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Assay of the Ca pump ATPase activity of intact red blood cells

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An assay for the Ca pump ATPase of intact human red blood cells (RBCs) was developed. The assay utilized a small volume (typically 10 μl) of packed RBCs in 1 ml of a buffer of known composition. The assay was based on the exposure of intact RBCs to the ionophore, A23187, in the presence of Ca. Such exposure caused a rapid degradation of ATP in RBCs. This degradation process is modeled in a numerical simulation in a companion paper (Vincenzi, F.F. and Hinds, T.R. (1992) Biochim. Biophys. Acta 1105, 63–70). The loss of ATP followed pseudo-first-order kinetics, and the rate constant for ATP degradation was taken as a measure of the capacity of the Ca pump ATPase. A number of variables were examined to optimize the activity of the ATPase. These variables included the concentrations of Ca and A23187. Because A23187 can promote loss of cellular Mg, it was necessary to include $MgCl_2$ in the incubation medium to optimize ATPase activity. Likewise, it was determined that inclusion of iodoacetic acid optimized the rate of ATP loss, presumably by preventing the resynthesis of ATP from ADP and inorganic phosphate. Cobalt inhibited the ionophore-dependent loss of ATP by apparent competition with Ca for binding to A23187. Results of many assays demonstrated substantial differences in the rate constant for ATP loss in RBCs from different individuals. RBCs were selected according to density. Density associated loss of Ca pump ATPase activity was observed both by the intact RBC assay, and by assay of Ca pump ATPase activity in saponin lysates of RBCs. The correlation coefficient between the two assays was 0.93. It is suggested that the rate constant for ATP loss in intact RBCs exposed to A23187 and Ca can be taken as a measure of the Ca pump ATPase activity. This may be useful when isolated membrane ATPase assays fail (e.g., dog RBCs). The intact cell assay can also be carried out on very small volumes of cells and may be of particular value when RBC volumes are limited.

Introduction

All eukaryotic cells, including red blood cells (RBCs) contain in their plasma membrane, a Ca pump that maintains low intracellular free Ca^{2+} (in this and the companion paper we will use 'Ca' to specify calcium without particular reference to its free or bound status and 'Ca^{2+}' to refer to free ionic calcium). Ca active transport is based on the activity of a membrane-bound (Ca^{2+} + Mg^{2+})-ATPase [2,3]. Activity of the Ca pump ATPase is increased by the binding of calmodulin (CaM) at the inner membrane surface [4,5]. The ATPase has been assayed in membranes from RBCs [2], or in saponin lysates [6,7]. However, certain species (e.g., dog) do not exhibit Ca pump ATPase activity in isolated membranes [8], although it is clear that dog RBCs actively transport Ca [9]. Thus, membrane isolation may cause loss of Ca pump ATPase activity.

To assay the Ca pump ATPase with a small volume of human RBCs, with a minimum of manipulation and without membrane isolation we have taken a different approach. We present here an assay based upon utilization of ATP by the Ca pump ATPase of intact cells. This is dependent on application of the divalent cation ionophore, A23187, to intact cells [10]. Other workers have reported that ATP is lost from cells exposed to Ca^{2+} and A23187 [11,12], but the rate of loss was not quantified. Here we demonstrate that ATP is consumed in human RBCs in a Ca^{2+} and A23187 dependent manner, that the consumption is rapid and follows pseudo-first-order kinetics. The ATP is consumed mainly by the 'ionophore-short-circuited' plasma membrane Ca pump ATPase. As shown in a companion paper [1], the rate constant for ATP consumption may be taken as an estimate of the Ca pump ATPase activity in intact cells. A similar assay system was applied to intact dog RBCs [10]. Preliminary results of

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similar data with human RBCs appeared in abstract form [13].

Materials and Methods

Iodoacetic acid (IAA) and ouabain were obtained from Sigma Chemical Company. Luciferin-luciferase was purchased from Los Alamos Diagnostics. Ficoll-Paque was obtained from Pharmacia, and A23187 was purchased from Calbiochem. Chemicals were analytical reagent grade.

Fresh RBCs were obtained from nine healthy human subjects by venipuncture. Heparinized blood was washed three times with cold saline, with careful removal of the buffy coat by aspiration to minimize RBC removal. The cells were then layered on Ficoll-Paque and centrifuged and cells were washed two more times with saline. Packed cells (85–90% hematocrit) were stored on ice until assayed. Assay of Ca pump ATPase in RBC saponin lysates was as described [14]. In some cases, 'top' and 'bottom' RBCs were separated according to density [15] in small glass tubes (SMI pipet 'D' tubes) in a microhematocrit centrifuge. We selected top and bottom populations of RBCs following 30 min centrifugation by cutting the top and bottom parts of the glass tube, respectively, after scoring with a diamond scribe. Packed RBCs in the short segments of glass tube were washed out using saline. By this method, the 'top' and 'bottom' cells represented approximately the 7% least and 7% most dense RBCs, respectively.

For assay of the Ca pump ATPase activity in intact cells, washed RBCs (10 μl) were added to 1.0 ml of buffer and pre-incubated for 5 min at 37°C. The buffer usually contained 20 mM Hepes (pH 7.4), 140 mM KCl, 2 mM $MgCl_2$, 1 mM IAA, 0.1 mM EGTA and, when added, 0.2 mM $CaCl_2$. Ca^{2+} was approximately 10^{-4} M when $CaCl_2$ was added. Ouabain, 0.1 mM was included in some experiments. IAA was made up fresh daily and was added to the cells at the time of pre-incubation. Following pre-incubation, at time zero 10 μl of A23187 in ethanol was added to the suspension to a final concentration of 3.8 μM (unless otherwise noted) while 'vortexing'. Incubation at 37°C was continued with removal of 10-μl aliquots every 2 min for 10 min (or as noted). Aliquots were immediately diluted in 1.0 ml of a lysing solution consisting of 0.5 mM $MgSO_4$ in 10 mM Tris buffer (pH 7.75). Then, 15 μl of the lysed aliquot of RBCs was added to 40 μl of solution containing luciferin-luciferase in 0.25 mM $MgSO_4$ in 5 mM Tris buffer (pH 7.75), in a Packard luminometer. The lysis and dilutions were performed to: (1) release ATP from the RBCs (this made ATP available for assay and also stopped the ATPase reaction by diluting the ATP in the large extracellular volume), (2) provide appropriate ionic conditions for the luciferase and (3) set the amount of ATP in the range in which the assay was sensitive and linear. A23187 exerted no effect on the luminometry assay. In most experiments, the ATP content of cells was not determined on an absolute, but rather on a relative scale to the best fit calculated initial ATP concentration. In the presence of A23187, ATP levels in the RBCs declined rapidly with pseudo first order behavior. The data were fitted with a first-order equation from which the rate constant was estimated.

RBC volumes were determined using a Coulter model Z_{Bi} cell counter and Channelyzer. Free Ca^{2+} levels in buffer solutions were measured by a Ca^{2+} electrode [16]. ATP standardization was carried out by standard additions to the samples. Concentrations of ATP in standard solutions were determined by absorbance at 260 nm, with a millimolar absorption coefficient of 15.4.

For determination of cellular Mg, 20 μl of cells was added to 4.0 ml of a solution containing $LaCl_3$, 10 mg, $SrCl_2$, 10 mg per liter in 0.1 M HCl with 1000 ppm of NaCl added. Following mixing and centrifugation at $3000 \times g$ at room temperature, the supernate was assayed for Mg using standard atomic absorption spectroscopy using Mg standards in the La/Sr diluent.

Results

The ATP concentration in intact RBCs was found to be 1.35 ± 0.40 mmol/l packed cells ($n = 4$). With added A23187 (3.8 μM), there was rapid pseudo first order loss of ATP. Typical data (Fig. 1) demonstrate that ATP was stable in RBCs in the presence of 5 mM EGTA, with A23187. By sharp contrast, rapid and pseudo-first-order loss of ATP occurred in RBCs ex-

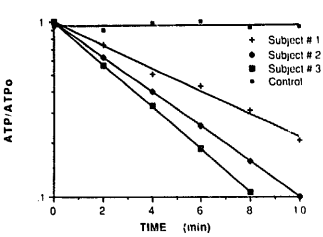

Fig. 1. Dependence on Ca and A23187 of the Ca pump ATPase of intact RBCs. Data are presented as a semilogarithmic plot of the ATP content, normalized to the ATP content at time zero. One control determined in the absence of added extracellular Ca and with 5 mM EGTA and in the presence of A23187 (□ ——— □), and assays of activity in samples from three different individuals are shown (other symbols). In each case the data were fitted with an exponential and the correlation coefficient was > 0.99. The slopes of the lines were the estimated rate constants, expressed as min^{-1}.

posed to A23187 (3.8 μM) in the presence of 10^{-4} M Ca^{2+}. Substantial inter-individual differences were apparent in RBCs from different subjects. Such differences are consistent with inter-individual variation observed using RBC saponin lysates and isolated RBC membranes in our laboratory over a number of years [7]. Under conditions of this assay, the rate of loss of ATP was not significantly different in the presence or the absence of 0.1 mM ouabain (not shown). It was previously determined that when the ATP level is very low then there is significant deviation from pseudo first order [10]. We routinely limited the loss of ATP to 90% of the original.

The rate constant for ATP loss in the intact cell assay was independent of the concentration of RBCs in the medium between 5 and 20 μl (data not shown). Thus, the rate constant should not be interpreted as a specific activity in the usual sense. Total ATP measured at time zero was dependent upon the total amount of cells, (not shown), but the rate constant for ATP loss was not significantly different at any RBC amount tested ($P > 0.05$).

As seen in Fig. 2, the rate of ATP utilization was dependent on the amount of A23187 up to 1 μM at which concentration, the rate of utilization of ATP became limiting; There was no statistically significant difference in rate between 1 μM and 10 μM A23187 ($P > 0.05$). The usual concentration of A23187 employed in the assay was 3.8 μM. Thus, the concentration of A23187 used in most assays was not limiting. The results also show that the rate of loss of ATP was small in the absence of added A23187.

The dependence of the rate of ATP loss on Ca^{2+} in the incubation medium was examined in the presence of 0.2 mM EGTA in the suspension buffer with various concentrations of added $CaCl_2$ (Fig. 3). Results

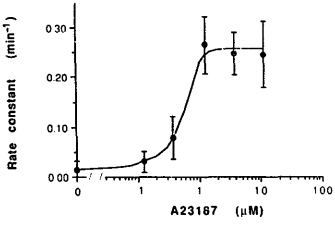

Fig. 2. Dependence on A23187 of the Ca pump ATPase of RBCs. A dilute suspension of washed RBCs was preincubated at 37°C for 5 min before the addition of different concentrations of A23187 in ethanol. The final concentration of ethanol was less than 3% in this series of assays, as contrasted with 1% in the usual assay. Results based on the means of blood samples from three different individuals. Vertical bars represent ± S.D.

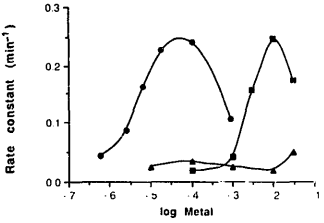

Fig. 3. Dependence on several divalent cations of the Ca pump ATPase of intact RBCs. RBCs in the medium containing various concentrations of Ca, Sr or Mn were incubated at 37°C for 5 min before the addition of A23187. Free Ca^{2+} was altered by varying $CaCl_2$ with added EGTA (0.2 mM). Free Ca^{2+} (●——●) was determined by Ca electrode. The apparent half-maximal and maximal concentrations of Ca^{2+} were $6 \cdot 10^{-6}$ M and $1 \cdot 10^{-4}$ M, respectively, and there was a prominent 'rollover' of activation at 10^{-3} M Ca^{2+} ($n = 2$). Sr (■——■) ($n = 3$) did, but Mn (▲——▲) ($n = 1$) did not activate the ATPase.

demonstrate that the usual concentration of 10^{-4} M Ca^{2+} in the incubation mixture promoted near maximal activation of ATP loss. Half-maximal activation was at about $3 \cdot 10^{-6}$ M Ca^{2+}. Sr activates the Ca pump ATPase of isolated membranes and is transported by the Ca pump of intact cells [2,17,18]. Sr (10 mM) in the incubation medium promoted maximal activation of ATP loss in the absence of Ca. Thus, Sr could replace Ca in the assay of the Ca pump ATPase of intact cells in the presence of Mg. Sr was as effective as Ca but its potency was approximately 500-times lower than that of Ca. By contrast with Sr, Mn did not promote activation of ATP loss (Fig. 3).

The loss of ATP of intact RBCs was inhibited by the addition of $CoCl_2$ in the presence of the ionophore (3.8 μM) and $CaCl_2$ (0.1 mM), with half-maximal inhibition occurring at 3.3 μM $CoCl_2$. Addition of three times less or five times more than normal A23187 shifted the concentration-effect curve; to the left with a $CoCl_2$ IC_{50} value of 1.9 μM, or to the right with a $CoCl_2$ IC_{50} value of 6.3 μM, respectively (data not shown).

As shown in Fig. 4, there was a broad dependence on Mg. Results show that 2 mM $MgCl_2$ supported essentially maximal activation of ATP loss. In the absence of added $MgCl_2$ or in the presence of 16 mM Mg, the rate of loss of ATP was decreased. The effect of A23187 (3.8 μM) on cellular Mg content was examined in incubation medium containing 2 mM $MgCl_2$. Addition of ionophore increased the cellular Mg content from an initial level of 2 mmol/l to 5.5 mmol/l. The Mg level reached a plateau within 4–5 min. Under assay conditions there was modest cell swelling, as

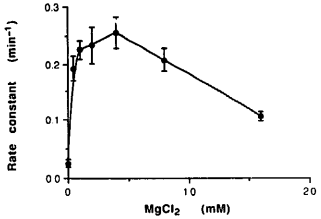

Fig. 4. Dependence on Mg of the Ca pump ATPase of intact RBCs. RBCs were preincubated with various concentrations of $MgCl_2$ at 37°C for 5 min before addition of A23187. Results based on the means of blood samples from three individuals. Vertical bars represent ± S.D.

determined in a cell counter (data not shown). Upon addition of A23187, the increase in RBC volume was about 10% during 10 min incubation. Cells incubated in the absence of A23187 swelled by approximately 2% during the 10 min period (data not shown).

The dependence of loss of ATP on IAA was examined, as shown in Fig. 5. The results demonstrate that 1 mM IAA in the incubation medium, the usual concentration employed, supported maximal activation of ATP loss. Presumably, at very low concentrations of IAA, some glycolytic synthesis of ATP occurred and reduced the apparent ATPase activity by about 20%. Significant inhibition was observed in the presence of 10 mM IAA, possibly because of direct effects on the Ca pump ATPase. However, between 0.3 and 3 mM IAA, the rate of loss of ATP was essentially constant and maximal.

It is generally accepted that in a population of normal RBCs the most dense (bottom) cells are the

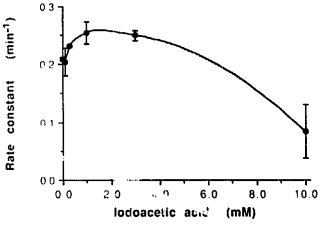

Fig. 5. Dependence on IAA of the Ca pump ATPase of intact RBCs. RBCs were preincubated for 5 min at 37°C in the standard medium, but with various concentrations of IAA. Results based on the means of blood samples from three individuals. Vertical bars represent ± S.D.

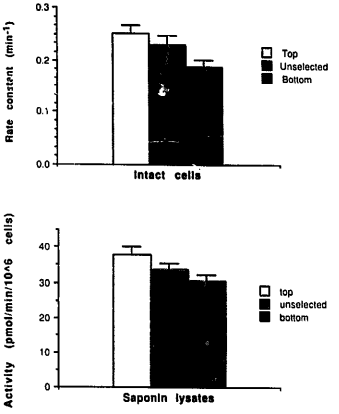

Fig. 6. Ca pump ATPase activities of intact RBCs and saponin lysates were measured as described in Materials and Methods in 'top' and 'bottom' fractions of RBCs. 'Top' (open), unselected (striped) and 'bottom' (solid) samples of RBCs gave respective rate constants of 0.251 ± 0.016, 0.230 ± 0.018 and 0.187 ± 0.013 min^{-1} (upper panel). The Ca pump ATPase activities of 'top' (open), unselected, (striped) and 'bottom' (solid) RBC saponin lysates were 37.8 ± 2.2, 33.6 ± 2.0 and 30.3 ± 1.9 pmol/10^6 cell per min, respectively (lower panel). Results based on the means of blood samples from eight individuals. Vertical bars represent ± S.D.

oldest, or at least the most senescent, while the least dense (top) cells are the youngest, or at least the least senescent [19]. Activities of the RBC Ca pump ATPase were determined by two different methods using unselected and top and bottom (selected by density) cells. Ca pump ATPase activities were expressed as the rate constant for intact cells and the specific activity for saponin lysates (Fig. 6). By both methods the activity of the top RBCs was significantly greater, and the activity of the bottom RBCs was significantly less, than that of the unselected RBCs ($P < 0.001$, paired t-test). Fig. 7 presents individual data points from Fig. 6. The data are plotted as a comparison of ATPase activity determined, on the one hand, by the saponin lysate technique and, on the other hand, by the intact cell assay. The results show excellent correlation between the two methods ($r = 0.93$).

Discussion

The present results support the suggestion that the capacity of the Ca pump can be estimated in intact RBCs by following the initial pseudo-first-order loss of

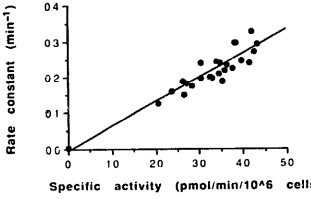

Fig. 7. Correlation of intact cell and saponin lysate assays of Ca pump ATPase in human RBCs (individual data points from Fig. 6). One typical Ca free control value (that illustrated in Fig. 1) is also plotted. The fit of the data shows an x intercept of 0.76 and a correlation coefficient of 0.93. The slope of the line ($6.8 \cdot 10^{-3}$) is the empiric relationship between the rate constant determined in the intact cell assay (expressed in min^{-1}) and the specific activity as determined in the saponin lysate assay (expressed in $pmol/10^6$ cell per min).

ATP in cells exposed to Ca and A23187. The dependencies of the rate of ATP loss on a number of variables were consistent with the interpretation that the majority of ATP consumption under the conditions of the assay was mediated by the Ca pump ATPase. Sr, which is carried by A23187, and which activates the Ca pump ATPase of isolated membranes, also activated ATPase in the intact cell assay. The Ca pump ATPase requires Mg as well as Ca, and in the presence of the ionophore, Mg (as well as Ca) movement is enhanced. For maximal activity, it was necessary to include Mg in the incubation medium. In the absence of added Mg cellular Mg was presumably depleted. Under usual conditions of the assay there was a modest increase in cellular Mg. Inclusion of IAA enhanced the maximal ATPase rate by approximately 20%. It is suggested that the increase in the rate of ATP loss caused by IAA was due to inhibition of glyceraldehyde-3-phosphate dehydrogenase [20]. Finally, there was an excellent correlation between assay of the Ca pump ATPase in intact RBCs and in saponin lysates. Results in our laboratory [13] and others [6,21] support the excellent correspondence of ATPase activities in saponin lysates and in isolated human RBC membranes, including inter-individual variation.

An estimate of the specific activity of the Ca pump ATPase in intact RBCs can be obtained from the empirical relationship between saponin lysate and intact cell assays, as shown in Fig. 7. This relationship was applied to the values for ATPase activities of top, unselected and bottom cells, as shown in Fig. 6. It was assumed that the RBC contains $6.6 \cdot 10^{-13}$ g of membrane protein [22]. The specific activities were thus 57.6 ± 3.67, 52.8 ± 4.14 and 43.2 ± 3.00 nmol/mg protein per min, respectively, for top, unselected and bottom cells. These values are similar to values reported for the calmodulin activated Ca pump ATPase in isolated membranes in a number of laboratories. Inhibition by trifluoperazine of ATP loss [10] is consistent with the interpretation that calmodulin activates the Ca pump ATPase in intact cells during this assay.

These estimates are based on the fact that the Ca pump ATPase is the rate limiting reaction for ATP consumption during the 10 min of the assay. Ca-activated ATPase activity in RBC membranes has been correlated with Ca transport with an apparent stoichiometry of 1 [26]. The cytosol of the human RBC expresses essentially no Ca-simulated ATPase activity [27]. These facts are the basis of the previously established assay of the Ca pump ATPase in saponin lysates of human RBCs [6,7].

There are several potential sources of error in the assay. Resynthesis of ATP, by adenylate kinase, would cause the rate of the Ca pump ATPase to be underestimated if there were significant resynthesis during the 10 min assay period. Adenylate kinase is strongly inhibited by K at concentrations that exist in the intact RBC [28]. Thus, it is difficult to know just how much resynthesis should be expected. This question is considered more fully in a companion paper [1]. Synthesis of ATP from 2,3-DPG is another potential way in which the activity of the Ca pump might be underestimated in this assay. However, at least one of the enzymes involved in that pathway, enolase, is inhibited by Ca^{2+} with a K_i of $7 \cdot 10^{-5}$ M [29]. Thus, the conditions of the assay inhibit the synthesis of ATP from 2,3-DPG. In short, over the limited time course of this assay, the major determinant of ATP loss is the Ca pump ATPase. The rate constant determined in this assay in any given blood sample is directly proportional to the enzymatic activity of the Ca pump ATPase determined by more traditional means using the same sample (Fig. 7). The mechanism of the pseudo-first-order loss of ATP is modeled in a companion paper [1].

Ca pump ATPase activity was dependent on Mg^{2+} (as well as Ca^{2+}) in intact RBCs (Fig. 6), as expected [2,24,25]. In the absence of added Mg, the rate constant for ATP loss was about 1/10 of its maximal value because the ionophore promoted loss of Mg into the large extracellular volume. The rate of ATP loss was increased by adding $MgCl_2$ to the external medium. The loss of ATP was less than optimal at medium concentrations higher than 8 mM $MgCl_2$. However, the usual concentration employed in the assay, 2 mM $MgCl_2$, which corresponded to approximately 5.5 mM of total cellular Mg after the addition of A23187 supported maximal activity of the Ca pump ATPase. Flatman and Lew reported that active Ca efflux was supported in the presence of 2 mM extracellular $MgCl_2$ [30]. Similarly, Ca pump ATPase activity was supported when Mg was introduced to Mg-depleted cells [24,25]. Ca dependence of the ATPase as determined in the

intact human RBC assay (Fig. 3) closely matched Ca dependence in a similar assay in intact dog RBCs [10] and of the CaM-activated Ca pump ATPase of isolated human RBC membranes determined in this laboratory [34]. This dependence also included the characteristic 'rollover' of activity at relatively high Ca^{2+} concentrations.

We found that about 10% swelling was associated with entry of Mg as did Flatman and Lew [30]. This was presumably due to the influx of K and water from the high KCl medium in response to the increased K permeability promoted by increased Ca^{2+} at the inner membrane surface (Gárdos effect) [35,36]. Results are consistent with the interpretation that, in the presence of ionophore, intracellular Ca^{2+} is substantially increased. Cell counts confirmed that essentially no hemolysis occurred during the 10 min exposure to A23187. Human RBCs can swell to a volume of approx. 119 fl before much hemolysis occurs [10].

The nature of the dependence on Ca^{2+} observed under these conditions is qualitatively similar to that reported for the Ca pump ATPase of isolated RBC membranes (5), and intact RBCs [10,24,37]. While there are differences in the absolute values of the Ca^{2+} sensitivity in various laboratories, we do not place great significance on such differences. Differing assumptions in buffer equations, the composition of buffers, purity of reagents, and other variables probably account for most of the apparent differences, in our opinion.

In the presence of A23187, Sr maximally activated the Ca pump ATPase of intact cells. Similar results have been shown in resealed ghosts [2,18], inside out vesicles [38], purified enzyme [39] and isolated membranes [17]. Mn is transported by A23187 [40] but is not transported by the Ca pump ATPase [3] and was found not to activate the Ca pump ATPase of intact RBCs. These results are consistent with the known ionic preferences of the Ca pump ATPase as determined in isolated membranes. Co is known to be rapidly transported by A23187 [41]. Co rapidly blocks ionophore-mediated Ca transport but it does not affect Ca transport by the Ca pump [42]. It appears that $CoCl_2$ effectively reduced the available A23187 that could carry Ca and thereby effectively inhibited the expression of Ca pump ATPase activity in intact RBCs. The addition of Co could potentially be useful for kinetic analysis in the whole cell assay.

Results in Figs. 6 and 7 support a previous report, based on saponin lysate assays, that there is a density-associated decrease in the activity of the Ca pump ATPase of human RBCs [43]. Similarly, decreased Ca pump ATPase activity was reported in the most dense RBCs of normal dogs, based on assay of Ca pump ATPase in intact cells [44]. The significance of this density associated loss of Ca pump ATPase activity is not yet known but may be related to cellular aging.

The present results can not be explained by simple Michaelis-Menten kinetics. Total intracellular ATP at time zero (1.35 mmol/l) is greater than what has been considered the low affinity K_m for the Ca pump ATPase in whole cells (500 μmol/l) [25], resealed ghosts (180 μM) [45], or 360 μM [46] or isotonic membranes (760 μM) [47]. In isolated membrane preparations there is less variability in the low affinity K_m, with values of about 140 μM [23]. Perhaps conditions in the intact cell favor a larger K_m. It is possible that some component is lost during membrane preparation or that some reorganization of the lipid or protein affects the ATPase activity [48]. In any event, although the K_m values found in intact cells are high they are not large enough to account for the first-order behavior of this assay [1].

We suggest that the ability to perform Ca pump ATPase assays on small samples of RBCs will be useful in certain circumstances. So far as we are aware, this method is the only method that can reliably assay the activity of this enzyme in the dog RBC [10]. This assay can be used to estimate the activity of the Ca pump ATPase using extremely small volumes. These can be typically in the range of 5–20 μl as described in this paper, but could be easily modified for smaller volumes by scaling down. The assay is sensitive without using radioisotopes. We have to measured the activity in cells separated according to their density in small glass tubes. When so applied the results [43] agree with data obtained by others using much more laborious techniques [49]. If one were to 'calibrate' the assay as in Fig. 7, then one could report the activities for the CaM-activated Ca pump ATPase in more usual terms. We suggest that this assay can be useful for the detection of inhibition of the Ca pump ATPase by natural products, circulating factors and drugs that are active on the outside of the cell and that would best be analyzed using an intact cell system. The system allows for minimal handling of the RBCs and limits the loss of integrity of the Ca pump ATPase that might occur during membrane isolation.

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