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Evidence for a Calmodulin-Activated Ca^{2+} Pump ATPase in Dog Erythrocytes (42290)

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Abstract. Previous work in several laboratories revealed little or no Ca^{2+} pump ATPase activity and little or no activation of the ATPase by calmodulin (CaM) in membranes isolated from dog red blood cells (RBCs). In the present work, intact RBCs from dogs were exposed to the ionophore, A23187, in the presence of Ca^{2+}. A rapid, apparently first order, loss of ATP occurred under these conditions. The first order rate constant was 0.0944 min^{-1}, or approximately 47% of that found in human RBCs under the same conditions. The anti-CaM drug, trifluoperazine, inhibited the loss of ATP and the Ca^{2+} activation curve of ATP loss in intact cells resembled that observed for CaM-activated Ca^{2+} pump ATPase in isolated human membranes. Taken together, these data are consistent with the interpretation that the dog RBC membrane contains a CaM-activated Ca^{2+} pump ATPase.

It is generally thought that all plasma membranes actively extrude Ca^{2+} from the cytosol, that this process is linked to the activity of a membrane-bound (Ca^{2+} + Mg^{2+})-ATPase or Ca^{2+} pump ATPase (1) and that this is important in the regulation of intracellular Ca^{2+} (2). It is also presumed, based on work in human red blood cells (RBCs) that most, if not all, plasma membrane Ca^{2+} pump ATPases are similar in being activated by calmodulin (CaM) in the presence of Ca^{2+} (1, 3). Direct interaction between the $CaM(Ca^{2+})_n$ complex and the Ca^{2+} pump ATPase of human RBCs has been demonstrated by several groups (4, 5). Recent attempts in our laboratory to show interaction with and activation of the Ca^{2+} pump ATPase by CaM in membranes isolated from dog RBCs were unsuccessful, in spite of several attempts to approach the problem (6, 7). By contrast, others (8, 9) reported that CaM was able to increase the rate of Ca^{2+} transport into inside-out vesicles (IOVs) prepared from dog RBCs. However, the specific activity of the transport reported by these workers was much lower than that for human IOVs, or for the ATPase of isolated dog RBC membranes (7).

The present work was undertaken to examine whether evidence for a CaM-sensitive Ca^{2+} pump ATPase could be demonstrated in intact dog RBCs, and to compare this with the well-studied human RBC enzyme under comparable conditions. In the present work, as in several previous studies (10, 11), the intrinsic permeability barrier of the cell to Ca^{2+} was circumvented by the divalent cation ionophore, A23187. This results in Ca^{2+}-dependent utilization of ATP (12, 13). Ion and ionophore-induced activation of the Ca^{2+} pump ATPase in intact RBCs, allowed study of the enzyme in the absence of gross biochemical changes brought about by membrane isolation procedures. Results are consistent with the interpretation that in *intact cells* the activity of the Ca^{2+} pump ATPase of dog RBCs is about 50% of that of human RBCs, and that the enzyme is activated by CaM, at least under these conditions.

Materials and Methods. Fresh RBCs were obtained from humans and mongrel dogs by venipuncture into heparinized tubes. Cells were washed five times in normal saline with careful removal of the buffy coat. Washed packed cells (85–90% hematocrit) were stored on ice until assayed. Packed RBCs (20 μl of dog and 10 μl of human) were then suspended in 1 ml of assay medium normally containing: Hepes, 20 m*M*, pH 7.4; KCl, 140 m*M*; $MgCl_2$, 2 m*M*; $CaCl_2$, 0.1 m*M*. When the concentration of free Ca^{2+} was varied, the amount of $CaCl_2$ was varied and various amounts of EGTA were added. Free Ca^{2+} was calculated using an iterative computer program. When human RBCs were assayed the above reaction mixture contained, in addition, 0.1 m*M* ouabain. The total osmolarity of the solution was determined to be 293 mosmole/kg. When present, the concentration of A23187 was 3.8 μ*M* and the concentration of iodoacetic acid (IAA) was 1 m*M*. Ionophore was dissolved in

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ethanol. The final concentration of ethanol in the incubation mixture with cells was always less than 1%.

RBCs were incubated in a shaking water bath at 37°C. Cells were preincubated for 5 min before addition of ionophore, which was defined as time zero. Ten-microliter samples were removed at various time points and rapidly added to 1 ml of 10 m*M* Tris, pH 7.8, 0.5 m*M* $MgSO_4$ to lyse the cells. Fifteen-microliter aliquots of this dilution were used for duplicate ATP determinations. The ATP content of RBCs was determined in a Packard luminometer using the luciferin–luciferase reaction. Trifluoperazine (TFP) was employed at various concentrations, as noted, and was usually added to the suspending medium prior to the addition of RBCs and ionophore. RBC volumes were determined using a Coulter Model ZB_I cell counter and Channelyzer.

The luminometer and the luciferin–luciferase assay were calibrated daily with fresh ATP standard. The count rate of the light produced, by the added ATP, was maintained within the linear response region of the instrument.

The protein content of dog RBC ghosts was determined as described previously (14) and is based on acetylcholinesterase activity of whole RBCs and ghost membranes. Hemolysis was measured by a cyanmethemoglobin method (15).

Trifluoperazine hydrochloride and ouabain were obtained from Sigma Chemical Company, St. Louis, Missouri. A23187 came from Calbiochem, San Diego, California, and the luciferin–luciferase reagent was obtained from Packard Instruments Company, Chicago, Illinois. All other chemicals were reagent grade.

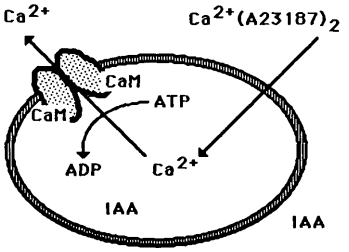

FIG. 1. Schematic diagram of the experimental system. A dilute suspension of washed RBCs (dog or human) was exposed to A23187 (3.8 μM) in the presence of Ca^{2+} and (usually) iodoacetic acid (IAA). At various times after addition of ionophore an aliquot of the suspension was removed for analysis of the ATP content. Because A23187 promoted an influx of calcium, there was activation of the Ca^{2+} pump and associated increase in ATP utilization.

Results and Discussion. The basic method employed in this paper consists of measuring the amount of ATP remaining in RBCs at specific times following an abrupt increase in Ca^{2+} permeability promoted by the divalent cation ionophore, A23187. A schematic diagram of the assay system is shown in Fig. 1. The internal Mg^{2+} concentration of the cell was maintained at an operationally defined optimum by inclusion of 2 m*M* Mg^{2+} in the external medium. Previous work performed in this laboratory has shown the importance of preventing net efflux of Mg^{2+} via the ionophore (13) when measuring ATP utilization in the intact RBCs. Dog RBCs do not contain ($Na^+ + K^+ + Mg^{2+}$)-ATPase (16) and we observed no effect on the metabolism of ATP if ouabain was or was not included in the incubation medium. By contrast, human RBCs do have ($Na^+ + K^+ + Mg^{2+}$)-ATPase and its activity can be inhibited by ouabain (17). This allows one to see Ca^{2+}-dependent utilization without the confounding influence of the ($Na^+ + K^+ + Mg^{2+}$)-ATPase.

As can be seen in Fig. 2, there was little or no loss of ATP in "resting" dog RBCs in the absence of A23187 (● and △) and no detectable change in ATP concentration was initiated in RBCs by the addition of A23187 when EGTA (1 m*M*) was added in excess of Ca^{2+} (0.1 m*M;* ▲). Similar results were found with human RBCs (data not shown). These findings may be at variance with those reported by Maretzki *et al.* (18) who found breakdown of ATP in washed human cells with an apparent half-life of approximately 3 hr.

When Ca^{2+} was introduced into RBCs via the ionophore there was a substantial time-dependent decrease in the level of ATP (○ and □). Under these conditions the major ATP-utilizing enzyme is the ($Ca^{2+} + Mg^{2+}$)-ATPase or Ca^{2+} pump ATPase (19). Iodoacetic acid was present to inhibit glyceraldehyde phosphate dehydrogenase (○). This treatment effectively blocks the synthesis of ATP, but due to its relatively mild reactivity, IAA does not react with other free sulfhydryl groups in the cell (20). The rate of ATP depletion appeared

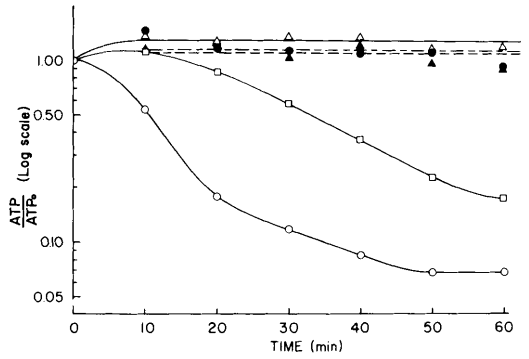

FIG. 2. Time-dependent loss of ATP in dog RBCs: influence of experimental conditions. Data are plotted as the logarithm of ATP content normalized to time = 2 min (first assay point), as a function of time in cells exposed to several different conditions. Various controls (△ = water, ▲ = 1 m*M* EGTA, ● = 1% ethanol) show the relative stability of ATP in dog RBCs in the absence of A23187. On the other hand, in the presence of A23187 (□), ATP was rapidly consumed. The simultaneous presence of IAA in the suspending medium (○) resulted in a more rapid loss of ATP. This was presumably due to inhibition by IAA of the resynthesis of ATP. The reaction conditions are as described under Materials and Methods.

to be faster in RBCs treated with IAA. When IAA was omitted from the reaction medium there was a lag in the loss of ATP (□). Apparently, resynthesis of ATP by residual substrates shifted the curve to the right and decreased the net rate of ATP loss as compared to the curve in the presence of IAA. The loss of ATP in these curves appeared to be first order until about 80% of the ATP was utilized.

The assays presented above were carried out in isotonic KCl buffer, in order to minimize Ca^{2+} movement on the Na^+/Ca^{2+} exchanger which is present in the dog RBC (21). The possibility existed that Ca^{2+} might bypass the pump and thereby decrease the measured activity. It has been shown that prolonged incubation of dog RBCs in KCl can cause hemolysis in the presence of Ca^{2+} (22). Dog RBCs contain about 7 m*M* K^+ (16) whereas human RBCs contain about 100 m*M* K^+ (23). Thus, dog RBCs are more susceptible to KCl-induced hemolysis via the "Gardos channel" than are human RBCs. KCl-induced hemolysis was avoided in the present work by relatively short incubation times. The volume of dog RBCs in the presence of A23187 and Ca^{2+} increased from 66 fl at time zero to 89 fl at the end of 60 min. Control cells (absence of A23187) increased to 71 fl over the same time. Based upon a cell surface area of 117 μM^2 (16) the theoretical maximum volume (sphere) before stretching the membrane would be 119 fl. At the end of 20 min dog RBC volumes increased to 77 fl (17% increase) for ionophore-treated cells and 68 fl (3% increase) for control cells. At the end of 20 min we observed less than 1% hemolysis of the ionophore-treated dog RBCs. Human RBCs under similar conditions increased from 89 fl (t = 0) to 97 fl (9% increase) at 20 min and 110 fl (13% increase) at the end of 60 min.

When assayed under appropriate conditions the decrease of ATP in RBCs was apparently first order. Typical first order decay profiles for ATP are shown in Fig. 3 for dog (■) and human (●) cells. We estimated that the first order decay of ATP in dog RBCs lasted for about 18 min whereas the rate of ATP loss in human RBCs was greater and was first order for about 12 min. After these times the rates decreased, presumably as the substrate became depleted. Therefore, assays were normally

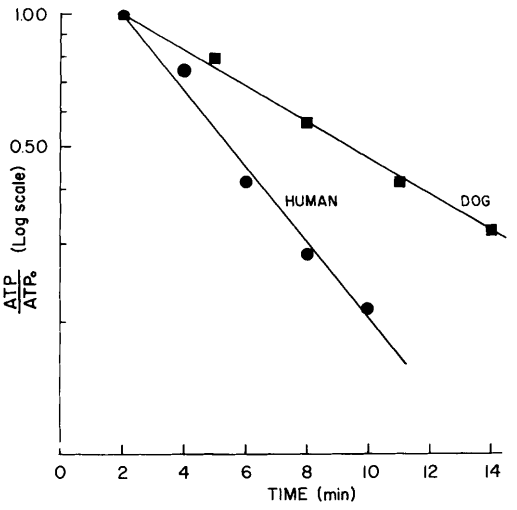

FIG. 3. Time-dependent loss of ATP from RBCs during exposure to A23187. Data are presented as a semilogarithmic plot of the ATP content, normalized to the ATP content at time = 2 min, of dog (■) and human (●) RBCs as a function of time during exposure to A23187. First order rate constants were obtained from the best-fit linear least-squares slopes, and are 0.0944 and 0.199 min^{-1} respectively. The reaction conditions are as described under Materials and Methods.

confined to the period of first order decay. The first sample was removed and analyzed 2 min after the addition of ionophore. Subsequent samples were analyzed at 3-min intervals for dog and 2-min intervals for human cells. Data points when plotted as log ATP/ATPo vs time show a first order process. First order rate constants were obtained from the slopes multiplied by −2.303. The slope is negative because the assay follows the decrease in ATP and the units of the first order rate constant are min^{-1}. The reaction becomes first order rapidly (i.e., within 2 min of the addition of ionophore) because of the limited amount of ATP contained within the cell, and because of the lack of resynthesis. We estimated that the initial ATP concentrations in fresh dog and human RBCs were about 0.85 and 1.6 mmole/liter packed RBC, respectively. When the quantity of ATP available falls below the K_m of the enzyme it then becomes rate limiting. The rate constant obtained for dog cells in Fig. 3 was 0.0944 min^{-1}. Under identical conditions the rate constant for human cells was 0.199 min^{-1}. By comparing the rate constants we estimated that the amount of $(Ca^{2+} + Mg^{2+})$-ATPase enzyme in the human cell is approximately twice that of the dog, on a *per cell basis.* This estimate assumes that the two enzymes are kinetically identical, i.e., that the two plasma membrane $(Ca^{2+} + Mg^{2+})$-ATPases have the same reaction mechanisms including Ca^{2+} and CaM dependency, assumptions for which we show support in Figs. 4 and 5.

The first order rate constant, k, for a simple Michaelis–Menten enzymatic reaction is given by

$$k = \frac{k_1 k_p [E]_t}{k_p + k_{-1}}$$

where k_1 is the rate constant for association between enzyme and substrate, k_{-1} is the rate constant for dissociation of enzyme and substrate, k_p is the rate constant of the forward reaction of the enzyme substrate complex and $[E]_t$ is the total concentration of enzyme (24), in this case the amount of enzyme in the cell. Based upon these assumptions, the ratio of first order rate constants is proportional to the ratio of the total amount of enzyme in the two species of cells. The predicted specific activity (nmole P_i/min/mg protein) of the $(Ca^{2+} + Mg^{2+})$ ATPase of isolated *membranes* of human RBCs would thus be only about 20% more than that of dog RBC membranes. This assumes that a human RBC yields 6.6×10^{-13} g protein/ghost (25) and a dog RBC yields 3.9×10^{-13} g protein/ghost, as determined in this laboratory (under Materials and Methods).

Further basic similarities between dog (■) and human (●) $(Ca^{2+} + Mg^{2+})$-ATPases are shown in Fig. 4. The two enzymes have essentially the same calcium sensitivity with 50% activation at an average value of 8.7×10^{-7} M Ca^{2+} and an average maximal activation at about 1×10^{-5} M Ca^{2+} in the external incubation medium. Both enzymes were inhibited at higher Ca^{2+} concentrations. The Ca^{2+} activation profile of the $(Ca^{2+} + Mg^{2+})$-ATPase of human RBC membranes shows 50% activation at 7.0×10^{-6} M Ca^{2+} and maximal activity at 4.0×10^{-5} M Ca^{2+} when the enzyme is activated by CaM (26). In the absence of CaM the Ca^{2+} profile does not have a maximum and tends to increase with increasing Ca^{2+} concentration. This has been substantiated by others (27) with 50% activity occurring at 4×10^{-6} M Ca^{2+} in the absence of CaM and maximal activity at 3×10^{-5} M Ca^{2+} with CaM present.

In previous studies we showed that intact, non-ATP-depleted human RBCs incubated in the presence of 0.10 mM Ca^{2+} and the ionophore, A23187, the Ca^{2+} concentration within

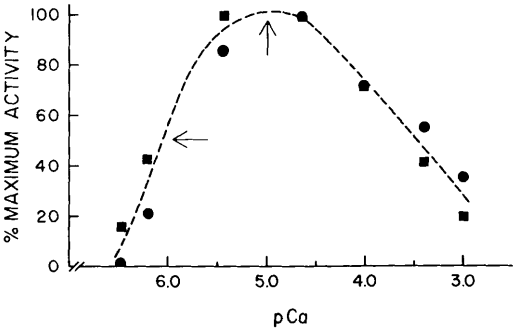

FIG. 4. ATP utilization in dog (■) and human (●) RBCs exposed to A23187: dependence on Ca^{2+}. The initial rate of the ATP utilization, as determined from the first order decay of ATP (see Fig. 3), and normalized to 100% for each species, was determined at various free Ca^{2+} levels. Arrows indicate the apparent half maximal (8.7×10^{-7} M) and maximal (1×10^{-5} M) Ca^{2+} concentrations, which were equal for each species. The reaction conditions are as described under Materials and Methods.

the cell increases to about 2.7 times the external concentration (28). With this in mind, the respective half maximal and maximal values for intracellular Ca^{2+} in the present assays would be 2.4×10^{-6} and 2.7×10^{-5} *M*. These values are in reasonable agreement with the previously noted half maximal activation values for isolated plasma membranes. The Ca^{2+} activation profile of Fig. 4 is also qualitatively the same as the CaM-activated form of the enzyme monitored in the isolated plasma membrane (Ca^{2+} + Mg^{2+})-ATPase of heart (29) and smooth (30) muscle.

Data in Fig. 4 can thus be taken to suggest that the Ca^{2+} pump enzyme is in a CaM-activated state during these assays. This is, of course, what one would expect for a CaM-dependent enzyme in the presence of Ca^{2+} and CaM. It has been shown that dog RBCs contain CaM; 47.1 vs 35.9 g/ml packed cells for human RBCs (based upon enzyme activation assays) (31). If the dog Ca^{2+} pump ATPase is CaM-activated under our assay conditions, then utilization of ATP should be inhibited by the anti-CaM drug, trifluoperazine. This would show up as a decrease in the first order rate constant. This is because CaM effects the V_{max} of the enzyme (1, 24), where $V_{max} = k_p[E]_t$. Inhibition could occur by affecting the concentration of the enzyme or by decreasing the forward rate constant k_p.

The concentration–effect curve of TFP on both dog (■) and human (●) RBCs is shown in Fig. 5. RBCs were preincubated at 37°C with TFP for 8 min prior to the addition of A23187 (10 min before the first assay sample). The results show significant inhibition by TFP of ATP utilization under these conditions. The dog and human activities had TFP IC_{50} values of 7.2×10^{-5} *M* and 6.6×10^{-5} *M*, respectively. These are in good agreement with the IC_{50} values reported by others: 1.8×10^{-5} *M* (32), 13.2×10^{-5} *M* (33), and 6.3×10^{-5} *M* (34) for isolated RBC membranes. This is further evidence that the (Ca^{2+} + Mg^{2+})-ATPase in the dog RBC is similar to that in the human RBC and that they each have properties similar to the enzyme in isolated (human, but not dog) plasma membranes.

The assays shown in Fig. 5 were conducted without measurable hemolysis. Above 2×10^{-4} *M* TFP, RBCs hemolyzed in the presence of A23187. The total amount of CaM

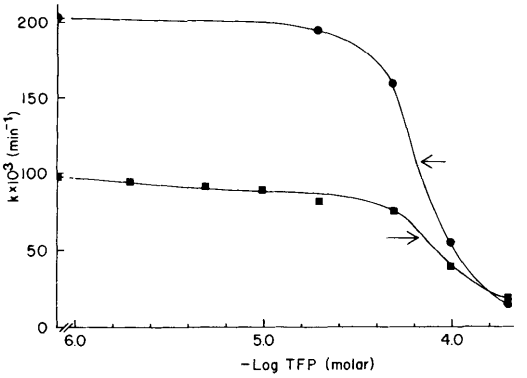

FIG. 5. Ca^{2+} pump ATPase: inhibition by TFP in dog (■) and human (●) RBCs. Data are expressed as first order rate constants of ATP decay, determined as in Fig. 3. The arrows indicate the IC_{50} values for the dog and human ATPase activities which are 7.2×10^{-5} *M* and 6.6×10^{-5} *M* respectively. The reaction conditions are as described under Materials and Methods.

inhibition was hard to assess because at high TFP concentrations basal enzyme activity was inhibited nonspecifically. Therefore, the inhibition at 2×10^{-4} *M* is likely to be the sum of the effect of binding of the drug to CaM in the presence of Ca^{2+} and nonspecific membrane perturbation (34). This was shown by measuring differential inhibition by TFP when it was added to cells prior to A23187 compared to when it was added after A23187 (the time at which, presumably, the concentration of CaM $(Ca^{2+})_n$ in the cell suddenly increased). Results of a typical experiment with dog and human RBCs can be seen in Table I. Two minutes preincubation with TFP produced much more effective inhibition than when it was added after the reaction was begun. This result is similar to that found for the isolated enzyme. Thus, TFP has been reported to be about 10 times more effective in inhibiting the CaM activation of the (Ca^{2+} + Mg^{2+})-ATPase when it is preincubated with CaM than when it is added during the reaction (33).

Calcium flux studies in RBCs using A23187 and TFP show a greater net influx of Ca^{2+} when TFP is added prior to the addition of a limiting amount of A23187 (35). Such results also support the notion that, besides the inhibition due to CaM binding, TFP causes membrane alterations. It is suggested that in the present work, at 100 μM TFP, 16% of the

TABLE I. ACTIVITY OF DOG AND HUMAN RBC Ca^{2+} PUMP ATPase: INFLUENCE OF ORDER OF ADDITION OF IONOPHORE AND TRIFLUOPERAZINE

Time of addition of TFP (100 μM)	−Rate constant (min^{-1})	Decrease (%)
Dog		
Control—no TFP	0.1104	—
−2.0 min	0.0532	52
+8.0 min	0.0926	16
Human		
Control—no TFP	0.0978	—
−2.0 min	0.0281	71
+6.0 min	0.0756	23

Note. Rate constants were determined as in Fig. 3. Results of a typical experiment show that TFP was more effective as an inhibitor of the Ca^{2+} pump ATPase in intact cells when given 2 min before, as compared to being given 6 or 8 min after, the ionophore. Dog pCa = 4.66 and human pCa = 4.02. This was a less-than-optimal rate for the human enzyme in order to allow a sufficient period of first order behavior to add TFP during the reaction. Addition of ionophore was defined as time zero.

inhibition was nonspecific in dog RBCs, and 23% was nonspecific in human RBCs. These estimates are based on the respective inhibitions of ATP loss caused by TFP addition after the ionophore (Table I). It is further suggested that the additional (specific) inhibition observed when TFP was added before the ionophore was due to TFP reacting with the $CaM(Ca^{2+})_n$ complex which formed upon the addition of A23187 and the associated influx of Ca^{2+}. Presumably, binding of TFP to the $CaM(Ca^{2+})_n$ complex (36) prevented interaction of $CaM(Ca^{2+})_n$ with the Ca^{2+}-pump ATPase because there was a great excess of TFP. Therefore, TFP had a higher probability of binding with $CaM(Ca^{2+})_n$ than did the Ca^{2+} pump ATPase under these conditions. When TFP is added after the CaM is bound to the enzyme the inhibition is less effective because of the slow off rate of CaM (37). Thus, from these and other results there appears to be a CaM-activated Ca^{2+} pump ATPase that is capable of being activated by influx of Ca^{2+} into the cell.

The plasma membrane Ca^{2+} pump is probably necessary for the cell to maintain the low intracellular Ca^{2+} levels that all cells appear to require (38). The dog RBC cell must have a functional Ca^{2+} pump ATPase because it possesses an exchanger for Na^+/Ca^{2+} which may allow influx of Ca^{2+}. There is also a functional Gardos channel in these cells which would allow K^+ influx [dog cells are normally low K^+ (16), because they lack a Na–K pump] in response to an increase in internal Ca^{2+} (22). Dog RBCs do not normally allow passive influx of K^+ which would cause them to swell (22). Other general "reasons" for the RBC Ca^{2+} pump have been reviewed (1).

In our hands, the specific activity of the human (Ca^{2+} + Mg^{2+})-ATPase is in the range of 8–15 nmole P_i/min/mg protein for the basal activity and 47–65 nmole P_i/min/mg protein for the CaM-activated activity (5, 32). Based on calculations shown above one would expect activity in dog membranes in the range of 38–52 nmole P_i/min/mg protein in the activated state. This is considerably different from the range of activities that have been previously reported in the literature for isolated membranes; 2.2–5.3 nmole P_i/min/mg protein (6, 7, 39). These reports also stated that the enzyme failed to respond to CaM stimulation.

Recently it was reported (8, 9) that IOVs prepared from dog RBCs by a one-step technique (40) had (Ca^{2+} + Mg^{2+})-ATPase activity that was CaM activatable. The authors reported a Ca^{2+} uptake rate of 383 μmole/liter cells/hour for basal uptake and CaM stimulation amounting to an average increase of 66% above this rate. The volume of the IOVs was about 50% of the original RBC volume. According to their logic, the Ca^{2+} pumping rate per liter of cells should be twice the uptake rate that was measured; or 766 μmole/liter cells/hr and the activated rate would be 1.27 mmole/liter cells/hr. In order to compare their data we used the following conversion factors; stoichiometry of Ca^{2+} pumped to ATP hydrolyzed = 1:1 (41), dog RBC volume = 66×10^{-15} l (determined in this laboratory), and a dog RBC ghost = 3.9×10^{-13} g protein (determined in this laboratory). The predicted values are thus 2.2 nmole P_i/min/mg protein for basal and 3.6 nmole P_i/min/mg protein. These values are as low as the values previously cited for isolated membranes, and in our mind do not reflect the true magnitude of the (Ca^{2+} + Mg^{2+})-ATPase that is present in the intact dog RBC. It appears that the act of membrane or IOV production causes a large loss in enzymatic activity. We believe that the values

reported in the present work are a truer reflection of the enzyme activity *in vivo*. Obviously, the present method measures the activity of enzyme in the intact cell and is less disruptive.

In contrast to our (6, 7) and others' (39) earlier results with *isolated membranes,* the present results obtained with *intact cells* support the interpretation that the Ca^{2+} pump ATPase of the dog RBC is a CaM-dependent enzyme and is similar to the human RBC enzyme. Based on assumptions of similar enzyme characteristics and on the apparent rate constants there appear to be approximately half as many Ca^{2+} pump sites in the dog RBC as in the human RBC. The mechanism of the lack of CaM sensitivity of the Ca^{2+} pump ATPase in isolated dog RBC membranes remains to be determined.

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