Distribution of plasma membrane Ca\textsuperscript{2+} pump activity in normal human red blood cells

Virgilio L. Lew*, Nuala Daw*, Deisy Perdomo*, Zipora Etzion**, Robert M. Bookchin** and Teresa Tiffert*

*Physiological Laboratory, University of Cambridge, Downing Street, Cambridge CB2 3EG, UK, and **Department of Medicine, Albert Einstein College of Medicine, Bronx, NY 10461, USA

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Corresponding author:
Dr V. L. Lew
Department of Physiology
University of Cambridge
Downing Street
Cambridge CB2 3EG, UK

Telephone: +44-(0)1223-333830
Facsimile: +44-(0)1223-333840
E-mail: VLL1@cam.ac.uk

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Abstract

The plasma membrane calcium pump (PMCA) is the only active Ca\textsuperscript{2+} transporter in human red blood cells (RBCs). Previous measurements of maximal Ca\textsuperscript{2+} extrusion rates ($V_{\text{max}}$) reported only mean values in the RBC population. Despite early evidence for differences in Ca\textsuperscript{2+} extrusion capacity among RBCs, the precise $V_{\text{max}}$ distribution remained unknown. It was important to characterize this distribution to assess the range and modality (uni- or multimodal) of PMCA $V_{\text{max}}$ variation and the likelihood of RBCs with elevated [Ca\textsuperscript{2+}], in the circulation participating in physiological and pathological processes. We report here the application of a new method to investigate the detailed distribution of PMCA $V_{\text{max}}$ activity in RBCs. The migrating profile of osmotic lysis curves was used to identify and quantify the fraction of cells which extrude a uniform Ca\textsuperscript{2+} load at different rates. The results revealed that RBCs from single donors have large variations in PMCA activity which follow a unimodal, broad distribution pattern consistently skewed towards higher $V_{\text{max}}$ values, suggesting an excess of cells with $V_{\text{max}}$ higher than the mean value. The method applied may provide a way of evaluating whether the observed variation in PMCA $V_{\text{max}}$ is related to cell age.
Introduction

The plasma membrane Ca\(^{2+}\) pump (PMCA), in its various isoforms, is expressed in all animal cells, where it plays a major role in the control of resting intracellular free Ca\(^{2+}\) levels ([Ca\(^{2+}\)]\(_i\)) \(^{1-4}\). The PMCA was originally discovered and extensively studied in human red blood cells (RBCs) in which it is the only active Ca\(^{2+}\) extrusion transporter. The maximal Ca\(^{2+}\) transport capacity (\(V_{\text{max}}\)) of the PMCA in human RBCs (\(\sim 10 \text{ mmol}(340 \text{ g Hb})^{-1} \text{ h}^{-1}\)) is high compared with the normal pump-leak turnover rate of Ca\(^{2+}\) (\(\sim 50 \mu\text{mol}(340 \text{ g Hb})^{-1} \text{ h}^{-1}\)) \(^3\). Although early work indicated that Ca\(^{2+}\) pump activity may vary from cell to cell over a much broader range than any other known hematological parameter \(^5\), available measurements of PMCA \(V_{\text{max}}\) in intact RBCs always reported mean values in the RBC population \(^6\); the actual distribution of PMCA \(V_{\text{max}}\) activity among RBCs was unknown. The aim of the present work was to characterize the distribution of PMCA \(V_{\text{max}}\) activity in normal human RBCs.

It is important to ascertain the extent of PMCA \(V_{\text{max}}\) variation among RBCs because differences in pump-leak [Ca\(^{2+}\)]\(_i\) levels may determine the degree of vulnerability of individual cells to certain physiological and pathological stresses. Human RBCs express in their plasma membrane a Ca\(^{2+}\)-sensitive K\(^+\) channel (the “Gardos” channel, \(^7-9\)). Hoffman et al., \(^10\) recently showed that the isoform hSK4 represents the small conductance Gardos channel of human RBCs. In conditions with elevated [Ca\(^{2+}\)]\(_i\), Gardos channel activation causes rapid net loss of KCl and water, leading to irreversible cell dehydration. Elevated [Ca\(^{2+}\)]\(_i\), has also been implicated in the activation of cell proteases and in the cross-linking of cytoskeletal proteins, with cumulative effects on cell ageing and viability \(^11-15\).

The first indirect evidence of substantial cell-to-cell variations in PMCA \(V_{\text{max}}\) arose when Tiffert et al. \(^16\) examined the dehydration response of RBCs uniformly permeabilized to Ca\(^{2+}\) with the ionophore A23187. As Ca\(^{2+}\) influx into RBCs was progressively increased by increasing the ionophore concentration in a stepwise manner, RBCs suspended in plasma-like media dehydrated following an all-or-none rather than a graded pattern: instead of all cells dehydrating at progressively faster rates - as expected with a uniform response - only a fraction of RBCs dehydrated, and this fraction increased with increasing Ca\(^{2+}\) influx. Further experiments showed that Gardos channel activity among the RBCs was fairly uniform and
could not have accounted for this all-or-none pattern. These results suggested that uniform Ca\textsuperscript{2+} permeabilization of RBCs did not generate uniformly elevated [Ca\textsuperscript{2+}], among the cells, and that the differences arose from variations in Ca\textsuperscript{2+} pump activity.

Further evidence for marked variations in Ca\textsuperscript{2+} pump activity among RBCs from single donors was provided by Garcia-Sancho and Lew who detected large differences in the total Ca\textsuperscript{2+} content of RBCs ([Ca\textsubscript{T}]) uniformly permeabilized to Ca\textsuperscript{2+} with A23187. As Ca\textsuperscript{2+} influx increased, so did the fraction of high-Ca\textsuperscript{2+} containing cells, indicating a gradual variation of some property among the RBCs rather than a sharp distinction between subpopulations. This unexpected behavior suggested that the measured total Ca\textsuperscript{2+} content of Ca\textsuperscript{2+}-permeabilized RBCs did not represent a uniform Ca\textsuperscript{2+} content, but rather the mean of extremely heterogeneous distributions. To interpret this phenomenon, Garcia-Sancho and Lew proposed a relatively wide distribution of PMCA $V_{\text{max}}$ activities among RBCs: the fraction of cells with $V_{\text{max}}$ below the set Ca\textsuperscript{2+} influx would gain Ca\textsuperscript{2+} continuously, while their pumps ran at the Ca\textsuperscript{2+}-saturated $V_{\text{max}}$ rate, consuming cell ATP. In those cells in which the $V_{\text{max}}$ of the pump exceeded the glycolytic capacity, ATP-depletion would eventually inhibit the pump and [Ca\textsuperscript{2+}], would approach the Ca\textsuperscript{2+} equilibrium distribution determined by the ionophore, with very high [Ca\textsubscript{T}]. On the other hand, in those RBCs with a pump $V_{\text{max}}$ higher than the set Ca\textsuperscript{2+} influx and with a glycolytic capacity to match ATP consumption by the pump, pump-leak balance would maintain [Ca\textsuperscript{2+}], at levels below pump saturation, in the micromolar or sub-micromolar range. More recent work provided additional evidence for $V_{\text{max}}$ differences among RBCs.

However, the actual distribution of PMCA $V_{\text{max}}$ among RBCs has never been directly investigated.

We report here an investigation of the distribution of PMCA $V_{\text{max}}$ in normal RBCs, using a new method developed ad hoc. The results exposed a broad unimodal variation of Ca\textsuperscript{2+} pump activity among RBCs from single donors, with a large coefficient of variation and a right skewed $V_{\text{max}}$ distribution. The significance of such a distribution is discussed.
Methods

Experimental design

Finding and characterizing RBCs which differ in the speed at which they can pump out an ionophore-induced uniform Ca\textsuperscript{2+} load\textsuperscript{20} requires a procedure to detect cells with different Ca\textsuperscript{2+} contents during the Ca\textsuperscript{2+}-extrusion process. We designed a method based on the property of RBCs with elevated [Ca\textsuperscript{2+}], to become rapidly dehydrated. The RBC components which participate in Ca\textsuperscript{2+}-induced dehydration are illustrated in Fig 1 (modified from Tiffert et al.,\textsuperscript{22}). The PMCA $V_{\text{max}}$ distribution method was based on the “Ca\textsuperscript{2+} load-extrusion” protocol designed by Dagher and Lew to measure the mean PMCA $V_{\text{max}}$ in RBCs\textsuperscript{6}. This is illustrated in the computer simulations of Fig 2a.

To detect $V_{\text{max}}$ heterogeneity and characterize its distribution, the load-extrusion protocol in Fig 2a was used as follows: At the points A to E on the model-generated Ca\textsuperscript{2+} load-extrusion curve, RBCs are sampled and delivered into a low-K\textsuperscript{+} medium (“LK” medium, see Solutions) consisting of a large volume of ice-cold, iso-osmotic buffer containing EGTA and no added Ca\textsuperscript{2+}, to prevent any Ca\textsuperscript{2+} gains; 1 mM vanadate to ensure complete pump inhibition; low-K\textsuperscript{+}; and 10 mM SCN\textsuperscript{-} to allow rapid dehydration of the cells with elevated Ca\textsuperscript{2+}. This medium instantly arrests Ca\textsuperscript{2+} fluxes mediated by ionophore and pump, so that each RBC retains the Ca\textsuperscript{2+} it contained at the time of sampling, which determines whether or not it will dehydrate.

When Gardos channels are fully activated at non-limiting anion permeabilities, full dehydration proceeds without profile changes in the haemolysis curves indicating that the distribution of Gardos channel-mediated dehydration capacity is fairly uniform among RBCs\textsuperscript{17}. Thus, any alterations in the profile of haemolysis curves from cells actively extruding a Ca\textsuperscript{2+} load could be attributed to differences in Ca\textsuperscript{2+} pump extrusion capacity among the cells. Let us assume provisionally that maximal Gardos channel activation is attained when [Ca\textsubscript{T}], exceeds 20 μmol (340 g Hb\textsuperscript{-1}, as indicated by the dashed horizontal line in Fig 2a. Then all RBCs with [Ca\textsubscript{T}] > 20 μmol (340 g Hb\textsuperscript{-1} will dehydrate maximally, whereas those with lower [Ca\textsubscript{T}] will dehydrate partially or not at all. If we induce Ca\textsuperscript{2+} loads much higher than the Gardos channel deactivation threshold, the time required to pump out the sub-threshold Ca\textsuperscript{2+} becomes
negligible in relation to the time required to pump out the full Ca\textsuperscript{2+} load, justifying the assumption that non-dehydrating cells have practically emptied their Ca\textsuperscript{2+} loads. After a brief incubation at 4°C in LK medium to allow the cells containing supra-threshold \([\text{Ca}\textsubscript{T}]_i\) to dehydrate maximally, the tubes are spun, most of the supernatant is discarded and the cell pellets are resuspended in the remaining medium for hemolysis curve sampling.

The predicted results were obtained in preliminary experiments whose real hemolysis curves, from samples taken at the times A to E in Fig 2a, are shown in Fig 2b. With samples A and E we see normal hemolysis curves since all the cells would have been essentially Ca\textsuperscript{2+}-free; in A, because they never experienced a Ca\textsuperscript{2+} load, and in E because the Ca\textsuperscript{2+} load had been fully extruded, even from the slowest pumping cells. The identity of the two curves rules out any significant effects of the Ca\textsuperscript{2+} loads implemented here on the critical haemolytic volume of normal RBC populations, as might have been expected from Ca\textsuperscript{2+}-induced membrane area losses \(^{23}\). Sample B gives the hemolysis curve corresponding to full dehydration of all the cells: Because RBCs dehydrated by net salt loss need to gain much more water than normal-volume cells to swell to their critical hemolytic volume, the curve is deeply left-shifted towards low relative tonicities. In samples C and D, variable fractions of high-\(V_{\text{max}}\) cells did not dehydrate since they had pumped out nearly all their Ca\textsuperscript{2+} at the time of sampling, whereas the slower pumping cells, retaining over 20 \(\mu\text{mol (340 g Hb)}^{-1}\) of \([\text{Ca}\textsubscript{T}]_i\), became fully dehydrated; the resulting lysis curves C and D are intermediate (between A and B) with an inflected, quasi-plateau region, indicating the development of a marked bimodality in the distribution of hydration states in the RBC population. As progressively more cells pump out their Ca\textsuperscript{2+} loads, the mixed lysis curves with the plateau pattern would migrate upwards towards the fully recovered pattern of sample E. The fraction of cells which emptied their Ca\textsuperscript{2+} loads at each sampling time may be estimated from the Y-intercept values read near the mid-point of the quasi-plateau region of the hemolysis curves. An example is shown in Fig 2b by the dashed vertical line at a relative tonicity of 0.3. The important consideration in the choice of relative tonicity is that the analysis to be applied should be largely independent of the value chosen to represent the plateau. In the experiments to be reported below the results agreed within a margin of ± 3% when the choice of relative tonicity for the plateau-Y intercepts was within the range 0.25 to 0.40. To estimate the percent cells with fully extruded Ca\textsuperscript{2+} loads at each sampling time (\(f_X\)) we must first define the 100% value. We use the ordinate values (Y)
at the intersection of curves A (or E) and B with the dashed vertical line to define $Y_A$ and $Y_B$. The full lysis curve migration interval (100%) is $(Y_A - Y_B)$. The percent cells with emptied Ca$^{2+}$ loads at the time of sample C ($f_C$) may be estimated from the ordinate value of curve C at a relative tonicity of 0.3 ($Y_C$), as follows: $f_C = 100 \frac{(Y_C - Y_B)}{(Y_A - Y_B)}$. And for D, $f_D = 100 \frac{(Y_D - Y_B)}{(Y_A - Y_B)}$.

Rapid sampling for lysis curves during the Ca$^{2+}$ extrusion phase of the load-extrusion protocol could thus provide a detailed characterization of $f_X$ as a function of the time interval between Co$^{2+}$ addition and emptying of the Ca$^{2+}$ load, $\Delta t_X$. This function is the integral of the distribution of PMCA $V_{\text{max}}$ values in the RBC population since for each $f_X$ the corresponding $V_{\text{max}}$ is computed from $(V_{\text{max}})_X = \frac{(\text{Ca}^{2+}\text{ load})}{\Delta t_X}$, where $\Delta t_X$ is the time interval between Co$^{2+}$ addition and sampling for X.

Solutions

The two basic solutions used (to which specific additions were made, as noted) were (i) "HK" (high-K$^+$) containing, in mM: KCl, 80; NaCl, 70; HEPES-Na, pH 7.5 at 37° C, 10, and MgCl$_2$, 0.15; and (ii) "LK" (low-K$^+$): NaCl, 140; NaSCN, 10; HEPES-Na, pH 7.5 at 37° C, 10, and MgCl$_2$, 0.15. Unless specified otherwise, the final concentrations of the most frequently added solutes was (in mM): EGTA, 0.1; CaCl$_2$, 0.14; inosine, 5; sodium orthovanadate, 1; CoCl$_2$, 0.4 in HK medium and 0.1 in LK medium; ionophore A23187 (from 2 mM stock in ethanol), 0.01. When radioactive $^{45}$Ca was used, the specific activity was set between $10^7$ and $2 \times 10^8$ cpm/µmol. All additions were done from stock solutions at least 100-fold more concentrated than in the final solutions or cell suspensions.

Preparation of cells

Venous blood was drawn from healthy volunteers into heparinized syringes after obtaining informed, written consent. RBCs were washed twice by centrifugation and resuspension in large volumes of ice-cold HK solution with added EGTA, and thrice more with solution HK alone. The buffy coat containing platelets and white cells was removed after each wash. After the last wash, the cells were suspended at 10% Hct in medium HK supplemented with inosine
and $^{45}$Ca(Ca$^{2+}$) or Ca$^{2+}$, as described in Figs 3 and 4, and incubated for about ten minutes at 37°C for temperature equilibration, before applying the load-extrusion protocol with the specific modalities described in the figure legends.

**Experimental Protocols**

Two sets of experiments were performed with slightly different protocols. The first set of experiments was designed to test the assumptions implicit in the design of the $V_{\text{max}}$ distribution method by comparing the release of Hb and $^{45}$Ca induced by hypotonic lysis (Fig 3 and Tables 1 and 2). The second set of experiments aimed to characterize the distribution of PMCA $V_{\text{max}}$ values among RBCs from normal subjects.

For the first set of experiments (Protocol 1, Fig 3) washed RBCs were suspended at 10 % Hct in medium HK, supplemented with inosine and high-specific activity $^{45}$Ca(Ca$^{2+}$) (2 x 10$^8$ cpm/µmol) and incubated for about ten minutes at 37°C for temperature equilibration. The ionophore A23187 was added at $t = 0$ followed two minutes later by CoCl$_2$. Samples (0.5 ml) were drawn once before ionophore addition, and twice or thrice after ionophore, and just before cobalt addition, to estimate the size of the Ca$^{2+}$ load. After cobalt, samples drawn every 30 seconds were delivered into centrifuge tubes containing 12 ml of ice-cold LK solution with added EGTA, and vanadate to inhibit the Ca$^{2+}$ pump (preliminary experiments had shown that exposure of the RBCs to 1 mM vanadate at 0-4°C produced irreversible and near complete Ca$^{2+}$ pump inhibition). In this medium the Ca$^{2+}$ content of the RBCs at the time of sampling was maintained, since low temperature, vanadate and intracellular cobalt prevented Ca$^{2+}$ loss through the ionophore or extrusion by the pump. After ~ 30 min incubation in the ice-bath, to allow maximal dehydration by the Ca$^{2+}$-containing cells, the RBCs were washed once by centrifugation and resuspension in 10 – 12 ml of LK+EGTA medium, and the cell pellet was finally resuspended in 1 ml LK+EGTA. The wash and dilution reduced external $^{45}$Ca to near background and rendered ~ 1 ml of a 5 % Hct suspension for each sample, whose intracellular $^{45}$Ca distribution was maintained from the time of sampling. Of this, 0.1 ml were centrifuged for 15 s in 1.5 ml microfuge tubes, and after discarding the supernatant the cell pellet was resuspended in 0.6 ml of 6 % trichloroacetic acid (TCA) and centrifuged again. From the clear TCA supernatant, 0.5 ml was taken for scintillation counting of $^{45}$Ca to estimate [Ca$_{\text{r}}$], (Fig 3a).
The remaining 0.9 ml of each sample were used for osmotic lysis curves (see below), after which the Hb and $^{45}$Ca$^{2+}$ released by hypotonic lysis was determined for each of the 12 different microwells.

**Osmotic lysis curves:** A solution containing 155 mM NaCl and 2 mM HEPES Na, pH 7.5 at room temperature, iso-osmotic with the HK and LK solutions, was diluted with a medium containing only 2 mM HEPES-Na to render 12 “lysis” solutions with the relative tonicities (RT) indicated in the figures. 250 µl of each of these lysis solutions were delivered into each of the eight wells of columns 1 to 12 of 96 x round-bottom microwell plates. 0.5 to 0.7 ml of the 0.9 ml 5% Hct sample were placed in a grooved container and, using a multichannel pipette, 10 µl were delivered and mixed into each of the 12 wells of the assigned row so that each sample provided a complete lysis curve. The round-bottom plates were centrifuged at 1950 rpm x 5 min, and 150 µl of the cell-free supernatant from each well were transferred to corresponding wells on 96 x flat-bottom microwell plates for measurement of Hb concentrations by light absorbance at 415 nm (Soret band) using a microplate reader (Molecular Devices, Sunnyvale, CA). The absorbance in the 2 mM HEPES-Na buffer (relative tonicity = “0”) was taken as 100 % lysis, and the readings at the higher tonicities were referred to it. After the Hb measurement, 130 µl of 11 % trichloroacetic acid (TCA) were added to each well, the plate was centrifuged to sediment denatured protein, and 200 µl of the clear TCA supernatant from each well were transferred to scintillation vials to measure the $^{45}$Ca released by lysis. These measurements provided a direct comparison of the Hb and Ca$^{2+}$ released from the RBCs at each relative tonicity and at each sampling time.

The second set of experiments (Protocol 2, Fig 4) differed from the first in that the original cell suspension was divided in two, one for measuring the mean $V_{\text{max}}$ of the PMCA by the Dagher-Lew method using low specific activity $^{45}$Ca(Ca$^{2+}$) (Fig 4a), the other for measuring lysis curve migration with high time-resolution in tracer-free conditions (Fig 4b). Except for the presence of tracer $^{45}$Ca in the aliquot for measurement of the mean $V_{\text{max}}$, the load extrusion protocol was the same for both suspensions. Samples for lysis curves (0.5 ml) from the tracer-free suspension were drawn at 10 second intervals after cobalt addition and processed as described above, except for the TCA treatment.
Results

We first tested the assumptions made for the experimental design shown in Fig 2: (i) that starting from a high, saturating and uniform Ca$^{2+}$ load, cell-to-cell differences in Ca$^{2+}$ pumping rates would generate a progressive heterogeneity of Ca$^{2+}$ contents among the RBCs; (ii) that deactivation of the Gardos channels would occur when [Ca$_{\text{T}}$]$_i$ fell below $\sim 20 \, \mu\text{mol}(340 \, \text{g Hb})^{-1}$ (dashed line in Fig 2a), and (iii), that the vertical migration of the quasi plateau region of hemolysis curves (Fig 2b) reports the progressive fraction of RBCs that have fully extruded the imposed Ca$^{2+}$ load.

A typical result is shown in Fig 3 and Tables 1 and 2. During the load extrusion protocol, parallel samples were taken at the indicated times to estimate the mean PMCA $V_{\text{max}}$ from the initial slope of the Ca$^{2+}$ extrusion curve after cobalt addition ($\sim 10 \, \text{mmol}(340 \, \text{g Hb})^{-1}(\text{h})^{-1}$, Fig 3a). Apparent Ca$^{2+}$ desaturation of the pump began after 4.5 min, when the mean [Ca$_{\text{T}}$]$_i$ was below $73 \, \mu\text{mol}(340 \, \text{g Hb})^{-1}$.

The losses of Hb and Ca$^{2+}$ induced by hypotonic lysis (Table 1 and Fig 3b) were compared for four selected samples, taken between 4 and 5.5 min of ionophore addition (2 and 3.5 min after addition of cobalt). With the 4 min sample (as with earlier samples taken after cobalt - not shown) the Hb and Ca$^{2+}$ release curves had very similar patterns (Fig 3b, open and solid circles, respectively). But the following three samples showed a progressive divergence between the Ca$^{2+}$ release curves and the Hb release curves. The change in Hb release pattern shows that the proportion of dehydrated cells decreased progressively from over 80 % in the 4 min sample to less than 20 % in the 5.5 min sample. In contrast, the $^{45}$Ca release pattern remained essentially unchanged; over 80 % of the Ca$^{2+}$ was released at relative tonicities below 0.20. Because dehydrated cells lyse only at these low relative tonicities, the Ca$^{2+}$ release pattern indicates that in all samples, over 80 % of the mean [Ca$_{\text{T}}$]$_i$ was always contained within cells that dehydrated during post-incubation. Thus, the increasing fraction of RBCs that failed to dehydrate during post-incubation in LK medium always contained less than 20 % of the RBCs' mean total Ca$^{2+}$ content.

These findings indicate that during PMCA-mediated Ca$^{2+}$ extrusion of a uniform Ca$^{2+}$ load, a
large and progressive heterogeneity of Ca$^{2+}$ contents is generated among the RBCs, which must be attributed to cell-to-cell differences in PMCA $V_{\text{max}}$.

In Fig 3c, the fractions of Hb and Ca$^{2+}$ released at each relative tonicity are compared directly. The progressive departures from single linearity expose developing heterogeneities of Ca$^{2+}$ content. At 4.5 min and later, with the emergence of low-[Ca$_T$] cells, the curves show two distinct slopes reflecting the progressive dissociation between lysis-induced Hb and Ca$^{2+}$ release. It can be seen that the increase in fractional Hb loss with time (steep slope component) is contributed by RBCs which contain less than 20% of the mean-[Ca$_T$] in the cell population.

Two additional samples in Fig 3c (dashed lines) were from cells post-incubated in HK media, so that they retained their original volume distribution from before the experiment. In these samples, as with all HK post-incubated RBCs, regardless of the sampling time during the load-extrusion protocol, the points fell within 5% of the unit slope line, indicating that when the original volume distribution of RBCs was retained during the load-extrusion protocol and subsequent sample processing, cells with different PMCA $V_{\text{max}}$ and [Ca$_T$]$_i$ remained evenly distributed in the cell population and cannot be distinguished by any of the properties that determine the normal lysis curve distribution, such as cell volume, area, Hb and total osmolyte content.$^{5,24}$ A slope of one was also obtained from samples drawn just before cobalt addition. Though not unexpected, since the $^{45}$Ca distribution was uniform at that stage, the result is important because it confirms an important assumption, that hypotonic swelling of cells to pre-lytic states, as must take place within all the microwells with hypotonic media, does not increase their Ca$^{2+}$ permeability.$^{18}$

The [Ca$_T$]$_i$ level at which Gardos channels deactivate was estimated from the analysis in Table 2 (from selected data in Table 1). Note that for each of the time points of partial Ca$^{2+}$ extrusion in Table 2 when the mean RBC [Ca$_T$]$_i$ was 73, 39 and 17 $\mu$mol(340 g Hb)$^{-1}$, and that of the dehydrated cells was 114, 95 and 78, respectively, the [Ca$_T$]$_i$ within the cells that did not dehydrate was 15, 6.2 and 2.7 $\mu$mol(340 g Hb)$^{-1}$. Thus, Gardos channel deactivation always occurred when [Ca$_T$]$_i$ fell below this chosen boundary of 20 $\mu$mol(340 g Hb)$^{-1}$. The results also document the large differences of Ca$^{2+}$ contents generated shortly after the initiation of net Ca$^{2+}$ extrusion which result from cell-to-cell variations in PMCA $V_{\text{max}}$. 
The detailed distribution of PMCA $V_{\text{max}}$ was investigated in RBCs from four donors, in five experiments which rendered very similar patterns (compiled in Table 3). Fig 4 shows a typical experiment in which the mean PMCA $V_{\text{max}}$, measured by linear regression through the first six time points after Co$^{2+}$ addition, was 12.8 mmol(340 g Hb)$^{-1}$h$^{-1}$ (Fig 4a). After the mean RBC $[\text{Ca}_T]$ fell below $\sim 100 \mu$mol(340 g Hb)$^{-1}$ the Ca$^{2+}$ extrusion rate dropped progressively. During Ca$^{2+}$ extrusion, frequent sampling for hemolysis curves gave the patterns shown in Fig 4b. Beginning about one minute after Co$^{2+}$ addition, there was a progressive increase in the fraction of RBCs which failed to dehydrate during post-incubation in low-$K^+$ media, indicating that they had pumped out their initial Ca$^{2+}$ loads to levels below those which activate the Gardos channels. The increase in the fraction of such cells was assessed by the upward progression of the quasi-plateau portions of the lysis curves (see Methods). The result is shown in Fig 4c, where the percent calcium-emptied cells, estimated from the readings at a relative tonicity of 0.3, was plotted as a function of time after Co$^{2+}$ addition. The experimental points were well fitted by a sigmoid, saturation-like curve (see legend of Fig 4c). About 90% of the RBCs emptied their Ca$^{2+}$ loads within 3 min of Co$^{2+}$ addition, while $\sim$10% took longer to complete Ca$^{2+}$ extrusion.

Fig 4d (solid line) shows the derivative of the curve-fit in Fig 4c, estimated by dividing the Ca$^{2+}$ load by the time interval between Co$^{2+}$ addition and Ca$^{2+}$ emptying, plotted as a function of $V_{\text{max}}$. This represents the distribution of PMCA $V_{\text{max}}$ activities among the RBCs.

The results show a broad distribution of PMCA $V_{\text{max}}$ values with a right skewed appearance. A symmetrical Gaussian distribution curve (Fig 4d, dotted line) was superimposed on the rising branch of the $V_{\text{max}}$ distribution to highlight its skewed nature. $V_{\text{max}}$ values varied about seven-fold in this experiment, between 5 and 35 mmol(340 g Hb)$^{-1}$h$^{-1}$, with mean and standard deviation of 12.9 and 5.9 mmol(340 g Hb)$^{-1}$h$^{-1}$, respectively, and a positive skew of 1.55. The mean $V_{\text{max}}$ was higher than the mode, as expected from the right skew of the distribution. In all five experiments of this series the $V_{\text{max}}$ distribution was unimodal, broad and positively skewed, with an identical pattern, and a range of $V_{\text{max}}$ variation similar to that shown for the experiment of Fig 4 (six- to nine-fold).
Discussion

The results presented here provide the first detailed description of the distribution of PMCA \( V_{\text{max}} \) activity in normal human RBCs. This distribution characterizes the differences in \( \text{Ca}^{2+} \) extrusion capacity amongst RBCs but carries no information on whether these differences result from disparities in the number of pumps, in the fraction of active pumps, in the factors controlling the turnover rate of the pumps in each cell, in a differential response of the two pump isoforms, or in a combination of these.

Earlier findings had indicated the existence of differences in \( \text{Ca}^{2+} \) pumping rate among RBC subpopulations, but the actual pattern and extent of variation was unknown. The present results show that there is a large variation in PMCA \( V_{\text{max}} \) among RBCs from single donors, and that this variation follows a unimodal, broad distribution pattern with a consistent positive skewness towards higher \( V_{\text{max}} \) values, suggesting an excess of cells with \( V_{\text{max}} \) higher than the mean value. The apparent range of \( V_{\text{max}} \) variation measured in the five experiments of this series was six- to nine-fold. However, it should be noted that, whereas the method can detect the early upward deflections in hemolysis curve profiles with high sensitivity (Figs 4b and 4c), the resolution in the late periods, when nearly all cells have emptied their \( \text{Ca}^{2+} \) loads, is less precise. Thus, the possibility cannot be ruled out that there are some RBCs with \( V_{\text{max}} \) values much lower than those apparent from the indicated range.

It is important to rule out a potential artifactual origin of the skewed distribution. Differences in PMCA \( V_{\text{max}} \) among RBCs may result in uneven ionophore -induced \( \text{Ca}^{2+} \) loads. The cells with the highest \( V_{\text{max}} \) would be expected to sustain a pump-leak balance with \([\text{Ca}_T]\), below the measured mean load. The early emptying of their \( \text{Ca}^{2+} \) load could result not only from high \( V_{\text{max}} \) values, but also from lower \( \text{Ca}^{2+} \) loads. Using the mean \( \text{Ca}^{2+} \) load to calculate their \( V_{\text{max}} \) could then overestimate their true \( V_{\text{max}} \) values, thus creating a false right-shift in the \( V_{\text{max}} \) distribution. However, Tiffert and Lew 25 (their Fig 8) showed that at the high ionophore and \([\text{Ca}^{2+}]_o\) concentrations used in the present experiments, the difference between measured mean \( \text{Ca}^{2+} \) loads in the presence and absence of 1 mM vanadate was negligible (this finding was repeatedly confirmed in other experiments, not shown). Vanadate at this concentration inhibited over 99.5 % of PMCA-mediated \( \text{Ca}^{2+} \) fluxes. Therefore, the differences in \( \text{Ca}^{2+} \) load
amongst RBCs must have been minimal and cannot account for the large positive skew observed here.

The three main properties of the PMCA $V_{\text{max}}$ distribution documented here are unimodality, wide spread and right-shifted skew. Unimodality indicates that the variations in PMCA $V_{\text{max}}$ in normal human RBCs are smooth and continuous, thus ruling out distinct cell subpopulations with large differences in $V_{\text{max}}$. The two isoforms of the PMCA known to be expressed in human RBCs may contribute to the observed variation in Ca$^{2+}$ extrusion capacity among the cells. The unimodal pattern imposes constraints on this potential contribution, but does not provide any information about possible functional differences between the two isoforms.

The question arises of how the large spread and positive skew of the PMCA $V_{\text{max}}$ distribution compares with those of other hematological and transport parameters of RBCs. Thus far, only a few constitutive and functional RBC properties have been examined for population variations. RBC volume, osmotic fragility, Hb content and Hb concentration all follow symmetrical Gaussian distributions, with coefficients of variation (in percent) of 13.7, 5.4, 12.5 and 7.0, respectively. RBC membrane transporters, whether highly expressed (anion exchanger - $1.1 \times 10^6$ per cell) or weakly expressed (Gardos channels, 100-200 per cell), also appear to have parameter distributions with coefficients of variation of less than 7%, indicating relative uniformity. On the other hand, prostaglandin E$_2$ was reported to induce Ca$^{2+}$-dependent Gardos channel activation, but only in about 15% of the RBCs, suggesting that receptor expression occurs in selected RBC subpopulations. Against this background, the PMCA $V_{\text{max}}$ distribution shows an unprecedented spread, with a coefficient of variation of about 50% (range of 45 to 53% in the five experiments (Table 3), 46% in the experiment of Fig 4), and a positive skew, unique amongst the distributions investigated.

What might be the origin of this unusual distribution? Does it represent a static, constitutive feature of mature RBC populations, generated during normal “production line” erythropoiesis, or is it a dynamic, age-related property, with an age-declining pattern? In other words, are individual RBCs “born” with the $V_{\text{max}}$ activity they will sustain throughout their ~120 days in the circulation, or does PMCA $V_{\text{max}}$ decline with age? At present, there is no conclusive evidence to distinguish between these two alternatives, and results of earlier experiments
testing variations in Ca$^{2+}$ pump $V_{\text{max}}$ in density-fractionated RBCs were conflicting $^{21,33,34}$.

If PMCA $V_{\text{max}}$ does decline with RBC age, the pattern would appear to differ from that of most other RBC transporters. In the transition from reticulocyte to mature RBC, the ion transport mediated by the sodium pump and the KCC symport become substantially reduced within the first two-to-three days in the circulation $^{35-39}$. On the other hand, there is no evidence of early decline in PMCA activity from reticulocyte to mature RBC $^{40}$. In the context of the age-decline hypothesis, the broad PMCA $V_{\text{max}}$ variation pattern points towards a progressive fall in pump activity throughout the life of the mature cell. The possible mechanism of $V_{\text{max}}$ decline with cell age, such as progressive protease-induced falls in viable pump units per cell $^{41}$, metabolically-linked $^{15,34}$, progressive glycation $^{42}$, or other, remains speculative. The method applied here to study the distribution of PMCA $V_{\text{max}}$, by enabling the segregation of RBCs with different $V_{\text{max}}$ values, may provide a way to investigate the possible age-$V_{\text{max}}$ relation more directly in the future.

A final consideration arising from the present results concerns the reduction in Ca$^{2+}$ extrusion rate at relatively high mean [Ca$_T$], levels, seen during the Ca$^{2+}$ extrusion phase of the load-extrusion protocol (Figs 3a and 4a). This pattern has been interpreted in the past as reflecting desaturation of Ca$^{2+}$ by the PMCA, and was used to derive kinetic parameters of the pump $^{21,43}$. As explained above in the analysis of Fig 2a, the wide PMCA $V_{\text{max}}$ distribution documented here (Fig 4d) fully accounts for the observed apparent Ca$^{2+}$ desaturation pattern which starts at high mean [Ca$_T$], levels. Thus, apparent desaturation at high [Ca$_T$], is an indicator of the presence of low-$V_{\text{max}}$ RBCs, and carries no information on the kinetics of PMCA activation by [Ca$^{2+}$]$_i$.

Acknowledgements
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Legends of Figures

Fig 1. Diagram of the RBC transport systems which participate in Ca\(^{2+}\)-induced dehydration.

The upper part of the figure shows the transporters of Ca\(^{2+}\), Cl\(^{-}\) and H\(^{+}\) responsible for the net gain of CaCl\(_2\) by RBCs when exposed to the ionophore A23187. With RBCs suspended in a Ca\(^{2+}\)-containing, plasma-like medium, addition of the ionophore A23187 triggers an electroneutral entry of Ca\(^{2+}\) in exchange for protons, with a Ca\(^{2+}\):2H\(^{+}\) stoichiometry. With CO\(_2\) concentrations at equilibrium across the RBC membrane, the anion exchanger (AE) and CO\(_2\) shunt operate jointly like an electroneutral Cl\(^{-}\):H\(^{+}\) cotransport 44, known as the Jacob-Stewart mechanism (JS) 45-47. Together, the ionophore and JS mediate net CaCl\(_2\) transport since the proton fluxes cancel out. Ca\(^{2+}\) transport is rate-limited by the ionophore concentration because the high constitutive expression of the AE ensures non-limiting speed in the co- and counter-ion transfers. The cytoplasmic Ca\(^{2+}\) buffering behavior of RBCs (B, CaB) may be approximated by [Ca\(^{2+}\)]\(_i\) = α[Ca\(_T\)]\(_i\), where α ~ 0.3 over a wide range of [Ca\(^{2+}\)]\(_i\) values, and [Ca\(_T\)]\(_i\) is the total calcium content of the cells 25,48. Thus, normal RBC [Ca\(_T\)]\(_i\) levels are hardly detectable, among the lowest of any cell in nature 49-51. At sufficiently high ionophore concentrations (usually > 10 \(\mu\)M in 10 % hematocrit RBC suspensions) and appropriate external Ca\(^{2+}\) concentrations ([Ca\(^{2+}\)]\(_o\) > 100 \(\mu\)M), the induced Ca\(^{2+}\) influx exceeds the \(V_{\text{max}}\) of the PMCA in all the RBCs causing their [Ca\(^{2+}\)]\(_i\) levels to approach equilibrium with [Ca\(^{2+}\)]\(_o\) in
a uniform manner. The ionophore-induced equilibrium sets 
\[
\frac{[Ca^{2+}]}{[Ca^{2+}]_o} = \left(\frac{[H^+]}{[H^+]_o}\right)^2
\]
Addition of Co\(^{2+}\) in excess of [Ca\(^{2+}\)]\(_o\) instantly blocks Ca\(^{2+}\) transport by the ionophore, allowing the PMCA to extrude the induced Ca\(^{2+}\) load. Co\(^{2+}\) is itself transported by the ionophore, but at normal [Mg\(^{2+}\)] levels it has no effect on PMCA-mediated Ca\(^{2+}\) fluxes. The lower part of the figure shows the transport systems which participate in the Ca\(^{2+}\)-induced, rapid dehydration response. Elevated [Ca\(^{2+}\)], triggers the dehydration process by activating the Gardos channels. Gardos channel activation hyperpolarizes the cell (E, membrane potential), driving Cl\(^{-}\) out, resulting in the net loss of KCl and water. With full Gardos activation, dehydration is rate-limited by the Cl\(^{-}\) permeability. Replacement of ~10 mM Cl\(^{-}\) by the more permeable anion SCN\(^{-}\) maximizes the dehydration rate because rapid SCN\(^{-}\):Cl\(^{-}\) exchange via AE, or SCN\(^{-}\) diffusion across the membrane (dashed line), continually replenish the intracellular SCN\(^{-}\) supply. Gardos-mediated dehydration has a very low temperature coefficient. With non-limiting anion movement, maximal dehydration of RBCs may be attained within 10 – 30 min at 0-4\(^\circ\)C.
Panel a: Computer simulation of the Ca\(^{2+}\) load-extrusion protocol in a RBC population whose symmetrical distribution of PMCA \(V_{\text{max}}\) activity is shown in the inset. Substrate-fed RBCs are assumed to be suspended at an hematocrit (Hct) of ~10% in an iso-osmotic buffer containing 80 mM K\(^+\) and 100 \(\mu\)M CaCl\(_2\) at 37°C with magnetic stirring. A high ionophore concentration is used to rapidly induce a large and uniform RBC Ca\(^{2+}\) load. After ~2 min (to prevent
significant reduction of cell ATP levels) Co²⁺ is added in excess of Ca²⁺ in the medium to block ionophore-mediated Ca²⁺ transport and expose the uphill extrusion of Ca²⁺ by the pump. [Caᵦ]ᵢ was estimated in timed samples with the use of ⁴⁵Ca by dividing the measured cell-contained ⁴⁵Ca radioactivity by the specific activity of ⁴⁵Ca (there are no endogenous Ca²⁺ pools which could dilute the specific activity set by the addition of tracer). Each of the Ca²⁺ extrusion curves was obtained by solving numerically the differential equation d[Ca²⁺]/dt = (Vₘₐₓ)([Ca²⁺][²/(K_D² + [Ca²⁺][²])), using a different Vₘₐₓ value for each curve and applying the conversion [Caᵦ]ᵢ = [Ca²⁺]/α (see legend of Fig 1). The thick line was computed as the weighted mean of the Vₘₐₓ distribution shown in the inset. Note that the Ca²⁺-extrusion curves with different Vₘₐₓ remain linear until [Caᵦ]ᵢ falls to about 2-4 µM, as expected from a Ca²⁺ desaturation kinetics with K_D values in the range of 0.2 – 0.5 µM ²,₄,₅₆. The mean-Vₘₐₓ curve also renders a linear segment with a slope close to the true mean Vₘₐₓ of the distribution (~ 14 mmol (340 g Hb)⁻¹(h)⁻¹) but with an apparent Ca²⁺ desaturation pattern starting at [Caᵦ]ᵢ ~ 100 µmol (340 g Hb)⁻¹, a level much higher than that of the single-Vₘₐₓ desaturation curves. This specious desaturation effect results from retention of Ca²⁺ within the low-Vₘₐₓ cells. Points A to E mark sampling times for hemolysis curves expected to have the patterns illustrated in Panel b. The horizontal dashed line is at the [Caᵦ]ᵢ level above which Gardos channels are assumed to open maximally. Panel b: Actual hemolysis curves from preliminary experiments illustrate the patterns expected with samples taken at the time points A (upright triangles) to E (circles) of the load-extrusion protocol simulated in Panel a.
Fig 3. Generation of $^{45}$Ca content heterogeneity during the Ca$^{2+}$ extrusion period of the load-extrusion protocol.

One experiment with Protocol 1 is shown (see Methods, Experimental Protocols) representative of three with similar results. Panel a: Measurement of the PMCA $V_{\text{max}}$ by the load-extrusion extrusion protocol. RBCs were suspended at 10 % Hct in medium HK supplemented with inosine, 5 mM, and $^{45}$Ca(Ca$^{2+}$), 140 µM. The ionophore A21387 was added at $t = 0$ (final concentration 10 µM), and CoCl$_2$ was added at $t = 2$ min (final concentration 0.4 mM). Panel b: Comparison of the release of Hb and $^{45}$Ca induced by hypotonic lysis, as a function of relative tonicity. The measurements were performed on aliquots of the same samples used for the $V_{\text{max}}$ estimates in Panel a, as per Protocol 1. All samples were post-incubated in LK medium. Solid and open symbols report percentages of $^{45}$Ca and Hb released at each relative tonicity, respectively. 100 % release corresponds to the values obtained at ~ 0 relative tonicity. Panel c: Direct comparison of lysis-induced release of Hb and $^{45}$Ca at each relative tonicity. The solid symbols and lines represent paired values from open and solid symbols of Panel b. The open symbols and dashed lines are from duplicate samples from the same experiment, treated as in Panel a except for post-incubation in HK medium, to preclude volume changes.
One experiment with protocol 2 is shown (see Methods, Experimental Protocols) representative of five with similar results. Panel a: Duplicate measurement of the PMCA $V_{\text{max}}$ by the load-extrusion protocol. The indicated mean $V_{\text{max}}$ values were obtained by linear regression through the first six time-points after cobalt addition. The mean rate of $\text{Ca}^{2+}$ extrusion fell progressively when $[\text{Ca}_{i}]$ dropped below $\sim 100 \mu\text{mol}(340 \text{ g Hb})^{-1}$. Panel b: RBCs from the same batch as those used for the $V_{\text{max}}$ measurements reported in Panel a underwent the load-extrusion protocol without $^{45}\text{Ca}$ tracer, and were sampled for hemolysis curves at the indicated times after ionophore addition (the number of curves exceeded the repertoire of available symbols in the software used leading to cyclic repetition; however, the bottom up sequence can be followed unambiguously). All samples were post-incubated in LK medium. There was a monotonic upward progression of the curves with time, from the fully dehydrated pattern (1min point) to the fully recovered pattern (7min point).
Panel c: Percent cells with emptied Ca\(^{2+}\) loads as a function of time after cobalt addition (\(\Delta t\)). The percent of Ca\(^{2+}\)-emptied RBCs was computed from the ordinate readings shown in Panel b at a Relative Tonicity of 0.3, taking the distance between lowest and highest reading as 100% (see Methods). The equation \(y = ax^n/(b^n + x^n)\) was found to give a good empirical fit of the experimental points in the five experiments of this series, slightly better than that obtained with other sigmoid saturation functions, as judged by eye and by least means squares analysis. The parameter values obtained by least mean squares fit to the equation in this experiment were: \(a = 100, b = 2.2\) and \(n = 5.5\). Panel d: Derivative of the curve-fit through the experimental points in Panel c (continuous line) compared with a Gaussian curve fit to the rising branch of the derivative curve (dotted line). Both curves are plotted as a function of \(V_{\text{max}}\) estimated from \((\text{Ca}^{2+}\text{ load})/\Delta t\). The Ca\(^{2+}\) load in the suspension used for hemolysis curve sampling (Panel b) was 390 \(\mu\text{mol}(340 \text{ g Hb})^{-1}\). Compared with the Gaussian curve, the derivative curve reporting
the actual $V_{\text{max}}$ distribution among RBCs shows a marked right-shifted skew, observed in all the experiments of this series (Table 3). The parameter values of the actual $V_{\text{max}}$ distribution are shown in the figure and were calculated as follows: mean = $\frac{\Sigma y_i x_i}{\Sigma y_i}$; SD = ($\frac{(\Sigma y_i (x_i - \text{mean})^2)}{\Sigma y_i})^{1/2}$; Skew = ($\frac{\Sigma ((y_i (x_i - \text{mean}))/\text{SD})^3}{\Sigma y_i}$).
Table 1

<table>
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<th>Relative Tonicity (%)</th>
<th>Percent $^{45}$Ca$^{2+}$ lost</th>
<th>Percent Hb lost</th>
</tr>
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<tr>
<td></td>
<td>4min</td>
<td>4.5min</td>
</tr>
<tr>
<td>0</td>
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<td>0</td>
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<tr>
<td>Plateau values mean ± SEM</td>
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<td>10±1</td>
</tr>
<tr>
<td>Mean [Ca$<em>{T}$]$</em>{\mu}$mol(340 g Hb)$^{-1}$</td>
<td>157</td>
<td>73</td>
</tr>
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</table>

Table 1. Simultaneous measurements of the fractions of Hb and Ca$^{2+}$ lost from AA RBCs by lysis in hypotonic media, at four sequential time points during the Ca$^{2+}$ extrusion stage of the load-extrusion protocol. The results are representative of those obtained in three similar experiments. The quasi-plateau values were calculated from the measured Hb and $^{45}$Ca losses (numbers in bold italics) in the relative tonicity range from 25 % to 45 %, and used as described in Table 2 to estimate the mean [Ca$_{T}$], retained within the cells that failed to dehydrate during post-incubation in LK medium. “Mean [Ca$_{T}$]” reports the measured mean total RBC Ca$^{2+}$ content at the indicated time points, as shown in Fig 3a.
Table 2

<table>
<thead>
<tr>
<th>Time of Sample (min)</th>
<th>Hb lost Quasi-plateau percent</th>
<th>$^{45}\text{Ca}^{2+}$ lost</th>
<th>Mean $[\text{Ca}_T]$ in the Cell population $\mu\text{mol}(340 \text{ g Hb})^{-1}$</th>
<th>Mean $[\text{Ca}_T]$ in the dehydrated cells $\mu\text{mol}(340 \text{ g Hb})^{-1}$</th>
<th>Mean $[\text{Ca}_T]$ in the non-dehydrated cells $\mu\text{mol}(340 \text{ g Hb})^{-1}$</th>
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<td>4.5</td>
<td>41</td>
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<tr>
<td>5.5</td>
<td>81</td>
<td>13</td>
<td>17</td>
<td>78</td>
<td>2.7</td>
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</table>

Table 2. Estimate of the mean $[\text{Ca}_T]$ retained within the RBCs that failed to dehydrate during post-incubation in LK medium. The estimates were applied to three samples taken at the indicated times after ionophore (A23187) addition, during the $\text{Ca}^{2+}$ extrusion stage of the load-extrusion protocol (Fig 3a), and are representative of the ranges found in three similar experiments. The results in the Table may be read and interpreted as illustrated in the following example using the 5 min data: At 5 min, in the plateau region of relative tonicities (25 to 45 %, Table 1) the mean lysis-induced losses of Hb and $^{45}\text{Ca}$ were 63 % and 10 %, respectively (Fig 3b). Since the mean $[\text{Ca}_T]$ in the cell population is partitioned between dehydrated and non-dehydrated cells, mean-$[\text{Ca}_T]$ = $f_d$ mean-($[\text{Ca}_T]$)$_d$ + $f_{nd}$ mean-($[\text{Ca}_T]$)$_{nd}$, where $f_d$ and $f_{nd}$ represent the fractions of dehydrated ($d$) and non-dehydrated ($nd$) cells, respectively, and mean-($[\text{Ca}_T]$)$_{(d \text{ or } nd)}$ is the mean $\text{Ca}^{2+}$ content within each $d$ or $nd$ subpopulation. Continuing with the 5 min example, 10 % of 39 $\mu\text{mol}(340 \text{ g Hb})^{-1}$ is within 63 % non-dehydrated cells and 90 % of 39 $\mu\text{mol}(340 \text{ g Hb})^{-1}$ is within 37 % of dehydrated cells, rendering mean-($[\text{Ca}_T]$)$_d$ = 95, and mean-($[\text{Ca}_T]$)$_{nd}$ = 6.2 in units of $\mu\text{mol}(340 \text{ g Hb})^{-1}$.
Table 3

<table>
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<th>Experiment</th>
<th>PMCA $V_{\text{max}}$ distribution parameters</th>
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<tr>
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<td>Mean $V_{\text{max}}$</td>
</tr>
<tr>
<td></td>
<td>(mmol(340 g Hb)$^{-1}h^{-1}$)</td>
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<td>11.4</td>
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<tr>
<td>0507</td>
<td>12.9</td>
</tr>
</tbody>
</table>

Table 3. Statistical parameters of the PMCA $V_{\text{max}}$ distribution: compiled results from five experiments with RBCs from four donors. The results were obtained as described in detail in the legend of Fig 4 (experiment 0507).
Distribution of plasma membrane Ca\textsuperscript{2+} pump activity in normal human red blood cells

Virgilio L Lew, Nuala Daw, Deisy Perdomo, Zipora Etzion, Robert M Bookchin and Teresa Tiffert