Ca^{2+}-ACTIVATED K^+ CHANNELS IN HUMAN RED CELLS

Comparison of Single-Channel Currents With Ion Fluxes

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ABSTRACT Exposure of the inner surface of intact red cells or red cell ghosts to Ca^{2+} evokes unitary currents that can be measured in cell-attached and cell-free membrane patches. The currents are preferentially carried by K^+ (P_K/P_{Na} 17) and show rectification. Increasing the Ca^{2+} concentration from 0 to 5 μM increases the probability of the open state of the channels parallel to the change of K^+ permeability as observed in suspensions of red cell ghosts. Prolonged incubation of red cell ghosts in the absence of external K^+ prevents the Ca^{2+} from increasing K^+ permeability. Similarly, the probability to find Ca^{2+}-activated unitary currents in membrane patches is drastically reduced. These observations suggest that the Ca^{2+}-induced changes of K^+ permeability observed in red cell suspensions are causally related to the appearance of the unitary K^+ currents. Attempts to determine the number of K^+ channels per cell were made by comparing fluxes measured in suspensions of red cells with the unitary currents in membrane patches as determined under comparable ionic conditions. At 100 mM KCl in the external medium, where no net movements of K^+ occur, the time course of equilibration of $^{86}Rb^+$ does not follow a single exponential. This indicates a heterogeneity of the response to Ca^{2+} of the cells in the population. The data are compatible with the assumption that 25% of the cells respond with $P_k = 33.2 \times 10^{-14}$cm^3/s and 75% with $P_k = 3.1 \times 10^{-14}$cm^3/s. At 100 mM external K^+ the zero current permeability of a single channel is 6.1×10^{-14}cm^3/s (corresponding to a conductance of 22 pS). Using appropriate values for the probability of a channel in the open state, we estimated that 25% of the cells in the population contain 11–55, and 75% of the cells 1–5 channels per cell that are activated in the time average (20°C, pH 7.6).

INTRODUCTION

In nearly all cell membranes studied so far, intracellular Ca^{2+} induces a selective increase of K^+ permeability (for recent reviews see Meech, 1976, Schwarz and Passow, 1983, Sarkadi and Gárdos, 1984). In human red cells, Ca^{2+} entry leads to an increase in K^+ permeability usually called the Gárdos effect. Applying the patch-clamp technique to human red cell membranes, Hamill (1981) has discovered the occurrence of unitary currents that indicate the existence of aqueous pores with a single-channel conductance of 20 pS. He suggested that these channels are activated by Ca^{2+} and responsible for the Gárdos effect. In the present paper, we pursued this suggestion. We tried to establish whether or not the channels meet a number of the criteria that are characteristic for the Gárdos effect as studied by means of flux measurements in red-cell suspension. First, it should be demonstrated that the presence of Ca^{2+} is indeed a necessary requirement for channel activation (Gárdos, 1958). Second, the channels should exhibit a high selectivity for K^+ over Na^+ (Passow, 1961, 1963). Third, their susceptibility to activation by Ca^{2+} should be reduced or abolished by preincubation of the red cells in a K^+-free medium (Heinz and Passow, 1980). Our results demonstrate that the channels are activated by Ca^{2+}. The interpretation of the other data still raises some questions, but on the whole there remains little if any doubt that the activation of these channels is responsible for the Gárdos effect.

Attempts to determine the number of Ca^{2+}-activated channels per red cell have been made previously (Lew et al., 1982). They showed that for inside-out vesicles made from human red cells only a fraction of the vesicles responds to Ca^{2+} with an increase in K^+ permeability. Assuming that the number of channels per cell is smaller than the number of vesicles, the authors infer that there are ~100–200 channels per cell. In the present work we have compared Ca^{2+}-induced single-channel K^+ currents with Ca^{2+}-induced tracer fluxes, both measured under similar conditions. From the data we estimated the upper limit of activable channels per red cell to be ~55 at 20°C.

METHODS

Human red cells were obtained from Rh^+O blood of healthy donors by washing the erythrocytes two to three times in solution II (see below). Ghosts were prepared as described by Bodemann and Passow (1972) and loaded with 70 mM KCl, 70 mM choline chloride, and 20 mM Tris (tris[hydroxymethyl]aminomethane) buffer adjusted to pH 7.6. All experiments were performed at 20–22°C.

Solutions

Solution I contained 150 mM KCl, 1 mM $CaCl_2$, and solution II 100 mM KCl, 50 mM NaCl, 10 μM free Ca^{2+}. All solutions were adjusted to pH 7.4–7.6 by 10 mM MOPS (morpholinopropane sulfonic acid) buffer. The

concentrations of free Ca^{2+} were usually established by buffering with EGTA (ethyleneglycol-bis[β-aminoethylether]N,N-tetraacetic acid) (see Barrett et al., 1982). Solutions with different ratios of K^+/Na^+ were obtained by replacing equivalent amounts of KCl by NaCl.

Patch-Clamp Experiments

Single-channel currents of Ca^{2+}-activated K^+ channels were recorded from membrane patches of red cells or ghosts in cell-attached configuration as described by Hamill et al. (1981). Gigaohm seals of 5–100 GΩ were formed with heat-polished pipettes (30–50 MΩ) made from borosilicate glass. Pipette solutions were usually Ca^{2+} free, but contained 1 mM $MgCl_2$ and were diluted to ~90% of the osmolarity of the bath solution. An elevated level of intracellular Ca^{2+} was achieved by addition of the ionophore A23187 (final concentration 1 μM) to the external bath medium containing 10 μM free Ca^{2+}. Occasionally, during patch formation, the membrane becomes leaky for Ca^{2+}. Therefore, at Ca^{2+} concentrations exceeding 5 μM in the external solution, the intracellular concentration of Ca^{2+} can increase to levels that activate the K^+ channels even in the absence of the ionophore. Cell-free, inside-out membrane patches were obtained after gigaseal formation by touching with the attached cell the bottom of the chamber or by briefly exposing the cell to the flow of distilled water. These procedures destroy the attached cell but leave the patched membrane and the seal intact.

The current records were filtered at 2.5 or 1.1 kHz and were either stored on analogue magnetic tape, or in digitized form (sampling rate 4–4,000 Hz) on floppy diskette. The off-line analysis was performed either by hand or by means of an LSI 11/23 computer (Digital Equipment Corp., Marlboro, MA). To calculate the probability of a channel in the open state (p), records of 125-ms duration with only one conducting level were displayed, and only times of open and closed states longer than one millisecond were taken into account. Essentially similar results are obtained for p when all currents with amplitudes larger than 50% of the mean single-channel current are assumed to represent current flow through open channels. Mean amplitudes of unitary currents were estimated either by eye or taken from the maximum of an amplitude histogram (resolution 0.2 pA) after base-line subtraction.

Measurement of $^{86}Rb^+$ Fluxes in Red Cells

In contrast to many of the K^+ channels that cannot be activated by Ca^{2+}, Ca^{2+}-activated K^+ channels exhibit a high permeability for Rb^+ (see e.g., Latorre and Miller, 1983; Schwarz and Passow, 1983); in red cell ghosts a ratio of 1.5 was reported for P_{Rb}/P_K (Simons 1976). We were unable to confirm this result under our experimental conditions. In double labeling experiments using $^{86}Rb^+$ and $^{42}K^+$ we found that P_{Rb}/P_K is 0.77 at zero net movements. This value is independent of the relative concentrations of Rb^+ and K^+ in cells and medium in the range of 0–10 mM RbCl at 100 mM KCl. Similar results for the permeability ratio have been observed in other cells (see Latorre and Miller, 1983) and even in red cells (La Celle and Rothstein, 1966), although in the latter under conditions different from ours. To load the erythrocytes with $^{86}Rb^+$ the cells were incubated at a hematocrit of 10% in solution II containing $^{86}Rb^+$. The rate of $^{86}Rb^+$ entry was accelerated by addition of 1 μM A23187. After 30 min at 37°C the cells were washed three times in solution II but without Ca^{2+}. During the first wash 0.5% bovine serum albumine was present. Finally, the red cells were resuspended at a hematocrit of 5% in solution II. Sufficient A23187 was added to give a final concentration of 1 μM, and the appearance of $^{86}Rb^+$ in the supernatant was followed at a temperature of 20°C.

Measurements of K^+ Loss From Red Cell Ghosts

Ca^{2+}-dependent K^+ loss was measured in ghosts that were either incubated in K^+-containing solution or in K^+-free solution for 90 min (see Heinz and Passow, 1980). For the incubation the ghosts were resuspended at a hematocrit of 0.25% in a solution of 140 mM choline chloride, 20 mM Tris buffer (pH 7.6), and 2 mM KCl; for K^+-free incubation KCl was ommitted. After addition of 10 μM free Ca^{2+} and 0.66 μM A23187, changes in the amount of intracellular K^+ were followed by flame photometric determinations.

RESULTS

Measurement of Unitary Currents in Membrane Patches

Using the patch-clamp technique as described by Hamill et al. (1981), single-channel events were observed in cell-attached membrane patches. Fig. 1 shows records for different voltage-clamp pulses as obtained during periods of high channel activity. These active periods are frequently interrupted by silent intervals in the range of seconds (see Fig. 2) similar to observations reported for Ca^{2+}-activated K^+ channels in other preparations (Barrett et al., 1982, Methfessel and Boheim, 1982). The activity observed during voltage-clamp pulses depends on the presence of Ca^{2+}. When the solution in the pipette and in the bath contained 1 mM $MgCl_2$ plus 0.1 mM EGTA, we never observed channel activity, while addition of $CaCl_2$ to the bath solution elicited single-channel responses, provided Ca^{2+} entry was promoted by the ionophore A23187 (1 μM) (see Methods). The Ca^{2+}-induced channel activity disappears when the $CaCl_2$ in the bath solution is replaced by EGTA plus the ionophore; this leads to the transfer of intracellular Ca^{2+} into the external medium.

The Ca^{2+} dependence is most easily demonstrated in cell-free membrane patches; Fig. 2 shows the effect of two different Ca^{2+} concentrations on the activity of single-channel currents. Channel activity at 5 μM Ca^{2+} is higher than at 1 μM and in Ca^{2+}-free solution the current

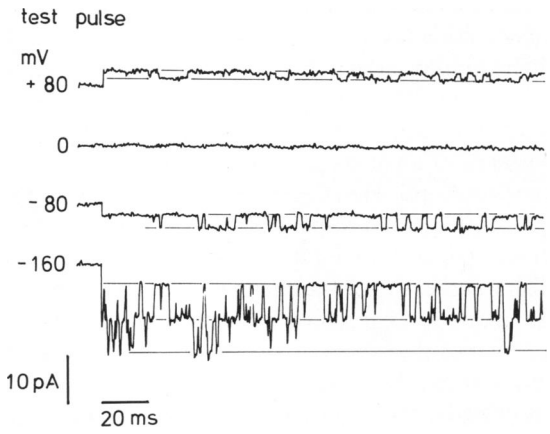

FIGURE 1 Voltage dependence of Ca^{2+}-activated single-channel currents in cell-attached membrane patches of human erythrocytes. Solutions in pipette and bath had the same composition: 140 mM KCl, 1 mM $CaCl_2$, 10 mM MOPS buffer adjusted to pH 7.4. Sampling rate 4 kHz, Temperature 20°C. Single-channel currents were recorded during different test potentials of 200-ms duration applied from a holding potential of 0 mV. The seal resistance was ~40 GΩ. At least 2 channels were present in this membrane patch. Experiment RC19AB.

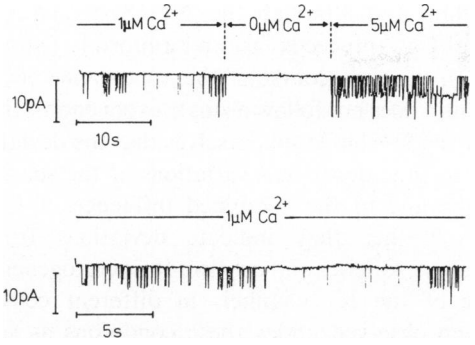

FIGURE 2 Effect of different Ca^{2+} concentrations on the single-channel activity in cell-free membrane patches. The solution in the pipette contained 140 mM KCl and was Ca^{2+} free; the bath solution contained 150 mM KCl and different concentrations of free Ca^{2+} as indicated in the figure. The single-channel currents were recorded at a holding potential of −150 mV. Sampling rate 10 and 20 Hz for the upper and lower trace, respectively.

fluctuations are totally inhibited. The effects are reversible, and the appearance and disappearance of channel activity occurs within less than a second.

No difference was observed in unitary currents of cell-attached and cell-free membrane patches. The single-channel currents exhibit a considerable inward rectification easily apparent in the current-voltage curve obtained with cell-attached patches (Fig. 3). The slope of the curve yields single-channel conductances of 40 and 10 pS at the lower and upper ends, respectively, of the voltage range covered (−280 to +160 mV). The conductance of ~25 pS at 0 mV, in 140 mM external K^+ is similar to the value of 18 pS at 100 mM K^+ reported by Hamill (1981) whose measurements were confined to the potential range of −90 mV to +50 mV where the rectification is not easily apparent.

The probability of a channel in the open state (p) increases with increasing the concentration of free Ca^{2+} as

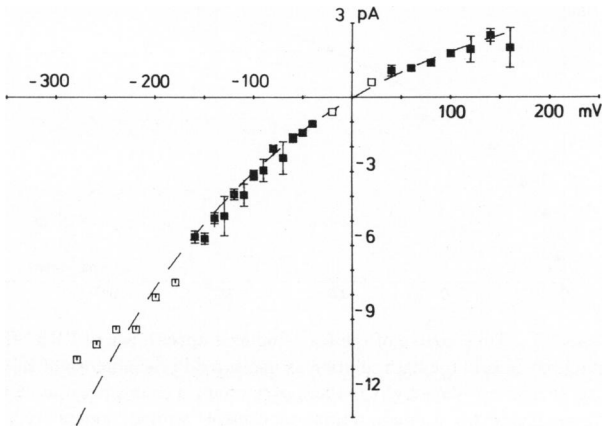

FIGURE 3 Current-voltage relation of single-channel events from cell-attached membrane patches. Closed symbols are mean values (±SEM) of 4 to 14 experiments; open symbols are from a single experiment. The line is fitted by eye to the data.

evident from Fig. 2. The steep concentration dependence of p agrees well with observations on the Ca^{2+}-induced K^+ loss from red cells (Heinz and Passow, 1980) (Fig. 4). At a concentration of intracellular free Ca^{2+} above 10 μM, both the rate of K^+ efflux and the single-channel activity reach a saturating level. At this level, determinations of p yielded values of 0.6 and 0.4 at −50 and +50 mV, respectively, for periods of activity.

Experiments in which the solution in the pipette contained Na^+ and K^+ at four different ratios show that the channels are preferentially permeable for K^+. Fig. 5 demonstrates that the dependence of the reversal potentials of unitary currents on external K^+ concentration deviates only slightly from a Nernst slope. Assuming intracellular concentrations of Na^+ and K^+ of 20 and 120 mM, respectively, one can derive from the reversal potentials at 10, 45, and 90 mM K^+ externally a mean permeability ratio P_{Na}/P_K of 0.06 ± 0.01.

Effect of K^+-free Incubation

A typical phenomenon of the Ca^{2+}-activated K^+ permeability responsible for the Gárdos effect is that the incubation of red cell ghosts in K^+-free solution irreversibly inhibits the activation of the K^+ channels by Ca^{2+} (Heinz and Passow, 1980). Fig. 6 demonstrates this inhibition; after 90 min of K^+-free incubation; the loss of K^+ from the ghosts is dramatically reduced. Patch-clamp experiments on ghosts confirm this effect; from two different batches of ghosts the following result was obtained. After incubation in K^+-containing solution four membrane patches out of eight showed unitary current events. This is a similar success rate found for membrane patches from intact red cells; in 46 ± 4% (±SEM, n = 35) of successful gigaseal formations single-channel currents were observed. Also the dependence of the unitary currents on Ca^{2+} concentration or voltage in membrane patches of ghosts are indistinguishable from those described in Figs. 2 and 3 for intact

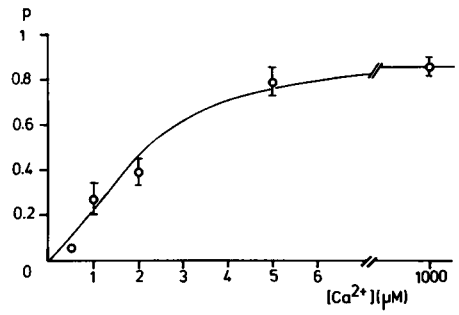

FIGURE 4 Dependence of the Ca^{2+}-activated K^+ permeability on intracellular Ca^{2+}. Symbols represent the probability of the open state (p) (±SEM) of unitary currents determined from cell-free membrane patches during channel activity; the membrane potential was −100 mV. The solid line (——) represents the rate constants of K^+ loss from red cell ghosts calculated from data of Heinz and Passow (1980, Fig. 9 *a*), and scaled to match the p values at saturating Ca^{2+} concentration.

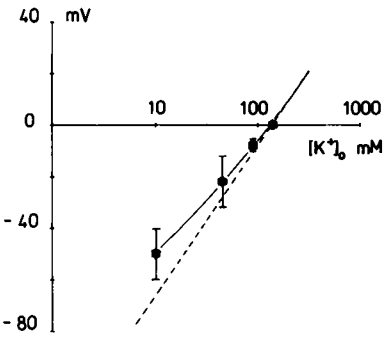

FIGURE 5 Reversal potentials of single-channel currents of cell-attached membrane patches with different K^+/Na^+ compositions in the pipette solution: 140 mM KCl/0 mM NaCl, 90 mM KCl/45 mM NaCl, 45 mM KCl/90 mM NaCl, 10 mM KCl/130 mM NaCl. The dotted line (---) represents the Nernst slope for a purely K^+-selective membrane patch. The permeability ratios P_{Na}/P_K that can be calculated from the data points at 90, 45, and 10 mK K^+ are 0.056, 0.064, and 0.052, respectively.

red cells. Incubation of the ghosts in K^+-free solution reduces the success rate, and only one membrane patch out of eight showed unitary current events.

Flux Measurements in the Absence of an Electrochemical Gradient Across the Membrane

Flux measurements in cell suspensions were performed at an external K^+ concentration of 100 mM/l, a concentration also used in the patch-clamp experiments. Under these conditions, net movements of K^+ are negligible. The changes in K^+ permeability were initiated by addition of the ionophore A23187 to the bath medium which was buffered to 10 μM free Ca^{2+} (solution II). The K^+ effluxes were measured by means of $^{86}Rb^+$ as a tracer (see Methods). Although the system is close to electrochemical equilibrium, and although the A23187-mediated equilibration of Ca^{2+} proceeds fast and uniformly (Simonsen et al., 1982), the time course of the appearance of $^{86}Rb^+$ in the medium does not follow a single exponential. In control experiments we convinced ourselves that the deviations are not due to time-dependent variations of the sensitivity of the membrane to the combined influence of Ca^{2+} and A23187. Rather they indicate deviations from two-compartment kinetics and confirm the heterogeneity of the response of the K^+ channels in different cells of the population observed under these conditions as seen previously by others (Hoffmann et al., 1980; Yingst and Hoffman, 1984, and for previous observations on the subject of heterogeneity see Riordan and Passow, 1973). The sum of two exponentials describes reasonably well the time course of appearance of $^{86}Rb^+$ in the supernatant (Fig. 7). From the two rate constants and the mean cellular volume two permeabilities, P_{K1} and P_{K2}, can be calculated possibly corresponding to two groups of cells in the population. From the larger rate constant ($k_1 = 0.143$ $min^{-1} \pm 0.019$, $n = 5$) we obtain $P^*_{K1} = 25.5\times10^{-14}$ cm^3/s; from the smaller rate constant ($k_2 = 0.0136$ $min^{-1} \pm 0.0028$, $n = 5$) we obtain $P^*_{K2} = 2.4\times10^{-14}$ cm^3/s. These values are obtained from measurements with $^{86}Rb^+$ as tracer and are multiplied by a factor of 1.3 (see Methods), thus $P_{K1} = 33.2 \times 10^{-14}$ cm^3/s and $P_{K2} = 3.1\times10^{-14}$ cm^3/s.

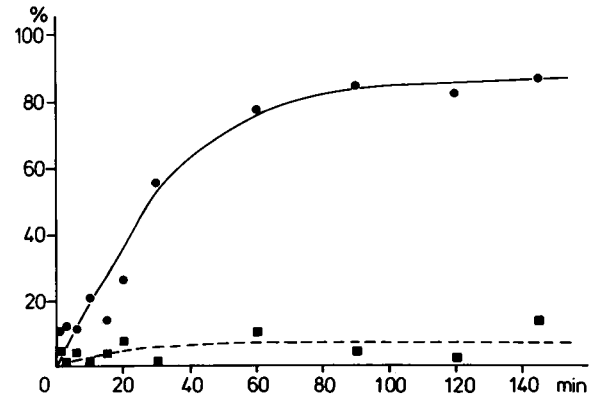

FIGURE 6 Net efflux of K^+ from red cell ghosts into a 2 mM K^+-containing medium. Circles (●) refer to ghosts incubated in 2 mM K^+, squares (■) refer to ghosts incubated in K^+-free solution before the Ca^{2+}-dependent K^+ loss was induced by application of A23187. The lines are drawn by eye.

DISCUSSION

The objective of the described experiments was to compare the single-channel currents measured with the patch-clamp technique with the Ca^{2+}-dependent K^+ fluxes in human red cells and red cell ghosts.

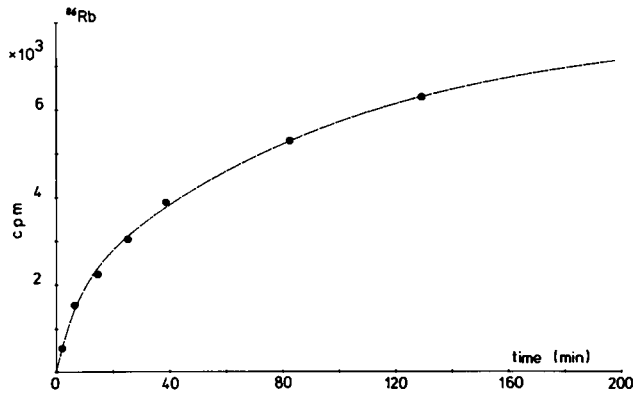

FIGURE 7 Time course of the Ca^{2+}-induced appearance of $^{86}Rb^+$ from intact red cells in the bath solution as measured in the absence of K^+ net flux. *Ordinate:* radioactivity in the supernatant (in counts per min [cpm]) was corrected for a small release in controls without ionophore. The drawn lines represent nonlinear least-squares fits to the data of the equation $A_1[1 - \exp(-k_1t)] + A_2(1 - \exp[-k_2t])$ with $A_1 = 175 \pm 10$ cpm, $k_1 = 0.144 \pm 0.018$ min^{-1}, $A_2 = 648 \pm 21$ cpm, $k_2 = 0.0096 \pm 0.0003$ min^{-1} (±SEM). Temperature 20°C. Experiment D7.

Common Pathway for Unitary Currents and K^+ Fluxes

(*a*) The experiments establish that the unitary currents, like the increase of K^+ flux, can only be elicited by the presence of Ca^{2+}. The intracellular Ca^{2+} concentrations required to elicit single-channel events and the K^+ fluxes measured isotopically are quite similar. Both phenomena rise from zero to maximal over the narrow concentration range of 15 μM (see Fig. 4).

(*b*) A typical characteristic of the Gárdos effect is the irreversible inhibition after incubation in K^+-free media. This inhibition has not been observed in other cells and thus seems to be a good indicator for the involvement of the Gárdos effect. The experiments demonstrate that parallel with the reduction of K^+ loss from K^+-free pretreated ghosts, the occurrence of unitary currents is reduced.

(*c*) An essential feature of the Gárdos effect is the selectivity of the Ca^{2+}-dependent permeability change for K^+(Passow, 1961, 1964; Kregenow and Hoffman 1962, Gárdos et al., 1977). The selectivity between K^+ and Na^+ is high (see Lew and Ferreira, 1978), and under conditions that come close to those used in our patch-clamp experiments, a lower limit of 40:1 was reported (Simons, 1976). Unpublished flux experiments from our laboratory suggest a selectivity ratio of at least 200:1. Our determinations of the reversal potential of the unitary currents yielded a permeability ratio of P_K/P_{Na} of ~17:1. This is clearly lower than inferred from flux measurements. We have no definite explanation for the discrepancy. In any event, the data show that an easily demonstrable preference for K^+ exists, as expected for a channel involved in the Gárdos effect.

In summing up the observations described in *a–c*, we think that the Ca^{2+}-activated unitary currents and the Ca^{2+}-activated permeability changes known as the Gárdos effect are mediated by the same pathways. This implies that the Gárdos effect does not involve carrier-mediated transport but represents ionic diffusion through aqueous pores with high K^+/Na^+ selectivity.

The Number of Ca^{2+}-induced K^+ Channels per Cell

When the Gárdos effect is elicited by 1 μM A23187 plus Ca^{2+} at concentrations used in our experiments, the individual cells of a population do not show identical responses. Some cells lose K^+ faster than others as could be demonstrated for example by fractionating the cells according to their density, which increases as a consequence of the K^+ loss (Hoffman et al., 1980, Yingst and Hoffman, 1984, see also Lew and Ferreira, 1978). The deviations of the kinetics of the Ca^{2+}-activated $^{86}Rb^+$ efflux from a single exponential in the absence of net movements of K^+ are obviously another reflection of the heterogeneity of the response of individual cells. This heterogeneity could either be due to different numbers of K^+ channels per cell or to channels with different kinetic characteristics resulting in different probabilities of the open state (p). The latter possibility does not seem very likely since at saturating Ca^{2+} concentration the high p values observed in individual cells never differ by an order of magnitude as the rate constants of the two exponentials of the time course of $^{86}Rb^+$ equilibration do (Fig. 7). Thus, assuming that the two exponentials refer to different numbers of K^+ channels in the two types of cells, the channel densities can be estimated. Combining Ohm's law and Goldman's flux equation allows the conversion of the single-channel conductance γ, into a single-channel permeability, π, $g(E - E_K) = \pi(EF^2/RT)(K_o - K_i \exp[EF/RT])/(1 - \exp[EF/RT])$. At an external K^+ concentration (K_o^+) of 100 mM, the zero-current conductance of a single channel is 22 pS and corresponds to a single-channel permeability of $\pi = 6.1 \times 10^{-14}$ cm^3/s. On the other hand, the permeability of a whole cell is $P_K = \pi Np$, where N is the number of channels per red cell. The probability p under zero-current conditions can only be estimated. Taking the mean from p at -50 (0.6) and $+50$ mV (0.4) gives a value of 0.5 for the probability of the open state in cell-free membrane patches. Because the periods of channel activity are interrupted by silent intervals this is an upper limit, though at saturating Ca^{2+} concentration these intervals are very brief; on the other hand, in the cell-attached patches, the similarly estimated p was smaller but usually not below 0.1. With these upper and lower limits of p one can calculate the number of channels for cells responding to Ca^{2+} with P_{K1} to 11 to 55 or responding with P_{K2} to 1 to 5 channels per cell. If we accept that the two exponentials used to describe the time course of $^{86}Rb^+$ movements refer to two groups of cells, then the relative amplitudes of the two exponentials refer to the percentages of cells with the lower and higher channel density; that means 75 ± 2% (n = 5) contain only 1–5 activable channels and 25 ± 2% (n = 5) contain 11–55 channels that are active in the time average. These values are significantly lower than the values of 100 to 200 channels per cell reported by Lew (1982).

Both the determinations of the channel numbers described here and by the other authors depend on certain assumptions whose significance can only be guessed. Our estimate is based on a rather arbitrary subdivision of the efflux into the two components that are represented by the two exponentials, although it would seem more likely that there exists more or less continuous distribution of rate constants amongst the individual cells, and on the assumption that the probability of the open-channel state and the single-channel conductance are the same under patch-clamp conditions and in intact cells. It is reassuring that in other cells the kinetics of single-channel activity were unaffected by the patch formation (e.g., Sigworth and Neher, 1980). The estimates of Lew et al. (1982) depend on the assumption that the vesiculation procedure does not

affect the transport system. While we determine the number of channels that are actually activated per time unit in intact red cells, the vesicles could contain channels that are silent in the intact cell but become activable by the vesiculation procedure. In addition, it is conceivable that at a temperature of 37°C (at which the experiments with the vesicles have been performed) the number of activable channels is higher than at 20°C (at which our own work has been done).

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