Dehydration response of sickle cells to sickling-induced Ca\superscript{++} permeabilization

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Interaction of hemoglobin S polymers with the red blood cell (RBC) membrane induces a reversible increase in permeability (P\textsubscript{sickle}) to (at least) Na\superscript{+}, K\superscript{+}, Ca\superscript{2+}, and Mg\superscript{2+}. Resulting changes in [Ca\superscript{2+}] and [H\superscript{+}] in susceptible cells activate 2 transporters involved in sickle cell dehydration, the Ca\superscript{2+}-sensitive K\superscript{+} ("Gardos") channel (K\textsubscript{Ca}) and the acid- and volume-sensitive K:Cl cotransport. We investigated the distribution of P\textsubscript{sickle} expression among deoxygenated sickle cell anemia (SS) RBCs using new experimental designs in which the RBC Ca\superscript{2+} pumps were partially inhibited by vanadate, and the cells’ dehydration rates were detected as progressive changes in the profiles of osmotic fragility curves and correlated with flow cytometric measurements. The results exposed marked variations in (sickling plus Ca\superscript{2+})-induced dehydration rates within populations of deoxygenated SS cells, with complex distributions, reflecting a broad heterogeneity of their P\textsubscript{sickle} values. P\textsubscript{sickle}-mediated dehydration was inhibited by clotrimazole, verifying the role of K\textsubscript{Ca}, and also by elevated [Ca\superscript{2+}]\textsubscript{i}, above 2 mM. Very high P\textsubscript{sickle} values occurred with some SS discocytes, which had a wide initial density (osmotic resistance) distribution. Together with its previously shown stochastic nature, the irregular distribution of P\textsubscript{sickle} documented here in discocytes is consistent with a mechanism involving low-probability, reversible interactions between sickle polymers and membrane or cytoskeletal components, affecting only a fraction of the RBCs during each deoxygenation event and a small number of activated pathways per RBC. A higher participation of SS reticulocytes in P\textsubscript{sickle}-triggered dehydration suggests that they form these pathways more efficiently than discocytes despite their lower cell hemoglobin concentrations. (Blood. 2002;99:2578-2585)

Introduction

The permeability pathway generated by the interaction of deoxygenated hemoglobin S polymers with the red blood cell (RBC) membrane (P\textsubscript{sickle}) plays a major role in the mechanism of dehydration of sickle cell (SS) anemia RBCs. The functional properties of this pathway, however, have yet to be determined. P\textsubscript{sickle} mediates changes in cell Ca\superscript{2+} and H\superscript{+} concentrations, which activate the 2 main transporters involved in sickle cell dehydration, the Ca\superscript{2+}-sensitive K\superscript{+} ("Gardos") channel (K\textsubscript{Ca}), expressed in RBCs of all ages,	extsuperscript{1,2} and an acid- and volume-sensitive K:Cl cotransport, expressed predominantly in young RBC and reticulocytes,	extsuperscript{3-8} with both leading to a net loss of KCl and water. In this sequence, P\textsubscript{sickle} remains the most obscure link—and the one most difficult to investigate—because the relevant membrane-polymer interactions are not yet accessible to direct morphologic and functional scrutiny.

Early work established that P\textsubscript{sickle} is a poorly selective permeability pathway for Na\superscript{+}, K\superscript{+}, Ca\superscript{2+}, and Mg\superscript{2+}.	extsuperscript{9,15} In reticulocytes, P\textsubscript{sickle} exhibits a peculiar heparin sensitivity	extsuperscript{16} and is partially inhibited by stilbenes.	extsuperscript{17} The Ca\superscript{2+} and Mg\superscript{2+} permeabilities through this pathway were shown to be similar in different density fractions of sickle cells (light, normal discocytes, and dense),	extsuperscript{15,16} suggesting that P\textsubscript{sickle} has little correlation with those variations in sickling morphology associated with different hydration states of the SS RBCs. A recent study	extsuperscript{18} revealed 2 fundamental properties of P\textsubscript{sickle} in SS discocytes: (1) P\textsubscript{sickle} remains open or active for the duration of each deoxygenation (deoxy) episode and therefore is the functional expression of a molecular structure that forms on deoxygenation and remains stable within each deoxy episode; and (2) consecutive deoxy pulses generate similar-sized fractions of RBCs with similar P\textsubscript{sickle} values, even after removal of high-P\textsubscript{sickle} cells from the previous pulse; this indicated that P\textsubscript{sickle} is stochastic in nature, with a conserved probability distribution in successive deoxy pulses. The actual distribution of P\textsubscript{sickle} values among deoxygenated SS cell fractions, however, remained to be characterized.

To investigate the range of expression of P\textsubscript{sickle}, we developed a new approach in which cell-to-cell differences in P\textsubscript{sickle} intensity were converted into differences in dehydration rates; these, in turn, were observed by following the progressive shape changes of the RBC osmotic fragility curves. This “profile migration method,” which we described in detail previously,	extsuperscript{18-21} is outlined below. In addition, a smaller, ancillary series of experiments was performed in which SS RBC dehydration was assessed by flow cytometric methods, as detailed below. Together, these measurements exposed marked variations in (sickling plus Ca\superscript{2+})-induced dehydration rates within populations of deoxygenated sickle cells that reflected a broad heterogeneity of their P\textsubscript{sickle} values.

Materials and methods

All chemicals were analytical reagent quality. Ethyleneglycoltetraacetic acid, HEPES, glucose, inosine, Na-orthovanadate, and dimethyl sulfoxide were from Sigma and were of the highest available grade.

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(DMSO) were obtained from Sigma Chemical (St Louis, MO). A23187 was obtained from Calbiochem-Novabiochem (La Jolla, CA).

**Composition of solutions**

Solution A contained 4 mM KCl, 140 mM NaCl, 0.15 mM MgCl₂, and 20 mM Na-HEPES (pH 7.5 at 37°C). Solution B differed from A by having 80 mM KCl, 64 mM NaCl, and 0.05 mM NaOH-neutralized ethyleneglycoltetraacetic acid. Solution C was the same as A with the addition of 5 mM glucose, 5 mM inosine, and 1% mEq bovine serum albumin. Solution D (used in flow cytometry experiments) contained 4 mM KCl, 135 mM NaCl, 5 mM NaSCN, 0.15 mM MgCl₂, 20 mM Na-HEPES (pH 7.4 at 37°C), and 1% human serum albumin.

**Preparation of RBCs**

Venous blood anticoagulated with Na-EDTA was obtained from patients with SS and normal (AA) controls after informed consent. AA blood was centrifuged, the plasma anduffy coat removed, and the packed cells washed thrice in solution B. SS blood was density-fractionated as previously described.²⁶ Briefly, the washed packed RBCs were suspended at about 50% hematocrit (Hct) in the same solution B and layered onto a discontinuous gradient of arabinogalactan in solution B. The reticulocyte-rich fraction with density (δ) ≈ 1.091 and the discocyte fraction with density 1.091 < δ ≈ 1.106 (which excluded the denser discocytes) were harvested and washed thrice in solution B. These broad density boundaries were chosen so as to incorporate most of the SS reticulocytes and discocytes within the corresponding density fractions.

**Experimental design**

To translate cell differences in P₅₀ in sickle into differences in cell dehydration rate, it was necessary to establish experimental conditions in which deoxygenation-induced sickle cell dehydration would be determined by P₅₀ in sickle. Because P₅₀ increases the Ca²⁺ permeability of the RBCs, their pump-leak [Ca²⁺]ι level will increase when they are deoxygenated in the presence of external Ca²⁺. Elevated [Ca²⁺], activates KᵥCa channels and, in low-K⁺ media, mediates net KCl loss and RBC dehydration. This dehydration is usually rate-limited by the relatively low electrodiffusional Cl⁻ permeability of the RBC membrane.²⁶,²⁷ It was therefore anticipated that the dehydration rate in each RBC will be determined by the KᵥCa channel activation at each P₅₀ in sickle. The aim was to induce minimal cell dehydration in control oxy conditions (when passive Ca²⁺ influx proceeds through the intrinsically low permeability of the RBC membrane) but to expose easily detectable dehydration when P₅₀ in sickle was activated by deoxygenation. In such conditions, variations in RBC dehydration rates could be attributed to and correlated with the P₅₀ in sickle distribution among the cells.

Based on the experimental design outlined above, the following protocol was used to measure the population distribution of dehydration rates with the profile migration method.¹⁹-²¹ Pairs of correspondingly labeled 96-well plates were used, with one of each pair having U-shaped well bottoms and the other being flat-bottomed for optical density measurements. Each row of the U-bottomed plate contained 250 μL of 12 solutions with different osmolarities, ranging from 1.0 to 0.01 relative tonicity (RT) units (lysis media). These were prepared by mixing appropriate volumes of 2 solutions, one containing 149 mM NaCl and 2 mM Na-HEPES (pH 7.5 at 20°C) and the other only 2 mM Na-HEPES (pH 7.5 at 20°C). For each set of measurements, 4 mL of a 2.5% (Hct) RBC suspension in solution C was equilibrated for the designated time periods at 37°C with water-saturated O₂ or N₂ in a tonometer (model 237, Instrumentation Laboratory, Lexington, MA). NaSCN was added to the suspension from a 1.5 M stock solution to a final concentration of 5 mM. Where indicated in “Results,” the following were added: ionophore A23187, from a 2 mM stock solution in ethanol/DMSO (4:1); final concentration of 100 μM RBC; valinomycin, from 12 mM stock in DMSO, final concentration 10 μM; orthovana date, from 100 mM stock, final concentration 1.0 mM; and calcium (from 1 M CaCl₂) stock, between 0.5 and 5.0 mM, corrected for albumin binding. Fifteen seconds before each sampling time, 0.6 mL of RBC suspension was transferred from the tonometer to a plastic incubation tray (Accutan disposable incubation tray, Schleicher & Schuell) at room temperature. At the exact sampling time, a 12-channel pipette (Finpipette, Thermo Labsystems, Helsinki, Finland) was used to deliver 10 μL samples of the suspension in the incubation tray to the row of 12 wells on a U-bottomed 96-microwell plate containing the 250 μL of the lysis media, and they were rapidly mixed by repeated vigorous squirts with the multichannel dispenser. Each timed sample generated a full hemolysis curve. To estimate the fraction of hemolysis in each well, the plate was centrifuged for 5 minutes at 1100 rpm, 150 μL samples of the supernatants were transferred with a 12-channel dispenser to the correspondingly labeled row on the flat-bottomed plate, and concentrations of hemoglobin were measured on a plate reader (Multispec Bichromatic type 348, Thermo Labsystems) by its absorption at 414 nm, the Sorette band, where its extinction coefficient is optimal.²⁸ Although the method proposed here was not used previously with SS RBCs, it had been thoroughly tested, as noted above, and shown to be reliable in various applications on normal human RBCs. The hemolysis curves are shown in the standard format, plotting the percent hemolysis as a function of RT. Conserved profiles, ie, constant shape of the migrating hemolysis curves, indicate uniformity of dehydration rates, whereas changing profiles reflect heterogeneities of dehydration rates among the cells. The population distribution of dehydration states may be appreciated more clearly from histograms representing the derivatives of the hemolysis curves. The histograms in Figure 5 report the increment in hemolysis (Δ% lysis) per increment in RT (ΔRT), normalized per ΔRT (Δ% lysis/ΔRT), for each ΔRT interval. The cumulative sum of the histogram columns from right to left accurately expresses the data from the measured hemolysis curves.

**Flow cytometry experiments**

An aliquot of SS discocytes with density 1.091 < δ ≈ 1.106 was prepared and washed as described above and suspended at 1% Hct in 1.5 mL of solution D containing, in addition, CaCl₂; to give a final concentration of between 1.0 and 2.0 mM [Ca²⁺]ι, (corrected for albumin binding), as indicated in “Results,” and equilibrated in the tonometer with water-saturated air for 10 minutes at 37°C. After baseline sampling, sodium vanadate was added from a stock solution to give a final concentration of between 0.1 and 1.0 mM, as indicated. Further equilibration was either with continuous air (oxy control) or with cycles of 10 minutes of N₂ gas followed by 5 minutes of air (deoxygenoxy cycling), for up to 45 minutes.

As with the profile migration studies, preliminary experiments were done to determine the optimal conditions that would inhibit the RBC Ca²⁺ pumps enough to permit relatively rapid, easily detectable SS cell dehydration when P₅₀ in sickle was activated by deoxygenation but would result in minimal dehydration during control oxy conditions. With the very dilute RBC suspensions used in these studies, this balance of Ca²⁺ pump

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inhibition was generally observed with vanadate concentrations of about 0.1 mM and [Ca\textsuperscript{2+}]\textsubscript{o} of about 1.0 mM but varied somewhat between samples from the same or different donors and had to be empirically determined for each experiment, as noted in “Results.”

RBCs from whole blood or density fractions were analyzed with a flow cytometry–based hematology analyzer, the Bayer-Technicon H\textsuperscript{+}3 RTX (Bayer Diagnostics, Tarrytown, NY), as described previously.\textsuperscript{26} Briefly, the RBCs are isovolumetrically sphered and the volume and hemoglobin concentration (HC) of each cell is determined by analysis of low- and high-angle laser light scattering. The H\textsuperscript{+}3 RTX instruments used in the present experiments differed from the commercial model of the H\textsuperscript{+}3 (and later generations of this instrument) in having only a manual rather than automatic sampling mode.

Using the manual mode, we could select the RBC conditions before H\textsuperscript{+}3 sampling. As observed before in preliminary experiments,\textsuperscript{26} after the experimental manipulations used, the standard H\textsuperscript{+}3 procedure of incubating for 15 minutes in the dye (oxazine) buffer solution, with or without the sphering detergent (TDAPS), resulted in RBC swelling, with broadening of HC distributions toward lower values. This swelling was avoided if the RBCs were read by the H\textsuperscript{+}3 immediately after sampling and mixing with the sphering agent, without incubation with the dye. Therefore, all RBC samples were sampled 2 ways in the H\textsuperscript{+}3 immediately after sphering (without RNA staining) and, then, to detect reticulocytes, after 15 minutes’ incubation with the dye in the appropriate buffer for the experiment, immediately after adding the sphering agent.

Results

Comparison of the dehydration response of SS discocytes and AA RBCs to deoxygenation and Ca\textsuperscript{2+} pump inhibition

Preliminary experiments confirmed earlier work showing that, in the absence of Ca\textsuperscript{2+} pump inhibition, sustained short-term deoxygenation produced minimal dehydration of SS RBCs.\textsuperscript{16} Figure 1 shows the effects of vanadate on hemolysis curve migration in oxy and deoxy conditions in the presence of 2 mM [Ca\textsuperscript{2+}]\textsubscript{o}. With AA RBCs there were negligible changes during the 1-hour incubation, oxy or deoxy (Figure 1A,B). With oxy SS discocytes (Figure 1C), addition of vanadate caused minor displacement of the hemolysis curves, apparently representing further dehydration of the relatively denser discocytes within this fraction whose osmotic resistance was highest initially. However, in deoxy conditions, addition of vanadate was associated with progressive migration of the hemolysis curves, ultimately affecting more than 95% of the RBCs (Figure 1D). The altered shape profile of the migrating curves, as compared with the control curve, indicated that there was substantial heterogeneity of dehydration rates.

Vanadate also inhibits the Na pump, which could result in RBC swelling and cause the hemolysis curve profiles to migrate to lower RTs. As seen in Figure 1, with both SS discocytes and AA RBCs this potential swelling effect was minimal within the time required to expose deoxy-induced dehydration.

**K\textsubscript{Ca} channel mediation of deoxy-induced dehydration of SS RBC: effects of varying external Ca\textsuperscript{2+} concentrations and of clotrimazole**

The effects of varying external Ca\textsuperscript{2+} concentrations ([Ca\textsuperscript{2+}]\textsubscript{o}) on vanadate-induced dehydration of deoxy SS discocytes are shown in Figure 2. In the absence of Ca\textsuperscript{2+} (Figure 2A) there was no dehydration and no difference between oxy and deoxy hemolysis curves. [Ca\textsuperscript{2+}]\textsubscript{o} levels of 0.5 and 1 mM produced moderate dehydration of the oxy RBCs and large oxy-deoxy differential dehydration at both 10 and 25 minutes (Figure 2B,C). However, when [Ca\textsuperscript{2+}]\textsubscript{i} was raised to between 2 and 5 mM, there was a progressive decrease in the extent of dehydration of the deoxy RBCs after 25 minutes’ incubation, with a corresponding reduction in the oxy-deoxy differential (Figure 2D-F). Together, these results show that the dehydration of SS RBCs elicited by deoxygenation and vanadate is strictly Ca\textsuperscript{2+}-dependent and that external Ca\textsuperscript{2+} has 2 opposing effects on the deoxy SS RBC: At low concentrations it elicits maximal dehydration, but at higher concentrations the rate of dehydration is reduced. Addition of a specific K\textsubscript{Ca} channel inhibitor, clotrimazole,\textsuperscript{29} prevented vanadate-induced dehydration of deoxy SS discocytes (Figure 3), confirming that the dehydration following deoxygenation-induced P\textsubscript{sickle} formation was mediated by K\textsubscript{Ca} channel activation.

**Effects of Ca\textsuperscript{2+}/K\textsuperscript{+} ionophores**

The observed differences in dehydration rates among SS discocytes could result either from (1) variations in their intracellular K\textsuperscript{+} concentrations, which would affect their driving gradients for dehydration, from (2) cell-to-cell differences in the number of K\textsubscript{Ca} channels or (3) the extent of their activation. To help distinguish between these possible mechanisms, we compared the pattern of dehydration of a single batch of SS discocytes exposed to valinomycin, a K\textsuperscript{+}-selective ionophore that...
produces uniform K\textsuperscript{+} permeabilization (Figure 4A), with that induced by deoxygenation plus vanadate (Figure 4D). Valinomycin caused maximal and uniform cell dehydration in more than 85% of the SS discocytes within 20 minutes, indicating that most of the SS RBCs in this density fraction retained normal driving K\textsuperscript{+} gradients for dehydration.

Addition of appropriate concentrations of the Ca\textsuperscript{2+} ionophore A23187 in the presence of external Ca\textsuperscript{2+} produces uniform Ca\textsuperscript{2+} loads in all the RBCs and induces maximal activation of their K\textsubscript{Ca} channels. 24 The dehydration pattern of the SS discocytes induced by A23187 plus Ca\textsuperscript{2+} (Figure 4B) was similar to that elicited by valinomycin, indicating that fully activated K\textsubscript{Ca} channels produce rapid and uniform dehydration in more than 85% of these SS RBCs, as seen previously with AA RBCs.30 The results with both the K\textsuperscript{+} and Ca\textsuperscript{2+} ionophores indicate that the grossly heterogeneous dehydration rates exhibited by SS discocytes deoxygenated in the presence of vanadate (Figure 4D) must reflect differences in their individual RBC [Ca\textsuperscript{2+}] levels, which in turn must result from variations in either their P\textsubscript{sickle} or their residual Ca\textsuperscript{2+} pumping.

P\textsubscript{sickle} distributions

Figure 5D-I illustrates the dynamic distribution of dehydration states of SS discocytes deoxygenated in the presence of Ca\textsuperscript{2+}, with their Ca\textsuperscript{2+} pumps inhibited by vanadate. Figure 5A,B reports the initial and final distributions in the oxy controls. Over the 45 minutes of deoxygenation, there is a progressive migration of RBCs into the fraction with maximal osmotic resistance. During the first 10 minutes of deoxy incubation, about 15% of the RBCs have dehydrated rapidly, newly appearing in the 0 to 15 RT columns (Figure 5F); these rapidly dehydrating RBCs appear to migrate from a wide range of initial RT columns (RT lytic ranges between 15-35). Over the next 45 minutes, portions of all but the lightest RBCs become progressively dehydrated, at apparently different rates, with some lagging behind or failing to dehydrate. About 8% to 10% of the lightest RBCs (RT > 35). Over the next 45 minutes, portions of all but the lightest RBCs become progressively dehydrated, at apparently different rates, with some lagging behind or failing to dehydrate. About 8% to 10% of the lightest RBCs (RT lytic range 40 to 65) fail to dehydrate altogether (Figure 5I) unless K\textsuperscript{+}-permeabilized with valinomycin (Figure 5C). Thus, dehydration rates appear to be distributed in a continuous gradation. To the extent that the heterogeneity of dehydration rates reflects the population variation in P\textsubscript{sickle}, the P\textsubscript{sickle} distribution follows a complex pattern with 2 major groups: a large group of intermediate to (relatively) high-density discocytes with a widely graded distribution of P\textsubscript{sickle} and an intermediate to very light group of RBCs in which P\textsubscript{sickle} is minimally or not activated and which is likely to contain most of the hemoglobin F cells present in this cell fraction.
Flow cytometric observations of the dehydration response of SS discocytes

For comparison with the results using the profile migration method, density-fractionated light SS discocytes were deoxygenated in the presence of vanadate and external Ca$^{2+}$ and the distribution of their HCs and volumes were followed with the Bayer-Technicon H$^+$3 flow cytometry system. As shown in Figure 6, it was possible to achieve a balance between the extent of inhibition of the Ca$^{2+}$ pump by vanadate and deoxygenation-induced $P_{\text{sick}}$ that resulted in little or no detectable RBC dehydration in the oxygenated control condition but distinct dehydration, with a markedly heterogeneous distribution, among the deoxygenated SS discocytes. In the conditions of that particular experiment, about half of the SS discocytes failed to shrink, while the other cells showed a wide range of dehydration responses in the first 30 minutes of incubation, with only small further changes over time. These results are qualitatively consistent with those obtained using the profile migration method: They show a fraction of SS discocytes that did not dehydrate in these test conditions and show a broadly distributed dehydration response among those RBCs that did dehydrate. No quantitative consistency is expected between these 2 methods, because they both depend on the balance achieved in each experiment between Ca$^{2+}$ pump inhibition by vanadate and the increased Ca$^{2+}$ influx generated by $P_{\text{sick}}$, and the effects of that balance among the RBC population.

Whereas the Hct of the cell suspensions used for the profile migration experiments was about 10%, the flow cytometry experiments were carried out at an Hct of about 1%. Preliminary experiments indicated that, to achieve the balanced level of Ca$^{2+}$ pump inhibition described above, the concentration of vanadate in the cell suspension had to be adjusted downward to preserve vanadate/cell ratios comparable to those in the 10% Hct suspensions. Therefore, in experiments with the same or different donors, it was found necessary to vary the concentration of vanadate empirically between 0.05 and 0.2 mM, but in each of 4 experiments, when that balance was achieved, the results were equivalent to those shown in Figure 6.

Deoxygenation-induced dehydration in reticulocyte-rich SS cell fractions

Using the same protocol as that applied to the SS discocyte fractions, we examined the effects of deoxygenation of light,
reticulocyte-rich SS cell fractions ($\delta \leq 1.091$) on the profile migration of hemolysis curves. As shown in the experiment of Figure 7 (representative of 3 similar experiments), the hemolysis curves of these light SS cell fractions extended over a much wider RT range than those of the SS discocytes. Importantly, in this low-density, low-mean-cell HC RBC fraction, initially high osmotic resistance is based not only on a high-density dehydration state, as in the SS discocytes, but also on the increased surface area/volume ratio of the reticulocytes. Because the reticulocytes must gain relatively more water to reach their critical hemolytic volume, they require lower RT values for hemolysis.

Upon K$^+$ permeabilization with valinomycin (Figure 7A), more than 80% of the cells in this light, reticulocyte-rich fraction dehydrated rapidly and maximally, indicating that they retained normal K$^+$ gradients for dehydration. However, about 18% of the light SS cell fraction used in this experiment and 30% and 22% in 2 similar experiments (not shown) showed no dehydration following exposure to valinomycin. These nondehydrating SS cells correspond to a recently discovered subpopulation of light, high-Na, low-K “valinomycin-resistant” RBCs that compose about 4% of the total SS cell population; their properties were described elsewhere and are not considered further here.

In oxy conditions (Figure 7B), the net effect of vanadate was rapid swelling of a substantial fraction of cells, attributable to inhibition of the reticulocytes’ Na pumps, which are more than 10-fold more active than those of mature RBCs. Swelling occurred in more than 80% of the cells but was much more prominent in about 35% of the cells that were most susceptible to osmotic lysis (other than val-res cells). Deoxygenation reversed the swelling in all the cells, countering the substantial differences in right-shift displacements. These shifts highlight patterns of $P_{sfcle}$.
heterogeneity unique to reticulocyte-rich cell fractions. However, the superposition of opposite effects with this method, together with the marked heterogeneity of osmotic resistance levels, limits further analysis of Psickle-induced dehydration responses in reticulocyte-rich SS cell fractions.

The vanadate-induced right shift observed in the hemolysis curves of the reticulocyte-rich cell fractions was either absent (Figure 1C) or minimal (Figure 4C) in oxy discocytes. Therefore, the pump-leak Na/K traffic in discocytes must have been too small to generate the counterbalancing, swelling effect observed with the reticulocyte-rich fraction and could not contribute significantly to the heterogeneity of the discocytes' Psickle response.

Discussion

The results of the present experiments show that the Psickle distribution generated within each deoxy episode is complex, often with sharp discontinuities between subpopulations (Figure 6), confirming earlier suggestions of unequal Psickle permeabilization of SS cell subpopulations during each deoxygenation episode. Among the SS discocytes, there appeared to be some RBCs with particularly high Psickle values; within this fraction, these RBCs were initially distributed over a broad range of densities (osmotic resistances), excluding only the lightest discocytes (Figure 5D-I).

Interpretation of the present results in terms of uneven distribution of Psickle values among sickle discocytes is consistent with our earlier observation on the stochastic nature of Psickle. As shown in Figure 1D, in vanadate-free controls in Cl medium (medium C), no significant dehydration of deoxy sickle cells was detected within 45 minutes. Using a high vanadate concentration to inhibit the Ca2^2+-dependent Na/K pump, it was possible to expose measurable patterns of Psickle-induced dehydration. The results demonstrate subpopulation differences in Ca2^2+-dependent, deoxy-induced dehydration rates, suggesting a wide distribution of Psickle values.

The relative decrease in the dehydration rate of SS discocytes observed when [Ca2^2+]_o was increased above 1 mM (Figure 2) is consistent with our earlier observations that external Ca2^2+ inhibited Psickle in SS reticulocytes, particularly the Na^+ component. Further studies by Joiner et al demonstrated that in the lightest 15% to 25% of SS RBCs, Ca2^2+ and other divalent cations inhibited the Na^+ component of Psickle more than the K^+ component, resulting in a net K^+ loss that occurred even in the absence of K^+ channel activation. Taken together, these observations indicate that Psickle is inhibited by external divalent cations and that the inhibitory effect has a low affinity, increasing from 1 mM to 5 mM for external Ca2^2+ (Figure 2). The mechanism underlying these Ca2^2+ (divalent cation) effects on Psickle remains to be determined.

The inhibitory effects of clotrimazole on Psickle-triggered dehydration of SS discocytes (Figure 3) confirm K^-mediation of the dehydration response. Valinomycin elicited uniform and maximal dehydration of more than 90% of discocytes and up to 80% of cells from the reticulocyte-rich fraction (Figures 4A and 7A), indicating that the slower and much more irregular dehydration pattern induced by Psickle could not be attributed to variations in K^-driving gradient among the cells. The known stochastic nature of Psickle, together with its heterogeneous distribution as documented here.
among the discocytes, are consistent with the view that the permeability pathway is generated by low-probability, direct or indirect interactions between hemoglobin S polymers and membrane or cytoskeletal components, affecting only a fraction of cells within each deoxy event, as if the number of activated pathways per cell was very small.

References

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