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Role of Magnesium in the ($Ca^{2+} + Mg^{2+}$)-Stimulated Membrane ATPase of Human Red Blood Cells

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Summary. ($Ca^{2+} + Mg^{2+}$)-stimulated ATPase of human red cell membranes as a function of ATP concentration was measured at fixed Ca^{2+} concentration and at two different but constant Mg^{2+} concentrations. Under the assumption that free ATP rather than Mg-ATP is the substrate, a value for K_m (for ATP) of 1–2 μM is found which is in good agreement with the value obtained in the phosphorylation reaction by A.F. Rega and P.J. Garrahan (1975. *J. Membrane Biol.* **22**:313). Mg^{2+} increases both the maximal rate and the affinity for ATP, whereas Ca^{2+} increases the maximal rate without affecting K_m for ATP.

As a by-product of these experiments, it was shown that after thorough removal of intracellular proteins the adenylate kinase reaction at approximately 1 mM substrate concentration is several times faster than maximal rate of ($Ca^{2+} + Mg^{2+}$)-ATPase in red cell membranes.

Both the active calcium transport and the underlying membrane bound Ca-activated ATPase of human red blood cells require the presence of Mg^{2+} ions on the internal membrane surface (Dunham & Glynn, 1961; Wins & Schoffeniels, 1966; Schatzmann, 1967, 1969; Schatzmann & Vincenzi, 1969). Ca-stimulated phosphorylation by ATP of a red cell membrane protein of 150,000 mol.wt. is thought to be a reflection of the Ca pump system (Knauf, Proverbio & Hoffman, 1974). Recently Rega and Garrahan (1975) have shown that this membrane phosphorylation is possible in the total absence of Mg^{2+} and that without Mg^{2+} the ATP concentration for half-maximal inorganic phosphate (P_i) uptake is 1.6 μM.[1] They further demonstrated that Mg is necessary for dephosphorylation, probably because it transforms the phosphorylated protein (EP) into a form which easily reacts with water.

1 ATP concentration for half-maximal P_i uptake is equal to that for half-maximal rate of ATPase under the special condition that hydrolysis of EP is prevented by omitting Mg^{2+} (*see* Discussion).

Mg^{2+} in their experiments increased ATP concentration for half-maximal P_i uptake.

Wolf (1972) had given a K_m of 43 μM for ATP in the (Ca + Mg)-stimulated ATPase reaction in the presence of MgATP and an excess of 2 mM $MgCl_2$ and claimed that free ATP cannot be accepted by the enzyme. The question arose whether the large difference between the two figures for apparent K_m[1] could be accounted for by an action of Mg^{2+}. Further examination of the role of Mg^{2+} therefore seemed warranted. In the present experiments the dependence of the activity of the (Ca + Mg)-stimulated membrane ATPase on ATP concentration was studied at two different and constant Mg^{2+} concentrations with the same constant Ca^{2+} concentration in both instances.

Materials and Methods

Human blood cells stored in the cold for 24 hr in citrate solution were washed five times with a solution of (mM) 150 NaCl, 5 KCl, 1 $MgCl_2$, the white cells being discarded. From the red cells membranes were prepared by hemolyzing an aliquot in a 9-fold volume of (mM) 20 tris-Cl, 20 sucrose, 10 NaCl, 5×10^{-3} $CaCl_2$ (pH 7.45 at 20 °C) at 0 °C and subsequent washing in (mM) 2 tris-Cl, 2 sucrose, 1 NaCl, 1 KCl, 5×10^{-3} $CaCl_2$ (pH 7.7 at 20 °C) at 0 °C until colorless (5 times). The membranes were rapidly frozen in this solution at −70 °C and thawed the next day immediately before the experiment.

Effect of Mg^{2+} at Constant Ca^{2+} Concentration

An aliquot of 0.2 ml membrane suspension (0.7–0.8 mg protein) was incubated for 10, 12 or 15 min at 37 °C in 9.8 ml medium (all concentrations are calculated per 10 ml). Medium plus membranes were prewarmed, the reaction started by adding ATP and terminated by adding 5 ml 10% cold trichloroacetic acid. The sample was removed from the water bath, another 5 ml trichloroacetic acid added and the resulting 20 ml filtered into ice-cooled flasks. 10 or 15 ml were assayed for *inorganic phosphate* according to Berenblum and Chain (1938). The samples contained (mM) 110 choline-Cl, 30 imidazole-Cl (pH 7.0 at 37 °C), 0.17 ouabain, 0.0125–0.2 tris-ATP and either (a) 4 $MgCl_2$, 0.04 $CaCl_2$ with Ca^{2+}-free controls having $CaCl_2$ replaced by 1 tris-EGTA, or (b) Ca-Mg-EDTA buffer (9 tris-EDTA, 8 $CaCl_2$, 1.45 $MgCl_2$ giving 0.04 Ca^{2+} and 0.42 Mg^{2+} in the medium) with Ca^{2+}-free controls having 3 EGTA-$MgCl_2$, giving 0.42 Mg^{2+}. Calculation for the Ca-Mg buffer is given in the Appendix.

Effect of Ca^{2+} at Constant Mg^{2+} Concentration

The assay medium contained variable concentrations of MgATP, 2 mM $MgCl_2$ and 0.05 mM $CaCl_2$ or 1 mM Ca-EGTA of [Ca^{2+}] 2×10^{-6} M. The other constituents were the same as above. Ca^{2+} free controls had 1 mM tris-EGTA instead of Ca.

Total ATP concentrations appearing in the graphs are the arithmetic mean between initial and final concentration, the latter being obtained by subtraction of P_i from ATP and corrected for adenylate kinase (*see* Appendix). The correction is slight and does not

influence the qualitative result. The mean ATP concentration was never less than 80% of the initial ATP concentration. In calculating free ATP not only the Mg-complex but also the Ca-complex was taken into account, assuming identical dissociation constants for both ($K = 10^{-3.67}$, Wolf, 1972). The correction for Ca was negligible in the high Mg experiments and 5–6% in the low Mg experiments.

Protein was measured according to Lowry *et al.* (1951). *Adenylate kinase* was assayed by incubating membranes (approx. 0.1 mg protein/ml) in a quartz photometer cuvette at 37 °C with AMP and ATP to produce ADP. Rate of formation of ADP was monitored by recording the reduction of NADH at 338 nm coupled to ADP rephosphorylation via the system phosphoenolpyruvate, pyruvate kinase, lactate dehydrogenase. The assay medium was composed according to Bergmeyer (1970) except for the presence of 1.5 mM $MgCl_2$, 0.4 mM tris-EGTA (to suppress Ca-Mg-ATPase) and 0.17 mM ouabain (to suppress Na + K-ATPase). Mg-ATPase was measured by omitting AMP and subtracted. Adenylate kinase was inhibited by diadenosinepentaphosphate (Feldhaus *et al.*, 1975).

Na_2ATP, Na_2AMP, diadenosinepentaphosphate, phosphoenolpyruvate cyclohexylammonium, Na salt of NADH, pyruvate kinase and lactate dehydrogenase were obtained from Boehringer Mannheim; EDTA and EGTA were from Siegfried Zofingen; choline-Cl and sucrose were the products of BDH Chemicals, Ltd; all other chemicals were analytical grade from Merck, Darmstadt or Fluka, Buchs. Tris-EDTA and tris-EGTA was made from the acids. Tris-ATP was made from Na_2ATP by ion exchange on an amberlite I.R. 120 column and freeze-dried. Its ADP content was 4% according to thin layer chromatography on polyethylene-imine impregnated cellulose MN 300 (Macherey and Nagel) in 2 M Na-formate, pH 3.4. The molecular weight was checked both by absorption at 260 nm and phosphate measurement after destruction in 70% perchloric acid at 180 °C. Mg-ATP was made from Na_2ATP on an amberlite I.R. 120 column before each experiment and the true concentration of the solution determined by perchloric acid destruction and P_i measurement. ATP solutions were neutralized with tris before adding them to the assay medium.

Results

ATP concentrations were corrected under the assumption that adenylate kinase activity is not lost during preparation of membranes (Sen & Post, 1964) and is large compared to (Ca + Mg)-ATPase activity (*see* Appendix (B)). Preliminary experiments showed that this is so. Adenylate kinase activity was measured in two membrane preparations and was found to be 11.6 and 19.4 μmole/(mg protein) × hr (ADP formed from ATP and AMP at approximately 1 mM concentration and 37 °C) which is 10 times faster than the maximal rate of (Ca + Mg)-ATPase, which under the same conditions was 1.8 and 2.3 μmole/(mg protein) × hr in these preparations. This finding demonstrates that adenylate kinase in human red cells is preserved under the conditions used for preparing membranes. 20 μM diadenosinepentaphosphate completely blocked the enzyme.

The composition of the Ca-Mg-EDTA buffer (9 mM EDTA, 8 mM $CaCl_2$, 1.45 $MgCl_2$) gives $[Ca^{2+}] = 0.04$ mM and $[Mg^{2+}] = 0.42$. In this

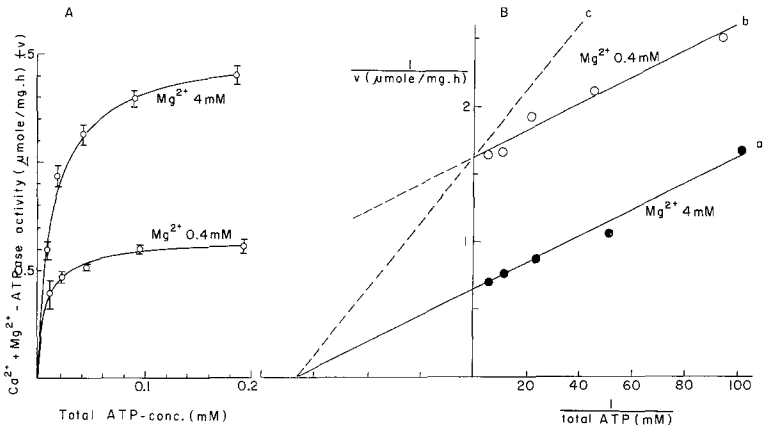

Fig. 1. (*A*) Ordinate: Rate of $(Ca^{2+}+Mg^{2+})$-stimulated ATPase (μmole P_i liberated per mg protein and hour) of human red cell membranes at Ca^{2+} concentration 0.04 mM and 37 °C. Samples contain 0.07–0.08 mg membrane protein/ml. For medium, *see* Methods. Abscissa: Mean total ATP concentration (=approximately 80% of added ATP; SEM smaller than circles). 4 expts. with 4 different batches of blood, vertical bars = ±1 SEM. (*B*) Lineweaver-Burk plot of the same data. Calculated regression lines solid. The difference in slope between line *b* and *c* is statistically significant ($P<0.001$), which shows that the *x*-axes intercepts of *a* and *b* are significantly different

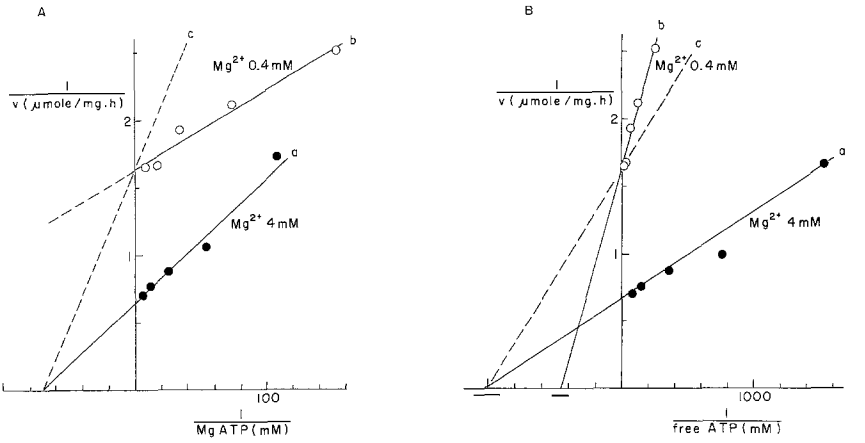

Fig. 2. Same data as Fig. 1 with mean MgATP conc. (*A*), or mean free Mg conc. (*B*) as independent variable. Solid lines are calculated regression lines. (*B*) Horizontal bars below *x*-axis are standard error of intercepts. Difference in slope between lines *b* and *c* is statistically significant ($P<0.02$), therefore decrease in K_m by Mg^{2+} seems real. For choice between *A* and *B* *see* Discussion

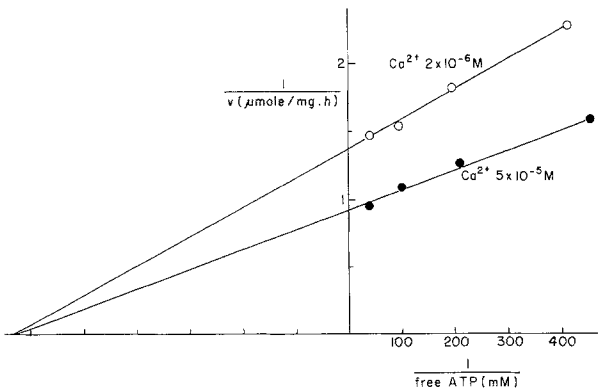

Fig. 3. Dependence of $(Ca^{2+} + Mg^{2+})$-ATPase on free ATP concentration. Lineweaver-Burk plot of a single experiment. Mg^{2+} conc. approx. constant (ATP added as MgATP + 2 mM $MgCl_2$) and Ca^{2+} conc. either 5×10^{-5} M (unbuffered) or 2×10^{-6} (buffered with 1 mM EGTA-Ca buffer). *Notice:* increase in v_{max} by Ca^{2+} but no effect of Ca^{2+} on K_m

buffer [CaEDTA] is nearly 8 mM, [MgEDTA] approximately 1 mM and free [EDTA] is vanishingly small. Since the dissociation constants for Ca and MgATP are 7 and 5 orders larger than those of the EDTA complexes even in the case of Mg the buffer capacity will easily outweigh the effect of 0.2 mM or less ATP. In the high Mg experiments no buffer was used because the large excess of Mg keeps the Mg^{2+} and Ca^{2+} concentration virtually constant. The influence of Mg^{2+} on the dependence of (Ca + Mg)-ATPase activity on ATP concentration is shown in Fig. 1. The main effect of a tenfold increase in Mg^{2+} concentration is an increase of v_{max} from 0.61 to 1.55 μmole/(mg protein) × hr. In addition there is an apparent increase of K_m from 5.96 to 15.4 μM. On the assumption that either free ATP or MgATP is the substrate for the enzyme the data can be replotted as shown in Fig. 2. The apparent decrease in affinity by Mg^{2+} is enhanced in the activity-MgATP plot. Converting the abscissa to free ATP shifts the apparent K_m by a factor corresponding to the (MgATP)/(ATP) ratio which is 1.6 for the low Mg^{2+} experiment and 14 for the high Mg^{2+} experiment. This leads to an apparent reversal of the Mg-effect on K_m. Table 1 summarizes the apparent K_m values according to the different hypotheses regarding the true substrate.

Fig. 3 demonstrates that a change in the Ca^{2+} concentration by a factor of 25 in the range where Ca^{2+} has a marked effect on the overall activity ($K_{Ca} \sim 1$ μM) alters the v_{max} by a factor of 1.5 but has no effect on the affinity for ATP.

Discussion

The behavior seen in Fig. 2 is compatible with either of two alternative assumptions: (I) MgATP is the substrate (whereby is meant that the affinity for MgATP is much larger than for free ATP) and Mg^{2+} decreases the affinity for MgATP, or (II) free ATP is the substrate and Mg^{2+} increases the affinity for free ATP. The possibility that both species are equally acceptable is dealt with below. A decision between the two alternatives on the basis of the present experiments is not possible. If we adopt the highly probable assumption that Ca sensitive phosphorylation of membranes by ATP is mainly due to the (Ca + Mg)-ATPase we are forced by the experiments of Rega and Garrahan (1975) to admit that the (Ca + Mg)-ATPase can react with ATP in the absence of Mg and hence that free ATP is or can be the substrate.

The possibility that both MgATP and free ATP serve equally well as substrate or that the affinity for MgATP is even higher than for free ATP is not compatible with the present finding that the apparent K_m at high Mg^{2+} concentration is larger than at low Mg^{2+} concentration when total ATP is considered to be the substrate. The possibility remains that the system has equal affinity for the two species and that Mg, bound to a second receptor site, lowers the affinity of the ATP accepting site in general. This assumption, however, leads to disagreement with Rega and Garrahan's finding of a low K_m, whereas alternative *II* (in Table 1) results in a K_m of 1–2 μM which agrees well with their figure of 1.6 μM. Alternative *II* (not taken absolutely but in the sense that the affinity for free ATP is higher than for MgATP) therefore is almost certainly preferable to alternative *III* (table) and seems more likely than alternative *I* (table).

Table 1. K_m values for ATP at low and high Mg^{2+} concentration for the different hypotheses regarding the substrate

$[Mg^{2+}]$ (mM)	Hypothesis		
	I. ATP_{total} is the substrate (μM)	II. free ATP is the substrate (μM)	III. MgATP is the substrate (μM)
0.42	5.96	2.27	3.67
4.0	15.4	1.02	14.37

For preference of hypothesis *II* *see* Discussion.

Rega and Garrahan measured ATP concentration for half-maximal P_i uptake in 30 sec, i.e., when equilibrium is reached, under the condition that decomposition of EP is blocked by removal of Mg. If the sequence of events is

$$E + \mathrm{ATP} \overset{K_1}{\rightleftharpoons} E \cdot \mathrm{ATP} \overset{K_2}{\rightleftharpoons} E \cdot P + \mathrm{ADP} (\rightleftharpoons \cdots E + P_i + \mathrm{ADP})$$

and if one makes the plausible assumption that the affinity for ADP is low and that the reactions of E with ATP and ADP are not rate limiting, [ATP] for half-maximal rate of ATP hydrolysis will be identical to [ATP] for half-maximal level of EP but it will not equal K_1 which is the conventional Michaelis-Menten constant.

Fig. 2. *B* shows that a tenfold increase in Mg^{2+} concentration increases rather than decreases the affinity for (free) ATP in a statistically significant way.

The increase by Mg^{2+} of the maximal rate indicates that it affects the enzyme at a step beyond ATP binding which is in agreement with the finding by Rega and Garrahan (1975) on the phosphorylation-dephosphorylation reactions. It cannot be decided whether the effect on v_{max} and on K_m are exerted by binding of Mg^{2+} to one or several sites.

The effect on K_m distinguishes Mg^{2+} from Ca^{2+} which seems to increase only v_{max} (Fig. 3). The absence of an affinity change by Ca^{2+} for ATP rules out the possibility that Ca binding is a prerequisite for ATP binding. Consequently the sequence of Ca and ATP binding is random or ATP occupies its site before Ca can be attached.

The present results support the idea that Ca-induced phosphorylation of human red cell membranes reflects the activity of the (Ca + Mg)-ATPase and hence the Ca-pump protein. They are in agreement with the claim that the important action of Ca^{2+} and Mg^{2+} is at a step beyond ATP binding to the system. The finding that in addition Mg^{2+} seems to favor ATP binding has probably no physiological significance since the system normally operates at an ATP concentration far above half saturation.

Wolf (1972) has concluded that free ATP cannot be the substrate because the pH dependence of v_{max} or K_m is inconspicuous at pH 6.5, i.e., the proton concentration corresponding to the proton dissociation constant of ATP. This conclusion may be fallacious if ionized groups are important, because the argument does not take into account the possible pH dependence of the receptor group, or erroneous because ionized groups might be of minor importance for ATP binding. At any rate it is difficult to reconcile it with the arguments advanced here.

Appendix

A. Calculation for the Composition of Ca + Mg-EDTA Buffer

To calculate the apparent metal dissociation constant

$$K'_{MeL}(\text{at pH } 7.0) = K_{MeL} \cdot \left(1 + \frac{H}{K_1} + \frac{H^2}{K_1 \cdot K_2} + \ldots \frac{H^n}{K_1 \cdot K_2 \ldots K_n}\right)$$

the proton dissociation constants for EDTA $K_1 = 5.5 \cdot 10^{-11}$, $K_2 = 6.9 \cdot 10^{-7}$ and $K_3 = 2.14 \cdot 10^{-3}$ and $K_{MgL} = 2.04 \cdot 10^{-9}$ and $K_{CaL} = 2.57 \cdot 10^{-11}$ were used (Wolf, 1973). By the following procedure of trial and error the composition of the buffer was found ($Ca = [Ca^{2+}]$, $Mg = [Mg^{2+}]$, $(CaL) = [\text{Ca-EDTA}]$, $(MgL) = [\text{Mg-EDTA}]$, $L = [EDTA_{free}]$, $L_T = [EDTA_{total}]$, $Ca_T = [Ca_{total}]$, $Mg_T = [Mg_{total}]$, K'_{CaL} = apparent dissociation constant of Ca-EDTA, K'_{MgL} = apparent dissociation constant of Mg-EDTA).

The following equations hold:

$$(CaL) = Ca_T - Ca, \tag{A1}$$

$$Mg_T = Mg + (MgL), \tag{A2}$$

$$(MgL) = L_T - L - (CaL), \tag{A3}$$

$$(Ca \times L)/(CaL) = K'_{CaL}, \tag{A4}$$

$$(Mg \times L)/(MgL) = K'_{MgL}, \tag{A5}$$

$$K'_{MgL}/K'_{CaL} = [(CaL) \times Mg]/[(MgL) \times Ca]. \tag{A6}$$

The desired values for L_T, Ca and Mg are chosen and a guess about ${}^aCa_T$ (superscript "a" means tentative value) is made. Then (A1) becomes

$${}^a(CaL) = ({}^aCa_T - Ca).$$

(A1) in (A4),

$${}^aL = K'_{CaL}({}^aCa_T - Ca)/Ca.$$

(A1) and (A4) in (A3),

$${}^a(MgL) = L_T - K'_{CaL}({}^aCa_T - Ca)/Ca - ({}^aCa_T - Ca). \tag{A7}$$

(A7) in (A2),

$${}^aMg_T = Mg + L_T - K'_{CaL}({}^aCa_T - Ca)/Ca - ({}^aCa_T - Ca).$$

Ca, Mg, ${}^a(MgL)$ and ${}^a(CaL)$ must satisfy (A6). If they do not, corrections in the appropriate direction are made on ${}^aCa_T$ or on ${}^aL_T$ and the

procedure repeated until (A6) holds. The method has the advantage that it makes no simplifying assumptions and with some practice is quite rapid.

B. Correcting ATP Concentration Taking into Account Adenylate Kinase Activity

Since adenylate kinase activity is large compared to (Ca + Mg)-ATPase activity (*see* text) equilibrium according to $\frac{\text{AMP} \times \text{ATP}}{\text{ADP}^2} = K$ is maintained at all times. For K a reliable value seems to be 0.76–0.88 (Markland & Wadkins, 1966). Simplifying, a value of 1 was inserted.

The following equations hold (M=[AMP]; T=[ATP]; D=[ADP] formed for a given amount of inorganic phosphate liberated; A_T=added [ATP]; P_i=concentration of liberated inorganic phosphate):

$$\frac{T \times M}{D^2} \sim 1. \tag{B1}$$

$$M + D + T = A_T. \tag{B2}$$

$$2M + D = P_i. \tag{B3}$$

Combining (B2) and (B3), $$D = 2A_T - P_i - 2T. \tag{B4}$$

Combining (B1) and (B3), $$T \cdot P_i = 2D^2 + T \cdot D. \tag{B5}$$

Inserting (B4) in (B5) and rearranging results in a quadratic equation in ATP,

$$6T^2 + (7P_i - 14A_T) \cdot T + (8A_T^2 - 8A_T P_i + 2P_i^2) = 0. \tag{B6}$$

[ATP] from Eq. (B6) differs from ($[\text{ATP}_{\text{total}}] - [P_i]$) by a factor of 1.01 if 10% of ATP appear as P_i, similarly by 1.07 at 30%, 1.23 at 50%, 1.86 at 75% and 3.85 at 90% P_i liberation.

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