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MODULATION OF THE ACTIVITY OF THE (Ca^{2+} + Mg^{2+})-DEPENDENT ADENOSINE TRIPHOSPHATASE OF THE HUMAN ERYTHROCYTE

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We previously reported that the activity of the (Ca^{2+} + Mg^{2+})-dependent adenosine triphosphatase (ATPase) of the human erythrocyte membrane is inhibited by micromolar or nanomolar concentrations of cyclic AMP. Our further studies have now indicated that the inhibition of (Ca^{2+} + Mg^{2+})-dependent phosphohydrolase activity requires the participation of a membrane-associated cyclic AMP-dependent protein kinase and a membrane-associated protein substrate that is distinct from the ATPase itself. We have furthermore, identified a 20 kDa membrane protein which undergoes phosphorylation that is promoted by micromolar, but not millimolar, concentrations of cyclic AMP and which, when phosphorylated, undergoes dephosphorylation that is promoted by Ca^{2+}. We suggest that this membrane component can participate in the modulation of the activity of the (Ca^{2+} + Mg^{2+})-dependent ATPase of the human erythrocyte.

Cyclic AMP-induced inhibition of the (Ca^{2+} + Mg^{2+})-dependent ATPase activity of the human erythrocyte membrane exhibits the identical distinctive concentration optimum as the phosphotransferase activity of the cyclic AMP-dependent protein kinase, which was isolated from the same source and purified in our laboratory. Both the phosphotransferase activity of the protein kinase [1] and the inhibition of (Ca^{2+} + Mg^{2+})-dependent ATPase activity [2] are maximal in the presence of micromolar or nanomolar concentrations of cyclic AMP, while millimolar concentrations are ineffective. We suggest that the inhibitory effect of cyclic AMP upon the (Ca^{2+} + Mg^{2+})-dependent ATPase activity of the human erythrocyte involves membrane protein phosphorylation catalyzed by the cyclic AMP-dependent protein kinase.

When erythrocyte membranes were exposed to 0.2% Triton X-100 over 70% of the (Ca^{2+} + Mg^{2+})-dependent ATPase activity was solubilized and this activity retained its sensitivity to micromolar or nanomolar concentrations of cyclic AMP with millimolar concentrations continuing to exert no effect as illustrated by data given in Table I. Since we had previously demonstrated that 0.2%Triton X-100 solubilizes both the cyclic AMP-dependent protein kinase of the erythrocyte membrane and membrane components that serve as its substrate [1], we concluded that our solubilized preparation of the (Ca^{2+} + Mg^{2+})-dependent ATPase contained in addition to the latter, the cyclic AMP-dependent protein kinase and a substrate that underwent phosphorylation and, thereby, became an inhibitor. Hence, we were able to demonstrate cyclic AMP-induced inhibition of the (Ca^{2+} + Mg^{2+})-dependent ATPase activity using this preparation. We also observed however,

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TABLE I

EFFECT OF CYCLIC AMP CONCENTRATION UPON ERYTHROCYTE ($Ca^{2+} + Mg^{2+}$)-DEPENDENT ATPase ACTIVITY

Membranes from human erythrocytes were isolated following lysis in 40 mosM phosphate buffer. For the purpose of enzyme solubilization, 0.2% Triton X-100 or, alternatively, 0.2% Trition X-100 with 0.1 mM Ca^{2+} was used. Particulate or solubilized preparations were examined for ($Ca^{2+} + Mg^{2+}$)-dependent ATPase (phosphohydrolase) activity under the following conditions. The reaction medium comprised 18 mM histidine- 18 mM imidazole buffer (pH 7.1), 3 mM magnesium chloride, 0.1 mM ouabain, 0.1 mM calcium chloride, the membrane preparation (0.8 to 5.0 mg protein/ml, detergent/protein ratio of 1 in solubilized preparations) and 3 mM disodium ATP (containing $3 \cdot 10^5$ cpm [γ-^{32}P]ATP) in a total volume of 500 μl. Following incubation for 20 min at 37°C enzymatic hydrolysis was terminated by the addition of 1.0 ml of 5% (w/v) trichloroacetic acid containing 2 mM potassium dihydrogen phosphate. Immediately thereafter, 1.0 ml of 1.25 M sulfuric acid containing 5% (w/v) ammonium molybdate was added followed by 5 ml of isobutanol/benzene (1 : 1, v/v). After thorough mixing for 30 s the resulting (triphasic) suspension was centrifuged and, subsequently, an aliquot of organic phase was transferred to a vial, Aquasol was added, and radioactivity was determined using liquid scintillation spectrometry. ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity is expressed in terms of nanomoles of inorganic phosphate liberated per mg protein per hour, with standard errors given. Nonenzymatic hydrolysis of ATP was assessed with each determination using a reaction medium that contained all of the components except the membrane preparation.

Cyclic AMP added	Phosphohydrolase activity (nmol P/mg per h)		
	Isolated membranes	Solubilized (Triton)	Solubilized (Triton + Ca^{2+})
None	373 ± 1	469 ± 2	1255–2
1 mM	370 ± 2	468 ± 2	1260 ± 1
10 μM	356 ± 1	440 ± 2	1255 ± 1
1 μM	324 ± 2	410 ± 1	1258 ± 2
10 nM	318 ± 1	412 ± 2	1261 ± 1
1 nM	337 ± 1	415 ± 2	1257 ± 1

that if the solubilization was conducted using a Triton solution that, in addition, contained 0.1 mM calcium chloride the solubilized enzyme now exhibited ($Ca^{2+} + Mg^{2+}$)-dependent phosphohydrolase activity that was enhanced approximately 3-fold, as compared to when the detergent solution lacked Ca^{2+}, but it was no longer sensitive to cyclic AMP, as illustrated by the data provided in the last column of the table. Subsequently, it was determined that if the preparation obtained following solubilization in the presence of Triton without Ca^{2+} was subjected to gel filtration on Sephacryl 300, the ($Ca^{2+} + Mg^{2+}$)-dependent phosphohydrolase activity obtained was, again, insensitive to cyclic AMP. In a typical experiment specific activities of preparations obtained following gel-filtration chromatography were 2314 nmol P/mg per h in the absence of added cyclic AMP and 2293 and 2312 nmol P/mg per h in the presence of 1.0 μM and 1.0 mM cyclic AMP, respectively. Our gel filtration procedure yielded a preparation of the ($Ca^{2+} + Mg^{2+}$)-dependent ATPase (125 kDa) that also included the cyclic AMP-dependent protein kinase (160 kDa) but it lacked protein components of 80 kDa or less. Since this preparation lacked all sensitivity to cyclic AMP it was implied that the inhibition of ($Ca^{2+} + Mg^{2+}$)-dependent phosphohydrolase activity requires not only a membrane-associated cyclic AMP-dependent protein kinase but also a membrane-associated protein substrate that is less then 80 kDa and distinct from the catalytic component of the ATPase.

In order to identify a membrane component that could, following its cyclic AMP-induced phosphorylation, function as an inhibitor of ($Ca^{2+} + Mg^{2+}$)-dependent ATPase activity, we examined the effect of cyclic AMP concentration upon patterns of erythrocyte membrane protein phosphorylation. Phosphorylation was assessed by monitoring the labeling of membrane proteins by [γ-^{32}P]ATP at short reaction times. In general, observed labeling reflects the extent of phosphorylation by [γ-^{32}P]ATP minus the extent of the dephosphorylation of labeled phosphoproteins. At very short reaction times, however, when con-

centrations of [γ-^{32}P]ATP are highest and concentrations of labeled phosphoproteins are virtually zero, observed labeling reflects protein phosphorylation with negligible impact by the dephosphorylation reaction. In these studies our reaction times were 10, 20, and 40 s. As illustrated by reproductions of autoradiograms shown in Fig. 1, micromolar concentrations of cyclic AMP promoted the phosphorylation of a series of membrane proteins. Focusing upon panels A, B, and C (prepared using an exposure time such that the incorporation of lesser amounts of label can be monitored), it is seen that the phosphorylation of a 20 kDa protein (band 8 according to the designations of Fairbanks et al. [3]), the 29 kDa and 28 kDa proteins, designated as 7a and 7b by Swislocki et al. [4]), a 35 kDa protein (band 6), and a 42 kDa protein (band 5) is, in each instance, promoted by micromolar but not millimolar concentrations of cyclic AMP. Micromolar, but not millimolar, concentrations of cyclic AMP also promote the phosphorylation of a 48 kDa protein (band 4.8), a 76 kDa protein (band 4.2), a 100 kDa protein (band 3), and a 215 kDa protein (band 2.1) as illustrated in panels a, b, and c which were prepared using an exposure time such that the incorporation of the greater amounts of the label could be monitored.

If cyclic AMP-induced phosphorylation of a membrane protein serves a regulatory function with respect to the activity of the (Ca^{2+} + Mg^{2+})-dependent ATPase, the dephosphorylation of the phosphoprotein must also be a critical reaction since it would permit reversal of the inhibition. Furthermore, the dephosphorylation of such a phosphoprotein should be subject to modulation just as the phosphorylation reaction is, in being promoted by cyclic AMP. Recently, we described for the first time a Ca^{2+}-dependent phos-

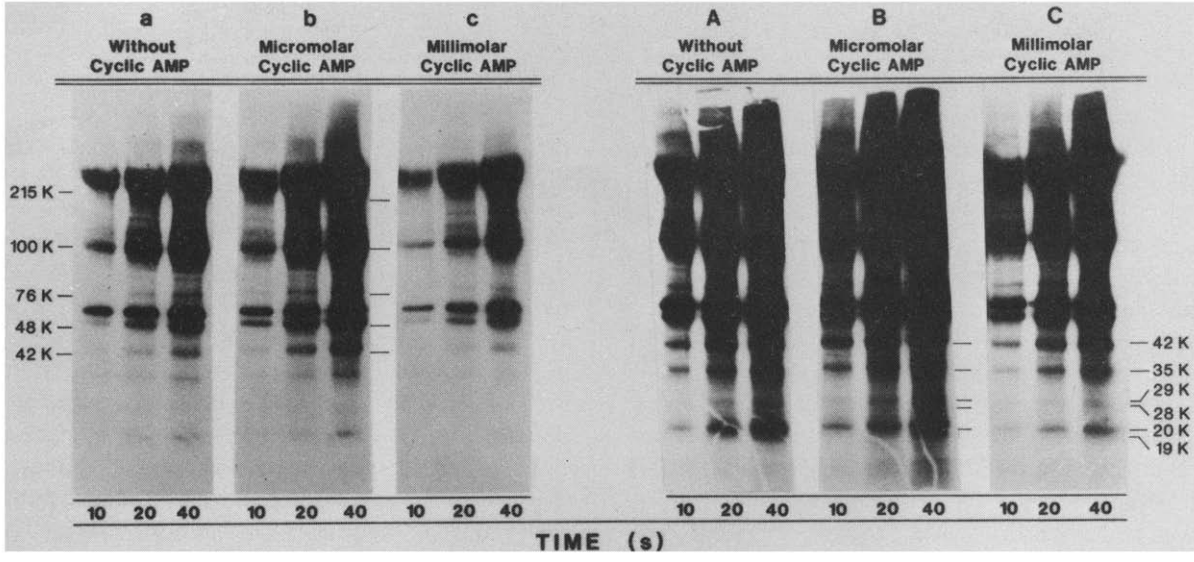

Fig. 1. Autoradiograms showing the effect of cyclic AMP concentration upon the phosphorylation of erythrocyte membrane proteins. Freshly isolated human erythrocyte membranes were incubated at 25°C in a medium containing 20 mM Tris-HCl buffer (pH 7.4), 5 mM magnesium acetate, 2 mM dithiothreitol, and 1 nM [γ-^{32}P]ATP ($5.6 \cdot 10^6$ cpm/ml, 30 Ci/mmol) in the absence of cyclic AMP (a and A), or with 1 μM cyclic AMP (b and B), or with 1 mM cyclic AMP (c and C). After 10, 20, or 40 s an aliquot of the reaction solution was removed and instaneously introduced into buffered sodium dodecyl sulfate. Subsequently, 80 μg of the protein were applied to a 5–20% gradient polyacrylamide gel and resolution was carried out using Tris-glycine buffer (pH 8.3) containing 0.1% sodium dodecyl sulfate. Following resolution, the protein components were stained with Coomassie blue and autoradiograms were prepared from the developed gels. Autoradiograms a and A, b and B, and c and C were, in each instance, prepared from the same gel. Autoradiograms a, b, and c were prepared using shorter exposure times, in order to monitor the behavior of membrane proteins that incorporate greater amounts of label. Autoradiograms A, B, and C were prepared using longer exposure times in order to monitor the behavior of membrane proteins that incorporate lesser amounts of label. Molecular weight markers were present on each gel.

phoprotein phosphatase that is associated with the rat erythrocyte membrane [4]. We have now determined that Ca^{2+}-dependent phosphoprotein phosphatase activity is also associated with the human erythrocyte membrane. Since Ca^{2+}-induced dephosphorylation of a phosphoprotein inhibitor of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase would allow the activity of this enzyme to increase in response to increasing intracellular Ca^{2+} concentrations, as would be desirable, a regulatory mechanism entailing cyclic AMP-induced protein phosphorylation coupled with Ca^{2+}-induced phosphoprotein dephosphorylation is an attractive one. We have, therefore, sought and we have found a membrane protein that undergoes both cyclic AMP-induced phosphorylation and Ca^{2+}-induced dephosphorylation. When human erythrocyte membrane proteins were phosphorylated in the presence of [γ-^{32}P]ATP and micromolar concentrations of cyclic AMP and then, subsequently, allowed to undergo dephosphorylation in the absence of ATP and Mg^{2+}, we observed that al-

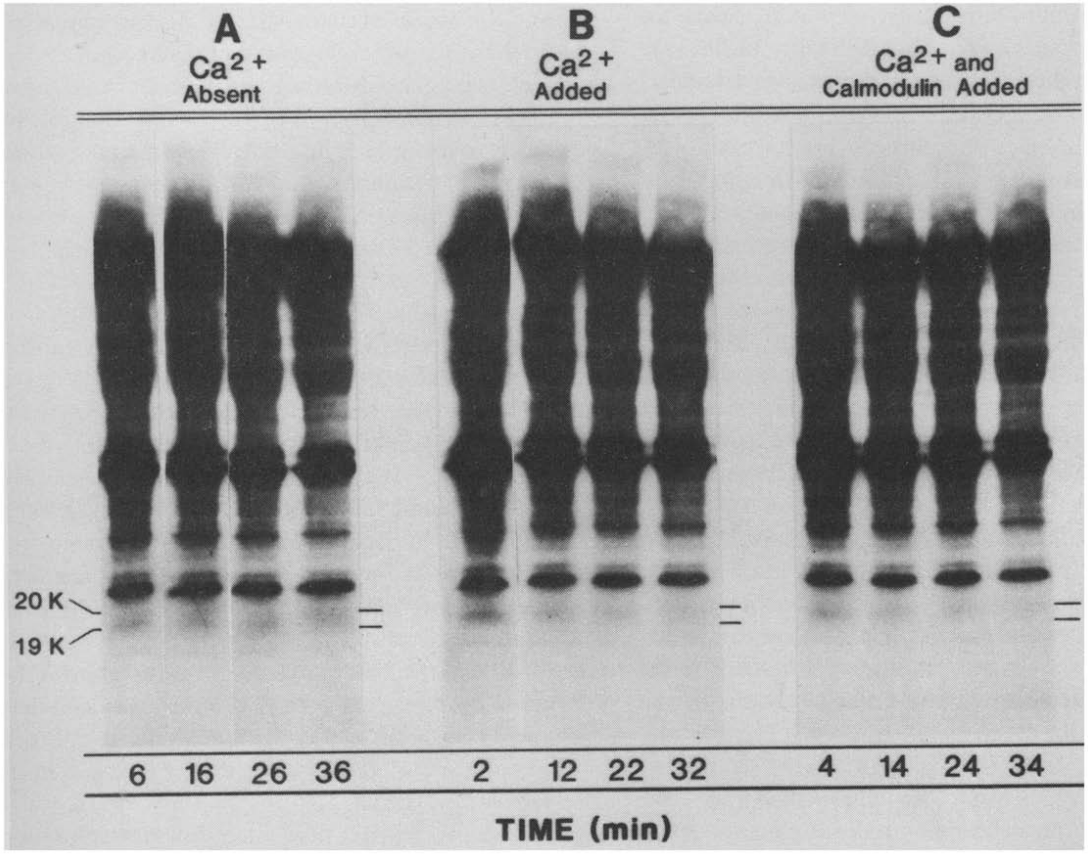

Fig. 2. Autoradiograms showing the effect of Ca^{2+} upon membrane phosphoprotein dephosphorylation. Freshly isolated human erythrocyte membranes were labeled by incubating them for 3 min at 37°C in a medium containing 20 mM Tris-HCl buffer (pH 7.4), 5 mM magnesium acetate, 2 mM dithiothreitol, 1 mM EGTA, 10 μM cyclic AMP, and 1 nM [γ-^{32}P]ATP ($5.6 \cdot 10^6$ cpm/ml, 30 Ci/mmol). Membranes were then centrifuged at 4°C in order to remove them from the presence of ATP and Mg^{2+} and, subsequently, aliquots were allowed to undergo dephosphorylation at 37°C in 20 mM Tris-HCl buffer (pH 7.4) containing 2 mM dithiothreitol and either 1 mM EGTA (A), 15 μM Ca^{2+} (B), or 15 μM Ca^{2+} and 100 units of purified (Boehringer Mannheim) calmodulin (C). In each instance aliquots were removed at the times indicated and each was instantaneously introduced into buffered sodium dodecyl sulfate. A portion of the resulting solution containing 80 μg of protein was then applied to a 5–20% gradient polyacrylamide gel and resolution was carried out using Tris-glycine buffer (pH 8.3) containing 0.1% sodium dodecyl sulfate. The developed gels were then stained with Coomassie blue and autoradiograms were prepared from these gels. Molecular weight markers were present on each gel.

though 1 μM Ca^{2+} exerted only a minimal effect upon the loss of label (data not shown), 15 μM Ca^{2+} primarily promoted the dephosphorylation of the 19 kDa and 20 kDa membrane proteins, as seen comparing panels A and B of Fig. 2. Because of our previous observation that exogenous calmodulin can potentiate the activity of the Ca^{2+}-dependent phosphoprotein phosphatase of the rat erythrocyte membrane, we determined the effect of the addition of calmodulin upon the Ca^{2+}-induced dephosphorylation of human erythrocyte membrane phosphoproteins. However, using the same concentrations as in the studies on the rat erythrocyte membrane, we were unable to observe potentiation by calmodulin in studies on the human erythrocyte membrane, as seen comparing panels B and C of Fig. 2. Our autoradiograms reproduced here were prepared using an exposure time which allowed us to monitor the dephosphorylation of membrane phosphoproteins that had, in the prior incubation, incorporated lesser amounts of the radioactive label. We could find no additional phosphoproteins that underwent Ca^{2+}-induced dephosphorylation when we examined companion autoradiograms prepared using shorter exposure times, designed to allow monitoring of the loss of label from those membrane proteins which had incorporated greater amounts of the label.

There have been no previous suggestions concerning the functions of the 20 kDa and 19 kDa membrane proteins which we designated as 8a and 8b, respectively. On the basis of our accumulated observations we suggest that the 20 kDa protein can, in the phosphorylated form, function as an inhibitor of the activity of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase. This small protein is separated from the $(Ca^{2+} + Mg^{2+})$-dependent ATPase by Sephacryl gel filtration. It undergoes phosphorylation promoted by micromolar, but not millimolar, concentrations of cyclic AMP, thus indicating that the inhibitory effect follows from covalent modification catalyzed by the membrane-associated cyclic AMP-dependent protein kinase. Furthermore, when the 20 kDa protein is phosphorylated, its dephosphorylation is promoted by 15 μM, but not 1 μM, Ca^{2+}, which would provide a mechanism by which the cyclic AMP-induced inhibition would be contravened in the event of inappropriately high intracellular concentrations of Ca^{2+}.

Addressing our observation that solubilized preparations of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase obtained in the presence of 0.1 mM Ca^{2+} were insensitive to cyclic AMP while those obtained in the absence of Ca^{2+} retained this sensitivity, we believe that the observed difference reflects a difference in the micellar environments of the catalytic component of the ATPase. The activity of a membrane-associated $(Ca^{2+} + Mg^{2+})$-dependent ATPase of the porcine erythrocyte is reported to be enhanced upon addition of phosphatidylinositol [5] while that of the enzyme purified from the human erythrocyte and reconstituted in the presence of phosphatidylserine no longer requires calmodulin in order to be maximal [6]. In each of these studies the phospholipid composition of the environment surrounding the $(Ca^{2+} + Mg^{2+})$-dependent ATPase was altered and we suggest that solubilization in the presence of Ca^{2+} is accompanied by a similar alteration. Under the conditions utilized in our studies an alteration in the composition of the phospholipids surrounding the solubilized $(Ca^{2+} + Mg^{2+})$-dependent ATPase could result because Ca^{2+} promotes the hydrolytic cleavage of membrane phosphatidylinositol phosphates [7]. We suggest that phospholipids closely associated with the catalytic component of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase facilitate its communication with modulators including the activator, calmodulin, and the 20 kDa inhibitor. Alterations in phospholipid composition, such as perturbing the phosphatidylinositol/phosphatidylinositol phosphate ratio, could disrupt communications, thus allowing the activity of the $(Ca^{2+} + Mg^{2+})$-dependent ATPase to be nonmodulated and maximal.

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