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Voltage Dependence of the Ca^{2+}-Activated K^+ Conductance of Human Red Cell Membranes is Strongly Dependent on the Extracellular K^+ Concentration

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Summary. The conductance of the Ca^{2+}-activated K^+ channel ($g_K(Ca)$) of the human red cell membrane was studied as a function of membrane potential (V_m) and extracellular K^+ concentration ($[K^+]_{ex}$). ATP-depleted cells, with fixed values of cellular K^+ (145 mM) and pH (~7.1), and preloaded with ~27 μM ionized Ca were transferred, with open K^+ channels, to buffer-free salt solutions with given K^+ concentrations. Outward-current conductances were calculated from initial net effluxes of K^+, corresponding V_m, monitored by CCCP-mediated electrochemical equilibration of protons between a buffer-free extracellular and the heavily buffered cellular phases, and Nernst equilibrium potentials of K ions (E_K) determined at the peak of hyperpolarization. Zero-current conductances were calculated from unidirectional effluxes of ^{42}K at $(V_m - E_K) \simeq 0$, using a single-file flux ratio exponent of 2.7. Within a $[K^+]_{ex}$ range of 5.5 to 60 mM and at $(V_m - E_K) \geq 20$ mV a basic conductance, which was independent of $[K^+]_{ex}$, was found. It had a small voltage dependence, varying linearly from 45 to 70 μS/cm^2 between 0 and −100 mV. As $(V_m - E_K)$ decreased from 20 towards zero mV $g_K(Ca)$ increased hyperbolically from the basic value towards a zero-current value of 165 μS/cm^2. The zero-current conductance was not significantly dependent on $[K^+]_{ex}$ (30 to 156 mM) corresponding to V_m (−50 mV to 0). A further increase in $g_K(Ca)$ symmetrically around E_K is suggested as $(V_m - E_K)$ becomes positive. Increasing the extracellular K^+ concentration from zero and up to ~3 mM resulted in an increase in $g_K(Ca)$ from ~50 to ~70 μS/cm^2. Since the driving force $(V_m - E_K)$ was larger than 20 mV within this range of $[K^+]_{ex}$ this was probably a specific K^+ activation of $g_K(Ca)$. In conclusion: The Ca^{2+}-activated K^+ channel of the human red cell membrane is an inward rectifier showing the characteristic voltage dependence of this type of channel.

Key Words Ca^{2+}-activated K^+ channel · human erythrocytes · voltage dependence · function of $[K^+]_{ex}$ · inward rectification

Introduction

During the past few years strong support has developed for the view that the Ca^{2+}-activated conductance of the human red cell membrane takes place via a K^+-specific, Ca^{2+}-activated ion channel. Applying the patch-clamp technique, Hamill (1981) showed that the currents across patches of Ca^{2+}-activated human red cell membranes occurred in discrete steps of unitary amplitude, indicating the existence of a K^+-specific ion channel with a single-channel conductance (γ) of ~18 pS (100 mM K^+, symmetrically, 19°C). In a later patch-clamp study of the human red cell membrane, Grygorczyk and Schwarz (1983) confirmed a γ value of ~20 pS (150 mM K^+, symmetrically, 20 to 22°C) and in addition they demonstrated that the channel showed inward rectification. The inward rectification of the K^+ channel of the human red cell membrane observed by Grygorczyk and Schwarz (1983) and later confirmed (Grygorczyk, Schwarz & Passow, 1984) is, however, not as pronounced as that of the inward rectifier of striated muscle membrane (Hodgkin & Horowicz, 1959) or a starfish egg cell membrane (Hagiwara & Takahashi, 1974).

The concept of a K^+-specific ion channel as the Ca^{2+}-activated conductance pathway received independent support by the demonstration that the unidirectional K^+ fluxes across the membranes of Ca^{2+}-activated human red cells did not obey the flux-ratio equation of Ussing (1949). Under net efflux conditions the ratio between the unidirectional K^+ fluxes followed the equation of Hodgkin and Keynes (1955) with a flux ratio exponent of ~2.7, and the conductance pathway thus exhibited single-file diffusion characteristics (Vestergaard-Bogind, Stampe & Christophersen, 1985*a*). The single-file exponent was found to be independent of the extracellular K^+ concentration within the range of 1 to 17 mM and probably up to 156 mM, independent of V_m and independent of the K^+ conductance. Thus in terms of the single-file model the channel does not resemble the inward rectifier of striated muscle membranes, where the exponent was found to be a function of the extracellular K^+ concentration (Horowicz, Gage & Eisenberg, 1968; Spalding et al., 1981).

A complex relationship between the rate con-

stant of the Ca^{2+}-activated net efflux of K ions from human red cells or ghosts and the extracellular concentration of K^+ and H^+ has been reported by Hoffman and co-workers (for a review *see* Hoffman et al., 1980) and Passow and co-workers (for a review *see* Schwarz & Passow, 1983).

The conductance pathway being a K^+-selective ion channel showing single-filing and inward rectification, the relationships between net or unidirectional fluxes and the membrane potential, extracellular K^+ concentration, etc., are not described by the constant-field regime and rate constants cannot easily be related to the K^+ conductance of the membrane. In the present paper we attempt to characterize in detail $g_K(Ca)$ at fixed values of cellular concentrations of K^+ (145 mM), and ionized Ca (~27 μM) as a function of the extracellular concentration of K^+ and the membrane potential. Since the Ca^{2+}-activated K^+ conductance varies markedly with cellular pH (Stampe & Vestergaard-Bogind, 1985), all experiments were made at a cellular pH value of ~7.1.

Materials and Methods

Chemicals

All inorganic salts (pro analysis) were purchased from Merck. Trizma base[1] 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 $MgCl_2$, pH ~ 7.4) containing 50 μM EGTA. The cells were depleted of ATP and 2,3-diphosphoglycerate as previously described (Vestergaard-Bogind & Stampe, 1984), and washed three times in high-K salt solution.

The cells were then suspended at a cytocrit of ~20% in high-K salt solution containing in addition 500 μM $CaCl_2$ (with ^{45}Ca). Ionophore A23187 was added to a concentration of 0.5 μmol per liter of cells. After 20 to 40 min of incubation at 25°C (*see below*) the cell suspension was centrifuged and the cells were washed once in 10 vol of high-K salt solution containing 5 μM of EGTA. By this incubation of ATP-depleted cells in the presence of A23187 and Ca the cells were loaded with Ca. The cellular content of Ca was determined from the cellular content of ^{45}Ca, and the concentration of ionized Ca in the intracellular phase was then taken as the total content of Ca per liter cells times 0.2 (*cf.* Stampe & Vestergaard-Bogind, 1985).

In our experience, ionophore A23187 once added to the cells cannot be washed out. In the presence of the very low concentration of ionophore A23187 used, even more than 1 hr of incubation did not result in an equilibrium distribution of Ca^{2+} between cells and medium. A predetermined cellular concentration of ionized Ca was therefore obtained in the following way. Samples of the incubation medium were taken at intervals of ~5 min and the cellular content of ^{45}Ca was determined by the phthalate method (*see later*). The values obtained were plotted against time, and the time necessary to obtain the chosen cellular concentration of ionized Ca (~27 μM) was found by linear extrapolation. The very low concentration of ionophore was used in order to avoid ionophore-mediated changes in the cellular concentration of ionized Ca during the experimental period. In the experimental series the CCCP-mediated electrochemical equilibration of protons established proton concentration gradients across the cell membranes from 0 up to 1.5 units of pH as a result of the various degrees of hyperpolarization of the membranes. The corresponding variation in ionophore A23187-mediated equilibrium distribution ratio of ionized Ca across the cell membranes would be from 1 to 1000 (Vestergaard-Bogind & Stampe, 1984), and it would be almost impossible to adjust the Ca^{2+} activity of the various extracellular phases so that Ca^{2+} would always be in equilibrium. In all experiments the extracellular phases were free of added Ca^{2+} and 5 μM EGTA was present to neutralize the Ca^{2+}-contamination from the various chemicals.

Phthalate Method

Cellular contents of K^+, Na^+, ^{42}K and ^{45}Ca and extracellular concentrations of K^+ were determined by the phthalate method as previously described (Vestergaard-Bogind et al., 1985*a*).

During an experiment 100-μl samples of the cell suspension (hematocrit $\simeq$ 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. The extracellular concentration of K^+ was determined by flame photometry on the 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 ^{45}Ca or ^{42}K content and flame photometric determination of the K^+ and Na^+ contents.

Membrane Potential

Changes in membrane potential (V_m) were determined according to the method of Macey, Adorante and Orme (1978). The experiments were carried out with cells suspended in buffer-free salt solution at a hematocrit of 3.1% in the presence of 20 μM of the protonophore CCCP, which mediates a fast electrochemical equilibration of protons across the cell membranes. Since the intracellular phase is heavily buffered, a change in V_m results in a shift in the extracellular pH to a new equilibrium value, determined by the constant intracellular proton activity and the membrane potential. 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 which remained constant during the experiment. Absolute V_m values were then

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

calculated from the differences between the peak values of extracellular pH and the corresponding cellular pH values (*see* Fig. 1).

Experimental Procedure

In all experiments one vol of packed cells loaded with about 27 μM of ionized Ca were transferred into 30 vol of buffer-free salt solution with a given K^+ concentration (156 mM (NaCl + KCl)), containing 20 μM CCCP, thermostatted at 37°C and vigorously stirred. Since the cells were transferred into the salt solutions with open K^+ channels the efflux started instantaneously.

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 net efflux of K^+ (or ^{42}K) from the cells, while extracellular pH was recorded. In Fig. 1 the results of a typical experiment are shown. At the end of an experiment samples were taken for determination of the total compartments of K^+ (^{42}K) and hemoglobin (compartments, whether intracellular, extracellular or total, are here taken 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 results presented in the following are all from experiments in which hemolysis was less than 1%. The experiments were terminated by addition of Triton X-100 to the suspension resulting within a few seconds in a decrease of pH to the value of the hemolysate, a value which is equal to the cellular pH. In each experimental series the initial cellular contents of water and K were determined on samples from the packed cells stored on ice.

Three main types of experiments were performed. In the first type the initial net efflux of K ions and the corresponding values of the Nernst equilibrium potential of K ions (E_K) and V_m were determined at various $[K^+]_{ex}$ values. In the second type of experiment cells were transferred into salt solutions containing 20 to 156 mM KCl and 25 μM DIDS. The cells were loaded with ^{42}K in addition to Ca (without ^{45}Ca). In these experiments, where only very small or no net fluxes occurred the initial unidirectional efflux of K^+ was determined. The chosen concentration of ionized Ca (~27 μM) was obtained by way of a parallel loading-incubation containing ^{45}Ca (*see* section on Cells). In the third type of experiment the Nernst potential and conductance of the dominating anion were varied (*see later*) and V_m and net efflux of K^+ were determined as described above.

Calculations

The net efflux of K^+ in mmol per liter cells per hr was calculated from the initial, constant decrease in cellular K^+ content. In the same way, the unidirectional effluxes were calculated from the initial, constant decreases in cellular ^{42}K content and the constant cellular K^+ content. The amount of cells in each experiment was determined from the concentration of total hemoglobin in the suspension. The value of the Nernst potential for K ions at the peak of hyperpolarization was calculated from the extracellular K^+ concentration at that time and the initially determined intracellular concentrations. Assuming that the initial loss in cellular K^+ takes place as an outflow of isotonic KCl (or KNO_3) solution the calculated change in $[K^+]_{cell}$ will lower the E_K value less than 1 mV within the first 30 sec, even in experiments with net effluxes of 3 mol per liter cells per hr.

The membrane potential (V_m) was calculated from the CCCP-mediated new electrochemical equilibrium of protons

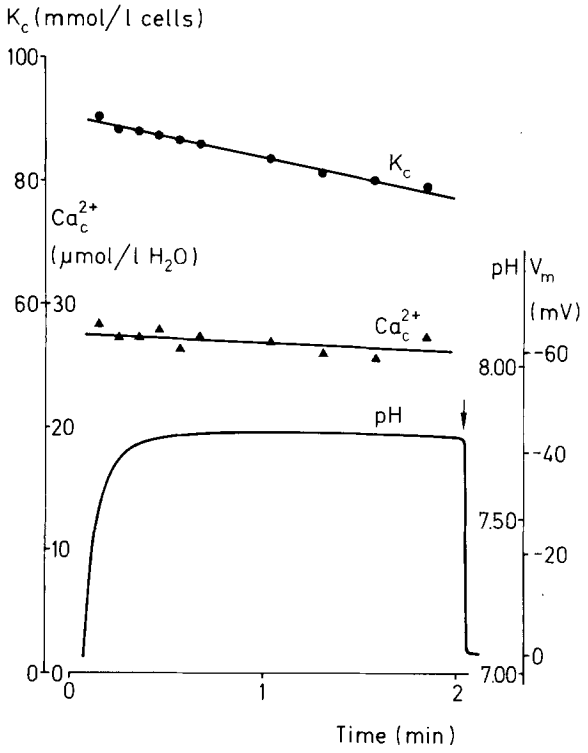

Fig. 1. Results of a typical experiment with K^+ net efflux from ATP-depleted cells preloaded with Ca (~27 μM). The abscissa is time in minutes and the ordinates are cellular content of K^+ in mmol per liter cells (K_c), cellular concentration of ionized calcium in μM (Ca_c^{2+}), pH and membrane potential (V_m) in mV. The arrow indicates addition of Triton X-100, which causes immediate hemolysis of all cells, the pH of the hemolysate becoming equal to the cellular pH. The membrane potential is calculated from the difference between the extracellular and the cellular pH (*see* section on Calculations). The net efflux of K^+ is calculated from the slope of the curve showing K_c. Note that the change in Ca_c^{2+} during the experiment is negligible

across the cell membranes (*see* Fig. 1 and section on methods). A pH difference (pH_{ex}-pH_c) of one unit was taken to be equivalent to a membrane potential of 61.5 mV (inside negative). It has previously been shown (Stampe & Vestergaard-Bogind, 1985) that the net efflux of K^+ is not affected by the increases in extracellular pH mediated by the CCCP-mediated redistribution of protons.

The Ca^{2+}-activated K^+ conductance $g_K(Ca)$ was, in the case of net efflux, calculated from the equation of Hodgkin and Huxley (1952)

$$I_K = J_K \cdot F = (V_m - E_K) \cdot g_K(Ca). \quad (1)$$

Here I_K is the current of K ions across the membrane, that is $J_K \cdot F$, where J_K is the net flux of K ions in μmol/(cm^2 · sec) and F is the Faraday constant. In the calculation of J_K per cm^2 of membrane it was assumed that the area of one liter of cells equals 1.75×10^7 cm^2. The conductance is accordingly obtained in μS/cm^2.

In the experiments, where the unidirectional efflux was determined at $V_m = E_K$, the zero-current conductance was calcu-

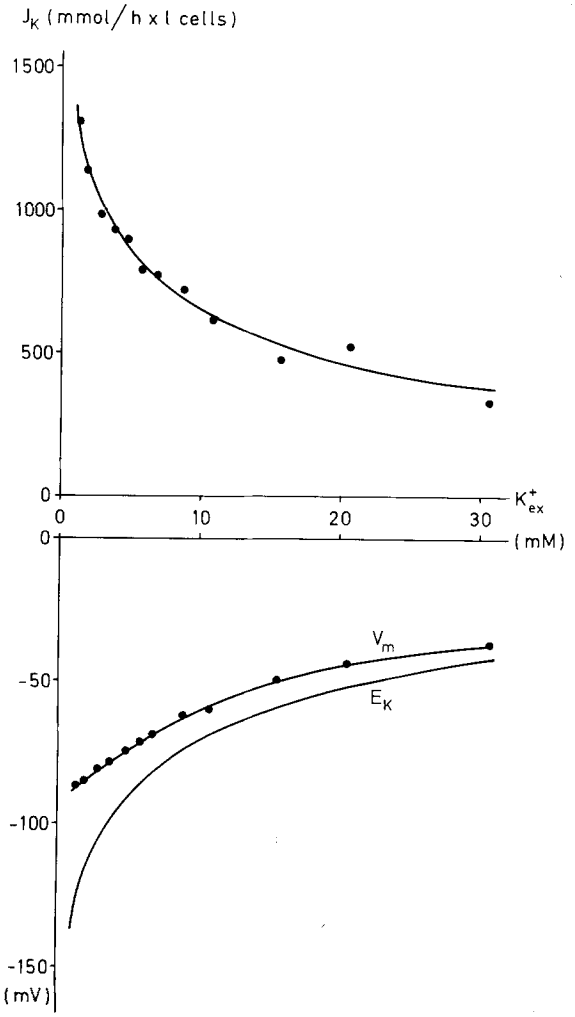

Fig. 2. K^+ net efflux (J_K) in mmol per hr per liter of cells, membrane potential (V_m) in mV, and calculated Nernst equilibrium potential of K ions (E_K) in mV versus extracellular potassium $[K^+]_{ex}$ in mM. Cellular concentration of K^+ was 145 mM

lated according to Hodgkin and Keynes (1955), using the equation:

$$g_K(Ca) = \frac{n \cdot F^2}{R \cdot T} \cdot J_{eff} \quad (2)$$

where J_{eff} is the unidirectional efflux and n is the flux ratio exponent for an ion channel exhibiting single-file diffusion. The previously determined n value of 2.7 (Vestergaard-Bogind et al., 1985*a*) was inserted in Eq. (2). R, T and F have their usual meaning.

Estimated Standard Deviations and Systematic Errors

The net effluxes, determined on the basis of the initial, constant decrease in cellular K^+ content, varied between 200 and 3000 mmol per liter cells per hr, the corresponding number of K^+_{cell} values obtained within the initial phase varying between 5 and 10, respectively (*compare* Fig. 1). The relative standard deviation of the fluxes was 2 to 5% depending on the magnitude of the flux.

In a series of control experiments where V_m was 40 to 60 mV the SD was less than 1% (n = 24) and the relative standard deviation on the determination of cellular K^+ content was less than 0.2% SEM (n = 10). In each experimental series the E_K values were calculated from the concentration of extracellular K^+ determined in each experiment and a separately determined cellular K^+ concentration of the stock of depleted cells (stored on ice).

Based on these values the standard deviation of the g_K(Ca) values was estimated to be maximally 5%, an error which in the present context is of minor importance. In addition to random errors there is probably a systematic error in the ($V_m - E_K$) values, predominantly originating in the E_K values. Thus, the activity coefficient of intra- and extracellular K^+ are assumed to be identical; the water content of the cells may, because of trapped extracellular volume and other factors, be systematically calculated too low or too high. In net flux experiments at ($V_m - E_K$) values of 1 to 2 mV we always found g_K(Ca) values higher than the zero-current conductances determined in unidirectional flux experiments. The uncertainty of the zero-current conductances is dominated by the standard deviation of the unidirectional flux estimate (*see above*) and the standard deviation of the mean of the flux-ratio exponent n. The latter has previously been determined to be 2.66 ± 0.04 (n = 16) (Vestergaard-Bogind et al., 1985*a*) and thus the error of the zero-current conductances should be low compared to the error on the outward-current conductances at low ($V_m - E_K$) values. These relationships indicate that the standard deviation is of minor importance, whereas the E_K values seem to be determined with a systematic error of about plus 1 mV. We have therefore omitted g_K(Ca) values determined in net flux experiments with ($V_m - E_K$) values < 3 mV.

Results

g_K(Ca) as a Function of $[K^+]_{ex}$

The influence upon g_K(Ca) of variation in the extracellular K^+ concentration has been determined over a wide span. The cellular concentration of ionized Ca was 27 μM, the cellular K^+ concentration was 145 mM and the cellular pH 7.1. Figure 1 shows the results of a typical flux experiment. The net effluxes and the corresponding V_m and E_K values are shown in Fig. 2. In Fig. 3 each filled circle represents the conductance calculated from the data shown in Fig. 2. The different $[K^+]_{ex}$ values were obtained by substituting KCl for NaCl maintaining a constant total concentration of NaCl + KCl of 156 mM. At all extracellular K^+ concentrations the net effluxes of K^+ were determined on the basis of the decrease in cellular K^+ content with time. The net efflux of K^+ in the absence of extracellular K^+ was determined on cells (not preloaded with Ca) washed several times in K^+-free Ringer's. The K^+ channels were

activated by addition of A23187 (8 μmol/liter cells) to the cell suspension (extracellular Ca concentration ~500 μM) as previously described (Vestergaard-Bogind, 1983). Thus, the initial net efflux of K^+ was determined while the cellular concentration of ionized Ca increased sharply, surpassing within ~5 sec the concentration necessary for maximal g_K(Ca) activation and the result is therefore not quite comparable to the main results. On the other hand, it was found to be the best way to establish an experimental situation with $[K^+]_{ex} \simeq 0$.

The filled triangles in Fig. 3 represent zero-current conductances determined at $V_m = E_K$. In these experiments the ^{42}K effluxes were measured at different extracellular K^+ concentrations. At 90 mM KCl extracellularly, we have $E_K = E_{Cl} = V_m$, and $(V_m - E_K)$ should be exactly zero. The conductance of K ions can therefore be calculated from the unidirectional efflux, using Eq. (2).

The experiments with extracellular K^+ concentrations different from 90 mM were performed in the presence of DIDS (25 μM), the decreased chloride conductance resulting in a $(V_m - E_K)$ value within ±2mV. Within the experimental period of ~2 min the net efflux of K^+ amounted to less than 3% of the corresponding unidirectional efflux and Eq. (2) was used for calculation of g_K(Ca).

In the experiment with $[K^+]_{ex} = 20$ mM the net efflux was 135 mM per liter of cells per hr, corresponding to more than 10% of the unidirectional efflux, and Eq. (2) was not applied. Instead, the unidirectional influx and then the flux ratio were calculated. Inserting the flux ratio and the flux ratio exponent (2.7) in Hodgkin and Keynes (1955) single-file equation:

$$\frac{J_{ef}}{J_{in}} = \exp \frac{n \cdot (V_m - E_K) \cdot F}{R \cdot T} \tag{3}$$

the driving force $(V_m - E_K)$ was now calculated (in a situation where the usual determination of the value would be too inaccurate). Finally, g_K(Ca) was calculated from the measured net efflux and the calculated $(V_m - E_K)$ value (*see* Fig. 7).

Assuming that the zero-current conductance is independent of $[K^+]_{ex}$ within the $[K^+]_{ex}$ range of 30 to 156 mM, an average value of 166 ± 2 μS/cm^2 (SEM, $n = 13$) was found.

The Conductance as a Function of V_m

All measurements have shown that during the experimental period the Na^+ content of the cells remained constant. Accordingly, the membrane potential of Ca-loaded red cells is determined by the Nernst potentials and conductances of potassium and the dominating anion, respectively. Under conditions where no net current crosses the membranes, that is at the peak of hyperpolarization, the membrane potential is given by

$$V_m = \frac{E_{An} \cdot g_{An} + E_K \cdot g_K(Ca)}{g_{An} + g_K(Ca)} \tag{4}$$

where An normally represents Cl ions, but in some of the experiments, in which all chloride had been replaced by NO_3 ions, represents the last mentioned.

Partly, the aim of the present study was to describe g_K(Ca) (at fixed cellular values of pH and concentrations of K^+ and ionized Ca) as a function of $[K^+]_{ex}$. Variation in the extracellular concentration

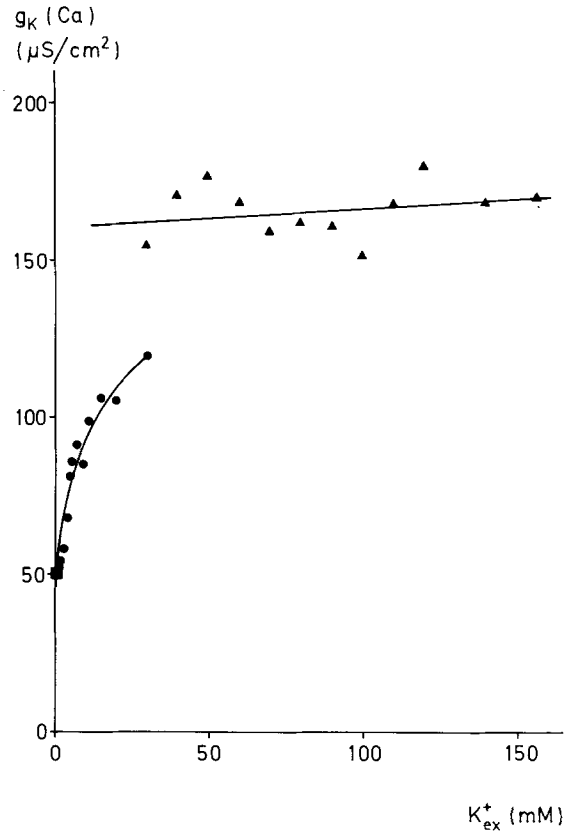

Fig. 3. K^+ conductance (g_K(Ca)) in μS per cm^2 as a function of extracellular potassium $[K^+]_{ex}$ in mM. ● represents the results calculated from the data shown in Fig. 2. ■ represents the result of a special experiment with cells not preloaded with Ca (*see* Results). ▲ represents g_K(Ca) values calculated from ^{42}K fluxes determined in experiments where the membrane potential was almost equal to the Nernst equilibrium potential of K ions. This condition was obtained by maximal DIDS inhibition of the Cl conductance (25 μM DIDS) (*see* section on Calculations)

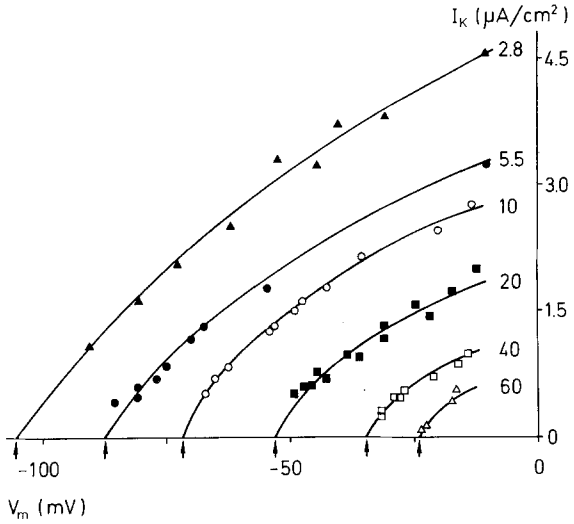

Fig. 4. Net current of K^+ (I_K) in μA/cm² as function of membrane potential (V_m) in series of experiments at different concentrations of extracellular K^+ (the numbers to the right of the curves) at a fixed value of cellular potassium of 145 mM. V_m was varied in the way described in the legend to Fig. 5

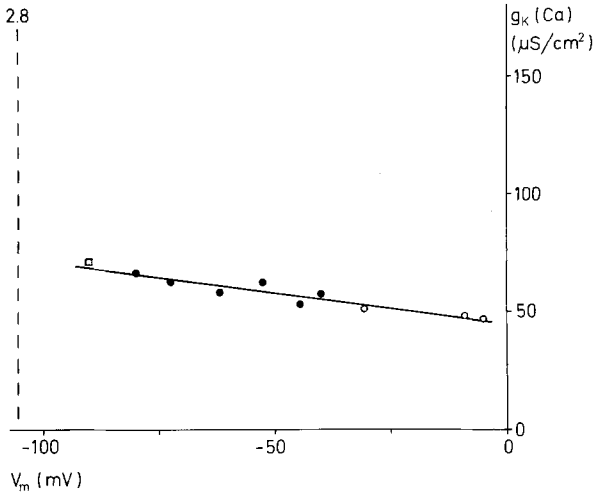

Fig. 5. Results from experiments where the conductance (g_K(Ca)) was determined at different membrane potentials (V_m). The concentration of extracellular potassium was 2.8 mM and the concentration of cellular potassium was 145 mM corresponding to a Nernst potential (E_K) of −105.4 mV. The vertical broken line indicates the K^+ equilibrium potential. Variation in V_m was obtained by means of DIDS-treatment (5 μM) (□), substitution of sucrose for extracellular NaCl (●) and substitution of sucrose for extracellular $NaNO_3$ in experiments where all Cl^- was replaced by NO_3^- (○)

of K^+ leads, however, to variations in E_K, which are directly reflected in a variation in V_m (Eq. 4). In series of experiments g_K(Ca) was therefore determined at different values of V_m at constant values of E_K.

Variations in the magnitude of V_m were achieved in two ways. In order to obtain values of V_m approaching E_K, g_{Cl} was decreased (*see* Eq. 4) by pretreatment of the cells with DIDS (0 to 25 μM) (Knauf et al., 1977). In this way g_{Cl} was varied from the normal value of ~25 μS/cm² (Bennekou, 1984) down to ~3 μS/cm². [Previously, it has been shown (Vestergaard-Bogind et al., 1985*a*) that there is no influence of DIDS upon the zero-current conductance of the K^+ channels.] To obtain V_m values close to zero mV, extracellular NaCl was replaced by sucrose, thereby shifting E_{Cl} from its normal value of −12 mV to initial values as high as +90 mV. In addition, all Cl ions were in some experiments replaced by NO_3 ions resulting in a higher anion-conductance (Vestergaard-Bogind et al., 1985*a*). Replacement of extracellular $NaNO_3$ by sucrose therefore had a more pronounced depolarizing effect than the corresponding replacement of NaCl.

In Fig. 4 the outward-going currents of K^+ (in μA per cm² of membrane) at various extracellular K^+ concentrations are plotted against the membrane potential. The intercepts with the abscissa axis of the extrapolated current curves, the E_K values, were the values calculated from the independently, directly determined concentrations of K^+ in the intra- and extracellular phases. The data of Fig. 4 have been used to calculate the K^+ conductances in Figs. 5 and 6. In Fig. 5 is shown the small, linear increase in g_K(Ca) with decreasing V_m found at an extracellular K^+ concentration of 2.8 mM. However, at increased extracellular K^+ concentrations quite a different picture was obtained.

In Fig. 6 g_K(Ca) at various extracellular K^+ concentrations are plotted versus the membrane potential, the zero-current conductances from Fig. 3 being represented by the dot-and-dash line. Since, with increasing extracellular K^+ concentrations, replacement of the remaining NaCl or $NaNO_3$ with sucrose resulted in Nernst equilibrium potentials of the anion which were less and less positive, then it became increasingly difficult to achieve V_m values close to 0 mV. All the same, it is obvious that with decreasing extracellular K^+ concentration the voltage range, within which a strong voltage dependence of g_K(Ca) exists, is displaced towards increasingly negative membrane potentials. In Fig. 7 g_K(Ca) as a function of V_m at an extracellular K^+ concentration of 20 mM is shown separately. For explanation of the broken line *see* Discussion.

Discussion

g_K(Ca) as a Function of $[K^+]_{ex}$

The relationship between g_K(Ca) of the human red cell membrane and the extracellular K^+ concentra-

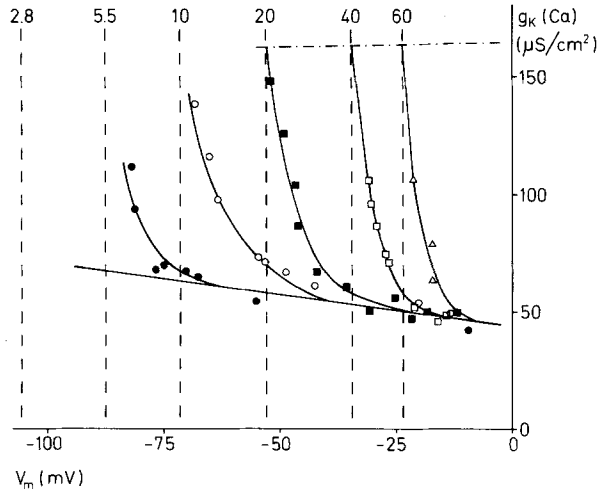

Fig. 6. Conductances (g_K(Ca)) calculated from the data shown in Fig. 4 are depicted as functions of membrane potential (V_m). The vertical broken lines indicate the K^+ equilibrium potentials calculated at the different extracellular potassium concentrations (the numbers on top of the broken lines). The dot-and-dash line represents the zero-current conductances from Fig. 3. The line representing the results at 2.8 mM of extracellular potassium (detailed in Fig. 5) is shown without the experimental points

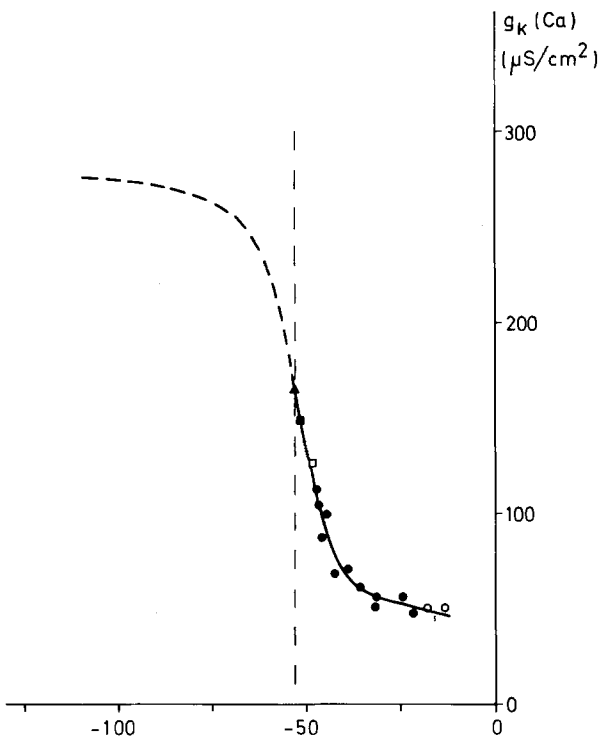

Fig. 7. Conductance (g_K(Ca)) as a function of the membrane potential (V_m) from two different experimental series, at an extracellular K^+ concentration of 20 mM. V_m was varied in the way described in the legend to Fig. 5. The zero-current conductance (extrapolated from Fig. 3) is marked by (▲). ■ represents the result of an experiment where g_K(Ca) was obtained from a measured net efflux and a calculated ($V_m - E_K$) value (*see* Results). The vertical broken line indicates the K^+ equilibrium potential. The other symbols are similar to the ones in Fig. 5. The broken line indicates the suggested shape of the g_K(Ca) curve at inward-going currents (*see* Discussion)

tion has not been dealt with previously, whereas in a number of studies the rate constants of net and unidirectional effluxes have been examined as a function of this parameter.

In Fig. 2 we have plotted the determined net effluxes and the corresponding V_m and E_K values versus the extracellular K^+ concentration in order to compare our data to the studies mentioned above. The result, a monotonically decreasing net efflux with increasing $[K^+]_{ex}$ does not conform to the results reported by Knauf et al. (1975), Hoffman et al. (1980) and Yingst and Hoffman (1984). They found that at pH values between 7 and 8 the rate constants of Ca^{2+}-activated net effluxes from depleted red cells or resealed red cell ghosts increased sharply as the extracellular K^+ concentration increased from zero to 2 to 3 mM and then decreased slightly with a further increase in $[K^+]_{ex}$. At pH 6.15 the maximum was displaced towards a $[K^+]_{ex}$ of ~30 mM (Hoffman et al., 1980).

We are at present unable to explain the discrepancy between our results and those cited above. It should thus be noted that a similar relationship between net efflux and $[K^+]_{ex}$ as that presented in Fig. 2 was found in experiments without CCCP.

Heinz and Passow (1980) have reported an almost total block of net efflux of K^+ from ghosts preexposed to a K^+-free salt solution. As seen from Fig. 3 such a block was not observed with intact cells with a normal intracellular K^+ concentration but suspended in a K^+-free salt solution. Under these conditions fluxes corresponding to g_K(Ca) values of ~50 μS/cm² were found.

In Fig. 3 the g_K(Ca) values at large outward-going currents (same experiments as in Fig. 2) and at zero current are shown as functions of the extracellular K^+ concentration. In abstract form, we have previously reported on g_K(Ca) as a function of $[K^+]_{ex}$ (Vestergaard-Bogind, Stampe & Christophersen, 1985*b*). At a cellular concentration of ionized Ca of ~12 μM, g_K(Ca) was determined in net efflux experiments where $[K^+]_{ex}$ varied between 1 and 30 mM, and zero-current conductance was determined at 90 mM extracellular K^+. g_K(Ca) was found to be a saturating function of $[K^+]_{ex}$. As will appear from the following, this reproducible, good fit (*not shown*) to a saturating function is fortuitous in the sense that it is a combined function of $[K^+]_{ex}$, E_K, V_m and correspondingly ($V_m - E_K$).

Since at ($V_m - E_K$) values below about 20 mV a strong voltage dependence is turned on (*see later*), it is important to notice that, for all g_K(Ca) values in Fig. 3 obtained at large outward-going currents, the

magnitudes of the driving force ($V_m - E_K$) were higher than 20 mV only at extracellular K^+ concentrations below about 3 mM (*see* Fig. 2). The activation of $g_K(Ca)$ by increasing the K^+ concentration on the *trans* side from zero up to about 3 mM might therefore represent a specific K^+ activation of the conductance and not the above-mentioned strong voltage dependence. This assumption is supported by the finding that as ($V_m - E_K$) increases above about 20 mV the $g_K(Ca)$ values determined at various $[K^+]_{ex}$ values (5.5 to 60 mM) approach the conductance determined for $[K^+]_{ex} \simeq 2.8$ mM at the V_m in question (*see* Fig. 6). We shall return to this problem in the discussion of the voltage dependence. It is questionable whether this phenomenon can be related to the reported sharp drop in single-channel conductance (γ) determined at large inward currents, at an extracellular K^+ concentration somewhere below 10 mM (Grygorczyk & Schwarz, 1983). It might well be important that in the patch-clamp experiments γ was determined as a function of the *cis*-side K^+ concentration whereas in the present experiments $g_K(Ca)$ was determined as a function of the *trans*-side K^+ concentration. Work is however in progress to characterize further the specific effect of $[K^+]_{ex}$ on $g_K(Ca)$.

As mentioned, the zero-current conductances were calculated from unidirectional fluxes at the reversal potentials using Eq. (2) (Hodgkin & Keynes, 1955), inserting the previously reported value of the flux ratio exponent of 2.7 (Vestergaard-Bogind et al., 1985*a*). As seen from Fig. 3 this conductance was perhaps slightly dependent on $[K^+]_{ex}$. In their patch-clamp studies Grygorczyk and Schwarz (1983) found that the zero-current conductance stayed almost constant at a γ value of about 20 pS as the extracellular K^+ concentration was varied between 10 and 140 mM. Thus, it is probable that the insignificant decrease in zero-current $g_K(Ca)$ with decreasing extracellular K^+ concentration (156 to 30 mM) reflects that, besides the single-channel conductance, the open-state probability p also is independent of $[K^+]_{ex}$, when $(V_m - E_K) \simeq 0$.

$g_K(Ca)$ as a Function of V_m

The results represented in Figs. 4 and 6 indicate that for outward-going currents the conductance becomes strongly voltage dependent as the membrane potential approaches the value of E_K. Alternatively formulated, the conductance is voltage dependent and with increasing extracellular K^+ concentration (*trans* concentration) the voltage dependence shifts towards less negative V_m values.

Unfortunately, it was impossible with suspended red cells and the present technique to determine inward currents at V_m values more negative than E_K, whereas zero-current values of $g_K(Ca)$ could be obtained from the unidirectional fluxes determined at zero net flux. Accordingly, the probable saturation value of $g_K(Ca)$ could not be determined. Neither has it been possible to determine outward currents at positive V_m values.

Taking into account the limited voltage range within which the strong increase in conductance could be followed it seems important first to analyze the reliability of the steep sections of the conductance curves. The net effluxes were determined with a small error (*see* discussion on errors in Materials and Methods). The problem here would be whether the determined net effluxes concealed an underlying small electro-silent net efflux, which resulted in an apparently high conductive net efflux of K ions at low ($V_m - E_K$) values. Since the cellular Na^+ content stayed constant during the experiments, KCl cotransport is the only likely candidate. However, in the experiments with critically low net effluxes the driving force of a possible KCl cotransport is at a minimum compared to the experiments with sucrose replacement of extracellular NaCl or the experiments at lower extracellular K^+ concentrations. Especially in the last-mentioned experiments the calculated outward currents from DIDS-treated cells should rise very steeply from the zero value at $V_m = E_K$ (Fig. 4), since the driving force of a possible KCl cotransport should be at its maximum, and since KCl cotransport is not inhibited by DIDS (Hoffmann, Sjøholm, & Simonsen, 1983). Furthermore, in experiments where all chloride was replaced by nitrate ions, the $g_K(Ca)$ values were of the expected magnitudes, yet cotransport of K^+ takes place only with Cl^- as the co-ion (Kramhøft, et al., 1986). Accordingly, we don't believe that quantitatively significant KCl cotransport takes place.

As V_m approaches E_K the uncertainty of the calculated $g_K(Ca)$ increases sharply with the decreasing ($V_m - E_K$). As discussed in Materials and Methods we believe that ($V_m - E_K$) values ≥ 3 mV are reasonably reliable. This assumption is supported by the fit of the independently determined E_K values to the net flux curves in Fig. 4 and the relatively good fit of the zero-current $g_K(Ca)$ values (which are determined on an experimentally and theoretically different basis) to the conductance curves in Fig. 6.

In Fig. 5 the linear V_m dependence of $g_K(Ca)$ found at a fixed extracellular K^+ concentration of 2.8 mM is shown. The slope corresponds to a weak voltage dependence of 0.25 $\mu S/(cm^2 \cdot mV)$. The conductances found at different $[K^+]_{ex}$ values at $(V_m - E_K) \geq 20$ mV (*see* Fig. 6) seem to fit the linear function represented in Fig. 5, and this function has

accordingly been used as the base line in Fig. 6. It shall be referred to as the basic conductance.

As mentioned above, these facts support the idea that at a concentration of about 3 mM the conductance is maximally activated by extracellular K^+. At ($V_m - E_K$) values higher than about 20 mV, where the voltage dependence of the gating process is negligible, the $g_K(Ca)$ values therefore all become identical to the basic conductance at the V_m in question. Thus, increasing $[K^+]_{ex}$ from 3 to, for example, 30 mM does not in itself lead to an increase in $g_K(Ca)$, but with increasing $[K^+]_{ex}$ the ($V_m - E_K$) values will decrease and voltage activation of $g_K(Ca)$ will subsequently become more and more pronounced. The result represented in Fig. 5 illustrates the opposite situation, namely that at an extracellular K^+ concentration of 2.8 mM it is impossible, even with high DIDS concentrations, to achieve ($V_m - E_K$) values below the critical ~20 mV.

In patch-clamp studies, at 5 μM ionized Ca and probably 140 mM K^+ symmetrically, the open-state probability increased by about 60% as V_m was changed from zero to −100 mV (Grygorczyk & Schwarz, 1983). The corresponding increase in $g_K(Ca)$ at ~27 μM ionized Ca and cellular and extracellular K^+ concentrations of 145 and 2.8 mM, respectively (Fig. 5), was about 55% and might thus reflect an increase in the open-state probability when ($V_m - E_K$) is larger than 20 mV.

In Fig. 7 an extrapolation of $g_K(Ca)$ at negative ($V_m - E_K$) values is shown. Within the range of extracellular K concentration of ~20 to 140 mM Grygorczyk and Schwarz (1983) found an almost constant ratio of about 1.7 between the single-channel conductances at large inward-going currents and at zero current. This ratio has been applied to our $g_K(Ca)$ values so that the saturating $g_K(Ca)$ values at large inward-going currents has been taken as 1.7 times the zero-current $g_K(Ca)$ values. One obvious objection to this procedure is the aforementioned fact that in the experiments of Grygorczyk and Schwarz (1983) the variation in K^+ concentration took place on the *cis* side whereas in the present experiments the *trans*-side K^+ concentration was varied. Another objection is that correction for the open-state probability as a function of voltage alone at large inward currents has not been included. There may be other objections, but semiquantitatively the function seems reasonable. It should be noted that, estimated in this way, the ratio between $g_K(Ca)$ at large outward net currents and at zero current becomes larger than the corresponding ratio for large inward currents and zero-current conductances.

A ($V_m - E_K$) dependence of the same type as that outlined in Fig. 7 has been reported by Hagiwara, Miyazaki and Rosenthal (1976) for the inward rectifier of the *Mediaster* egg membrane. If the independence of $[K^+]_{ex}$ of the zero-current conductance $g_K(Ca)$ can be taken as a reflection of a corresponding $[K^+]_{ex}$ independence of the zero-current single-channel conductance γ, it seems reasonable to assume that the marked voltage dependence (*cf.* Figs. 6 and 7) reflects a voltage dependence around the E_K value of the gating process only. Thus the results represented in Fig. 6 support the concept that inward rectifiers open with steep voltage dependence on hyperpolarization, the voltage dependence of their gating depending on the extracellular K^+ concentration, shifting along the voltage axis with the quantity $R \cdot T \cdot \ln[K^+]_{ex}$ (Hille, 1984). It is important to notice, however, that in the case of the human red cell K^+ channel we deal with a Ca^{2+}-activated inward rectifier which in addition to the voltage-gated conductance, possesses a Ca^{2+}-activated basic conductance. Furthermore, Ca^{2+}-activated K^+ channels in general are voltage dependent in the opposite direction: They open at depolarization and close at hyperpolarization (Latorre & Miller, 1983; Hille, 1984).

Conclusion

The K^+ channel of the human red cell membrane seems to combine several central characteristics of K^+ channels in a unique way. It is a Ca^{2+}-activated ion channel and at the same time an inward rectifier. In a flux ratio analysis this inward rectifier shows single filing with a flux ratio exponent of 2.7 independent of $[K^+]_{ex}$. This is similar with the delayed rectifier of squid axons but is in contrast to the inward rectifier of striated muscle membrane where the flux ratio exponent increases from 1 to 2 as the extracellular K^+ concentration increases.

The voltage dependence resembles that of an inward rectifier and is opposite to that of Ca^{2+}-activated K^+ channels in general. Our results indicate that, like the inward rectifier of a starfish egg membrane (Hagiwara & Takahashi, 1974), a strong voltage-dependent change in conductance around the zero-current value takes place within a relatively narrow range of about ± 20 mV around E_K. However, in contrast to the starfish egg cell inward rectifier the maximally Ca^{2+}-activated rectifier of the red cell membrane shows in addition a substantial basic conductance which is independent of $[K^+]_{ex}$ from 60 mM and down to at least 5.5 mM. This basic conductance is only weakly dependent on membrane potential (0 to −100 mV).

Finally, since no disagreement exist between our results and those obtained with the patch-clamp technique it should be possible to calculate the average number of open channels per cell. With a zero-

current $g_K(Ca)$, at saturating level of cellular ionized Ca of ~27 μM, of about 165 $\mu S/cm^2$ and a zero-current γ of about 20 pS (Grygorczyk & Schwarz, 1983) this number is about 15.

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