Active Uptake of Ca\(^{++}\) and Ca\(^{++}\)-Activated Mg\(^{++}\) ATPase in Red Cell Membrane Fragments

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ABSTRACT Isolated human red blood cell membrane fragments (RBCMCF) were found to take up Ca\(^{++}\) in the presence of ATP.\(^1\) This ATP-dependent Ca\(^{++}\) uptake by RBCMCF appears to be the manifestation of an active Ca\(^{++}\) transport mechanism in the red cell membrane reported previously (Schatzmann, 1966; Lee and Shin, 1969). The influences of altering experimental conditions on Ca\(^{++}\)-stimulated Mg\(^{++}\) ATPase (Ca\(^{++}\) ATPase) and Ca\(^{++}\) uptake of RBCMCF were studied. It was found that pretreatment of RBCMCF at 50\(^{\circ}\)C abolished both Ca\(^{++}\) ATPase and Ca\(^{++}\) uptake. Pretreatment of RBCMCF with phospholipases A and C decreased both Ca\(^{++}\) ATPase and Ca\(^{++}\) uptake, whereas pretreatment with phospholipase D did not significantly alter either Ca\(^{++}\) ATPase or Ca\(^{++}\) uptake. Both Ca\(^{++}\) ATPase and Ca\(^{++}\) uptake had ATP specificity, similar optimum pH's, and optimum incubation temperatures. From these results, it was concluded that Ca\(^{++}\) uptake is intimately linked to Ca\(^{++}\) ATPase.

INTRODUCTION

It has been previously shown that the red cell membrane has an active Ca\(^{++}\) extrusion mechanism (Schatzmann, 1966; Schatzmann and Vincenzi, 1969; Lee and Shin, 1969). Also, it was found that the fragmented red cell membrane splits ATP in the presence of Mg\(^{++}\) and this Mg\(^{++}\) ATPase activity is greatly stimulated by Ca\(^{++}\). Thus, this Ca\(^{++}\)-stimulated Mg\(^{++}\) ATPase (Ca\(^{++}\) ATPase) was suggested to be intimately associated with the active Ca\(^{++}\) transport mechanism (Schatzmann and Vincenzi, 1969; Vincenzi and Schatzmann, 1967). However, no conclusive evidence is available as to the suggested intimate relationship. In the present investigation, an attempt

\(^1\) The following abbreviations are used in this paper: EDTA, ethylenediamine tetraacetate; EGTA, ethylene glycol bis (\(\beta\)-aminoethyl ether)-N\(^{\prime}\),N\(^{\prime}\)-tetraacetic acid; ATP, adenosinetriphosphate; CTP, cytidine triphosphate; ITP, inosine triphosphate; GTP, guanosine triphosphate; UTP, uridine triphosphate.
was made to correlate this Ca++ ATPase and the active transport mechanism in the red blood cell membrane. The data presented here strongly indicate that in the red cell membrane the Ca++ ATPase is intimately linked to the active transport of Ca++.

**METHODS**

*Preparation of Ca++ ATPase of Red Cell Membrane* All procedures were performed at 0–4°C. Citrated human red blood cells (RBC) were washed four times with six times their volume of 0.9% NaCl, and after centrifugation the white buffy layer overlaying RBC was eliminated as completely as possible. After the last washing, 1 volume of packed RBC was hemolyzed in 5 volumes of a solution containing 1 mM EDTA and 1 mM Tris buffer (pH 7.0). About 15 min after hemolysis, the whole homogenate was centrifuged at 20,000 × g for 15 min. The precipitate (ghost cells) was sequentially washed twice with 3 volumes of the lysing solution, once with 3 volumes of 10 mM Na₂EDTA solution, and once again with 3 volumes of the lysing solution. This precipitate was washed once with 3 volumes of 2.0% NaCl solution, 4 times with 0.5 mM histidine-imidazole buffer, pH 7.0, and was finally suspended in 0.5 mM histidine-imidazole buffer (pH 7.0). The final suspension, which consisted of red blood cell membrane fragments (RBCMF), contained 3–5 mg protein per ml and was stored at −20°C before use.

*Measurement of ATPase Activities* The compositions of standard reaction mixtures for ATPase activities are shown in Table I, and any deviation from these compositions is indicated in the text. The procedure for measurement of ATPase activity was as follows: The reaction mixture (total volume 5 ml) containing all components except ATP and including RBCMF, was pre-equilibrated at 37°C for 3 min, and the reaction was started by adding ATP to the mixture. 30 min after the incubation at 37°C, the reaction was stopped by adding 1 ml of cold 20% trichloric acid or perchloric acid. Then the mixture was centrifuged and the amount of inorganic acid (Pi) in the supernatant was measured by the method of Fiske and Subbarow (1929). The difference of ATPase activities between Mg++ ATPase and Ca++ ATPase was considered to be due to activation by Ca++. All ATPase activities are expressed in micromoles Pi liberated per milligram protein per hour.

**TABLE I**

COMPOSITION OF THE STANDARD REACTION MIXTURES FOR MEASUREMENT OF ATPase ACTIVITIES

|                      | Mg++ ATPase | Ca++ ATPase |
|----------------------|-------------|-------------|
| mM                   | mM          | mM          |
| KCl                  | 120         | 120         |
| CaCl₂                | —           | 0.5         |
| EGTA                 | 0.5         | —           |
| Histidine-imidazole buffer, pH 7.0 | 30 | 30 |
| MgCl₂                | 5           | 5           |
| ATP                  | 2           | 2           |
Measurement of ATP-Dependent Ca\textsuperscript{++} Uptake by RBCMF  
In the standard type of experiment, RBCMF (0.8–1 mg/ml reaction mixture) were incubated in a reaction mixture which contained (mM) 120 KCl, 30 histidine-imidazole buffer (pH 7.0), 5 MgCl\textsubscript{2}, 1 ATP, and 0.08\mu Ci of \textsuperscript{45}Ca. The \textsuperscript{45}Ca contributed only about 10 \textsuperscript{-6}M Ca++ which was much less than the contaminating calcium in the reaction mixture (see Results section). The mixture, whose total volume was 2.5 ml, including 0.5 ml of RBCMF suspension, was incubated at 37°C for various periods, and the reaction was stopped by blowing 5 ml of ice cold 0.5 mM histidine-imidazole buffer into the reaction tube, which was immediately placed in an ice bath. RBCMF were centrifuged at 20,000 g for 15 min. The precipitate was washed twice with 5 ml of the ice cold histidine-imidazole buffer (0.5 mM, pH 7.0), and the final precipitate was suspended in 2 ml of 0.1 M NaOH solution. The final precipitate suspension (0.5 ml) was applied to a planchet and dried, and the radioactivity was measured in a thin window Nuclear Chicago gas flow counter. Also, the radioactivity of the original supernatant of the incubation mixture was measured in the same way. Any deviation from standard conditions is described in the text or figures.

Pretreatment of RBCMF with Phospholipases  
1 ml of RBCMF suspension was incubated with phospholipase A (1 mg), phospholipase C (1 mg), or phospholipase D (100 units) for 20 min, at 37°C. The mixture was then chilled in an ice bath and centrifuged. The precipitate was washed once with 0.5 mM histidine-imidazole buffer and resuspended in the original volume of the histidine-imidazole buffer. Phospholipase A was obtained from Calbiochem (Los Angeles, Calif.), and phospholipase C and D were obtained from Nutritional Biochemical Corp., Cleveland, Ohio.

Measurement of Free Calcium in Reaction Mixture  
The free concentration of Ca++ in the reaction mixture was adjusted and measured by the EGTA-CaEGTA buffer system and the murexide method, respectively, as described by Klaus and Lee (1969).

RESULTS

ATP-Dependent Ca\textsuperscript{++} Uptake of RBCMF  
The Ca++ uptake by RBCMF was measured in the following four different media: (a) the standard mixture; (b) the standard mixture except that ATP was omitted; (c) the standard mixture except that the free Ca++ concentration was maintained at 1 \muM by a Ca++-EGTA buffer system; (d) the standard mixture except that the free Ca++ concentration was maintained at 1 \muM and no ATP was added. After 5 min incubation, 5 ml of ice-cold 0.5 mM histidine-imidazole buffer was blown into the reaction tube, which was then placed in an ice bath. The reaction mixture was equally divided into five tubes and centrifuged. The subsequent precipitates were washed zero to five times with 5 ml of histidine-imidazole buffer (0.5 mM, pH 7.0) and the radioactivity of the final precipitate was measured in the manner described in the Methods section. The results are shown in Fig. 1. The unwashed RBCMF had the highest \textsuperscript{45}Ca activity. The first washing resulted in a marked decrease in \textsuperscript{45}Ca activity of RBCMF, but the subsequent washings caused a slow decrease of the radio-
activity of RBCMF, both in the presence and absence of ATP. The extrapolation of values obtained during the washing procedure gives the theoretical value of $^{46}$Ca taken up by RBCMF before washing. It was noted that the standard mixture in which only a trace of $^{46}$Ca was added was more favorable for measurement of $^{46}$Ca uptake than the mixture in which the free Ca$^{++}$ concentration was maintained at 1 $\mu$m by the Ca-EGTA buffer system. It should be mentioned, however, that since the specific activity of $^{46}$Ca in the EGTA buffer medium and that in the standard mixture were different, the comparison of absolute amounts of Ca$^{++}$ taken up by RBCMF in these media is not made here, and is unknown. The "contaminating" Ca$^{++}$ concentration of the standard mixture was found to be approximately
6 μM as measured by the murexide method. The findings presented in Fig. 1 clearly indicate that there is an ATP-dependent Ca\(^{++}\) uptake by RBCMF which is not lost significantly by the washing procedure.

In view of the above data, most subsequent experiments on \(^{45}\)Ca uptake were performed under standard conditions in which RBCMF were incubated in the medium to which no exogenous Ca\(^{++}\) was added except \(^{46}\)Ca. The radioactivity of twice washed precipitates was measured, since these values represented reasonably well the ATP-dependent Ca\(^{++}\) taken up by RBCMF.

Effects of ATP Concentrations on \(^{45}\)Ca Uptake The calcium uptake by RBCMF was measured in the presence of ATP in varying concentrations, with other conditions being standard. The results shown in Fig. 2 indicate that under the present experimental conditions the optimum ATP concentration for Ca\(^{++}\) uptake is 1 mM.

![Graph showing effects of ATP concentration on \(^{45}\)Ca uptake](image-url)

**Figure 2.** Effects of ATP concentration on \(^{45}\)Ca uptake. Each point represents an average of three experiments, performed with the same RBCMF. Condition of incubation is standard, except that ATP concentration was varied. Radioactivity is expressed in terms of counts per minute per milligram of protein of RBCMF.
Effect of Incubation Time on 45Ca Uptake The 45Ca uptake of RBCMF was measured after the mixture was incubated for various intervals at 37°C under standard conditions (Fig. 3). The uptake of 45Ca proceeded rapidly during the initial 5 min of incubation, and then leveled off slowly. The maximum 45Ca uptake was reached only after 40 min of incubation.

Effect of Oxalate on the ATP-Dependent Ca++ Uptake Since the time-dependent 45Ca uptake of RBCMF in the presence of ATP was similar to the ATP-dependent Ca++ uptake by the sarcoplasmic reticulum from the muscle tissue (Hasselbach, 1964; Lee, 1965, and others), the effect of oxalate, which increases the Ca++ uptake by the sarcoplasmic reticulum, was studied in the present preparation. In this experiment, oxalate was added to the reaction mixture in a concentration of 5 mM, and the Mg++ concentration was 15 mM. All other conditions were standard. Results presented in Fig. 4 show that the presence of oxalate in the medium increased the ATP-dependent 45Ca uptake of RBCMF in a manner which was similar to that observed with muscle sarcoplasmic reticulum fragments.

Effect of Addition of Ca++ during Incubation on 45Ca Uptake RBCMF were incubated under standard conditions and nonradioactive Ca++ was suddenly introduced into the reaction mixture 20 min after the initiation of reaction. The addition of nonradioactive Ca++ in a concentration of 1 mM decreased the radioactivity of RBCMF (Fig. 5). This finding indicated that some of the 45Ca previously taken up by RBCMF was replaced by the nonradioactive Ca++ introduced in the middle of the experiment.

Mg++ ATPase and Ca++ ATPase Under the standard conditions (see
Figure 4. Effect of oxalate on $^{46}$Ca uptake. RBCMF were incubated under standard conditions, with and without oxalate and/or ATP. Concentration of oxalate, 5 mM. Average of three experiments.

Figure 5. Effects of the addition of Ca$^{++}$ during incubation in $^{46}$Ca uptake experiments. Condition of incubation, standard. At the arrow, unlabeled Ca$^{++}$ in a concentration of 1 mM was added to the reaction mixture. Average of three experiments.
Methods section), activities of Mg++ ATPase and Ca++-stimulated Mg++ ATPase (Ca++ ATPase) were found to be 0.33 μmole ± 0.02 (SE) and 1.65 μmoles ± 0.12 (SE) Pi released per mg protein per hr (20 experiments), respectively.

It was previously suggested that the Ca++ transport mechanism in the red cell membrane appeared to be intimately related to this Ca++ ATPase of RBCMF (Schatzmann and Vincenzi, 1969; Vincenzi and Schatzmann, 1967). In the following experiments, the effects of altering experimental conditions on 46Ca uptake and Ca++ ATPase of RBCMF were studied in order to investigate the relationship between these two functions of RBCMF. In all experiments, 46Ca uptake and Ca++ ATPase were measured after 30 min incubation.

**Effects of Mg++ Concentration on 46Ca Uptake and Ca++ ATPase of RBCMF**

46Ca uptake and Ca++ ATPase activity of RBCMF were determined under standard conditions except for varying concentrations of Mg++. These results are shown in Fig. 6. The effect of the Mg++ concentration on 46Ca uptake in the absence of ATP was also investigated to ascertain the possible competition between Mg++ and Ca++ in the absence of ATP. It was found that, in the absence of ATP, an increase in the concentration of Mg++ was accompanied by a decrease in the 46Ca uptake. On the other hand, in the presence of ATP, increasing the Mg++ concentration resulted in an increase
in $^{45}\text{Ca}$ uptake. Similarly, an increase in Mg++ concentration led to an increase in Ca++ ATPase activity. These findings strongly support the idea that $^{45}\text{Ca}$ uptake is intimately associated with Ca++ ATPase and that an increase in $^{45}\text{Ca}$ uptake very closely parallels Ca++ ATPase activity.

**Effects of Preincubation at Different Temperatures on $^{45}\text{Ca}$ Uptake and Ca++ ATPase of RBCMF**

The suspension of RBCMF was preincubated at various temperatures for 30 min, and the Ca++ ATPase activity and $^{45}\text{Ca}$ uptake of the RBCMF were then measured under standard conditions (at 37°C, for 30 min). Preincubation of RBCMF for 30 min at temperatures up to 40°C had no significant effect on both Ca++ ATPase and $^{45}\text{Ca}$ uptake (Fig. 7).

However, preincubation of RBCMF at 50°C resulted in an abolition of both the Ca++ ATPase activity and the $^{45}\text{Ca}$ uptake.

**Effects of the Incubation Temperature on Ca++ ATPase and $^{45}\text{Ca}$ Uptake**

Experiments on $^{45}\text{Ca}$ uptake and Ca++ ATPase were carried out under standard conditions, except that the incubation temperature was varied from 0°C to 50°C. These results are shown in Fig. 8. There was a very close parallelism between the effects of incubation temperature on these two parameters, and 40°C appears to be the optimal temperature for both these functions of RBCMF.

**Effects of Pretreatment of RBCMF with Lipases**

The RBCMF suspensions were pretreated with phospholipases A, C, and D in the manner described in
the Methods section, and $^{46}$Ca uptake and Ca$^{++}$ ATPase of the treated RBCMF were investigated under standard conditions. The pretreatment with phospholipases A or C markedly depressed both $^{46}$Ca uptake and Ca$^{++}$

![Graph](image.png)

**Figure 8.** Effects of incubation temperature on $^{46}$Ca uptake and Ca$^{++}$ ATPase activity. Both $^{46}$Ca uptake and Ca$^{++}$ ATPase activity were measured under standard conditions, except for the incubation temperature. Average of four experiments.

| Treatment          | Radioactivity, cpm per mg protein of RBCMF | Ca$^{++}$ ATPase, μmole per mg protein per hr |
|--------------------|---------------------------------------------|-----------------------------------------------|
| None               | 1901                                        | 1.70                                          |
| Phospholipase A    | 905                                         | 0.62                                          |
| Phospholipase C    | 832                                         | 0.59                                          |
| Phospholipase D    | 1544                                        | 1.55                                          |

Phospholipase-treated RBCMF were used under the standard conditions of measurements. Average of five experiments.

ATPase (Table II). However, phospholipase D did not significantly influence either $^{46}$Ca uptake or Ca$^{++}$ ATPase.

**Specificity of Nucleoside Triphosphates** It was found that ATP is the specific substrate for both $^{46}$Ca uptake and Ca$^{++}$ ATPase of RBCMF (Table III). CTP, GTP, and ITP caused negligible uptake and were very poorly hydrolyzed as compared with ATP. This specificity of ATP for $^{46}$Ca uptake and
TABLE III
NUCLEOSIDE TRIPHOSPHATE SPECIFICITY ON
\(^{45}\text{Ca} \) UPTAKE AND Ca\(^{++}\) ATPase OF RBCMF

| Function            | Nucleotide | ATP  | CTP  | GTP  | ITP  |
|---------------------|------------|------|------|------|------|
| \(^{45}\text{Ca} \) uptake | RBCMF only | 3209 | 154  | 205  | 154  |
|                     | RBCMF + original supernatant | 3269 | 2671 | 3479 | 3405 |
| Ca\(^{++}\) ATPase  | RBCMF only | 1.84 | 0.21 | 0.09 | 0.15 |
|                     | RBCMF + original supernatant | 2.67 | 2.94 | —    | 2.99 |

\(^{45}\text{Ca} \) uptake is expressed in terms of counts per minute per milligram of protein of RBCMF. Ca\(^{++}\) ATPase is expressed in micromoles of Pi liberated per milligram of protein per hour. Average of six experiments. All nucleotides were in 1 mm.

Ca\(^{++}\) ATPase was in contrast with results obtained previously, which showed Ca\(^{++}\) extrusion in resealed red blood cells in the presence of CTP, GTP, and ITP. In order to investigate this aspect further, the following experiments were conducted: The first supernatant from the whole cell hemolysate (at the first hemolysis in the lysing solution of 1 mM EDTA) was saved, and 0.5 ml of this original supernatant was added to the standard reaction mixture in addition to RBCMF (0.5 ml) for measurements of Ca\(^{++}\) ATPase and \(^{45}\text{Ca} \) uptake. The results are shown in Table III. It is clear that the presence of the original supernatant enables CTP, GTP, and ITP to promote \(^{45}\text{Ca} \) uptake and to be hydrolyzed.

DISCUSSION

It has been previously shown that the red cell membrane has a very active calcium extrusion mechanism, which is ATP-dependent (Schatzmann, 1966; Schatzmann and Vincenzi, 1969; Olson and Cazort, 1969; Lee and Shin, 1969). When the red cell membrane is fragmented in the manner described here, red cell membrane fragments take up significantly more Ca\(^{++}\) from the medium in the presence of ATP than in the absence of ATP.

The fact that the \(^{45}\text{Ca} \) uptake observed here is clearly ATP-dependent and requires Mg\(^{++}\) is supported by the following data. In the absence of ATP, an increase in Mg\(^{++}\) concentration is accompanied by a decrease in \(^{45}\text{Ca} \) uptake by RBCMF, which would be expected in the case of competition between Mg\(^{++}\) and Ca\(^{++}\) for nonspecific binding to RBCMF (see Fig. 6). On the other hand, in the presence of ATP, an increase in Mg\(^{++}\) concentration results in an increase in \(^{45}\text{Ca} \) uptake by RBCMF, which indicates that
the \(^{45}\)Ca uptake requires ATP and Mg++. As the Mg++ concentration is increased, the Ca++-stimulated Mg++ ATPase (Ca++ ATPase) activity increases in a manner parallel with an increase in \(^{45}\)Ca uptake. These data strongly indicate that the \(^{45}\)Ca uptake is an active process involving Mg++-catalyzed ATPase activity.

The optimum Ca++ uptake by RBCMF occurs with an ATP concentration of 1 mM, under the present experimental conditions. Once Ca++ is taken up by RBCMF, the affinity is such that washing releases the Ca++ taken up from RBCMF somewhat, but not markedly (see Fig. 1). The \(^{45}\)Ca uptake process resembles many membrane transport phenomena, in that it is time-dependent and reaches a maximum after 40-80 min of incubation (see Fig. 3). These findings indicate that the observed Ca++ uptake is associated with the Ca++ transport phenomenon, which was previously reported by Schatzmann (1966) and by Lee and Shin (1969).

The causal relationship between Ca++ uptake and Ca++ ATPase is indicated by results obtained in studies altering the experimental conditions. Preincubation of RBCMF at 50°C markedly diminished both Ca++ uptake and Ca++ ATPase activity. Preexposure of RBCMF to temperatures lower than 50°C did not alter either of these functions. Both functions of RBCMF have the same optimal temperature of incubation. Pretreatment of RBCMF with phospholipase A or C, which decreased Ca++ ATPase, also decreased the Ca++ uptake. On the other hand, phospholipase D, which did not influence Ca++ ATPase activity, also did not alter Ca++ uptake. Both Ca++ uptake and Ca++ ATPase reach maximum activity at similar Mg++ concentrations (3-10 mM). Thus, in all experiments performed in the present study, Ca++ uptake parallels Ca++ ATPase activity. Data presented in Tables II and III indicate that ATP is the specific substrate for both \(^{45}\)Ca uptake and Ca++ ATPase. However, CTP, GTP, and ITP can substitute for ATP for both Ca++ ATPase activity and \(^{45}\)Ca uptake in the presence of other factors found in the original supernatant from the hemolysis of the red blood cell. All the above findings strongly support the hypothesis that the Ca++ ATPase is intimately involved with the Ca++ transport mechanism in the red cell membrane.

With regard to the nature of Ca++ uptake by RBCMF, it is unknown, at present, whether the Ca++ taken up by RBCMF is transported into the vesicle lumen or is bound to membrane. The more likely possibility is that Ca++ is bound to membrane in the presence of ATP through the mechanism associated with the Ca++ transport system. The sudden introduction of a large amount of unlabeled Ca++ into the reaction mixture during the process of \(^{45}\)Ca uptake decreased the radioactivity of RBCMF (see Fig. 5). This finding may be due to the replacement of \(^{45}\)Ca previously taken up by unlabeled Ca++
during continued uptake, and may suggest that calcium taken up is bound to the membrane, which in turn is in equilibrium with calcium in the medium. On the other hand, the possibility that the Ca\textsuperscript{2+} uptake is a reversed phenomenon of the outward Ca\textsuperscript{2+} transport mechanism, reported previously by Schatzmann (1966) and others, cannot be ruled out. Recent studies of Penninger and Green (1968) and Steck et al. (1970) showed, through electron microscopic and other morphological investigations, that the red cell membrane, when fragmented under certain experimental conditions, formed vesicles which were inside out. If such inside-out vesicles are formed under the present experimental conditions, then the outward Ca\textsuperscript{2+} transport observed in the normal red cell membrane would be expressed as the Ca\textsuperscript{2+} uptake by the reversed RBCMF. It was found that, in the presence of oxalate, the ATP-dependent \textsuperscript{45}Ca uptake was greater. This finding may suggest the possibility that \textsuperscript{46}Ca may be transported into vesicles, and oxalate may facilitate \textsuperscript{45}Ca uptake by forming insoluble calcium-oxalate intravesicularly, as is the case with the sarcoplasmic reticulum (Hasselbach, 1964; Hasselbach and Makino, 1961). Another possibility is that some vesicles are inside out and some are not, and the conflicting phenomena mentioned above are due to the fact that the present preparation is the mixture of these different vesicles. However, at present, no evidence is available for these possibilities. Thus, further studies are required for elucidation of the mechanism of this Ca\textsuperscript{2+} uptake by RBCMF.

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