Age Decline in the Activity of the Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} Channel of Human Red Blood Cells

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The Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel of human red blood cells (RBCs) (Gardos channel, hIK1, hSK4) was implicated in the progressive densification of RBCs during normal senescence and in the mechanism of sickle cell dehydration. Saturating RBC Ca\textsuperscript{2+} loads were shown before to induce rapid and homogeneous dehydration, suggesting that Gardos channel capacity was uniform among the RBCs, regardless of age. Using glycated hemoglobin as a reliable RBC age marker, we investigated the age–activity relation of Gardos channels by measuring the mean age of RBC subpopulations exceeding a set high density boundary during dehydration. When K\textsuperscript{+} permeabilization was induced with valinomycin, the oldest and densest cells, which started nearest to the set density boundary, crossed it first, reflecting conservation of the normal age–density distribution pattern during dehydration. However, when Ca\textsuperscript{2+} loads were used to induce maximal K\textsuperscript{+} fluxes via Gardos channels in all RBCs ($F_{\text{max}}$), the youngest RBCs passed the boundary first, ahead of the older RBCs, indicating that Gardos channel $F_{\text{max}}$ was highest in those young RBCs, and that the previously observed appearance of uniform dehydration concealed a substantial degree of age scrambling during the dehydration process. Further analysis of the Gardos channel age–activity relation revealed a monotonic decline in $F_{\text{max}}$ with cell age, with a broad quasi-Gaussian $F_{\text{max}}$ distribution among the RBCs.

INTRODUCTION

A Ca\textsuperscript{2+}-sensitive K\textsuperscript{+} channel (Gardos channel, hIK1, or hSK4) is expressed in the plasma membrane of human red blood cells (RBCs) (Gardos, 1958; Ishii et al., 1997; Vandorpe et al., 1998; Hoffman et al., 2003). At saturating intracellular Ca\textsuperscript{2+} concentrations, Gardos channel activation increases the mean K\textsuperscript{+} flux through the plasma membrane by three orders of magnitude (Gardos, 1958; Simons, 1976; Lew and Ferreira, 1978), causing the loss of an alkaline, hypertonic effluent containing KCl and KOH (Lew and Bookchin, 1986; Freeman et al., 1987). Gardos channel activation plays an important role in the mechanism of sickle cell dehydration (Lew and Bookchin, 2005a) and may also participate in the physiological densification of aging RBCs (Clark, 1988; Lutz, 1988).

Early estimates of the number of Gardos channels per cell described values ranging from 1 to 200, with claims of wide differences among RBC subpopulations (Lew et al., 1982; Grygorczyk and Schwarz, 1983, 1985; Grygorczyk et al., 1984; Alvarez and García-Sancho, 1987; Wolff et al., 1988; Brugnara et al., 1993; Lew et al., 2004, 2005). However, when Ca\textsuperscript{2+} ionophores were used to induce rapid and uniform Ca\textsuperscript{2+} loads (Simonsen et al., 1982), triggering instant and maximal Gardos channel activation in all the cells ($F_{\text{max}}$ at saturating Ca\textsuperscript{2+} loads), the distribution of dehydration rates in the RBC population, as followed by flow cytometry, proceeded to lower mean volumes with no significant variation in standard deviation or appearance of multimodality (Lew et al., 2005).

This suggested uniformity of RBC dehydration rates following maximal Gardos channel activation. The discrepant estimates of the early experiments were attributed to unrecognized differences in intracellular RBC Ca\textsuperscript{2+} concentrations due to cell to cell variations in Ca\textsuperscript{2+} pump-leak balance (Lew et al., 2003, 2005). We use $F_{\text{max}}$ throughout to represent the experimental condition in which saturating Ca\textsuperscript{2+} loads maximally activate the K\textsuperscript{+} fluxes via Gardos channels in each RBC in any given sample of RBCs. Thus defined, $F_{\text{max}}$ equals the $nP_{o}$ product, where $n$ represents the number of active Gardos channels per cell, $P_{o}$ is the open state probability condition of the channels in each cell, and $f$ is the unit channel flux at saturating Ca\textsuperscript{2+} levels, as determined by single channel conductance.

The apparent uniformity of Gardos channel capacity among the RBCs was surprising because it did not reveal the expected decrease in driving force for dehydration among the aging, denser RBCs, which would be seen as a tail of slower dehydrating cells in the migrating Gaussian curves. Age-dependent changes in RBC metabolism, composition, and transport have been well documented (Beutler, 1985; Lutz et al., 1988; Chasis et al., 1989; Mohandas and Groner, 1989; Romero and Romero, 1997; Boas et al., 1998; Lew et al., 2003; Kuypers and de Jong, 2004; Arrese et al., 2005). The unexpected finding that Gardos channel activity appeared uniform,

Abbreviations used in this paper: DEP, diethylphthalate; Hb A1c, glycated hemoglobin; Hct, hematocrit; RBC, human red blood cell.
Regardless of RBC age, was puzzling and prompted a re-evaluation. To further investigate possible age variations in Gardos channel $F_{\text{max}}$ among the cells, we used glycated hemoglobin (Hb A1c) as an age marker for RBCs from normal, nondiabetic subjects (Bookchin and Gallop, 1968; Bunn et al., 1976; Bosch et al., 1992).

MATERIALS AND METHODS

Experimental Design and Procedure

To detect age-related variations in Gardos channel activity among RBCs, we designed experimental conditions in which the RBC dehydration rates were determined by the Gardos channel $F_{\text{max}}$ and by the driving gradient for dehydration in each cell, and the relative ages of the fastest and slowest dehydrating RBCs were assessed by measuring their Hb A1c contents.

Venous blood anticoagulated with EGTA was obtained from healthy donors after informed written consent. The RBCs were harvested above diethylphthalate oil (DEP oil, $\delta = 1.117 \text{ g/ml}$), and spun at 10,000 $g$ for 1 min to separate the RBCs that exceeded that density at each time interval after Gardos channel activation. The RBCs harvested from the pellets and from the top of the oil were analyzed for hemoglobin (Hb) to estimate the proportion of RBCs recovered from each fraction, and for Hb A1c by HPLC as follows.

The Hb concentration in the RBC lysates was determined by light absorption at 415 nm (the Soret band) using a plate reader (Spectramax 190, Molecular Devices). The Hb yields of the density-segregated cell fractions were expressed as a percent of the total Hb in the unfraccionated sample. Hb A1c was measured on code-labeled samples by ion exchange HPLC (Abraham et al., 1984; Bosch et al., 1992) at the Biochemistry Laboratories, Addenbrooke’s Hospital, Cambridge, UK (Project 719) and at Jacobi Medical Center, Bronx, NY, using Variant II instruments (Bio-Rad Laboratories). Reproducibility of the measurements was estimated on 20 aliquots of a single blood sample, whose mean ± standard deviation was 5.71% ± 0.02, giving a coefficient of variation of 0.35%. Thus the intrinsic measurement error of the reported Hb A1c values was within the size of the points in the figures below.

In selected experiments the RBC Na$^+$ content was measured by flame photometry (Corning 455). The cells were washed three times in 10 volumes of isotonic MgCl$_2$ lysed, and diluted in distilled water to the required optimal reading range and measured against standards with similar Na$^+$ concentrations. Additional experiments on valinomycin-treated RBCs are detailed in the Results. The osmotic fragility measurements for the experiments reported in Fig. 3 below were performed as previously described (Lew et al., 1995; Raftos et al., 1996; Raftos et al., 1997). In brief, pairs of 96-well plates were used, one of each pair having a U-shaped well bottom, and the other flat bottomed for optical density measurements. Each row of the U-bottomed plate contained 250 $\mu$l each of 12 lysis media solutions with different osmolarities, ranging from 1.0 to 0.01 relative tonicity (RT) units, prepared by mixing appropriate volumes of one solution containing 149 mM NaCl and 2 mM Na-HEPES (pH 7.5 at 20°C), and a second solution containing only 2 mM of Na-HEPES (pH 7.5 at 20°C). 15 s before each sampling time, 0.5 $\mu$l of cell suspension was transferred to a groove in a plastic incubation tray (Accutran disposable incubation tray, Schleicher & Schuell) at room temperature. At the exact sampling time, a 12-channel pipette (Finnpipette, Lab Systems) was used to deliver 10-$\mu$l samples of the suspension in the incubation tray to the row of 12 wells on a U-bottomed 96-microwell plate containing the 250 $\mu$l of the lysis media, and rapidly mixed by repeated vigorous squirts with the multichannel dispenser. Each timed sample generated a full osmotic fragility curve. To estimate the fraction of hemolysis in each well, the plate was centrifuged for 5 min at 1,500 $g$, 150-$\mu$l samples of the supernatants were transferred with a 12-channel dispenser to the correspondingly labeled row on the flat-bottomed plate. The concentrations of Hb were measured on the SPECTRAMAX plate.
RESULTS

Fig. 1, typical of three similar experiments, reports the time-dependent changes in RBC fraction and Hb A1c content of the RBCs recovered above the DEP oil after the onset of the Ca^{2+} load. As the fraction of RBCs remaining above the oil declined (Fig. 1 A), their Hb A1c fraction increased (Fig. 1 B). Fig. 1 C shows that there was an inverse correlation between the percent Hb A1c and the fraction of cells with density <1.117, indicating that those RBCs slowest to become dense had the highest Hb A1c values.

The results in Fig. 1 are in striking contrast with those expected from the known age-density distribution of RBCs, and from the assumption that RBCs of all ages would have comparable Gardos channel $F_{\text{max}}$ activity, which would have been the simplest explanation of the observed conservation of the volume distribution profile during dehydration (Lew et al., 2005). If that explanation had been valid, then during Gardos channel-mediated dehydration, the RBCs expected to exceed the oil density first would have been the densest and oldest cells nearest to the density boundary (Fig. 2, Predicted).

The observed pattern (Fig. 1) was the opposite; the youngest RBCs became dense first and the oldest RBCs last. These results suggest that RBC Gardos channel activity is not uniform, but rather that it declines with cell age.

A close analysis of the factors influencing the age distribution of Gardos-dehydrating RBCs suggests alternative possibilities. Three main factors determine the speed at which K^+ permeabilization, the RBCs dehydrate and their increasing density is represented by the shift to the right of the Gaussian curves. The standard deviation is shown conserved representing previous experimental results (Bookchin et al., 2000; Lew et al., 2005). Those findings led to the suggestion (Predicted) that dehydration rates were similar in all the RBCs, with conservation of the original RBC age distribution within the migrating Gaussians (Predicted). The present results showed that this pattern is valid only with valinomycin-induced K^+ permeabilization. By contrast, for Gardos channel-mediated K^+ permeabilization, the results suggest that conservation of the standard deviation conceals a substantial degree of age scrambling within the migrating Gaussian curves (Observed), with the younger RBCs passing the older cells that started as most dense in the “Initial condition”; thus the RBCs that first pass the set density boundary are much younger than those reaching it last, reflecting a declining pattern of Gardos $F_{\text{max}}$ with RBC age.
and to cell-to-cell differences in driving gradient for dehydration, not to variations in induced K⁺ permeability among the RBCs.

Valinomycin, a K⁺-selective ionophore (Harris and Pressman, 1967; Mueller and Rudin, 1967), has been extensively used to increase the K⁺ permeability of cell membranes, but for our purposes it was necessary to establish first whether the valinomycin-induced K⁺ permeability was uniform among the RBCs. If it could be shown that valinomycin rapidly equilibrated between treated and untreated RBCs, this would indicate that valinomycin distributes itself rapidly and in equal concentration per unit RBC membrane area, thus generating a uniform K⁺ permeability among the RBCs. To investigate this point, we mixed valinomycin-treated with untreated RBCs, and followed the dehydration response in the mixture using the profile migration method, in which the uniformity of the RBC dehydration is monitored by the extent of slope conservation during the left-shifted displacement of the osmotic fragility curves (Raftos et al., 1996). Fig. 3 A, the control condition, shows the fairly rapid and parallel displacement of the osmotic fragility curves generated by valinomycin addition to a suspension of RBCs in low-K⁺ media, as expected from uniformity of dehydration rates. Whether this uniformity was indeed the result of uniform K⁺ permeabilization was explored in the condition shown in Fig. 3 B. Comparison of the dehydration rates in Fig. 3 (A and B) shows that the ∼50% untreated RBCs in the mixed suspension dehydrated to the same final condition as the control cells at a rate similar to that of the original population (A), indicating a very rapid redistribution of valinomycin between treated and untreated RBCs. The results are representative of two experiments with blood from two donors.
rates result from their initial density distribution or from differences in their driving K⁺ gradients. Fig. 4 A shows that the first RBCs to enter the pellets (filled circles, lower yields) had relatively high Hb A1c contents. These are the fastest dehydrating cells in the presence of valinomycin and must contain a considerable portion of aged RBCs, a trend opposite to that observed with Gardos-dehydrated RBCs (Fig. 1). Comparison of the range of Hb A1c values in Fig. 1 B and Fig. 4 A shows a much narrower variation among the valinomycin-treated cells than among the Ca²⁺-loaded cells, a consistent observation in all the experiments of this series. This indicates a more substantial age mix in the fractions recovered from valinomycin-treated RBCs than from those dehydrated by Gardos channel activation.

The Hb A1c of the RBCs above the oil (open circles) did not differ significantly from the mean values, in any of the three experiments of this series, except for a tiny RBC fraction recovered after maximal dehydration (Fig. 4 A, empty symbol at the lowest yield). The latter RBCs also had the highest Na⁺ content (Fig. 4 B), sufficient to lower the driving gradient to an extent that they would not dehydrate beyond the 1.117 g/ml density boundary. The yield of these high-Na⁺ cells varied from 0.3 to 0.6% in the three experiments. In Fig. 1 B, at the highest yields, the mean RBC Na⁺ content was 7–10 mmol(340 g Hb)⁻¹. At yields from 5 to 25% the mean Na⁺ content was ~10 to 16 mmol(340 g Hb)⁻¹, indicating a minor but progressive increase in mean Na⁺ content with RBC age, and consequent reduction in the driving gradient for dehydration.

These results show that for uniformly K⁺-permeabilized (valinomycin-treated) RBCs, the main determinant of dehydration rate was their initial density, i.e., their initial distance from the set density boundary (Fig. 2, top). There was also a progressive contribution of Na⁺/K⁺ gradient dissipation, slowing the older RBCs’ dehydration and thus generating a large age mix in the density-separated fractions, with reduced variations in Hb A1c. Gradient dissipation became extreme in a minute, high-Na⁺ fraction of cells. Thus, following uniform K⁺ permeabilization with valinomycin, the oldest and densest cells pass the density boundary first, but because of their weaker driving force for dehydration they soon become admixed in the pellets with younger RBCs. Thus in these conditions, dehydration rates are determined by the weak opposing effects of initial density distribution and progressively reduced driving gradient.
whereas with Ca\(^{2+}\)-induced dehydration it is the variation of Gardos channel \(F_{\text{max}}\) among the RBCs that dominates the dehydration rates, generating an inverse age–density distribution (Fig. 2, bottom).

Furthermore, the experimental need of relatively high Hct (10% in our experiments) to harvest sufficient RBCs for measurements of initial and final Hb Alc and [Na\(^+\)] also contributed to the larger age mix seen with valinomycin. In such concentrated cell suspensions, the net KCl loss generates a large rapid increase in the extracellular [K\(^+\)], so that the faster dehydrating RBCs are driven by a higher outward K\(^+\) concentration gradient than those slower to dehydrate. With valinomycin, the two main contributors to the rate at which dehydrating RBCs approach the density boundary are the initial cell density distribution and the strength of the driving gradient. Initial cell density places the older RBCs nearer the density boundary, but the driving gradient of the younger RBCs (which start further from the boundary) is stronger. The younger cells dehydrate faster and the K\(^+\) they release slows the rate at which the older cells approach the boundary, thus further mixing the ages of dehydrating, valinomycin-treated RBCs relative to their original age–density distribution. With Ca\(^{2+}\)-induced dehydration, on the other hand, the rise in extracellular [K\(^+\)] during dehydration amplifies their age differences in Gardos \(F_{\text{max}}\), further slowing the older RBCs’ dehydration rates.

The protocol in Fig. 1 can now be applied to analyze the distribution of Gardos channel \(F_{\text{max}}\) among the RBCs. Fig. 5 A shows the compiled results of three experiments like that in Fig. 1. For a rough estimate of the Gardos channel \(F_{\text{max}}\) distribution, we assume that the time it takes for any RBC to reach the boundary density is determined primarily by its Gardos channel \(F_{\text{max}}\), and that its initial density and the status of its driving gradient have negligible effects. With these assumptions, we can apply the Lew-Bookchin red cell model (Lew and Bookchin, 1986) to simulate the present experimental conditions and compute the time needed for RBCs with different K\(^+\) permeabilities (P\(_K\)) (representing Gardos channel \(F_{\text{max}}\) variations) to attain a density of 1.117 g/ml. The simulation in Fig. 5 B plots P\(_K\) as a function of time. Using “Time” as the parametric variable, and the fit in Fig. 5 B, we can now plot cell frequency as a function of P\(_K\) from the data in Fig. 5 A, and obtain the relation shown in Fig. 5 C. The best fit empirical curve in Fig. 5 C represents the integral of the distribution of Gardos channel \(F_{\text{max}}\) among the RBCs. Its derivative, representing the distribution of Gardos channel \(F_{\text{max}}\) is shown in Fig. 5 D. The dashed line shows the best Gaussian fit of the derivative curve, rendering a mean \(P_K\) of 1.117 g/ml. The simulation in Fig. 5 B shows the best Gaussian fit of the derivative curve, rendering a mean \(P_K\) of 1.117 g/ml, and a coefficient of variation of 37%. Fig. 5 E reports the variation in Hb Alc in the RBCs recovered above the oil at the time intervals shown after the Ca\(^{2+}\) load. Again, using “Time” as the parametric variable for Fig. 5 (B and E) we can plot the percent Hb Alc as a function of P\(_K\) (Fig. 5 F); this reveals an inverse relation between percent Hb Alc and P\(_K\) corresponding to an inverse relation between RBC age and Gardos channel \(F_{\text{max}}\).

With caution, given the simplifying assumptions inherent in this analysis and the unavoidable age mix in the samples, these results show that the Gardos channel \(F_{\text{max}}\) declines monotonically with RBC age (Fig. 5, E and F), and that the \(F_{\text{max}}\) values among the RBCs follows a fairly broad, approximate Gaussian distribution (Fig. 5 D) with a very high mean P\(_K\). It is important to note that even the lowest P\(_K\) value in the oldest RBCs is \(\sim 10^{-1}\), still three to four orders of magnitude above the ground K\(^+\) permeability of the human RBC (Beaugé and Lew, 1977; Lew and Beaugé, 1979).

**DISCUSSION**

The present results show that Gardos channel activity, measured at saturating Ca\(^{2+}\) loads, declines sharply throughout the lifespan of human RBCs. However, these results do not allow us to discriminate between the separate contributions of \(n\) or \(P_o\) to the age decline in Gardos channel \(F_{\text{max}}\). A reduction in \(n\) only is equivalent to a cumulative and progressive all-or-none inactivation of Gardos channels within each cell. Such an all-or-none type of inactivation was observed experimentally with the Ca\(^{2+}\)-Mg\(^{2+}\)-ATPase of isolated RBC membranes exposed to high glucose concentrations (Gonzalez Flecha et al., 1999). These authors showed that glycation of a single essential Lys residue, probably located near the catalytic ATP site of the ATPase, caused full inhibition, whereas those molecules without glycation of the vulnerable Lys residue remained functionally normal. The possible effects of glycation on Gardos channel function have not yet been explored. A reduction in \(P_o\) with cell age may perhaps be inferred from the peculiar temperature response of the Gardos channels observed in excised membrane patches from RBC membranes (Grygorczyk, 1987). At saturating Ca\(^{2+}\) levels, \(P_o\) was shown to fall sharply, by >90%, when the temperature was increased from 30°C to 35°C. On the other hand, when Gardos channel–mediated K\(^+\) fluxes were measured in intact RBCs, the fall in efflux rate constant between 27°C and 37°C was only about half (Hoffman et al., 2003). Hoffman et al. (2003) considered the possibility that this discrepancy in the magnitude of the temperature effect may result from heterogeneity in the response of intact RBC populations. Taken together with the present results, the discrepancy may be explained if what has been detected as a differential temperature effect reflects in fact a decline in \(P_o\) with RBC age. In this interpretation, the \(P_o\) of young RBCs would be high and show little change with temperature. As RBCs age, their channels undergo structural or regulatory
changes that can be exposed experimentally as a progressive decline in $P_o$ at temperatures $>30^\circ C$. Thus, age heterogeneity in the RBC population would generate a mean temperature differential that could mask the extreme high and low $P_o$ values at $37^\circ C$ in young and old RBCs, respectively. This interpretation assumes that the reported patch clamp data were obtained preferentially from aged RBCs with low $P_o$ responses at $35^\circ C$. Further research on excised patches from age-regulated RBCs is needed to investigate whether the temperature effect is related to RBC age and to assess the contribution of $P_o$ to $F_{\text{max}}$ decline.

To the extent that the homeostatic changes in aging RBCs are driven by elevated $[\text{Ca}^{2+}]$, the effects of decline in Gardos channel activity and of the minor reductions in driving force for dehydration, as quantified above, would have negligible effects on age-associated densification. The pattern of $Na^+$ gain in aging RBCs (Fig. 4B) suggests a slow and gradual $Na^+$ gain, with a sharp late increase leading to the formation of the light, high-$Na^+$ cells in a terminal homeostatic condition (Bookchin et al., 2000).

The effect of age-related decline in Gardos channel $F_{\text{max}}$ may be relevant to the pattern of sickle cell dehydration (Lew and Bookchin, 2005). The interaction of the polymers of deoxygenated haemoglobin S with the membrane of sickle cells activates a poorly selective cation permeability pathway, Pickle, with a stochastic distribution of $\text{Ca}^{2+}$ permeabilities in the RBC population in each deoxygenation event (Lew et al., 1997). The variability increased $[\text{Ca}^{2+}]$, activates Gardos channels, triggering RBC dehydration and acidification. In sickle reticulocytes and young sickle cells, which retain a high activity of acid-sensitive KCl cotransporters (Brugnara et al., 1986), Gardos-induced acidification stimulates further KCl-mediated dehydration, segregating sickle cells into slow- and fast-track dehydrating cells, with a predominance of young cells in the densest fraction of sickle cells, rich in irreversible sickle cells (Bookchin et al., 1991; Lew et al., 1991). The age distribution of Gardos channel $F_{\text{max}}$ documented here for normal RBCs reveals extraordinarily high $F_{\text{max}}$ values in young RBCs. Young cells also have the highest driving gradients for dehydration via K$^+$ permeabilization. These features may contribute to the increased vulnerability of young SS cells to dehydration observed by us and others, and they provide an additional mechanism for enhancing the differences between slow- and fast-track dehydrating SS RBCs.

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