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Evidence for a random entry of Ca^{2+} into human red cells

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ABSTRACT

Under the influence of a Gardos channel activator, NS309, acting through an increase of the channels Ca^{2+} sensitivity, it is found that the single population behavior of a suspension of human red cells, showing normal distributed osmotic resistance and density, after addition of NS309 in a time dependent manner changes to a two population distribution, with an increasing fraction of cells having high osmotic resistance or high density.

The increase with time of the high resistance fraction can be fitted to an exponential, with a time constant corresponding to about 50 min. Since the 'remaining' cell fraction is practically unchanged, this points to a sudden random activation of the individual cells, caused by Ca^{2+} entry through a channel like pathway.

1. Introduction

Under physiological or near physiological conditions, the concentration of free Ca^{2+} in the red cell cytosol is maintained at a very low level, probably about 30 to 60 nM [1,2] by the powerful CaATPase [3], thereby maintaining the activity of the Ca^{2+} activated K^+ channel, the Gardos channel, very close to zero. It has been shown that the passive calcium influx, which if not countermanded would activate the Gardos channel, can be described by a saturating and a linear component, where the linear component is negligible in fresh cells. The saturating component has been described by a 'hyperbole' with $K_{0.5}$ about 1 mM, and an influx at this extracellular concentration of 50 μmol/(l_{cell} h) [4,5]. Trans-acceleration was observed, and it was concluded, that a major part of the Ca^{2+} influx, under physiological conditions was carrier mediated.

A serious problem for the characterization of the passive Ca^{2+} influx, especially at low extracellular Ca^{2+} concentrations, is the lack of specific CaATPase (PMCA) inhibitors. Commonly used methods have been metabolic depletion or vanadate treatment. However, in both cases a residual pumping activity remains [6] and furthermore vanadate seems to increase the passive Ca^{2+} influx [7,8].

In the intact red cell, the Ca^{2+} sensitivity for activation of the PMCA and the Gardos channel is of the same order of magnitude, in the range 0.5 to 1.0 μM. However, in recent years, a number of Gardos channel agonists have become available [9,10], and one of these, NS309 seems up to now to be the most powerful with regard to a decrease of the $K_{1/2}(Ca^{2+})$ for the Gardos channel. In the present work, NS309 has been used to hypersensitize the Gardos channel, causing activation at a, possibly subphysiological, Ca^{2+} level, where the PMCA can be assumed to be almost inactive [5]. In a low potassium Ringer, the Gardos channel activation resulting from a spontaneous Ca^{2+} influx will then cause a hyperpolarization due to the K^+ conductance increase, and the concomitant loss of KCl and water will cause the cells to shrink, increasing the density and the osmotic fragility.

2. Materials and methods

2.1. Reagents

CCCP (Carbonylcyanide-m-chloro-phenyl-hydrazone), nitrendipine, clotrimazole and NS309 (6,7-dichloro-1*H*-indole-2,3-dione 3-oxime) were from Sigma and NS1652 ((2-(*N*′-trifluoromethylphenyl) ureido)benzoic acid) was synthesized at NeuroSearch [14]. All compounds were prepared as stock solutions in DMSO. Salts and sucrose (Sigma) for the Ringers were of analytical grade or better. The low Cl^- solution (SR 2/0) contained 264 mM sucrose and 2 mM KCl, and the normal Ringer (nR) 154 mM Na^+ and 2 mM K^+ as chlorides unless otherwise stated. Diethylphthalat (DEP), d. 1.117–1.119 g/ml and dibutylphtalat, d.1.045–1.047 (DBP) were from Merch–Schuchardt.

2.2. Erythrocytes

Blood from healthy human donors (the authors) was drawn into heparinized vacuum tubes and centrifuged. The buffy coat and plasma

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were removed, and the cells (hRBC) were washed 3 times in nR before storage as packed cells on ice.

2.3. Membrane potential

To 3000 µl experimental solution, thermostatted at 38 °C, were added CCCP (final concentration 20 µM) and NS1652 (final concentration 10 µM). The pharmacological agents were added as described for the individual experiments. The total concentration of DMSO in the experimental solution never exceeded 0.3%, a concentration that had no effect on either fluxes or membrane potentials. The experiments were initiated by the injection of 100 µl packed cells into the medium to a final cytocrit of 3.2%.

The membrane potential was estimated from the CCCP mediated pH-change in the buffer free extracellular solution [11] as $V_m = 61.5\ mV*(pH_{in} - pH_{out})$. pH_{in} was determined as the pH in the solution after the cells were lysed by addition of 100 µl 1% Triton X-100 in 3 M NaCl and the conductances were calculated as:

$$g_{K^+} = g_{Cl}\frac{E_{Cl} - V_m}{V_m - E_{K^+}} \quad (1)$$

2.4. Osmotic resistance

500 µl packed cells suspended in 4500 µl of Ringer (nR) were incubated at 38 °C. The experiment was initiated by addition of 100 µM NS309 (final concentration). At varied times 35 µl suspension was transferred to a series of Eppendorf tubes containing NaCl solutions of 0, 0.05, 0.1, 0.175, 0.2, 0.3, 0.35, 0.4, 0.45, 0.5, 0.55, 0.6 and 0.8% NaCl, shaken and centrifuged for 30 s at 20,000 *g*. Note that due to the salt content in the sample, 0.027% NaCl should be added to the nominal NaCl %. The supernatants were transferred to disposable cuvettes and the optical densities at 540 nM were measured. Assuming the hemolysis of red cells to be normally distributed around a mean value of NaCl concentration of the hemolyzing solution, the optical density (the absorbance) of the supernatant, which is proportional to the liberated hemoglobin as function of the NaCl concentration was fitted to:

$$\mathrm{Abs}(s\%) = A \times \mathrm{erfc}\left\{\frac{s\% - B}{C}\right\} \quad (2)$$

where *A* is the half of the optical density at total hemolysis, *B* is the mean (the concentration of NaCl in W/v-%, s%, corresponding to 50% hemolysis) and *C* the SD of the corresponding Gauss-distribution. *B* corresponds to the mean value, μ and *K* to the width, σ, of the corresponding normal distribution. Thus 68% of the cells hemolyze in the interval *B* +/− *K* [12]. Should stochastic events cause some of the cells to switch from the initial state to a new hydration state, two or more populations will coexist in the suspension. In the case of two populations the osmotic fragility can now be described by:

$$\mathrm{Abs}(s\%) = A_1 \times \mathrm{erfc}\left\{\frac{s\% - B_1}{C_1}\right\} + A_2 \times \mathrm{erfc}\left\{\frac{s\% - B_2}{C_2}\right\} \quad (3)$$

where *A*, *B* and *C* have the same meaning as above.

The data can be normalized by division by $2*(A_1+A_2)$, yielding an initial normalized absorption value of 1.

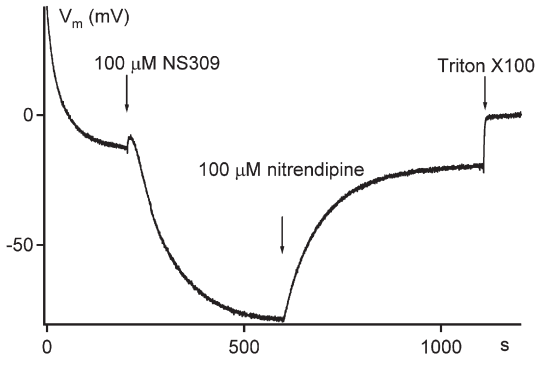

Fig. 1. NS309 induced hyperpolarization in nR supplemented with 10 µM NS1652 and 20 µM CCCP. Arrows indicate addition of NS309, nitrendipine and triton X-100.

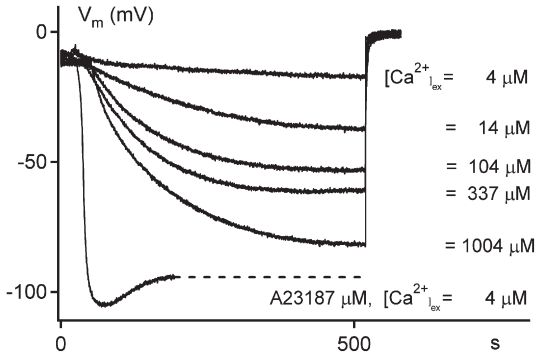

Fig. 2. NS309 (10 µM) induced hyperpolarization as function of the extracellular Ca^{2+} concentration. nR supplemented with 10 µM NS1652, 20 µM CCCP and Ca^{2+} as indicated in the fig. Trace with partly broken line, A23187 induced hyperpolarization.

2.5. Heavy cell fraction

5000 µl of experimental Ringer and 200 µl cells (final cytocrite of 3.84%) were incubated at 38 °C and the experiment initiated by addition of NS309. At varied times, 200 µl of the suspension was transferred to Eppendorf tubes containing 300 µl heavy oil (DEP) and 800 µl nR. The total amount of hemoglobin was estimated in 300 µl light oil (DBP) and 800 µl nR at $t=0$. The Eppendorf tubes were centrifuged for 15 s at 20,000 *g*, and the supernatants (including oil and Ringer) were removed. The pellet was dissolved in 1000 µl of water, and 800 µl of the sample was transferred to cuvettes and the optical density (OD) measured spectrophometrically at 540 nM.

3. Results

Following injection of the packed red cells into the unbuffered experimental medium (nR), the extracellular pH settles at a value corresponding to the normal red cell membrane potential of about −10 mV. Immediately following addition of NS309, the membrane potential hyperpolarizes dose-dependently (not shown). At 100 µM NS309, the membrane potential hyperpolarizes to about −80 mV, corresponding to a K^+ conductance of about 5 µS/cm^2, a hyperpolarization which can be reversed by addition of 10 µM nitrendipine, see Fig. 1, and clotrimazole (not shown). It should be noted, that the potential response is enhanced, since the chloride conductance is blocked about 90% in the presence of 10 µM NS1652.

In the presence of 10 µM NS309, the induced hyperpolarization showed a marked dependence on the extracellular calcium concentration, with the hyperpolarization reaching the same level at 1 mM Ca^{2+} as seen with 100 µM NS309 in the presence of only contaminating amounts of Ca^{2+} (about 4 µM). However, the hyperpolarization following NS309 addition, is relatively slow at all concentrations of $[Ca^{2+}]_{ex}$ compared to the hyperpolarization induced by the action of the calcium ionophore A23187 at the lowest $[Ca^{2+}]_{ex}$, see Fig. 2.

In order to test whether the enhanced hyperpolarization in the presence of increasing amounts of extracellular Ca^{2+} could be due to an ionophore effect of NS309, red cell uptake of ^{45}Ca and partition of ^{45}Ca into a water/*n*-octanol phase +/−100 µM NS309 were determined.

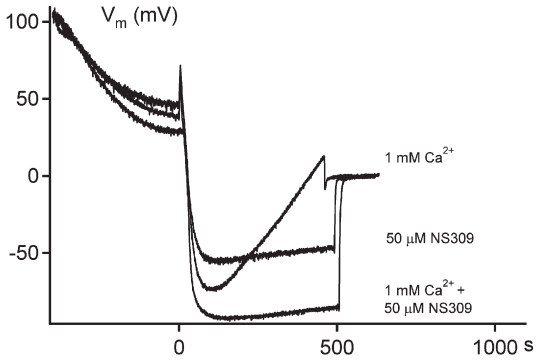

Fig. 3. hRBC preincubated for 7 min in SR, supplemented with 20 μM CCCP and 10 μM NS1652. At $t=0$, either Ca^{2+}, NS309 or Ca^{2+} and NS309 as indicated in the fig. were added.

However, no indication of ionophore properties was observed. When hRBCs are suspended in a sucrosed substituted medium, nominally Ca^{2+} free (about 4 μM as contamination), the initial depolarization activates the non-selective voltage dependent cation channel (the NSVDC channel) resulting in a repolarization. When stationarity is reached, after about 7 min, addition of Ca^{2+} (1 mM) gives rise to a hyperpolarization, due to Ca^{2+} entry through the NSVDC channel causing activation of the Gardos channel. This hyperpolarization is followed by a redepolarization, caused by the voltage dependent closing of the NSVDC channel mediated Ca^{2+} influx and the pump extrusion of the Ca^{2+} (see Fig. 3). If instead of Ca^{2+}, 50 μM NS309 is added as hyperpolarization is again observed, but in this case not transient, which is the case too, if Ca^{2+} and NS 309 is combined, but the hyperpolarization reaches more negative values.

In the presence of 100 μM NS309, the original monophasic osmotic fragility curve, which can be fitted to a complementary error function (Eq. 2), reflecting the underlying normal distribution, changes into a biphasic curve, which can be described by a sum of two weighted complementary error functions (Eq. 3), see Fig. 4, left panel. The fraction of the cells, which changes into the high resistance fraction (hrf) vs. time, can be fitted to a mono exponential function, see Fig. 4 right panel and Table 1.

As illustrated in Fig. 4, left panel and Fig. 5, the cells either transit to a high resistance state, or retains relatively unchanged the osmotic resistance, corresponding to the initial (control) state, as illustrated by Fig. 5, right panel, which shows the % NaCl solution at which half of the remaining cells have hemolyzed and the interval in % NaCl in which 68% hemolysis occurs.

An alternative approach to the determination of the fraction of osmotic high resistance cells in the presence of NS309, is an isolation of the dense cells by centrifugation of the cell suspension through a heavy phathalate oil, where the fraction of cells, which has experienced a massive loss of KCl and water forms a pellet below the oil. At the normal chloride conductance (20 μS/cm^2) the first arrival of heavy cells is observed after a lag time of about 4 min, see Fig. 6. However, if the chloride conductance is lowered to about 10% of the normal value, the lag time before the first arrival is increased to about 30 min. In both cases, the increase of the heavy fraction with time can be fitted to mono exponential functions having nearly identical rate constants.

4. Discussion

Following the addition of NS309 to a suspension of red cells, a hyperpolarization, estimated by the CCCP method, is observed. This hyperpolarization, which is dose dependent, can be reversed by the action of the Gardos channel blockers nitrendipine [13] and clotrimazole [14], see Fig. 1. Since the hyperpolarization is dependent on the concentration of NS309, as well as the concentration of extracellular calcium, a possible explanation for at least part of this observation could be that NS309 had calcium ionophore properties. However, since NS309 neither caused an increase of Ca^{2+} influx into red cells nor gave rise to a ^{45}Ca transport from a Ringer solution into an *n*-octanol phase, this possibility seems to be excluded. This demonstrates that NS309 acts as a Gardos channel activator in intact human red cells, as has previously been shown to be the case for hIK and SK channels in expression systems, where NS309 has been shown to increase the channel sensitivity towards Ca^{2+} [10].

In the human red cell two major transport systems are activated by Ca^{2+}, the Gardos channel and the PMCA, both of which have Ca^{2+} sensitivities in the same range. It has previously been shown, that a non-selective cation pathway, the NSVDC channel, which is permeable to Ca^{2+} too, is activated by depolarization caused by suspension of the cells in a sucrose substituted Ringer. Subsequent addition of Ca^{2+} activates the Gardos channel, followed by deactivation, due to calcium extrusion by the PMCA [15]. Addition of NS309 to a suspension of cells in a sucrose substituted Ringer containing contaminating Ca^{2+} only, (about 4 μM), causes likewise a hyperpolarization, but contrary to the calcium induced hyperpolarization in the absence of NS309, this is not transient, see Fig. 3, indicating that the Ca^{2+} sensitivity of the PMCA is unaffected by NS309. This is further supported by the finding that NS309 did not enhance liberation of inorganic phosphate from inside-out vesicles in the presence of ATP and calmodulin.

Since the Gardos channel Ca^{2+} sensor have been shown to be constitutively bound calmodulin [16] and the PMCA is activated by association to a calcium–calmodulin complex, it seems probable that the NS309 agonist effect is caused by direct interaction with the

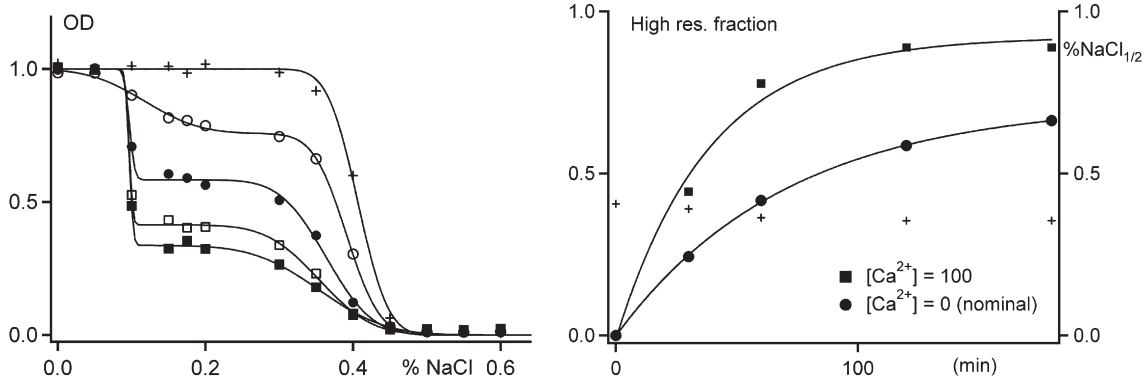

Fig. 4. Left panel: Osmotic resistance vs. time, nR supplemented with 100 μM NS309. + control, ○ 30 min, ● 60 min, □ 120 min, ■ 180 min. Unbroken lines are fit to Eq 2, respective Eq. 3. Right panel: Fraction of cell with resistance below 0.2 % NaCl, upper curve (■) + 100 μM Ca^{2+}, lower curve (●) 0 μM Ca^{2+} (nominal), corresponding to the left panel. Unbroken curves are fit to mono exponential functions.

Table 1
Parameters from fits to a mono exponential function of fraction of cells having high osmotic resistance vs. time

$[Ca^{2+}]_{out}$	0 μM (nominal)	SD	100 μM	SD	
Inf. hrf	0.719	0.0092	0.92	0.049	Fraction
Rate const.	0.0142	0.0005	0.0259	0.0048	min^{-1}

Curves shown in Fig. 4, right panel.

Gardos channel, and not by an action on calmodulin per se. This implies, that the activation of the Gardos channels, under the influence of NS309, in the intact cells, are indicators for changes in intracellular free calcium at concentration levels where the Ca^{2+} pump is almost inactive.

A consequence of an activation of the Gardos channel in low K^+ Ringers is a concomitant loss of KCl and water, tending to increase the osmotic resistance due to the shrinkage. Contrary to what has been observed at maximum activation of the Gardos channel [17] and the NSVDC channel [18] where the cells behave as one population, the osmotic resistance curve under the influence of NS309 becomes biphasic, see Fig. 4. The curves seem to be composed of a fraction which increases with time of high resistance cells and a fraction of 'remaining', almost unchanged cells (Fig. 5), which points to a fast transit from the normal state to the high resistance state. The fraction of high resistance cells vs. time in the presence of about 4 μM Ca^{2+} (contamination) and 100 μM NS309 can be fitted to mono exponential functions with time constants of about 70 and 40 min (see Table 1), but with a high resistance fraction at infinite clearly below 1.0. At 100 μM extracellular Ca^{2+} the high resistance fraction reaches about 0.92 (Fig. 4) and at even higher extracellular Ca^{2+} the fraction approaches 1.0.

It has been shown, that the PMCA capacity for Ca^{2+} extrusion varies considerably within the cell population [3], from 2–60 mmol/(l_{cells} h) which would cause $[Ca^{2+}]_{cell}$ to vary between 15 and 80 nM under physiological conditions with a Ca^{2+} influx of 50 μmol/(l_{cells} h) [19]. The NS309 enhanced Gardos channel sensitivity could then cause the cells with the highest Ca content to respond immediately, with subsequent recruitment of cells in the low capacity pumping range. However, under the present experimental conditions, at about 4 μM Ca^{2+} in the extracellular solution, the mean influx of Ca^{2+} can be assumed to be appreciably lower, and a graded activation of the Gardos channels in the susceptible fraction should be reflected by a marked broadening of the width of the osmotic resistance distribution for the 'remaining' cells, which is not observed (see Fig. 5).

An alternative approach to the determination of the osmotic resistance as an estimate of the Gardos channel activation is the measurement of the fraction of cells becoming very dense, in the present experiments cells reaching a density above 1.118, see Fig. 6. It can be calculated, that the cells must loose about 65 mmol KCl/(l original cells) to reach this density. At the normal chloride conductance, which is about 20–25 μS/cm^2 [20] the first arrival of cells in the dense state occurs after about 3–4 min. This implies that the flux rate is above 1000 mmol/(l original cells h) of KCl, very close to the flux rate observed at maximum Gardos channel activation. However, the time constant for the exponential increase of the dense fraction is about 50 min, not significantly different from the time constant found for the change in the osmotic resistance at 4 μM extracellular Ca^{2+}. If the chloride conductance is lowered to about 10% of the physiological value by the action of 10 μM NS1652 [21], the first arrival of cells into the dense state is after about 30 min, consistent with the obtainable loss rate at maximum or near maximum Gardos channel activation at a chloride conductance of about 2 μS/cm^2, but again with a time constant for the exponential of about 50 min, see Fig. 6.

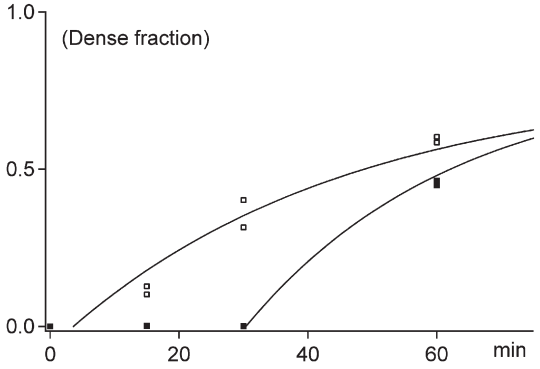

Fig. 6. Fraction of dense cells vs. time, nR supplemented with 100 μM NS309. □ control, ■ as control, supplemented with 10 μM NS1652. Unbroken curves are fits to a mono exponential function.

An exponential rise in the high resistance or dense cell fraction is consistent with a random activation of the individual cell. Since Ca^{2+} is a prerequisite for Gardos channel activation, this could be caused by an opening of a calcium pathway working in an on/off mode, that is channel like. Such a behaviour has been observed previously for sickle cells, run through a series of oxygenation/deoxygenation cycles leading to cells becoming irreversibly sickled, and has been ascribed to the opening of a randomly active cation pathway, P_{cat}, in deoxygenized cells [22].

The waiting time for first arrival of cells into the dense fraction of 3–4 min at the normal chloride conductance, and about 30 min with inhibition to about 10% of the normal value, signifies that the Gardos

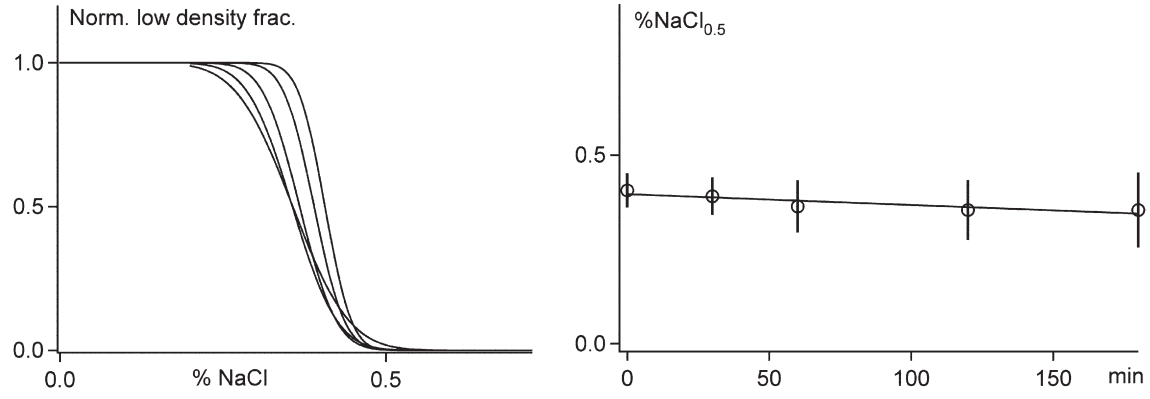

Fig. 5. Remaining cells, corresponding to Fig. 4, left panel. Normalized osmotic resistance of low resistance fraction, right panel: Half hemolysis NaCl % (unbroken line) and the width of the distribution are indicated as vertical bars.

channels in the activated cells become maximally or near maximally activated, corresponding to about 60 to 80 μS/cm^2 [23,24]. Since not all cells are activated instantaneously, as is the case under the action of NS309, the intracellular free calcium concentration in the major fraction of the cells must be too low, due to the active extrusion, to cause Gardos channel activation, even in the presence of NS309.

It has been shown, that the Gardos channel activation induced by A23187 is instantaneous and near maximal. As can be seen from Fig. 2, the activation caused by NS309 results in an apparent hyperpolarization, which is lower and develops more slowly. The probabilistic activation by NS309 of the individual cells, as demonstrated in Fig. 4 explains the phenomenon. If the cells, instead of being homogeneously activated, are either activated, which means that they hyperpolarize, or remains in the resting state, the change in the extracellular pH cannot be used directly for a membrane potential calculation, but only in a qualitative sense. The cells with maximally activated Gardos channels will hyperpolarize to about −90 mV respectively −108 mV at g_{Cl} ~2 μS/cm^2. The cells which remain in the resting state are equivalent to a buffered extracellular medium relative to the activated cells, and changes in the cellular pH will occur, both for the cells in the activated and resting state [11]. The membrane potential estimated from the suspension pH thus becomes a weighted mean value, where the weighting factors are the fractions in the respective states.

Although the average Ca^{2+} influx has been shown to be dominated by a carrier like mechanism, infrequent random opening of a calcium entry pathway in the individual cell, could lead to an abrupt increase of the $[Ca^{2+}]_{cell}$, causing the Gardos channels in this cell to open, whereby the membrane potential hyperpolarizes. However, the lag times for the first arrival of cells in the dense state indicate that a cell, once activated stays activated for 4 or 30 min, which at maximum Gardos channel activation is necessary for the cell to reach a density above 1.118 g/ml cells. This seems to imply, that the resting pump-leak cellular Ca^{2+} concentration is below the threshold for Gardos channel activation, but following a Ca^{2+} burst the PMCA cannot lower the Ca^{2+} concentration sufficiently to deactivate the Gardos channel again, as directly seen with cells suspended in sucrose Ringer (Fig. 3). Tentatively this might be ascribed to the establishment of a new steady state at a higher concentration, due to the hyperpolarization. It should be noted, that since the Gardos channel activation probably is dependent on at least the 2nd power of the $[Ca^{2+}]_{cell}$, as has been shown for IK and SK channels [25,26] a lowering of the half activation concentration for Gardos channel activation to about 50 nM gives a very steep activation curve, almost resembling a square function.

At present, it is unclear, why the fraction of cells recruited to the high resistance state at infinity seems to be dependent on the extracellular calcium concentration. A number of explanations can be guessed at: 1) a distribution over the cell population of the Gardos channel calcium sensitivity, 2) a distribution over calcium permeability and 3) a distribution over PMCA activity at low or subphysiological calcium concentrations. The most probable explanation seems to be the distribution over the pump activity. It has been shown, that the V_{max} for the pump covers a wide range of values, from about 5–35 mmol (340 g Hb)$^{-1}$h^{-1} [3]. If this spread in V_{max} is paralleled by the pump rates at physiological or subphysiological $[Ca^{2+}]_{in}$ the increased influx at an elevated $[Ca^{2+}]_{out}$ once a 'calcium channel' opened would lead recruitment of the cells with the highest pump rates, too.

The molecular nature of these putative Ca^{2+} channels is at present unknown. Apart from the NSVDC channel, which is permeable to Ca^{2+} [27,15] functional Ca^{2+} channels have been identified in patch clamp experiments [28]. These channels, which were characterized as B-channels, seem to have very long closed periods, interrupted by occasional bursts of activity. Furthermore, immunological analysis has shown the presence of voltage dependent Ca^{2+} channels in the human red cell membrane, and it was shown that a range of Ca^{2+} blockers inhibited the Ca^{2+} influx [29].

5. Conclusion

Through a pharmaca-induced increase of the Gardos channel Ca^{2+} sensitivity, the activation of this channel becomes an indicator for passive Ca^{2+} influx. The time dependence of the activation can be described by an exponential, which is consistent with a random opening of a Ca^{2+} pathway, which causes a $[Ca^{2+}]_{cell}$ increase above the mean pump-leak level, and above the threshold for the enhanced Gardos channel activation. This indicates the presence of a functional channel like pathway for Ca^{2+} in the human red cell membrane.

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