Cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in the plasma membranes of different blood cells

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Plasma membrane preparations from lymphocytes, platelets and red cells were phosphorylated in the presence of [γ-^{32}P]ATP. The dissociated catalytic subunit of cyclic AMP-dependent protein kinase increased the ^{32}P-labelling of proteins and polyphosphoinositides in lymphocyte, platelet and in some red cell membranes. In the majority of red cell membrane preparations the ^{32}P-labelling of proteins and polyphosphoinositides seemed to be stimulated by the catalytic subunit of the endogenous protein kinase, since the phosphorylation was not increased by the addition of the catalytic subunit but it was decreased by the heat-stable inhibitor protein of the protein kinase. Different sets of ^{32}P-labelled proteins were shown by SDS-gel electrophoresis in the membranes of the 3 cell types. A 24000-M_r protein was the only one which was phosphorylated by the catalytic subunit in each membrane.

Cyclic AMP *Protein kinase* *Membrane phosphorylation*
Polyphosphoinositide *Phosphatidylinositol metabolism* $M_r = 24000$ *phosphoprotein*

1. INTRODUCTION

We have reported that cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in lymphocyte plasma membranes [1]. Phosphatidylinositol metabolism and polyphosphoinositides have been suggested to play some role in the transducing mechanism of several hormones and other agents using calcium as second messenger [2–5]. The calcium and cyclic AMP messenger systems are intimately interrelated [6,7] and the formation of polyphosphoinositides may be involved in this interaction. In the approach of this problem it is important to know whether the stimulatory effect of cyclic AMP-dependent protein kinase on polyphosphoinositide formation is restricted to lymphocyte membranes or whether is is a common mechanism in a variety of cells.

This paper demonstrates that the cyclic AMP-dependent protein kinase also increased the polyphosphoinositide formation in platelet and in red cell membranes. Although different phosphoprotein sets were found in the membranes of the 3 cell types, a 24000-M_r protein was phosphorylated by the cyclic AMP-dependent protein kinase in each membrane. This 24000-M_r phosphoprotein may be responsible for the polyphosphoinositide formation stimulated by cyclic AMP-dependent protein kinase.

2. EXPERIMENTAL

Plasma membrane preparations from human blood lymphocytes and red cells were obtained as in [8,9]. Human platelet membranes were isolated as in [10] and the preparations were analyzed by electron microscopy. They were practically

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homogeneous and free of intracellular organelles. The dissociated catalytic subunit of cyclic AMP-dependent protein kinase was prepared as in [1]. The conditions of incubation, the methods of lipid extraction and measurement of ^{32}P-radioactivity in proteins and lipids were carried out as in [1]. The heat-stable inhibitor protein of cyclic AMP-dependent protein kinase was purified as in [11]. Thin-layer chromatography (TLC) of phospholipids was carried out as in [1], except that before use, the plates were impregnated with 1% potassium oxalate and activated for 1 h at 110°C. For measurements of ^{32}P-incorporation, the silica gel was scraped (2.5-mm samples) from the TLC plates and radioactivity was determined in a liquid scintillation spectrometer.

Membrane proteins (100–200 μg) were separated on 7.5% polyacrylamide gels [12]. From 3 identical gels one was stained with Coomassie blue R-250, and the other two were cut into 2.5–5-mm slices for the measurement of ^{32}P-radioactivity. M_r standards were trypsinogen (24000), pepsin (34000), egg albumin (45000) and bovine serum albumin (66000). In addition, red cell membrane proteins spectrin 2 (M_r = 22000) and band 3 (100000) were regarded as standards [13].

3. RESULTS AND DISCUSSION

We compared the effects of the dissociated catalytic subunit of cyclic AMP-dependent protein kinase on the phosphorylation of plasma membrane preparations from lymphocytes, platelets and red cells. Representative results from a series of experiments are shown in table 1.

When lymphocyte membrane preparations were tested, the catalytic subunit increased the phosphorylation of membrane proteins and the ^{32}P-labelling of membrane lipids in 10 preparations without exception. The increase of ^{32}P-labelling caused by the catalytic subunit in the different preparations varied between 50–300% both in membrane proteins and lipid fractions.

The catalytic subunit also stimulated the

Table 1

Effects of the catalytic subunit of cyclic AMP-dependent protein kinase and its heat-stable inhibitor protein on the phosphorylation of membrane preparations from lymphocytes, platelets and red cells

Cell type	Addition	^{32}P-incorporation into membrane	
		Lipid	Protein
Lymphocyte	None	12.5	10.8
	Inhibitor	12.0	10.5
	Catalytic subunit	20.3	29.6
	Catalytic subunit + inhibitor	12.7	14.1
Platelet	None	3.1	4.4
	Inhibitor	3.1	4.4
	Catalytic subunit	5.3	35.5
	Catalytic subunit + inhibitor	3.5	6.2
Red cell	None	24.4	25.3
	Inhibitor	13.3	15.4
	Catalytic subunit	24.6	24.8
	Catalytic subunit + inhibitor	13.0	16.0

^{32}P-incorporation is expressed as pmol phosphate/mg membrane preparation containing 1 mg protein. The incubation period was 5 min

phosphorylation of platelet membrane proteins and lipids in the majority of preparations (in 5 out of 7 preparations). In platelet membranes the increase in protein phosphorylation was in all cases more marked (50–800%) than the increase in lipid phosphorylation (30–100%). The amount of phosphate incorporated into lipids of platelet membrane was always smaller than in the lipids of lymphocyte or red cell membranes.

In erythrocyte membranes we could not observe the stimulation of phosphorylation by the exogenous catalytic subunit in 5 out of 8 preparations. However, ^{32}P-incorporation into protein and lipid fractions of these membranes was significantly decreased by the heat-stable inhibitor protein [11] in the absence of the exogenous catalytic subunit (Table 1). Since the inhibitor protein is specific for the cyclic AMP-dependent protein kinase, these results indicate that in the majority of red cell membrane preparations the phosphorylation of proteins and lipids was stimulated by the catalytic subunit of a cyclic AMP-dependent protein kinase present in the membrane. The regulatory subunit presumably dissociated during the procedure of membrane isolation, since cyclic AMP never increased the phosphorylation of these membrane preparations. Nevertheless, in 3 preparations the stimulatory effect of the exogenous catalytic subunit was positively established (fig.1,2).

Phosphorylated membrane lipids were analyzed by TLC. We have reported that in lymphocytes the increase of ^{32}P-labelling stimulated by the catalytic subunit was found exclusively in the position of polyphosphoinositides, though a small amount of ^{32}P-labelled phosphatidic acid was also demonstrated [1]. The ^{32}P-labelled lipid patterns of platelet and red cell membranes were even more simple. ^{32}P-labelled lipids were found exclusively in the position of polyphosphoinositides when these membranes were incubated with [γ-^{32}P]ATP either in the presence or absence of the catalytic subunit (fig. 1). Thus, in our experiments any change in the phosphorylation of membrane lipids was due to the change in the formation of polyphosphoinositides.

Cyclic AMP-dependent protein kinase phosphorylates proteins only. Therefore the increased polyphosphoinositide formation is due to another enzyme which is activated by phosphorylation. We

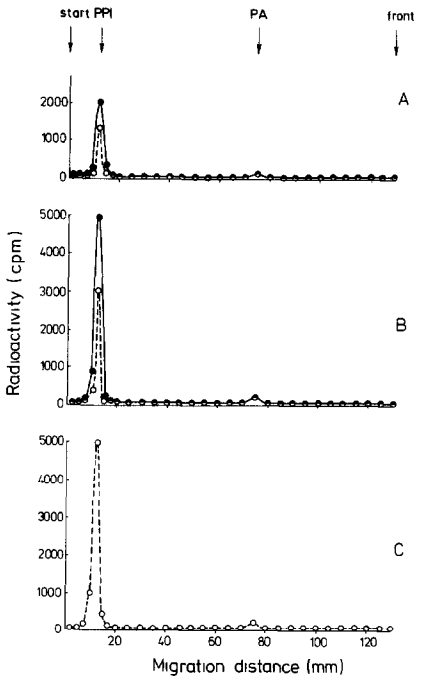

Fig.1. Thin-layer chromatographic patterns of the ^{32}P-labelled lipids extracted from membrane preparations obtained from platelets (A) and red cells (B,C). The membranes were phosphorylated in the absence (○---○) or presence (●——●) of the dissociated catalytic subunit. Parts A and B show data from 1 of 3 similar experiments. Part C shows the phospholipid pattern of a red cell membrane preparation in which the phosphorylation was not stimulated by the exogenous catalytic subunit. PA, phosphatidic acid; PPI, diphosphoinositide and triphosphoinositide.

compared the phosphoproteins of the membranes obtained from the 3 cell types. SDS-gel electrophoresis of proteins from the 3 membranes showed different phosphoprotein sets (fig.2). In lymphocyte membranes, ^{32}P-labelled proteins were found at the positions of $M_2 = 24000$, 35000, 70000 and in the region of about 150000. In some preparations a 45000–50000-M_r phosphoprotein was also found.

The electrophoretic pattern of platelet membranes showed phosphoprotein bands at the positions of $M_r = 24000$, 45000–50000, 70000 and about 100000. In lymphocyte and platelet membrane preparations each phosphoprotein band was affected by the catalytic subunit but the most

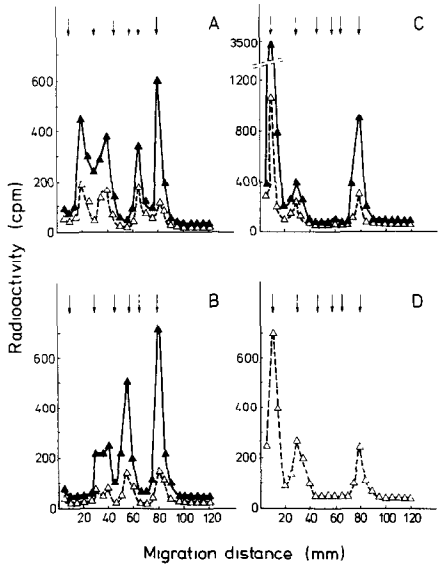

Fig.2. SDS-gel electrophoretic patterns of the ^{32}P-labelled proteins of lymphocyte (A), platelet (B) and red cell membranes (C,D). The membranes were phosphorylated in the absence (△—△) and presence (▲—▲) of the dissociated catalytic subunit. Parts A, B and C show data from 1 of 3 similar experiments. Part D shows the phosphoprotein pattern of a red cell membrane preparation in which the phosphorylation was not stimulated by the exogenous catalytic subunit. The arrows indicate the positions of proteins of M_r = 220000, 100000, 66000, 45000, 35000 and 24000, respectively.

significant increase was found in the phosphorylation of the 24000-M_r protein.

In red cell membranes 3 phosphoprotein fractions were observed. The phosphorylation of a 24000-M_r protein was markedly stimulated by the catalytic subunit. The phosphorylation of spectrin was also increased by this enzyme, in agreement with [14]. ^{32}P-labelling was also found in the region of band 3 [13].

While the formation of polyphosphoinositides was stimulated by the catalytic subunit in each cell type, the 24000-M_r phosphoprotein was the only protein which was demonstrated in each membrane as a substrate for the cyclic AMP-dependent protein kinase. This result suggests that the 24000-M_r phosphoprotein is responsible for polyphosphoinositide formation stimulated by the catalytic subunit. In platelets, a 22000-M_r [15] or 24000-M_r [16] membrane protein has been reported to be phosphorylated by the cyclic AMP-dependent protein kinase and to play an essential role in the regulation of the intracellular Ca^{2+} level. This protein is probably present in both the plasma membrane and the intracellular membranes of platelet and it has been regarded to be similar to phospholamban [16] demonstrated in studies on sarcoplasmic reticulum [17]. However, differences between phospholamban and the phospholamban-like protein of platelets have also been described [18]. We put forward the hypothesis that the 24000-M_r phosphoprotein is an enzyme which is involved in the formation of polyphosphoinositides.

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