Phosphorylation of the Red Blood Cell Membrane during the Active Transport of Ca++

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ABSTRACT The phosphorylation of red blood cell membrane fragments (RBCMF) during Ca++ transport was investigated. When red cell membrane fragments are incubated with [γ-32P]ATP under the experimental condition which minimizes the phosphorylation of Na+-K+-ATPase, RBCMF are labeled in the presence of Mg++ without Ca++. When Ca++ is added, the labeling decreases due to dephosphorylation of RBCMF. The initial reaction of phosphorylation is reversed in the presence of excess ADP. The treatment of RBCMF with n-ethylmaleimide (NEM) does not interfere with the initial phosphorylation reaction, but blocks the dephosphorylation in the presence of Ca++. These data suggest that the enzymatic sequence of the Ca++ transport mechanism may be very similar to that of the Na+ transport mechanism.

INTRODUCTION

It has been shown previously that the red blood cell membrane has an active transport mechanism for outward extrusion of Ca++ (Schatzmann and Vincenzi, 1969; Lee and Shin, 1969), and ATP is the specific energy source of the Ca++ transport system (Vincenzi and Schatzmann, 1967; Cha et al., 1971). Previously, it was found that when red blood cells are fragmented, red blood cell membrane fragments (RBCMF) were found to take up Ca++ and this Ca++ uptake is accompanied by ATP hydrolysis by Mg++-activated ATPase (Lee and Shin, 1969; Cha et al. 1971). It has been suggested that this Ca++ uptake is due to the presence of inside out vesicles in the present system although not all of the vesicles are inside out (Steck et al., 1970; Weiner and Lee, 1972). RBCMF were found to have both Na+-K+-ATPase and Ca++-ATPase which are intimately associated with the sodium and calcium transport mechanisms, respectively (Schatzmann, 1966; Skou, 1965; Lee and Shin, 1969).

Numerous studies on the sodium transport mechanism in the red blood cell membrane indicate the involvement of phosphorylated intermediates during the enzymatic process (Albers, 1967; Albers et al., 1968; Post et al., 1969). Therefore, an attempt has been made to investigate the possibility that the similar phosphorylation of RBCMF may also be involved with the calcium transport mechanism. It was found that the calcium transport mechanism appears to share similarities with the sodium transport mechanism with regard to phosphorylation mechanisms involved with the enzymatic action.
METHODS

Preparation of RBCMF

RBCMF were prepared according to the procedure used previously in this laboratory (Cha et al., 1971).

Measurement of ATPase Activities

The Ca++-ATPase activity (in the presence of Mg++) was measured by incubating RBCMF in the standard medium for 10 min at 37°C. The standard medium for Ca++-ATPase contained: 120 mM KCl; 30 mM histidine-imidazole buffer, pH 7.0; 3 mM MgCl₂; 2 mM ATP; and 0.5 mM CaCl₂. The Na+-K+-ATPase was measured by incubating RBCMF for 10 min at 37°C in the medium containing: 100 mM NaCl; 10 mM KCl; 30 mM histidine-imidazole buffer, pH 7.0; 3 mM MgCl₂; and 2 mM ATP.

Measurement of ATP-Dependent Ca ++ Uptake by RBCMF

The Ca++ uptake of RBCMF was measured according to the method of Cha et al. (1971) employing ⁴⁰Ca. In this method, the radioactivity of RBCMF after incubation of RBCMF with ⁴⁰Ca in the presence of ATP was measured.

Measurements of Phosphorylation of RBCMF

In phosphorylation experiments, RBCMF (1 mg protein/ml reaction mixture) were incubated at 37°C in a standard medium for phosphorylation which contained 120 mM KCl, 30 mM histidine-imidazole buffer (pH 7.0), 0.1 mM EGTA (ethylene diamine tetraacetic acid), 2 μM radioactive ATP (1 μCi/M), and 3 mM MgCl₂. Before the actual incubation, RBCMF were equilibrated at 37°C for 5 min in the standard medium without MgCl₂ and the reaction was started by adding MgCl₂ into the mixture after the equilibration. The absence of Na+ from the reaction mixture in the presence of KCl was essential to exclude any possibility that the phosphorylation might result from Na+-dependent phosphorylation of Na+-K+-ATPase present in RBCMF. After various periods of incubation the reaction was stopped by adding 5 ml of ice-cold 5% trichloroacetic acid (TCA) (with 0.01 mM ATP and 0.1 mM P_i) to the reaction mixture and placing it in an ice bath immediately. When Ca++-dependent dephosphorylation was desired, Ca++ (0.2 mM) was introduced at an appropriate time after the reaction had been started, and the incubation was continued until the reaction was stopped by adding TCA. The mixture was then centrifuged at 2,000 g for 10 min at 4°C. The precipitate was washed twice with 5 ml of the same TCA solution, and the final precipitate was suspended in 2 ml of 0.1 N NaOH. A half milliliter of the suspension was applied to a planchet or put into a scintillation vial with 10 ml Bray's solution (Bray, 1960), and counted either in a thin-window Nuclear Chicago gas flow counter or in a Packard scintillation counter (Packard Instrument Co., Inc., Downers Grove, Ill.), respectively.

RESULTS

ATPase Activities and ATP-Dependent Ca++ Uptake

The Na+-K+-activated and Ca++-activated ATPase (in the presence of Mg++) of RBCMF were 0.45 and 1.68 μmol P_i/mg protein/h, respectively. The Ca++ uptake of RBCMF in the presence and absence of ATP after 1 h of incubation were 45.2 and 6.1 nmol/mg protein, respectively. This indicates that the present RBCMF has an active Ca++ transport mechanism.
Binding of $[\alpha^{32}\text{P}]\text{ATP}$ and $[\gamma^{32}\text{P}]\text{ATP}$ to RBCMFe

Radioactive ATP, labeled at $\alpha$ or $\gamma$ position was incubated in the standard medium for phosphorylation for 30 and 60 min with RBCMFe, and the radioactivity taken up by RBCMFe during the incubation period was measured. Results are shown in Table I.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Time incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>30 min</td>
</tr>
<tr>
<td>$[\alpha^{32}\text{P}]\text{ATP}$</td>
<td>558</td>
</tr>
<tr>
<td>$[\gamma^{32}\text{P}]\text{ATP}$</td>
<td>5962</td>
</tr>
</tbody>
</table>

$^{32}\text{P}$-labeling is expressed in terms of counts per minute per 0.25 mg protein of RBCMFe.

As can be seen in this table, the radioactivity of RBCMFe after TCA treatment was significant with $[\gamma^{32}\text{P}]\text{ATP}$, but not with $[\alpha^{32}\text{P}]\text{ATP}$. This indicates that RBCMFe is phosphorylated with $[\gamma^{32}\text{P}]\text{ATP}$ only, and not by $[\alpha^{32}\text{P}]\text{ATP}$. In all the following experiments, only $[\gamma^{32}\text{P}]\text{ATP}$ was employed for all phosphorylation experiments. It should be noted that the phosphorylation of RBCMFe by $[\gamma^{32}\text{P}]\text{ATP}$ occurs in the medium which contains 120 mM KCl and no NaCl, the condition which minimizes the possibility of phosphorylation of Na$^+$-K$^+$-ATPase by ATP.

Influence of Mg$^{++}$ and Ca$^{++}$ on Phosphorylation

Radioactive labeling of RBCMFe was measured after incubation of RBCMFe in mixtures containing the basic components (BC) of 120 mM KCl, 30 mM histidine-imidazole buffer (pH 7.0), 0.1 mM EGTA, 2 $\mu$M radioactive ATP (1 $\mu$Ci/M) and the following variations: BC only, (no Mg$^{++}$, no Ca$^{++}$ in Fig. 1), BC + 3 mM MgCl$_2$ (Mg$^{++}$ in Fig. 1), BC + 0.5 mM CaCl$_2$ (Ca$^{++}$ but no Mg$^{++}$ in Fig. 1), BC + 3 mM MgCl$_2$ + 0.5 mM CaCl$_2$ (Ca$^{++}$ at zero time in Fig. 1). Also, in some experiments with BC + 3 mM MgCl$_2$ (Mg$^{++}$ in Fig. 1) 0.5 mM CaCl$_2$ was added 5 min after the incubation started. Results are shown in Fig. 1. In the presence of Mg$^{++}$ alone (Mg$^{++}$ in Fig. 1), RBCMFe were very rapidly phosphorylated in the first few seconds, and then continued at a diminished rate during the remainder of the 10-min incubation period. Without Mg$^{++}$ in media (no Mg$^{++}$, no Ca$^{++}$, and Ca$^{++}$ but no Mg$^{++}$, in Fig. 1), no significant labeling of RBCMFe occurred. Although it is not shown in Fig. 1, the addition of KCl up to 5 min after the incubation in Mg$^{++}$ solution did not have any effect on the labeling of RBCMFe. When both Ca$^{++}$ and Mg$^{++}$ were present from the start of the reaction (Ca$^{++}$ at zero time in Fig. 1), RBCMFe were phosphorylated slightly within the first few seconds and progressed slowly during the rest of the incubation time. These results indicate that the presence of Mg$^{++}$ and Ca$^{++}$ are required for phosphorylation and dephosphorylation of RBCMFe, respectively. Thus, the greatest
labeling of RBCMF occurs when media contained Mg ++ only, and the labeling decreases when Ca ++ is also present in media.

Influence of Addition of ADP on Labeling during Incubation

Experiments were performed where 0.2 mM ADP was added 5 min after RBCMF were incubated in the standard medium for phosphorylation. It should be mentioned that the standard medium is the same as the medium noted

"Mg ++" in Fig. 1. As shown in Fig. 2, the addition of ADP caused an initial rapid fall of radioactivity of RBCMF, which was followed by an increase in labeling at the same rate as that of the control with Mg ++ alone. In Fig. 2, the effect of addition of nonradioactive ATP (0.2 mM) during the incubation is also shown. The labeling was diluted by formation of nonradioactive phosphate intermediaries, when cold ATP was added. Since Ca ++ is absent in the medium, the effect of ADP in decreasing the labeling may be due to the shift of reaction $E + ATP \rightleftharpoons ADP + E - P$ to the left, although other unknown mechanisms maybe responsible for this phenomenon.

Influence of EDTA on the Response of RBCMF to the Addition of ADP or Ca ++

Five minutes after RBCMF were incubated in the standard medium for phosphorylation, EDTA (ethylene diamine tetraacetate) in a concentration of 5 mM was added to the reaction mixture. Results are shown in Fig. 3. As can be seen in this figure, the addition of EDTA is followed by the cessation of further labeling of RBCMF. This probably is due to the chelation of Mg ++ by EDTA, which
results in the lack of Mg\textsuperscript{++} necessary for the phosphorylation reaction. When 0.2 mM ADP is added immediately after EDTA, no effect of ADP is observed. This supports the suggestion made previously that the effect of ADP is due to the reversal of the initial phosphorylation reaction, which requires Mg\textsuperscript{++}. The addition of Ca\textsuperscript{++} after EDTA still causes the decrease in labeling of RBCMF probably because the dephosphorylation requires the presence of Ca\textsuperscript{++} but not Mg\textsuperscript{++}.

Figure 2. Effects of ADP and ATP on labeling of RBCMF. Experimental conditions: Incubation medium contained 120 mM KCl, 30 mM histidine-imidazole buffer (pH 7.0) 0.1 mM EGTA, 2 \mu M radioactive ATP, and 3 mM MgCl\textsubscript{2}. ADP (0.2 mM) or ATP (0.2 mM) was added 5 min after incubation was started.

Influence of ADP and Ca\textsuperscript{++} on Labeling of NEM-Treated RBCMF

RBCMF were treated with 0.2 mM NEM at 37°C for 20 min before the incubation. The NEM-treated RBCMF were then incubated with [\gamma-\textsuperscript{32P}]ATP in the standard medium for phosphorylation and either ADP or Ca\textsuperscript{++} was added 5 min after incubation. The radioactivity of RBCMF during various stages of experiments is shown in Fig. 4. As can be seen in this figure, the addition of Ca\textsuperscript{++} does not influence the labeling of NEM-treated RBCMF. The addition of ADP, however, still causes a fall in labeling of NEM-treated RBCMF similar to that observed with nontreated RBCMF. This suggests that NEM treatment blocks dephosphorylation in the presence of Ca\textsuperscript{++}, but does not block the effect of ADP, which acts on the initial phosphorylation step.
**Addition of \(^{45}\text{Ca}\) to the Previously Phosphorylated RBCMF**

Five minutes after RBCMF were incubated in a medium containing 20 \(\mu\text{M}\) radioactive ATP in the presence of \(\text{Mg}^{++}\) alone, \(^{45}\text{Ca}\) (1 \(\mu\text{Ci}/\text{mM}\)) was introduced into the reaction mixture. The 10-times greater concentration of ATP above the ATP concentration used in the phosphorylation experiments was used here, because a significant amount of \(^{45}\text{Ca}\) uptake was needed in this experiment. As can be seen in Fig. 5, the level of phosphorylation decreased to a certain level and remained so, while the \(^{45}\text{Ca}\) was continuously being taken up by the RBCMF. Thus, it appears that a steady level of phosphorylation is maintained during the active \(\text{Ca}^{++}\) uptake in RBCMF, although further investigation is required to ascertain this aspect.

**DISCUSSION**

It has been established that \(\text{Na}^{+}\text{-K}^{+}\)-activated ATPase and \(\text{Ca}^{++}\)-activated ATPase are intimately associated with outward extrusion of \(\text{Na}^{+}\) and \(\text{Ca}^{2+}\), respectively (Skou, 1957 and 1965; Hoffman, 1961; Schatzmann, 1966; Albers, 1967; Post et al., 1969; Schatzmann and Vincenzi, 1969; Lee and Shin, 1969). Since the present RBCMF has both of the above two ATPase activities, an attempt has been made to investigate the similarity of these two active transport systems in molecular mechanisms. The sequence of enzymatic steps involved in \(\text{Na}^{+}\text{-K}^{+}\)-ATPase is intensively studied by many investigators, whereas very little is known.
with regard to the enzymatic sequence of the Ca\textsuperscript{++} transport system of Ca\textsuperscript{++}-ATPase in RBCMFB.

With Na\textsuperscript{+}-K\textsuperscript{+}-ATPase, evidence suggests that the following sequential reaction takes place (Bader et al., 1966; Albers et al., 1968; Post et al., 1969):

\[
\text{ATP} + E_1^{\text{Na}^+ \text{Mg}^{++}} \rightarrow E_1'P + ADP
\]

Mg\textsuperscript{++} blocked by NEM

\[
P_i + E_2 \rightarrow E_2'P + H_2O
\]

In the present study the possibility that the Ca\textsuperscript{++} transport mechanism of RBCMFB may go through a similar sequence has been explored.

The radioactivities of RBCMFB incubated in the standard medium for phosphorylation with either [\textalpha\textsuperscript{32P}]ATP or [\textgamma\textsuperscript{32P}]ATP are shown in Table I. The data indicate that the terminal phosphate of ATP forms an acid stable complex with RBCMFB under experimental conditions.

The participation of phosphorylated intermediates in Na\textsuperscript{+} transport has been investigated by numerous investigators, and it is known that labeling of the Na\textsuperscript{+}-activated membrane ATPase occurs during the transport of Na\textsuperscript{+} (Avruch and Fairbanks, 1972; Post et al., 1969; Blostein, 1968; Knauf et al., 1974 a, b).

Although the present RBCMFB has both Na\textsuperscript{+}-K\textsuperscript{+}-ATPase and Ca\textsuperscript{++}-ATPase, the experimental conditions make it unlikely that this labeling of RBCMFB is due to the phosphorylation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. This incubation medium for phosphorylation contains no Na\textsuperscript{+} which is needed for phosphorylation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. On the other hand, the medium has K\textsuperscript{+} (120 mM) which mediates dephosphorylation of Na\textsuperscript{+}-K\textsuperscript{+}-ATPase. Furthermore, the addition of K\textsuperscript{+} to the labeled RBCMFB did not have any effect, whereas the addition of Ca\textsuperscript{++} decreased
the labeling (Fig. 1). These data indicate that the labeling of RBCMF under the present experimental condition is due to the phosphorylation of Ca\(^{++}\)-ATPase of RBCMF.

Data in Fig. 1 show that the phosphorylation of RBCMF proceeds throughout the experiment in the presence of Mg\(^{++}\) only. Thus the initial phosphorylation step appears to require Mg\(^{++}\), but not Ca\(^{++}\). The addition of Ca\(^{++}\) to labeled RBCMF is followed by a rapid fall of radioactivity of RBCMF. The rapid fall of radioactivity of RBCMF may be due either to the inhibition of phosphorylation or acceleration of dephosphorylation. The inhibition of phosphorylation probably due to competition of Ca\(^{++}\) with Mg\(^{++}\) appears unlikely because in NEM-treated RBCMF, the addition of Ca\(^{++}\) has no effect whatsoever on the phosphorylation as indicated by the continuous labeling of RBCMF (Fig. 4). Thus the fall of labeling after the addition of Ca\(^{++}\) probably is due to dephosphorylation of phosphorylated intermediates. Since, as discussed above, the Ca\(^{++}\) transport ATPase and not Na\(^{+}\)-K\(^{+}\)-ATPase is phosphorylated under the experimental conditions, it appears that the fall of labeling after the addition of Ca\(^{++}\) is due to dephosphorylation of Ca\(^{++}\) transport ATPase in the presence of Ca\(^{++}\). In this connection, results obtained in the recent work by Knauf et al. (1974 b) should be mentioned. These workers measured phosphorylation of red blood cell membrane in media containing Mg\(^{++}\) and Na\(^{+}\) (or Na\(^{+}\)-K\(^{+}\)) at 0°C, and found that the presence of Ca\(^{++}\) increased phosphorylation. Two possibilities were suggested. One possibility was that the Ca\(^{++}\) phosphoprotein in their study may be different from that of Ca\(^{++}\) transport ATPase, since conditions of their study (tempera-
ture, ATP concentrations, etc.) were different from those of previous works on the Ca++ transport ATPase. Another possibility suggested was that the Ca++ phosphoprotein characterized in their work may represent an intermediate in the Mg++-Ca++-ATPase involved with Ca++ transport. If this is the case, then the different results obtained in their and the present study may be reconciled by assuming that Ca++ is required for the step from $E_1P$ to $E_2P$. Since the Ca++ transport ATPase is very sensitive to temperature and no dephosphorylation occurs at 0°C (Cha et al., 1971), the presence of Ca++ would increase phosphorylation by forming $E_2P$ without dephosphorylation of this intermediate at 0°C in the study of Knauf et al. (1974 b). As discussed by the authors in their study, another cause of increased phosphorylation by Ca++ was the inhibition of dephosphorylation of Na+-K+-ATPase by Ca++. In the present study, on the other hand, this condition of increasing phosphorylation by Ca++ does not exist, since no phosphorylation of Na+-K+-ATPase presumably occurs in the present experimental conditions. Thus the present Ca++ transport ATPase seems to require Mg++ and Ca++ for the initial phosphorylation and dephosphorylation, respectively. Another aspect to be considered with the phosphorylated intermediates of the Ca++ transport system in the RBCMF in this study is to compare the present system with that of sarcoplasmic reticulum. Previously, the participation of phosphorylated intermediates in the Ca++ transport system in sarcoplasmic reticulum of skeletal muscle has been reported (Hasselbach and Makinose, 1962; Yamamoto and Tonomura, 1967; Martonosi, 1967, 1969). There are many differences between phosphorylation of the Ca++ transport system in sarcoplasmic reticulum and in RBCMF. In sarcoplasmic reticulum, Ca++ increases phosphorylation and Mg++ decreases the level of phosphorylation, whereas in RBCMF it is the opposite, namely, Mg++ and Ca++ are required for phosphorylation and dephosphorylation, respectively. Thus, Martonosi (1969) found that phosphorylated intermediates of sarcoplasmic reticulum appear in the presence of Ca++ only and almost no phosphorylated intermediates with Mg++ alone, whereas the opposite is true with the RBCMF in this study. Furthermore, the concentrations of ATP and pH of media for the optimal phosphorylation of sarcoplasmic reticulum are quite different from those of RBCMF.

It is interesting to note that when Ca++ is present at zero time along with Mg++, labeling of RBCMF occurs very slowly, since the steady state of phosphorylation and dephosphorylation is reached from the beginning of the experiment. Also it is noted that the considerable initial phosphorylation is found in the absence of Ca++ and Mg++. This may be due to the presence of endogenous Mg++ and Ca++ in RBCMF.

The effect of ADP on the phosphorylated RBCMF is shown in Fig. 2. The addition of ADP initially causes a rapid fall of labeling, then the relabeling of RBCMF proceeds at the same rate as the control. Since this medium does not contain Ca++, dephosphorylation would not occur. The mechanism of this effect of ADP is not known at present. However, the following possibility is suggested. This effect of ADP in decreasing the labeling (Fig. 2) may be due to the shift of the initial phosphorylating step: $E + ATP \rightarrow E-P + ADP$ to left. This is supported by the finding that ADP decreases the labeling of RBCMF only.
temporarily (Fig. 2), and the ADP effect is not observed when Mg$^{++}$ is not present (Fig. 3, after addition of EDTA). Furthermore, the effect of ADP is observed in the medium which does not contain Ca$^{++}$. However, the addition of Ca$^{++}$ after EDTA still brings about the decrease in labeling of RBCMF (Fig. 3). This again indicates that the dephosphorylation of Ca$^{++}$ ATPase requires the presence of Ca$^{++}$, but not Mg$^{++}$.

In NEM-treated RBCMF, the addition of Ca$^{++}$ did not have any effect on the labeling of RBCMF. This suggests that NEM blocks somewhere along the enzymatic processes which lead to the dephosphorylation of Ca$^{++}$-ATPase. This suggests that NEM blocks the reaction somewhere after the phosphorylation and before the dephosphorylation. The effect of ADP in decreasing the labeling is still observed in NEM-treated RBCMF, and this indicates that NEM does not interfere with the initial phosphorylating step. Thus, the enzymatic sequence of the calcium transport mechanism (Ca$^{++}$ ATPase) may be presented in the following scheme:

\[
\text{ATP} + E \xrightarrow{\text{Mg}^{++}} E-P + \text{ADP} \\
\text{Ca}^{++} \downarrow \text{NEM-sensitive} \\
E + P.
\]

Thus, the enzymatic sequence of the Ca$^{++}$ transport system is very similar to that of the Na$^{+}$ transport mechanism in the red blood cell membrane.

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