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Ca^{2+}-activated K^+ permeability in human erythrocytes: Modulation of single-channel events

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Abstract. Elevated levels of intracellular Ca^{2+} activate a K^+-selective permeability in the membrane of human erythrocytes. Currents through single channels were analysed in excised inside-out membrane patches. The effects of several ions that are known to inhibit K^+ fluxes are described with respect to the single-channel events. The results suggest that the blocking ions can partly move into the channels (but cannot penetrate) and interact with other ions inside the pore. The reduction of single-channel conductance by Cs^+, tetraethylammonium and Ba^{2+} and of single-channel activity by quinine and Ba^{2+} is referred to different rates of access to the channel. The concentration- and voltage-dependent inhibition by ions with measurable permeability (Na^+ and Rb^+) can be explained by their lower permeability, with single-file movement and ionic interactions inside the pore.

Key words: Red cell, K^+ channel, Ca^{2+} dependence, single-channel current

Introduction

Increasing the activity of intracellular Ca^{2+} in human erythrocytes produces a marked increase in membrane permeability (Gardos 1958) with a high selectivity for K^+ over Na^+ (Passow 1963). Subsequently, such Ca^{2+}-activated and K^+-selective channels have been discovered in nearly all cell membranes (for reviews see e.g. Schwarz and Passow 1983; Sarkadi and Gardos 1984), but the human erythrocyte is still the preparation where this system has been studied in most detail. The information available is nearly exclusively based on measurements of tracer fluxes, but the development of the improved patch-clamp technique (Hamill et al. 1981) and its recent application to erythrocytes (Hamill 1983; Grygorczyk and Schwarz 1983) now allows electrophysiological measurements on this system. It could be demonstrated by this technique that the Ca^{2+}-activated K^+ permeability is mediated by channels that exhibit inward rectification for the single-channel conductance of up to 40 pS (Grygorczyk and Schwarz 1983), and this even with identical solutions on both sides of the membrane. Replacement of K^+ by Na^+ reduces the single-channel conductance, and also affects the reversal potential indicating that, in contrast to flux measurements on cells in suspension, under patch-clamp conditions the channels exhibit low but measurable permeability for Na^+ with a selectivity ratio $P_{Na}:P_K$ of about 1:15; the selectivity obtained by double tracer experiments (unpublished data by H. Passow, see also Grygorczyk et al. 1984) is higher by an order of magnitude. Though we have no definite explanation for this discrepancy, the measured fluxes and currents pertain to the same channel; this could be demonstrated by several features of the Ca^{2+}-activated K^+ permeability of cells in suspension that were also observed in membrane patches (Grygorczyk and Schwarz 1983; Grygorczyk et al. 1984).

This paper reports on further common properties. The effects of several factors that are known to modulate the permeability of red cells in suspension will be shown to modulate single-channel events recorded from excised membrane patches. These factors alter the single-channel activity or inhibit the single-channel conductance. The results also demonstrate that the Ca^{2+}-activated K^+ channels in human erythrocytes have characteristic features in common with K^+-selective channels in other types of cells. Part of these results have been briefly reported previously (Grygorczyk and Schwarz 1984).

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Materials and methods

Human red cells were obtained from Rh^+0 blood of healthy donors and washed two to three times in isotonic KCl solution buffered to pH 7.6 by morpholinopropane sulfonic acid (MOPS).

Solutions

Our standard solution contained 150 mmol/l KCl, 1 mmol/l $MgCl_2$, 10 μmol/l $CaCl_2$, and was adjusted to pH 7.4 to 7.6 by 10 mmol/l MOPS buffer. Concentrations of free Ca^{2+} below 5 μmol/l were usually established by buffering with 80 μmol/l ethyleneglycol-bis[β-amino-ethylether]N,N-tetracetic acid (see Barrett et al. 1982). If desired, modifying agents were added or K^+ ions were partially replaced as indicated in the results section and in the figure legends. Solutions in the pipette were prepared without $CaCl_2$.

Patch-clamp recordings

The giga-seal patch-clamp technique (Hamill et al. 1981) was applied to record single-channel currents primarily in cell-free, inside-out membrane patches. Giga-ohm seals of 10–50 GΩ were formed with heat-polished pipettes (20 MΩ) made from borosilicate glass. After giga-seal formation excised membrane patches were obtained by either touching the bottom of the chamber with the cell attached to the pipette or the tip of another pipette connected to a vibrating piezo crystal.

The gating of the Ca^{2+}-activated K^+ channels does not exhibit potential-dependent inhibition, therefore currents could be recorded continuously during longer lasting holding potentials. The data were stored on analog magnetic tape, and were later transferred to a brush recorder (Gould) for evaluation by hand, or records were digitized at a sampling rate of 1 or 2 kHz, transferred to floppy diskettes and analysed by means of an LSI 11/23 computer (Digital Equipment; see also Nagy et al. 1983). If closed time histograms were constructed, only records with single openings were analysed. Open-time histograms were occasionally constructed from records with more than one active channel. In this case single-channel events were treated as if the channel that opens first also closes first which yields the same result as if we assumed that the channel that opens first closes last. In addition, dwell times longer than 60 ms were not considered for histograms, while for the determination of the probability of a channel being open the longer dwell times were also considered. The distribution of dwell times in histograms shows a large variability from experiment to experiment; but qualitative conclusions can be drawn by comparison of open and closed times obtained from the same membrane patch.

Results

Since the Ca^{2+}-activated K^+ channels exhibit inward rectification, outward currents do not usually exceed 1 pA even in solutions containing high K^+ on both sides of the membrane. Therefore, inhibitory effects on the single-channel conductance were usually studied on inward currents. Outward currents were occasionally analysed, but only if the sidedness is to be demonstrated or if instead of the conductance the single-channel activity is modulated.

Modulation of single-channel activity

Increasing the intracellular activity of Ca^{2+} in the micromolar range up to 10 μmol/l produces an increase in the K^+ permeability of human red cells. This is achieved by an increase in the probability of a channel being open (p) rather than by an increase in the single-channel conductance (Fig. 1 A, see also Grygorczyk and Schwarz 1983). Elevated values of p may be referred to increased dwell times at the conducting level or to reduced dwell times at the non-conducting level. Figures 1 B and 1 C show, for three different Ca^{2+} activities, histograms of the open and closed times of single channels. While the open-time distribution (Fig. 1 B) can be fitted by a single exponential:

$$N_o = P_o \cdot \exp(-t/\tau_o) \qquad (1\,a)$$

the closed-time distribution (Fig. 1 C) must be described by the sum of at least two exponentials:

$$N_c = P_{c1} \cdot \exp(-t/\tau_{c1}) + P_{c2} \cdot \exp(-t/\tau_{c2})\,. \qquad (1\,b)$$

While the open time constant (corresponding to the rate of channel closing) increases with increasing Ca^{2+} activity, the two exponentials describing the closed-time distribution do not significantly depend on the Ca^{2+} activity (see Fig. 1 B, C and Table 1).

Table 1. Fit parameters of Eq. (1) for the histograms of Fig. 1 B and C (± SEM)

Ca^{2+} (μmol/l)	2	5	10
P_o	91 ± 2	92 ± 2	66 ± 3
τ_o (ms)	5.6 ± 0.2	7.2 ± 0.3	8.5 ± 0.5
P_{c1}	100 ± 5	131 ± 6	152 ± 8
τ_{c1} (ms)	0.85 ± 0.07	0.72 ± 0.05	0.76 ± 0.06
P_{c2}	5.8 ± 1.4	5.6 ± 1.1	10.3 ± 1.9
τ_{c2} (ms)	13.0 ± 3.4	15.6 ± 3.7	13.0 ± 2.7

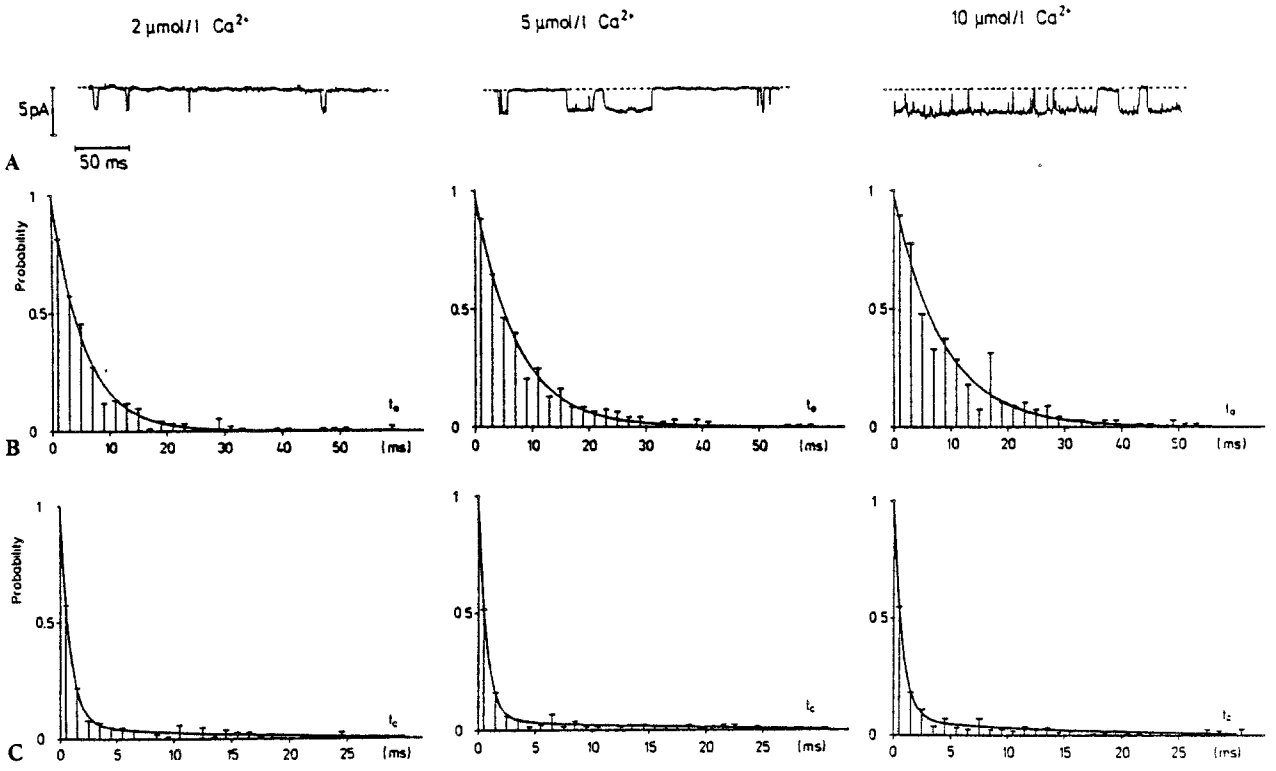


Fig. 1A–C. Ca^{2+} dependence of the gating of single K^+ channels in excised membrane patches. Both sides of the membrane were in contact with 150 mmol/l K^+ and the membrane potential was set to −100 mV. The activity of Ca^{2+} at the internal membrane surface is given above each panel. (**A**) Records of single-channel inward currents (downward deflections). (**B**) Histograms of the probability of dwell times of the open state. (**C**) Histograms of the probability of dwell times of the closed state. The curves in **B** and **C** represent least-squares fits of Eqs. (1 a) and (1 b), respectively, to the data; fit parameters are given in Table 1 (RC 137)

Even at a fixed activity of Ca^{2+} in the solution in contact with the internal membrane surface the activity of single-channel events can be modulated by different factors. Figure 2 demonstrates that internal Mg^{2+} alters the susceptibility of the K^+ channels for Ca^{2+}. The higher the level of internal Mg^{2+} the higher are the activities of Ca^{2+} that are necessary to produce the same single-channel activity expressed as the probability of a channel being open.

K^+ channels of many cells are blocked by quinine (inset Fig. 3A) or quinidine (for reference see e.g. Hermann and Gorman 1984). Figure 3 demonstrates that the single-channel activity in human erythrocytes is inhibited by quinine without affecting the single-channel conductance; 200 µmol/l of internal quinine reduce the probability of a channel being open at −100 mV by more than 50% (Fig. 3A) without significant effect on the amplitude of the single-channel currents (Fig. 3B). The reduction of the p value by quinine is primarily due to increased dwell times of the non-conducting states while the average dwell time of the conducting state does not change significantly. The degree of inhibition depends on membrane potential and trans concentration of K^+. Outward current (at +50 mV) is more potently blocked by internal quinine than inward current (at −100 mV) (see Fig. 3A); on the other hand inward current is more potently blocked if quinine is applied externally (not shown). Increasing the trans concentration of K^+ obviously counteracts the inhibitory effect by quinine (compare the two curves at +50 mV in Fig. 3A).

Modulation of single-channel conductance

Impermeable small cations inhibit K^+ currents in a characteristic concentration and voltage-dependent manner as if the blocking ion acts by moving part of the way across the channel, and then forming a blocking complex inside the channel. This inhibition can frequently be described by a Boltzmann distribution for the ratio of inhibited (R) to uninhibited ($1-R$) current

$$R/(1-R) = ([B^{z+}]/K_B) \cdot \exp(-z^* EF/RT), \qquad (1)$$

where $[B^{z+}]$ is the activity of the blocking ion, K_B the dissociation constant of the blocking complex,

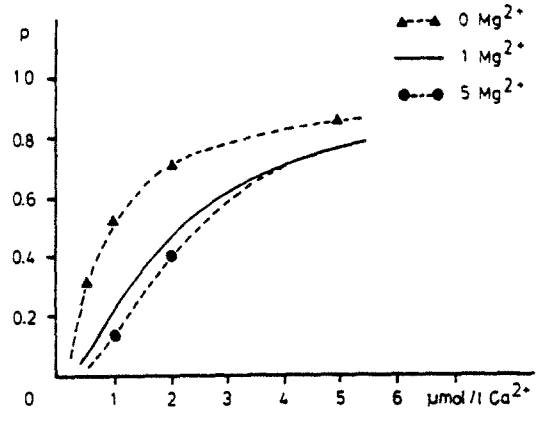


Fig. 2. Dependence of the probability, p, of a channel being open on the internal Ca^{2+} activity at three different internal Mg^{2+} concentrations. The solid line (1 mmol/l Mg^{2+}) is an approximation of data published previously (see Grygorczyk et al. 1984, Fig. 4). Both sides of the membrane were in contact with 150 mmol/l K^+ and the membrane potential was set to −100 mV (RC 206, 226)

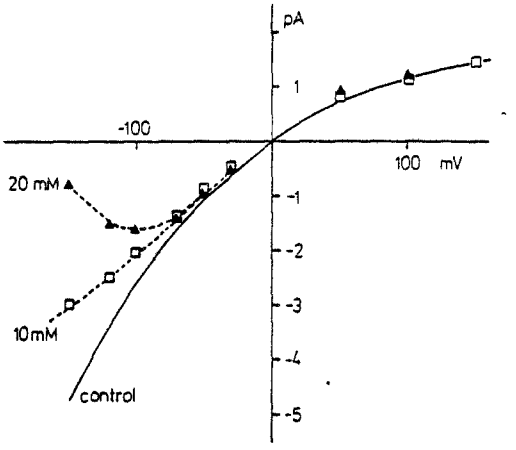
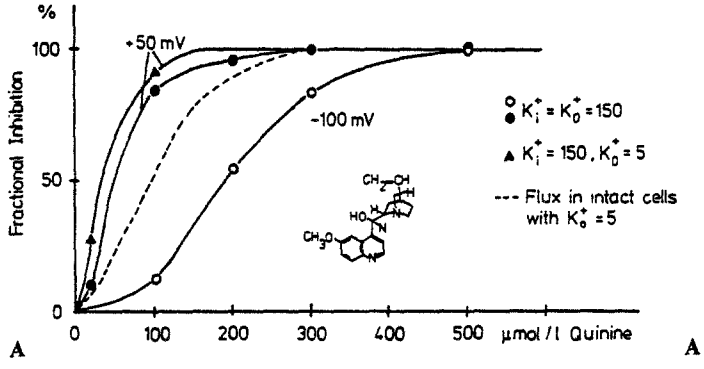


Fig. 4. Voltage dependence of single-channel currents at two different concentrations of Cs^+ (10 and 20 mmol/l) added to the standard pipette solution. The control curve is the same as published previously (Grygorczyk et al. 1984, Fig. 3)



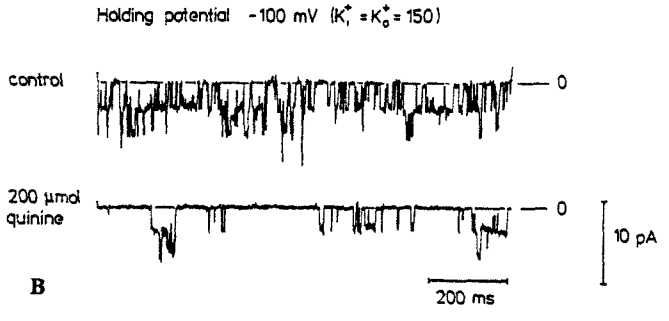
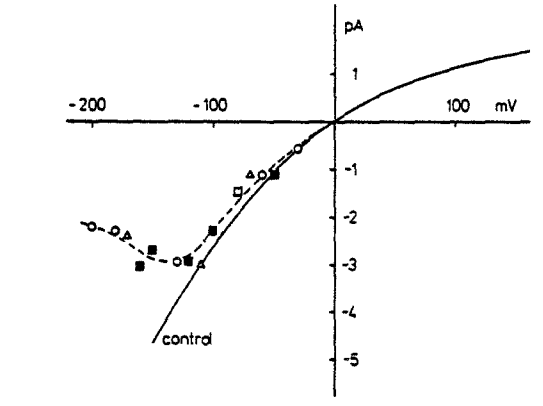


Fig. 3 A and B. Inhibition of single-channel activity by internal quinine (inset). (**A**) Dependence of the degree of inhibition on quinine concentration at membrane potentials of +50 and −100 mV. The K^+ concentration on both sides of the membrane were as indicated in the figure; for 5 mmol/l K_0^+, K^+ was partially replaced by Na^+. The dotted line was redrawn from flux data published by Reichstein and Rothstein (1981). (**B**) Single-channel inward currents (downward deflections) without and with 200 μmol/l internal quinine (RC 220)



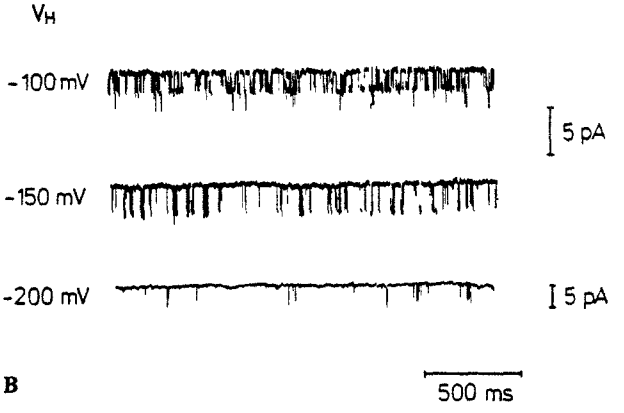


Fig. 5 A and B. Voltage dependence of single-channel events with 0.5 mmol/l $BaCl_2$ added to the standard solutions on both sides of the membrane. (**A**) Current-voltage data from three different experiments. The control curve is the same as in Fig. 4. (**B**) Single-channel inward currents (downward deflections) at three different holding potentials (RC 225)

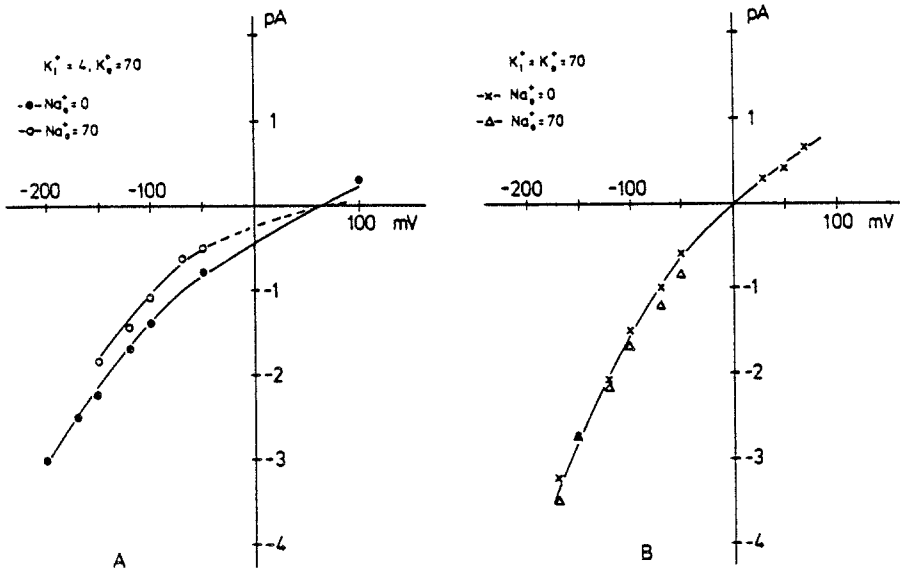


Fig. 6 A and B. Current-voltage curves of single-channel currents at different Na^+-K^+ compositions. For data with 0 Na^+, Na^+ was replaced by choline. (**A**) 4 mmol/l internal K^+ and (**B**) 70 mmol/l internal K^+

and the effective charge z^* is the product of the valence z of an external blocking ion and the fraction of the membrane potential acting on the ion while it is moving into the channel. The blocking ion studied in most detail is Cs^+ (for ref. see e.g. Latorre and Miller 1983). Our patch-clamp experiments on human erythrocytes reveal the same characteristics as found for K^+ channels in other cell membranes. Figure 4 shows the effect of two different external Cs^+ concentrations on the current-voltage relation of single Ca^{2+}-activated K^+ channels in the erythrocyte. The characteristic voltage- and concentration-dependent block of inward current by external Cs^+ is qualitatively as expected from the above equation; for higher Cs^+ concentrations and for more negative potentials that increase the driving force for inward current the degree of inhibition increases. Outward current (at positive membrane potential) seems to be almost unaffected by the external Cs^+.

Concentration- and voltage-dependent block of inward K^+ current is also observed with external Ba^{2+}. However, in contrast to Cs^+ not only is the single-channel current reduced (Fig. 5A) but the single-channel activity is also inhibited. While under control conditions the single-channel activity increases with more negative potentials (Grygorczyk and Schwarz 1983), in the presence of Ba^{2+} the single-channel activity decreases with more negative potentials (see Fig. 5B); this can be attributed to altered dwell times of both conducting and non-conducting states. More detailed analysis of dwell time distributions showed that under control conditions τ_{c1} is nearly constant (about 0.8 ms) between -150 and $+70$ mV; application of Ba^{2+} increases τ_{c1} at -100 mV to 2.4 ms and at -150 mV to 5.7 ms. In addition, in the presence of Ba^{2+}, the mean open time, τ_o, decreases from 4.5 ms at -100 mV to 1.6 ms at -150 mV.

Another typical inhibitor of K^+ currents is tetraethylammonium (TEA) that usually blocks inward currents from the outside and vice versa. Like Cs^+, the single-channel conductance is reduced without significant effect on the kinetics of opening and closing. In an experiment with an outside-out patch, application of 10 mmol/l TEA to the bath solution reduced the single-channel current at 70 mV by about 60%.

Also Na^+, which has a low permeability for the K^+ channel apparently reduces the single-channel conductance for inward current if applied to the solution in contact with the external membrane surface. Figure 6A demonstrates that replacement of 70 mmol/l external choline by Na^+ reduces the inward current through single channels. This inhibition is counteracted by elevation of the trans K^+ concentration (compare with Fig. 6B).

For flux measurements Rb^+ is often used as a tracer. Replacement of all K^+ by Rb^+ in the pipette solution reduces the single-channel conductance for inward current to about 50% (see Fig. 7). In addition, the larger the driving force for inward current the larger is the reduction of inward current compared to pure K^+-containing solution in the pipette leading to a slight voltage-dependent inhibition. Though this suggests that Rb^+ is much less permeable than K^+, only small effects on the reversal potential can be detected. For the permeability ratio calculated from a possible shift in the reversal potential of less than 7 mV (see Fig. 7) a lower limit of $P_{Rb}:P_K$ can be estimated at only 1 : 1.3.

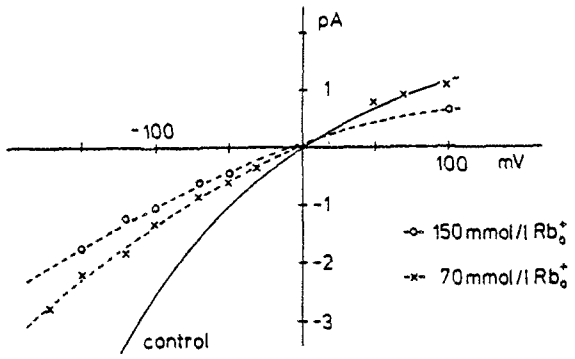


Fig. 7. Current-voltage curves of single-channel currents at different external Rb^+ concentrations in the pipette solution with K^+ replaced by Rb^+. The control curve is the same as in Fig. 4

Discussion

Compared to flux measurements in cell suspensions patch-clamp experiments on human red cells have the advantage that the Ca^{2+}-activated K^+ channel can be investigated independently of other transport systems that may contribute to the measured fluxes or influence the K^+ fluxes; these therefore, have to be deducted from the measured values (see Yingst and Hoffman 1984). The possibility of working with excised membrane patches of human erythrocytes also allows one to determine quite easily the sidedness of the action of inhibitors. In the following, we would like to demonstrate that comparison of the results obtained from single-channel measurements with those derived from flux measurements may help to provide a more detailed picture of the activation and inhibition of K^+ channels and of ion permeation in the red cells. We also intend to show that the Ca^{2+}-activated K^+ channel in human red cells is in many respects similar to K^+ channels in other cell membranes.

Modulation of channel gating by Ca^{2+}

Gating of the K^+ permeability in the human red cells is only possible if Ca^{2+} has bound to an intracellular receptor. Since the distribution of the closed times (related to the rate of channel opening) has to be described by two exponentials at least two different closed states have to be assumed. Taking into account closed states lasting longer than 60 ms, calculation of the mean value of dwell times yields decreasing τ_{mc} values with increasing Ca^{2+} (see Grygorczyk and Schwarz 1983) as expected from a simple activation model where the Ca^{2+} has to bind to a receptor to open a channel. The steepness of the concentration dependence of single-channel activity and also of the rate of K^+ fluxes (see Grygorczyk et al. 1984) suggests that two Ca^{2+} ions have to bind for channel activation. Interestingly, the time constants of the two exponentials are not significantly affected by increasing Ca^{2+}, and the reduced mean dwell time of the closed state at higher Ca^{2+} activities is primarily due to a reduced dwell time of a long lasting ($t_c > 60$ ms) closed state. This is similar to observations by Magleby and Pallotta (1983) on Ca^{2+}-activated K^+ channels in cultured rat muscle. The higher probability of the open channel state is also partly due to an increase of the mean dwell time of the open state, τ_{mo}. Dependence of τ_{mo} on Ca^{2+} was considered by Methfessel and Boheim (1982) as evidence for the existence of charged gating particles and that compensation of the charges by Ca^{2+} is involved in keeping open Ca^{2+}-activated K^+ channels. This suggests an additional open state (see also Latorre et al. 1983) though in the time distribution of the open state only one component has been resolved; but because of limited time resolution we cannot rule out an additional open state with mean dwell times less than 0.5 ms. Such a short-living and Ca^{2+}-independent state was described for the Ca^{2+}-activated channel in rat muscle (Magleby and Pallotta 1983).

The inhibitory effect of internal Mg^{2+} on single-channel activity is similar to results obtained from flux measurements (Simons 1976; Garcia-Sancho et al. 1982), and supports the view that Mg^{2+} may compete with Ca^{2+} for the internal binding site but without activating the channel.

Selectivity of the K^+ channel

The Ca^{2+}-activated channel in human erythrocytes is highly selective for K^+. Like K^+ channels in other preparations (for review see e.g. Latorre and Miller 1983) this channel is impermeable for Cs^+, has a low permeability for Na^+ but a high permeability for Rb^+. From measurements of the reversal potential a selectivity $P_K > P_{Rb} > P_{Na} \gg P_{Cs}$ is obtained with permeabilities relative to P_K of 1.0, 0.77, 0.06, ~0.0, respectively. Because of the high permeability of Rb^+ to nearly all K^+ channels (see e.g. Chandler and Meves 1965; Hille 1975; Coronado et al. 1980; Reuter and Stevens 1980; Gorman et al. 1982; Blatz and Magleby 1984) Rb^+ is often used as a tracer for K^+ fluxes. Also from flux measurements using $^{86}Rb^+$ and $^{42}K^+$ as tracer a permeability ratio of 0.7 has been determined under conditions of zero net ion movements (Grygorczyk et al. 1984). But experiments with Rb^+ also demonstrate (see Fig. 7 and e.g. Clay and Shlesinger 1983; Yellen 1984) that Rb^+ blocks net K^+ movements. If Rb^+ is applied externally inward currents are reduced slightly more than outward currents; this means that the use of

Rb^+ as a tracer has to take into account this apparent voltage-dependent selectivity. The discrepancy of the selectivities calculated from the reversal potential and from the currents measured at a fixed driving force indicate deviations from the independence principle. Such voltage-dependent inhibition can be expected if Rb^+ and K^+ moved in single-file through the pore and interacted inside the pore (Hille and Schwarz 1978), a typical characteristic of K^+ channels in other preparations.

Na^+-K^+ interactions

If applied to the external membrane surface, Na^+ reduces inward current. Though the blocking effect is much less pronounced than in most other K^+ channels, qualitatively similar observations have been made with respect to Na^+-K^+ interactions (Chandler and Meves 1965; Bergman 1970; Bezanilla and Armstrong 1972; Marty 1983; Yellen 1984b). Elevation of the K^+ concentration not only increases the conductance (compare slopes of the I–V curves in Fig. 6A and B), but elevation of internal K^+ also counteracts the inhibitory effect of external Na^+ on the inward current. In other preparations these phenomena have been explained by interaction of the ions inside a single-file pore with multiple occupancy (e.g. Yellen 1984b). In human erythrocytes similar observations have been made for the effect of internal Na^+ and external K^+ on the rate of K^+ efflux (e.g. Riordan and Passow 1973; Yingst and Hoffman 1984) but have been explained by an allosteric interaction between an internal binding site for Na^+ and an external binding site for K^+; if internal Na^+ is bound, activation of the channel is inhibited, externally bound K^+ is necessary for activation and in addition reduces the inhibitory effect of the trans Na^+. At the moment we cannot distinguish whether Na^+ and K^+ do directly interact inside the channels or via an allosteric effect. Since in flux experiments the interaction has been observed only with internal but not with external Na^+ (Passow 1969) a combined mechanism seems most likely to us.

Inhibition by impermeable ions

Pronounced inhibition of K^+ currents can be observed with impermeable cations that are assumed to enter the channel, with a barrier inside the pore that can be passed by K^+ but not by the blocking ion. If a membrane potential is applied that drives inward current an externally added blocking ion may enter the channel and will be moved by the electric field into the channel until it reaches the barrier and blocks further inward movements of ions, but not outward movements. The channel only becomes permeable for inward current if the blocking ion jumps out again; but this is less probable the stronger the electric field leading to the characteristic voltage-dependent inhibition (e.g. Fig. 4). With respect to single-channel currents two extremes can be distinguished. If the rates of blocking and unblocking are too fast to be resolved by the electronics, an apparent reduction of the single-channel conductance will be the result as observed with Cs^+ (see also Schwarz et al. 1981; Yellen 1984a, b). If one calculated the effective charge of the Cs^+ ion (z^*) accordingly to Eq. (1), an apparent effective charge larger than one would be obtained for this monovalent ion; for example, for 20 mmol/l Cs^+ the maximum steepness of inhibition could be described by $z^* = 1.1$. This is strong evidence that at least two ions can simultaneously occupy the channel (see Hille and Schwarz 1978). If on the other hand, the rates are slow enough, additional closings may be observed while the channel is in the open state. This will result in altered distribution in the open and closed-time histograms. Such behaviour and voltage-dependent inhibition was observed with quinine (Fig. 3) which suggests that quinine like Cs^+ blocks by plugging the channel. The inhibitory effect of externally applied quinine on outward K movement in cell suspensions (Reichstein and Rothstein 1981) could, therefore, be explained if quinine penetrated the membrane in its neutral form and produced its effect from the internal membrane surface in its charged form (pK values of the amino groups, see inset Fig. 3A, are 5.1 and 9.7). Such a mechanism is also supported by the fact that the inhibition of efflux by external quinine (see dotted curve in Fig. 3A) has a similar concentration dependence as the inhibition of outward current by internal quinine and that the inhibition is also counteracted by external K^+. A similar proposal was made by Hermann and Gorman (1984) about the effect of externally applied quinidine on the Ca^{2+}-activated K^+ current in molluscan neurons. A blocking mechanism by plugging is also supported by the counteraction of trans K^+, similar to the observations on the Na^+-K^+ interaction.

Voltage-dependent inhibition is also observed with Ba^{2+}. For this ion fast and slow steps are obviously involved in the blocking reaction, since both the single-channel conductance and the mean open time are reduced (Fig. 5). This may be explained by a fast exchange of Ba^{2+} between the external solution and a first binding site inside the channel leading to the apparent voltage-dependent inhibition of the single-channel conductance, and by a slower second binding step further inside the channel that leads to the additionally observed fluctuations of single-channel current with reduced open

and increased closed times. A similar explanation was given for the two effects of Cs^+ on single-channel conductance and on current fluctuations in K^+ channels of skeletal muscle (Schwarz et al. 1981). From the steepness of the voltage-dependent reduction of the single-channel conductance an effective charge of the Ba^{2+} ion during the first fast binding step of about 2 can be determined.

Conclusion

The data presented give evidence that the Ca^{2+}-activated K^+ channel in human erythrocytes has several characteristics of K^+ channels in other cell membranes that can be described by a structure (see Latorre and Miller 1983; Yellen 1984a) of internal and external openings large enough to also allow large ions like quinine to enter the pore or to allow fast access of smaller blocking ions; but there is also a narrow part inside the pore that can be penetrated only in single-file with a high selectivity for K^+. Despite these similarities to other K^+-selective channels, there may be additional characteristics that are typical for the K^+ channel in erythrocytes, for example the sided effects of Na^+ and K^+ on the K^+ permeability.

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