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Numerical simulation of assay of the calcium pump of intact red blood cells

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An assay of the Ca pump ATPase of intact human RBCs is described in a companion paper (Wu, L., Hinds, T.R. and Vincenzi, F.F. (1992) Biochim. Biophys. Acta 1106, 56–62). The assay is based on the rapid loss of ATP in RBCs that occurs when the cells are exposed to the ionophore, A23187, in the presence of Ca. An unexpected finding was that the initial loss of ATP follows pseudo-first-order kinetics. This was unexpected because the ATP content of RBCs is somewhat higher than the K_m of the Ca pump for ATP. Thus, the initial loss of ATP would be expected to follow zero-order kinetics; at least if the Ca pump ATPase operated with Michaelis kinetics. We performed a series of computer simulations of the Ca pump ATPase to investigate the possible cause of the unexpected pseudo-first-order behavior. The results confirmed that the data can not be accounted for by Michaelis kinetics of the Ca pump ATPase. Possible effects of adenylate kinase were tested and were also not found to account for the pseudo-first-order behavior of an ATPase operating with Michaelis kinetics. The enzymatic properties of the Ca pump ATPase were re-examined. It was found that the Ca pump ATPase exhibits positive cooperativity toward ATP. The apparent cooperativity was 1.91. In simulations it was found that positive cooperativity of the Ca pump ATPase in the range of 1.5 to 2.0 could account for the pseudo-first-order behavior. Excellent fit of the simulation data to first-order behavior was true with or without any contribution from adenylate kinase. Rate constants of ATP loss were thus examined using cooperativity of 2.0. Over a wide range the rate constant of the loss of ATP was directly proportional to the assumed V_{max} of the Ca pump ATPase, but only if the data were limited to loss of less than 67% of the initial ATP. It is suggested, therefore, that the rate constant for the initial loss of ATP in intact RBC, as stimulated by the ionophore A23187, can be taken as a measure of the capacity of the Ca pump ATPase.

Introduction

We have described an assay of the plasma membrane Ca pump ATPase of intact red blood cells (RBCs) [1–3]. The technique is based upon exposure of a suspension of RBCs to a divalent metal ionophore (A23187) in the presence of Ca^{2+}. In the presence of the ionophore, there is a massive influx of Ca into the RBCs. This results in maximal activation of the plasma membrane Ca pump ATPase. In the presence of 1 mM iodoacetic acid (IAA), to limit glycolytic synthesis of ATP, there is a rapid loss of ATP in the RBCs upon the addition of ionophore. This loss of ATP is monitored by sampling of the RBCs every 1 or 2 min and determining cellular ATP by luminometry.

ATP disappears from RBCs under these conditions according to pseudo-first-order kinetics. That is, the logarithm of ATP content decreases approximately linearly for about 10 min or until about 90% of the initial ATP is consumed [1]. Thereafter ATP consumption slows and there is a significant departure from pseudo-first-order kinetics. We have suggested that the rate constant (slope) of the initial disappearance of ATP in RBCs under such conditions may be taken as a measure of the maximal capacity of the Ca pump ATPase of the cells. This forms the basis of the assay of the Ca pump of intact RBCs as described in the companion paper [3].

First-order disappearance of ATP is not what would be expected, at least a priori. Typical RBC content of ATP is about 1.3 mmol/l [4]. Roufogalis reported that the K_m of the Ca pump ATPase in intact RBCs is 0.5 mmol/l [5]. Thus, one might predict that ATP should disappear from RBCs exposed to Ca and A23187 initially by a zero order process, and then by mixed order kinetics (as the concentration of ATP falls below the K_m). However, this is not what is seen experimentally.

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The initial disappearance of ATP from ionophore exposed cells follows pseudo-first-order kinetics. The ATPase assays of isolated RBC membranes and the numerical simulations presented here were performed to help evaluate the possible contributions of adenylate kinase and cooperativity of ATP to the unexpected pseudo-first-order loss of ATP.

Methods

Assay of Ca pump ATPase activity of intact RBCs was carried out as described in the companion paper [3]. Ca pump ATPase activity of isolated RBC membranes was determined by previously described methods [6,7] with the modification that the incubations and colorimetric determinations of phosphate were all carried out in 96 well immunoassay plates. Assay of RBC content of ATP, ADP and AMP was carried out by HPLC essentially as described by Payne and Ames [8].

Simulations were carried out using STELLA modeling software (High Performance Systems, Inc., Hanover, NH) operating on a Macintosh IIci computer (Apple Computer Co., Cupertino, CA). Cricket Graph (Cricket Software, Malvern, PA) was used to prepare figures, and for fitting of data. Values of slope (rate constant) and fits (expressed as the coefficients of determination, r^2) of various data points for straight line functions were obtained from Cricket Graph. The coefficient of determination is equal to the correlation coefficient squared [9]. Equations for the enzymatic activity of the Ca pump ATPase when operating in a cooperative fashion were according to the Hill equation as described by Segel [10]. Strictly speaking, at a cooperativity value of greater than 1.0, the concentra-

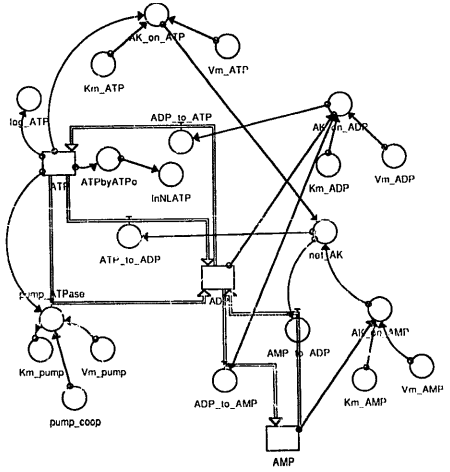

Fig. 1. Schematic model of the STELLA simulation of the assay of the Ca pump ATPase of intact RBCs. Boxes represent quantities (and, for the 1 liter RBCs assumed, concentrations) of ATP, ADP and AMP, respectively. Broad arrows connecting quantities represent the chemical reactions. For example, the broad arrow labeled 'pump-ATPase', represents the conversion of ATP to ADP, as mediated by the Ca pump ATPase. The features of reactions are dependent on variables that feed in by small solid arrows (e.g., concentration of ATP, K_m of the ATPase (K_m-pump), V_{max} of the ATPase (V_{max}-pump) and cooperativity (coop-pump)). Other reactions simulated here included both the forward and backward reactions of the adenylate kinase. As with the ATPase, the reactions are determined by substrate concentration, K_m and V_{max}. Because the conversion of 2 ADP molecules into 1 ATP is linked to the conversion into 1 AMP, the reactions are linked into one rate. This reaction rate is specified by 'AK-on-ADP'. The reverse reaction, conversion of 1 AMP and 1 ATP to 2 ADP molecules must also be considered. Again the reactions are linked and specified by 'net-AK'. When the effect of only the Ca pump ATPase was examined the V_{max} values for 'AK-on-ADP' and 'net-AK' were set to zero. To simulate the activation of the Ca pump ATPase by addition of ionophore to RBCs, the V_{max} of the Ca pump ATPase was changed from zero to (for example) 0.3 mmol/min per liter. This resulted in rapid loss of ATP. In the presence of finite adenylate kinase activity, there was also net conversion of ADP into AMP. In the absence of activation of the Ca pump ATPase the system maintained steady state concentrations of 0.5, 0.5, and 0.1 mmol/l of ATP, ADP and AMP, respectively. As the V_{max} of adenylate kinase was increased the rate of interconversion of the nucleotides was increased, but there was no net change until activation of the Ca pump ATPase.

tion of substrate at half-maximal activity of an enzyme should be designated by a term other than K_m (that term being reserved for Michaelis kinetics, i.e., cooperativity = 1.0). Segel [10] suggested k'. However, in keeping with common parlance and understanding we have simply used the label 'K_m' in the STELLA model to apply to a range of cooperativity values from 1.0 to 3.0. In keeping with the units utilized in a recent numerical simulation of Ca pump ATPase of normal human RBCs in vitro (no added ionophore) [11], we have expressed concentrations in mmol/l RBC, and rates in mmol/min per liter RBC.

Results and Discussion

A schematic diagram of the simulation model is presented in Fig. 1. This diagram shows the complete model, but in some cases the activity of certain pathways was set to zero. For example, when conversion of ATP to ADP by the Ca pump ATPase (pump-ATPase) alone was modeled, V_{max} values of other reactions were set to zero. K_m-pump and V_{max}-pump were set at values that gave a halflife of ATP that agreed reasonably with that typically measured values in intact RBCs. Initially we assumed Michaelis kinetics of the Ca pump ATPase, i.e., the cooperativity of ATP as a substrate was set at 1.0.

In this and essentially all similar simulations, the RBC content of ATP was reported at 1 min intervals. Similar to our approach in the laboratory [3], the first measurement of ATP was made after 'addition of ionophore' (i.e., a sudden increase in V_{max} of Ca pump ATPase from zero to the selected V_{max} value). ATP values from 1 min to 10 min after addition of ionophore were used to determine the function of the loss of ATP. The ATP content, as in our laboratory assay, was expressed as the ratio of ATP to the time zero value of ATP (ATPbyATP$_0$). These values were also converted to the natural logarithm (lnNLATP). When plotted as a function of time, the natural logarithmic values were used to determine the slope of the best fit to a straight line (single-exponential function). When calculated in this manner, the slope of the line is equal to the apparent rate constant for the process. It is explicitly recognized that the determination of apparent rate constant by this approach is valid only if the data reasonably fit a first-order process. One of the first questions we asked concerned the goodness of fit of simulation data to a first-order process as dependent on the assumptions of the model.

The V_{max} of Ca pump ATPase was, unless otherwise specified, assumed to be 0.3 mmol/min per liter RBCs. This is the V_{max} value we have used for modeling normal human RBCs in vitro [11]. When the cooperativity was set at 1.0, the disappearance of ATP followed what would be expected for simple Michaelis-Menten kinetics. That is, the natural log of ATP/ATP$_0$ decreased, but in a convex downward fashion, as shown in Fig. 2. This general shape of the curve is what would be expected if consumption of ATP were initially zero order and then tending toward first order. Clearly, this model did not produce a simulation that resembled actual laboratory data. Typical laboratory data on the assay of the Ca pump ATPase of RBCs are included in Fig. 2 for comparison.

Two major kinds of questions are typically raised when the intact RBC Ca pump assay is described. First, is the apparent rate constant indeed related to the V_{max} of the enzyme? We will come back to that issue. Second, what about interference from other ATP utilizing or synthesizing enzymes? In particular, the question of adenylate kinase is raised. This enzyme promotes the conversion of 2 ADP molecules into one ATP and one AMP molecule. Haslam and Mills [12] reported activities of 1378 and 2069 mmol/min per liter, respectively, for the forward (2 ADP → AMP + ATP) and backward (AMP + ATP → 2 ADP) reactions of the enzyme in extracts of RBCs. However, intact RBCs showed little or no activity. According to Levin and Beutler [13], the specific activity of adenylate ki-

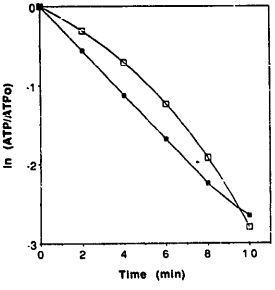

Fig. 2. Simulation of ATP loss by the Ca pump ATPase assuming Michaelis-Menten (cooperativity = 1) kinetics. In this and similar plots the natural logarithm of the ATP concentration, normalized to the initial concentration, is plotted as a function of time following activation of the Ca pump ATPase. In this plot, as in the companion paper, the data points were collected every 2 min. In other figures, data points were determined every minute. The assumed V_{max} of the Ca pump in this simulation was 0.28 mmol/min per liter. The data points in this and similar figures are connected to emphasize the shape of the pattern. Data points for the simulation (□——□) followed a convex downward pattern, and the fit to a single exponential (straight line, not plotted) was poor. Thus, considering the Ca pump ATPase only, Michaelis kinetics, could not account for data typically observed in the laboratory. Typical laboratory data (■——■) of an actual assay are included for comparison. These data were well fitted by a single exponential ($r^2 = 0.998$) which, for simplicity, is not plotted.

nase in RBC lysates is 834 mmol/min per liter RBC at 30°C. The ratio of adenylate kinase at 30°C compared to 37°C is 0.770 [14] Thus, the apparent activity of adenylate kinase measured by Levin and Beutler was 1083 mmol/min per liter. Beutler [14] subsequently reported an activity of 877 mmol/min per liter at 37°C. Seitz [15], reported the activity of adenylate kinase in human RBCs at 1119 mmol/min per liter. It should be noted that these activities were measured only after removal (by centrifugation) of an inhibitor. It is suggested that a value of 1000 mmol/min per liter is a generous overestimate of the specific activity of adenylate kinase in intact RBCs.

Several questions arose. Would adenylate kinase render the assay useless by swamping out the relatively small V_{max} of the Ca pump ATPase? Alternatively, was it possible, that resynthesis of ATP by adenylate kinase was just sufficient to make the mixed order loss of ATP appear to be first order (initially resynthesizing at a low rate, and later at a high rate)? In modeling the activity of adenylate kinase, it was important that the system be stable in the absence of activation of the Ca pump ATPase. The ATP, ADP and AMP contents of RBCs are stable in vitro, at least until the addition of ionophore. This is true even in the presence of IAA, which can be expected to limit glycolytic synthesis of ATP by inhibition of glyceraldehyde-3-phosphate dehydrogenase [16].

The relative K_m and V_{max} values for the forward and backward reactions of adenylate kinase were simulated such that the concentrations of ATP, ADP and AMP were maintained at 1.5, 0.5 and 0.1 mmol/l, respectively. Using this simulation model it was found that K_m values for the AK-on-ATP, AK-on-AMP and AK-on-ADP reactions that maintained ATP, ADP and AMP at these respective levels were 0.5, 8 and 8 mmol/l, respectively. Haslam and Mills [12] reported a K_m ADP value of about 1.5 mmol/l. We will deal with the question of the adenylate kinase K_m later. Once the relative K_m values were determined that maintained stable levels of ATP, ADP and AMP in our model (in the absence of ionophore), then the V_{max} value of AK-on-ADP was varied from 0 to 10000 (and corresponding change in V_{max} of AK-on-AMP and AK-on-AMP of 0 to 50000) without a net change in the respective concentrations of the nucleotides. In further references to the activity of adenylate kinase, we will refer to the V_{max} of the AK-on-ADP reaction. In each such case (except zero), the relative V_{max} values of the AK-on-ATP and AK-on-AMP reactions are 5-fold greater. The rates of the interchange of ATP, ADP and AMP increased as the V_{max} values were increased, but the steady-state levels did not change.

Simulations were performed of activation of the Ca pump ATPase over a range of adenylate kinase V_{max} values from zero to 10000. Some of these simulations are plotted in Fig. 3. As can be seen in Fig. 3, adenylate kinase activity slowed the loss of ATP slightly. However, because of the poor fit to single exponentials, little can be said about the quantitative impact of adenylate kinase. Also, because of the poor fit to single exponentials, adenylate kinase can not account for the observed pseudo-first-order loss of ATP during assays of the Ca pump ATPase of intact RBCs in the laboratory.

Michaelis kinetics, either in the absence or presence of adenylate kinase activity, could not account for the laboratory data. Thus, we decided to reexamine the activation of the Ca pump ATPase by the substrate. Activation by ATP of ATP splitting in RBC membranes has been interpreted to be a reflection of one high-affinity and one low-affinity site for ATP [17]. However, we obtained data that were compatible with the interpretation of simple cooperativity. As shown in Fig. 4, Ca pump ATPase activity was determined in the presence of calmodulin (CaM). CaM activated enzyme is the proper model for the situation that exists in RBCs exposed to Ca and A23187 [1]. This is because in RBCs exposed to A23187 in the presence of Ca^{2+}, the cell is loaded with Ca and the binding of CaM to the Ca pump ATPase is dependent on Ca^{2+} with a half-maximal value in the micromolar range [18]. A Hill plot of the data for 10–90% of maximal activity of the Ca pump ATPase (Fig. 5) provided an estimate for the cooperativity of ATP of 1.91. Therefore, it was assumed that activation of the ATPase activity was coop-

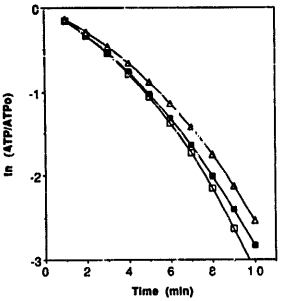

Fig. 3. Simulation of the effect of adenylate kinase on ATP loss by the Ca pump ATPase assuming Michaelis (cooperativity = 1) kinetics. In this case, the data points follow a convex downward pattern. Again, the fit of the data to a single exponential was poor. As the V_{max} of adenylate kinase was increased from zero (□——□), to 0.1 (■——■) and 1 (△——△) mmol/min per liter, respectively, there was a less rapid loss of ATP. Beyond 1 mmol/min per liter, further increases in V_{max} of adenylate kinase exerted no further effect on the rate of loss of ATP.

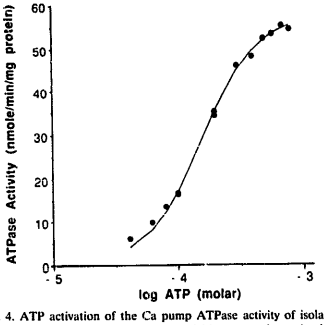

Fig. 4. ATP activation of the Ca pump ATPase activity of isolated human RBC membranes. ATPase activities were determined in isolated membranes over a range of ATP concentrations in the presence of 30 nM of calmodulin. The solid line plotted is a plot of the equation representing the best fit to a Hill plot of the data, as shown in Fig. 5.

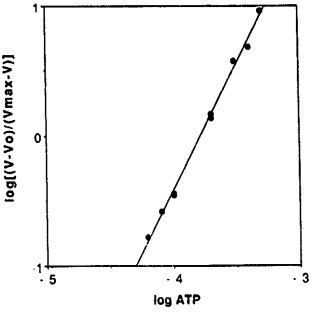

Fig. 5. Hill plot of the data shown in Fig. 4. The data points between 10 and 90% of V_{max} were fitted. V_0 and V_{max} values were assumed to be 2 and 58 nmol/min per mg protein, respectively. The equation for the best fit to the Hill plot had a slope (apparent cooperativity) of 1.91 and a gave a half-maximal rate at 0.16 mmol/l.

erative, and that the value is 2. Considering that the Ca pump ATPase exists in the membrane as a dimer [19–21], we suggest that this interpretation is quite plausible.

Fig. 6 presents the ATP disappearance for simulations with cooperativities of 1.0, 2.0 and 3.0. Fig. 7 presents the coefficient of determination (r^2) as a function of the assumed cooperativities of a series of simulations. The fit to a single exponential was quite good between assumed cooperativities of about 1.5 to 2.0. Thus, the simulations offer insight into the apparent pseudo-first-order loss of ATP from intact RBCs under conditions of assay of the Ca pump ATPase. However, these simulations were carried out on only the Ca pump ATPase. What is the possible influence of adenylate cyclase on the apparent rate of ATP loss?

We performed a series of simulation in which the cooperativity of the Ca pump ATPase was 2.0 and the V_{max} was held constant (at 0.3 mmol/min per liter). These simulations were performed over a wide range of adenylate kinase activities. As shown in Fig. 8, there was a good fit of the data to single exponentials in all cases ($r^2 > 0.99$). As the V_{max} of adenylate kinase was increased from 0 to 10 mmol/min per liter there was a slight decrease in the apparent rate constant determined from the slope of the line. Beyond 10 mmol/min per liter, increasing the V_{max} of adenylate kinase had essentially no effect on the apparent rate constant. However, it appeared that there was a slight 'dip' in the value of the apparent rate constant at 1 mmol/min per liter.

A series of simulations was performed with V_{max} values of adenylate kinase ranging from 0 to 10000 mmol/min per liter. Particular attention was paid to the region around 1 mmol/min per liter. The resulting data are shown in Fig. 9. As previously, there was no

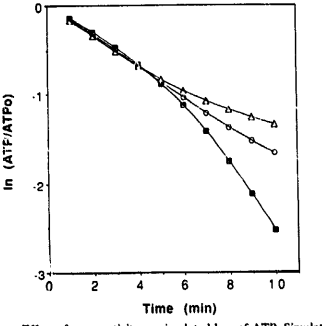

Fig. 6. Effect of cooperativity on simulated loss of ATP. Simulations were performed in the absence of adenylate kinase activity to assess the influence of cooperativity on the simple system. As previously seen, for cooperativity of 1 (■ ——— ■), the data points follow a convex downward course, and the fit to a single exponential was poor ($r^2 = 0.972$). For a cooperativity of 2 (○ ——— ○), the r^2 of the fit to a single exponential was 0.996. At a cooperativity of 2, there was a tendency toward convex upward when a large fraction of initial ATP was consumed. This pattern is qualitatively similar to what is seen in laboratory assays of the Ca pump ATPase of intact RBCs (Fig. 2). At a cooperativity of 3 (△ ——— △), the data points followed a convex upward course, and the r^2 of a fit to a single exponential was 0.973.

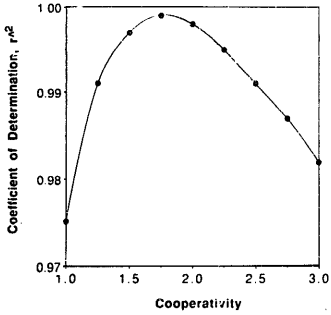

Fig. 7. Effect of cooperativity on the goodness of fit of ATP loss to single exponential. Cooperativity of the Ca pump ATPase was varied and the simulated loss of ATP was fitted to a series of single exponentials. The r^2 value of each fit is plotted against the cooperativity. It is apparent that good fit to single exponential loss of ATP was predicted when the cooperativity of the Ca pump ATPase was approximately 1.5–2.0.

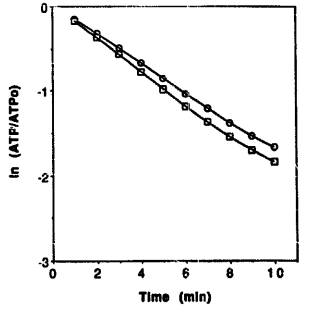

Fig. 8. Effect of adenylate kinase on simulated loss of ATP: Cooperative kinetics. The data for adenylate kinase activity of zero (□——□) and 1000 (○——○) mmol/min per liter are plotted. For adenylate kinase activities between 0 and 1000 mmol/min per liter, rates of ATP loss were similar, as shown in Fig. 9.

effect of adenylate kinase beyond 10 mmol/min per liter. The difference in apparent rate constant for ATP loss in the presence of high adenylate kinase activity versus none amounted to only 8.5%. Curiously, there was a 'dip' in the apparent rate constant for the loss of ATP as a function of the V_{max} of adenylate kinase with the minimal value being at approx. 0.6 mmol/min per liter. It must be emphasized that the effect shown in Fig. 9 is small. The minimal rate constant was only about 10.6% less than the maximal value. Considering the potential capacity of adenylate cyclase in intact RBCs (about 1000 mmol/min per liter), it is suggested that any contribution of adenylate kinase to the apparent rate constant of ATP loss as mediated by the Ca pump ATPase is essentially constant. These data are consistent with the interpretation adenylate cyclase has only a negligible to slight effect to decrease the apparent rate constant of ATP loss (compared to no contribution from adenylate kinase). Qualitatively similar results were obtained if it was assumed that the K_m of adenylate kinase was assumed to be 1.5 mmol/l, as reported by Haslam and Mills [12]. For these simulations, K_m values for the AK-on-ATP AK-on-AMP and AK-on-ADP were 0.09, 1.5 and 1.5 mmol/l, respectively. The difference in the rate constant for ATP loss between assumed V_{max} values for adenylate kinase of zero and 1000 mmol/min per liter, respectively, was less than 6% (data not shown). It may not be intuitively clear why the effect of adenylate kinase is so small. The 'bottom line' is that, during the short time of the simulation, most of the ATP that is lost accumulates as ADP, but that (because of the ratio of the K_m values of the forward and backward reactions) there is relatively little net conversion of ADP to AMP (and ATP).

In the face of a more or less constant contribution by adenylate kinase can the apparent rate constant for the initial loss of ATP be taken as a measure of the V_{max} of the Ca pump ATPase? A series of simulations was performed in which the adenylate kinase activity

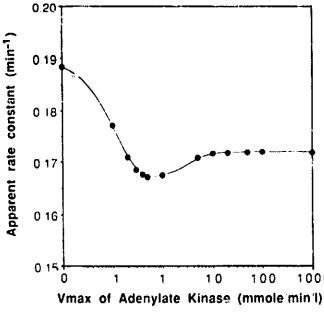

Fig. 9. Effect of adenylate kinase on the apparent rate constant for loss of ATP. Data obtained from simulations like those shown in Fig. 8. In each case, the fit of data points to a single exponential was acceptable ($r^2 > 0.99$). As the V_{max} of adenylate kinase was increased from zero, the apparent rate constant decreased, displayed a minimum value and then increased slightly and became constant at a value of 91.5% of the maximum.

was assumed to have a V_{max} of 1000 mmol/min per liter. Cooperativity of 2 was assumed. The V_{max} of the Ca pump ATPase was varied over a wide range. The data are presented in Fig. 10. At low V_{max} values for the Ca pump ATPase the apparent rate constants were low, and the fit of the data to single exponentials was good. At high values of V_{max} the apparent rate constants were high, but could not be adequately judged because of relatively poor fit to single exponential (as low as 0.969 at $V_{max} = 0.6$ mmol/min per liter). The poor fits at high V_{max} values were obviously due to the convex upward pattern of the data points.

The pattern observed at high V_{max} values is similar to what we have seen in the laboratory. That is, as most of the ATP in RBCs is consumed, the rate of ATP loss slows. We have typically limited the data points used in the curve fitting to values representing not more than 90% consumption of the initial ATP in the 10 min following addition of the ionophore (see companion paper). However, with the possible exception of rabbit RBCs (which, by inference, appear to have very active Ca pump ATPase activity) we have not observed rates of loss of ATP as rapid as those which were simulated in Fig. 10.

The curves representing high rates in Fig. 10 showed significant deviation from first-order behavior even when data points were limited to those representing less than 90% loss of ATP. Thus, this is probably not a sufficiently selective criterion for high rates of ATP

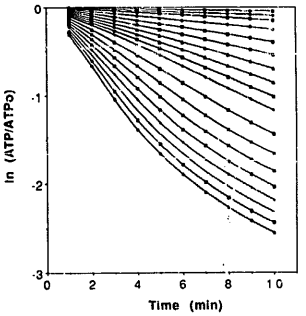

Fig. 10. Effect of V_{max} of Ca pump ATPase on simulated loss of ATP. Cooperative kinetics (2.0) and adenylate kinase (1000 mmol/min per liter) were included in these simulations. Increased V_{max} of the Ca pump ATPase, over the range of 0.01 to 0.6 mmol/min per liter, was associated with increased rate of loss of ATP. At high V_{max} values, the fit of the data to a single exponential was poor ($r^2 < 0.98$). However, if limited to those points that represented no more than 67% loss of the initial ATP the data were well fitted with a single exponential ($r^2 > 0.998$).

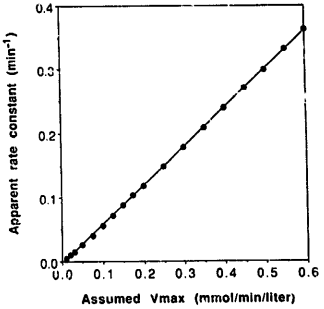

Fig. 11. Relationship between assumed V_{max} of the Ca pump ATPase and the apparent rate constant of the initial loss of ATP. Apparent rate constants determined in Fig. 11 are plotted versus the V_{max} of the Ca pump ATPase. The relationship is a straight line with a near zero intercept. Thus, according to the model, the apparent rate constant of the initial loss of ATP can be used as a measure of the V_{max} of the Ca pump ATPase of intact RBCs.

consumption. However, if data points representing more than 67% loss of ATP were eliminated then the data were well fitted with single exponentials. In each case the r^2 of the fitted data was at least 0.999. It must be emphasized that these excellent fits to first-order behavior were obtained in the presence of the contribution of adenylate kinase with a V_{max} of 1000 mmol/min per liter. Elimination of data points representing more that 67% loss of ATP required discarding a number of points at high V_{max} values. At the highest V_{max} value simulated (0.6 mmol/min per liter) only three points were included. Of course, considering the scatter of real data points, the anticipated fit of laboratory determined ATP loss will be less. The results of the simulations are interpreted to mean that it is reasonable to determine an apparent rate constant only for the initial disappearance of ATP under these conditions.

Acceptable fit to first order of the initial portions of the curves presented in Fig. 10 justifies comparison of the various apparent rate constants. We compared the apparent rate constants with the assumed V_{max} values for the Ca pump ATPase. The result is shown in Fig. 11. The result is a straight line relationship ($r^2 = 1.00$) with a nearly zero intercept (0.00389). The slope of the line (0.609) is the relationship between the rate constant (min^{-1}) and the V_{max} (mmol/min per liter). This is similar to that determined empirically in the companion paper [3]. In that paper, the operationally defined Ca activated ATPase activity of saponin lysates was taken as a measure of the Ca pump ATPase

activity. If RBC volume is assumed to be 90 fl [4], then the experimental data are equivalent to a slope of 0.607 min^{-1}/mmol per min per liter. Thus, the model is in good agreement with the experimental findings.

In summary, according to our measurements, the Ca pump ATPase exhibits positive cooperativity of about 2 toward ATP. In our simulations using such cooperativity there was a direct relationship between the V_{max} of the Ca pump ATPase and the rate constant of the initial disappearance of ATP. Thus, it is proposed that determination of the apparent rate constant of the initial loss of ATP in intact RBCs can be taken as a measure of the V_{max} of the Ca pump ATPase.

Various reactions for synthesizing and degrading ATP exist in RBCs. Under conditions of the assay simulated in this work, the Ca pump ATPase is the dominant ATP utilizing reaction. Thus the V_{max} of this enzyme becomes the major determinant of the loss of ATP in the RBCs, at least for a few minutes. Because good fit to first order behavior is observed when a cooperativity of 2 for the Ca pump ATPase is assumed, we suggest that under conditions of the assay the Ca pump exhibits positive cooperativity in intact RBCs. We further suggest, given these considerations, that the apparent rate constant for the initial loss of ATP can be used as a measure of the V_{max} of the Ca pump ATPase of the intact RBC.

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