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CALCIUM-CALMODULIN DEPENDENT PHOSPHORYLATION OF ERYTHROCYTE PYRUVATE KINASE

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In the red cell incubated with ortho-[^{32}P] phosphate, $CaCl_2$ and calcium ionophore A 23187, phosphorylation of erythrocyte pyruvate kinase was demonstrated using the double antibody technique and autoradiography. Phosphorylation was inhibited by calmodulin inhibitors, trifluoperazine or $ZnCl_2$. In the presence of purified erythrocyte calmodulin, $CaCl_2$ and [γ-^{32}P] ATP, the partially purified erythrocyte pyruvate kinase containing cytozol protein kinases was phosphorylated. This was also inhibited by trifluoperazine or $ZnCl_2$. From these results, it was concluded that erythrocyte pyruvate kinase is phosphorylated by a calcium-calmodulin dependent process.

INTRODUCTION

The phosphorylation of L-type and erythrocyte pyruvate kinases [EC 2.7.1.40] decreased the affinity of the enzymes for the substrate, phosphoenolpyruvate *in vitro* (1-10). The phosphorylation was demonstrated *in vivo* (5,10). This suggests that pyruvate kinase activity may be regulated by a phosphorylation-dephosphorylation process. In the liver, cyclic AMP might be a second messenger for phosphorylation of L-type pyruvate kinase because adenylate cyclase is activated and L-type pyruvate kinase is phosphorylated by some hormones, for example, glucagon (2,3,5). But in the red cell, this physiological event which requires extracellular stimulation does not occur because the matured human red cell contains little or no adenylate cyclase or cyclic AMP-phosphodiesterase (11,12). Cyclic AMP enters the red cell *in vitro* (13-15) and achieves a sufficiently high intracellular concentration to activate the cyclic AMP dependent protein kinase for phosphorylation of erythrocyte pyruvate kinase (9).

Abbreviations used : TFP, trifluoperazine; SDS, sodium dodecylsulfate

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There might be a mechanism to control the pyruvate kinase activity through cyclic AMP transport but, in this case, cyclic AMP is not a second messenger.

Calcium ion is another intracellular second messenger and intracellular calcium receptor, calmodulin, mediates the effects of calcium ions on the activities of many intracellular enzyme systems (16,17). In the red cell, the calcium-calmodulin system was reported to have an important role in regulating Ca-ATPase (18,19). These findings suggest the presence of a possible process in which calcium-calmodulin dependent phosphorylation of erythrocyte pyruvate kinase occurs. In this report, we present the calcium-calmodulin mediated phosphorylation of erythrocyte pyruvate kinase.

MATERIALS AND METHODS

Cyclic AMP was assayed using the Yamasa Cyclic AMP Assay Kit according to the instruction manual.

Human erythrocyte calmodulin was purified using the method of Jarret et al. (20) and its homogeneity verified by SDS polyacrylamide gel electrophoresis.

Phosphorylation of erythrocyte pyruvate kinase using red cells and ortho-[^{32}P] phosphate was confirmed by the method reported previously (9), in the presence of 0.1 mM cyclic AMP, 0.1 mM cyclic GMP, 0.1 mM $CaCl_2$, 0.1 mM $CaCl_2$ + 0.2 μM A 23187 or 0.1 μM A 23187. Procedures for pyruvate kinase recovery, SDS polyacrylamide gel electrophoresis and autoradiography were the same as those of the previous reports (9,10).

Inhibition of the phosphorylation was observed by addition of 50 μM TFP or 50 μM $ZnCl_2$ to tubes containing 0.1 mM $CaCl_2$ + 0.2 μM A 23187 or 0.1 mM cyclic AMP.

Phosphorylation of erythrocyte pyruvate kinase in a cell-free system using partially purified human and rat erythrocyte pyruvate kinases and [γ-^{32}P] ATP was done using the method reported previously (9) in the presence of 0.1 mM $CaCl_2$, 1 ug/ml of purified human erythrocyte calmodulin and 50 μM TFP or 50 μM $ZnCl_2$.

RESULTS AND DISCUSSION

The autoradiogram of the SDS polyacrylamide gel electrophoresis is shown in Fig. 1. Erythrocyte pyruvate kinase was significantly phosphorylated in the presence of cyclic AMP or $CaCl_2$ + A 23187, and slightly in the presence of $CaCl_2$ or A 23187. These data confirmed our previous results of cyclic AMP stimulation of pyruvate kinase phosphorylation (9) and also indicated that calcium ion entered the cell in the presence of A 23187 and induced the phosphorylation of pyruvate kinase. Calcium ion itself was reported to damage the red cell membrane and metabolism

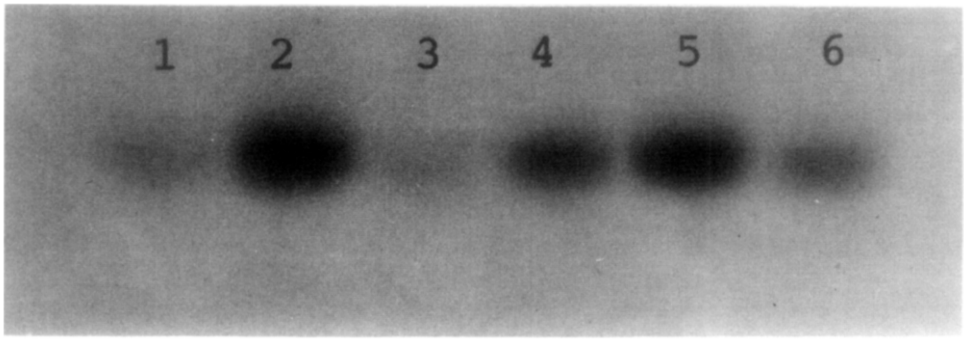

Fig. 1. Autoradiogram of SDS-polyacrylamide gel electrophoresis of the immune complex of phosphorylated erythrocyte pyruvate kinase of the red cells incubated with (1) none, (2) 0.1 mM cyclic AMP, (3) 0.1 mM cyclic GMP, (4) 0.1 mM $CaCl_2$, (5) 0.1 mM $CaCl_2$ + 0.2μM A 23187 and (6) 0.2 μM A 23187.

and create morphological changes and membrane protein cross-linking (21,22). But under experimental conditions, concentrations of $CaCl_2$ and A 23187 are low enough to prevent such damage. As shown in Fig. 2, this calcium-induced phosphorylation was inhibited by inhibitors of calcium-activated calmodulin function, TFP or $ZnCl_2$. But cyclic AMP dependent phosphorylation of erythrocyte pyruvate kinase was not significantly inhibited by TFP or $ZnCl_2$ (Fig. 2). Phenothiazine derivative also has a toxic effect on the red cell (23). But 50 μM TFP was not high enough to cause stomatocyte formation. No elevation of cyclic AMP concentraion in the red cell incubated with $CaCl_2$ and A 23187 was observed. It is reasonable to consider that calcium ion entered the cell in the presence of A 23187 and bound with calmodulin and the calcium-bound calmodulin

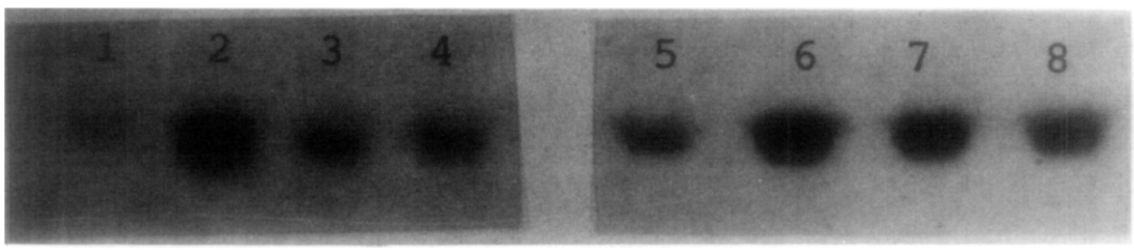

Fig. 2. Autoradiogram of SDS-polyacrylamide gel electrophoresis of the immune complex of phosphorylated erythrocyte pyruvate kinase of the red cells incubated wtih (1) none, (2) 0.1 mM $CaCl_2$ + 0.2 μM A23187, (3) 0.1 mM $CaCl_2$ + 0.2 μM A 23187 + 50 μM TFP, (4) 0.1 mM $CaCl_2$ + 0.2 μM A 23187 + 50 μM $ZnCl_2$, (5) none, (6) 0.1 mM cyclic AMP, (7) 0.1 mM cyclic AMP + 50 μM TFP and (8) 0.1 mM cyclic AMP + 50 μM $ZnCl_2$.

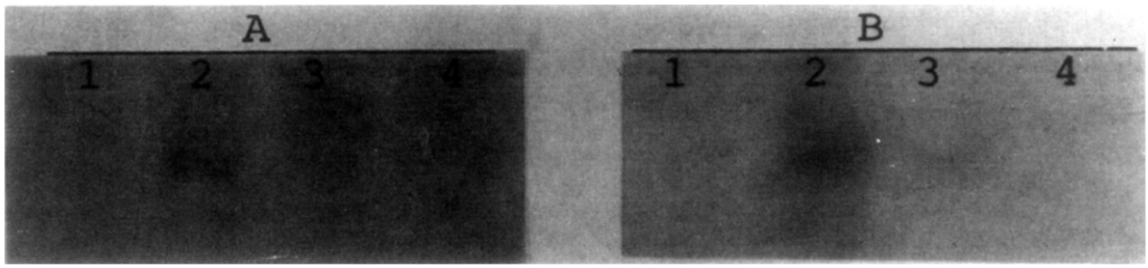

Fig. 3. Autoradiogram of SDS-polyacrylamide gel electrophoresis of the immune complex of phosphorylated (A) human and (B) rat erythrocyte pyruvate kinases in the reaction mixtures of (1) no addition, (2) 0.1 mM $CaCl_2$+ 1 μg/ml calmodulin, (3) 0.1 mM $CaCl_2$ + 1 μg/ml calmodulin + 50 μM TFP and (4) 0.1 mM $CaCl_2$ + 1 μg/ml calmodulin + 50 μM $ZnCl_2$.

activated a protein kinase to phosphorylate erythrocyte pyruvate kinase. This was prevented by the inhibiting effects of calcium-activated calmodulin function of TFP or Zn^{2+}. In Fig. 3, phosphorylation of erythrocyte pyruvate kinase in the presence of $CaCl_2$ and purified calmodulin was inhibited by TFP or $ZnCl_2$. In the reaction of a cell-free system, the nonspecific effects of TFP and $CaCl_2$ on the red cell were completely eliminated, so TFP and $ZnCl_2$ can be regarded as inhibitors of calcium-activated calmodulin function as reported in previous literature (17,24-26). It can be concluded that the calcium-calmodulin dependent process mediates the phosphorylation of erythrocyte pyruvate kinase.

Erythrocyte pyruvate kinase is phosphorylated by cyclic AMP-dependent protein kinase. Cyclic AMP easily enters the red cell *in vitro* (13-15) and achieves a sufficiently high intracellular concentration to activate the cyclic AMP dependent protein kinase for phosphorylation of erythrocyte pyruvate kinase (9), but calcium ion almost never enters the fresh red cell without calcium ionophore. There might be a mechanism to control the red cell biological activity through cyclic AMP transport. But this might be relatively independent of calcium-calmodulin dependent process for phosphorylation of erythrocyte pyruvate kinase. At this time, the results strongly suggest that there is a calcium-calmodulin dependent protein kinase which phosphorylates erythrocyte pyruvate kinase. The properties of the protein kinase remain to be elucidated.

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