mentioned case the V_m variation was obtained by variation of the extracellular Cl^- concentration. Thus the lowest concentration of extracellular K^+ used in these experiments was 25 mM. Horowicz et al. (1968) suggested that potassium ions move through the membrane by associating with a mobile carrier system.

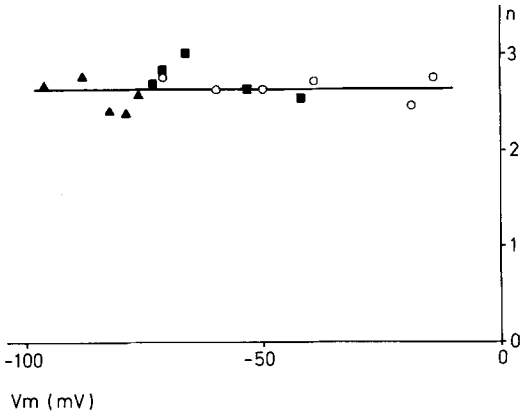

Fig. 4. The flux ratio exponent n versus membrane potential V_m at fixed E_K. The points represent all the results from Table 1. n = 2.66 ± 0.04 (SE, n = 16). Table 1A ▲. Table 1B ○. Table 1C ■

In a later paper, Spalding et al. (1981) extended this study. They determined the ratios between the unidirectional fluxes in frog striated muscle fibers loaded with 305 mM K^+ and 120 mM Cl^-. At $(V_m - E_K)$ values below 40 mV, the exponent n was again found to be equal to 2. At $(V_m - E_K)$ values >40 mV the value of n decreased towards a value of 1 as the K^+_{ex} decreased towards 2 mM; that is, the ratio of the unidirectional fluxes approached the value expected from Ussing's flux ratio equation. They suggested that the inward rectifier was a potassium channel containing two or more activating sites within the membrane that bind K^+ and are accessible from the outside (Spalding et al., 1981).

Sjodin (1965) determined the flux ratio of potassium ions in frog sartorius muscle at an extracellular K^+ concentration of 0.2 mM. The intracellular K^+ concentration was 120 to 150 mM, the membrane potential was about −112 mV and a substantial net potassium efflux accordingly took place. A small, but significant deviation from the results predicted in Eq. (3) was found, but the result was considered to favor a transport mechanism obeying the independence principle.

According to the studies of Horowicz et al. (1968) and Spalding et al. (1981) the flux ratio exponent and the conductance of the inward rectifier are related, so that at lower values of conductance (low extracellular K^+ concentrations) Ussing's flux ratio equation is valid, whereas at higher values of conductance (extracellular K^+ concentrations ≥ 25 mM) the flux ratio follows Eq. (6) with an exponent n equal to 2. According to the K^+-channel model of Hille and Schwarz (1978) the conductance and flux ratio exponent n of a multi-ion, single-file channel

should increase as the K^+ activity on the outside of the membrane increases. In the E_K series of experiments the extracellular concentration of K^+ varied from 1 to 17 mM and no significant variation in n was found (Table 2, section A). In addition, an n value of 2.97 was found in the one experiment in the double-tracer series (K^+_{ex} = 156 mM) where ($V_m - E_K$) attained a value of −6.7 mV, allowing a calculation of n. These results indicate that most probably there is no significant dependence of n on the extracellular K^+ concentration within the range of 1 to 156 mM.

In the experimental series in which g_K variations were induced by loading the cells with different concentrations of ionized Ca, a variation in g_K from 10 to 80 $\mu S/cm^2$ (not shown in the Table) was achieved. The result, an average n value of 2.66 (Table 1) indicates that n is independent of g_K within the above-mentioned range.

It should be noted that in a separate experiment with preloaded cells (20 μM Ca^{2+}) g_K was diminished to 14 $\mu S/cm^2$ by the addition of quinine (50 μM). The corresponding n value was 2.84.

As mentioned above, this is to our knowledge the first systematic study of the flux ratio in a Ca^{2+}-activated K^+ channel. However, one previous study of the flux ratio of the K^+ channel of the human red blood cell membrane has been reported by Kregenow and Hoffman (1972). K^+ flux ratios were determined in experiments on energy-depleted red cells in which the Ca^{2+}-activated K^+ channels were activated by a passive leak of Ca^{2+} into the cells. They found that the ratio between the measured unidirectional fluxes appeared to satisfy the flux ratio equation for passive diffusion, provided that the membrane potential (which at that time could not be determined) was equal to E_{Cl}. With fully activated K^+ channels this provision should not be valid, but according to the published data the net efflux of K^+ in the experiments was maximally about 135 mmol per liter cells per hr, which should result in a hyperpolarization of only 5 to 10 mV, that is an absolute value of V_m of −15 to −20 mV. This should correspond to a value of ($V_m - E_K$) of about 60 mV in these experiments, and with an n value of 2.7 a flux ratio of ~1000 should be found, as compared to the reported flux ratio of about 30. The explanation of this large discrepancy may well be that in experiments where the Ca^{2+} activation of the K^+ channels takes place by a slow leak of Ca^{2+} into energy-depleted cells, there could be a low (on average), slowly increasing conductance which is very unevenly distributed within the cell population. Furthermore, taking into account later reports indicating a high Ca-binding capacity of the intracellular phase (*see* α value in Materials and Methods), the average cellular concentration of ionized Ca in these experiments may well have been about 0.5 to 1.5 μM, values well below the K_m value (*cf.* Yingst & Hoffman, 1984).

Conclusion

The ratio between the unidirectional fluxes of K^+ through the Ca^{2+}-activated K^+ channels of the human red cell membranes has been determined over a wide range of ($V_m - E_K$), V_m and E_K, respectively. The flux ratios were well described by the Hodgkin-Keynes single-file equation, with a striking similarity between the flux ratio exponent (n = 2.7) and the exponent found by Hodgkin and Keynes (1955) in the delayed rectifier of squid axons (n = 2.5). Thus, the Ca^{2+}-activated K^+ channel of the human red cell membrane seems to be a multi-ion, single-file channel with at least three sites.

The flux ratio exponent was found to be independent of V_m (−16 to −96 mV), extracellular K^+ concentration (1 to 17 mM, and probably up to 156 mM) and g_K (10 to 80 $\mu S/cm^2$).

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