# Cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in lymphocyte plasma membrane

B. Sarkadi, Ágnes Enyedi, Anna Faragó*, G. Mészáros*, T. Kremmer+ and G. Gárdos

*National Institute of Haematology and Blood Transfusion, *1st Institute of Biochemistry, Semmelweis University Medical School* and +*National Oncological Institute, Research Institute of Oncopathology, Budapest, Hungary*

Received 15 December 1982

Inside-out vesicles from lymphocyte plasma membrane were phosphorylated in the presence of [$\gamma$-$^{32}$P]ATP. The dissociated catalytic subunit of cyclic AMP-dependent protein kinase stimulated both membrane protein and membrane lipid phosphorylation, indicating the presence of a phosphorylation cascade. The phosphorylated membrane lipids were analyzed by thin-layer chromatography. Increase of $^{32}$P-labelling stimulated by the cyclic AMP-dependent protein kinase was found exclusively in polyphosphoinositides.

*Cyclic AMP* *Protein kinase* *Membrane phosphorylation* *Polyphosphoinositide* *Phosphatidylinositol metabolism* *Lymphocyte*

## 1. INTRODUCTION

Cyclic AMP-dependent phosphorylation and subsequent dephosphorylation is a well documented way of cellular regulation. In the initiation of lymphocyte growth both cyclic nucleotides and calcium ions have been implicated as second messengers [1–3] and an attractive possibility is the involvement of membrane phosphorylation in such regulation [3–4]. The presence of cyclic AMP-dependent and -independent protein kinases in lymphocytes [5–6] and the activity of such membrane-bound enzymes [7–8] have also been positively established. In [9] we described the preparation of sealed inside-out vesicles (IOVs) from the plasma membrane of human lymphocytes. We report here that in this membrane preparation, the catalytic subunit of cyclic AMP-dependent protein kinase stimulated both protein and lipid phosphorylation indicating the presence of a phosphorylation cascade. The observed increase in lipid phosphorylation was due to the formation of polyphosphoinositides from phosphatidylinositol.

## 2. EXPERIMENTAL

Plasma membrane IOVs were prepared from lymphocytes [9] and from red blood cells [10]. The percentage of sealed IOVs was 75–78 in the case of red cell and 30–35 in the case of lymphocyte membrane.

The dissociated catalytic subunit of cyclic AMP-dependent protein kinase was prepared from calf thymus extract. The extract was incubated in the presence of cyclic AMP and DEAE-cellulose chromatography was carried out in the presence of cyclic AMP as in [11]. Fractions containing the dissociated catalytic subunit were pooled and applied onto a phosphocellulose column. Elution was performed using NaCl concentration gradient in 10 mM potassium phosphate (pH 7.5). The catalytic subunit eluted at about 0.2 M NaCl. The spec. act. of the preparation obtained was about 60 nmol phosphate transferred $\times$ min$^{-1}$/mg protein when measured with H2b histone as substrate.

Incubation media for phosphorylation contained 100 mM KCl, 25 mM Tris–HCl (pH 7.5), 2.5 $\mu$M [$\gamma$-$^{32}$P]ATP (spec. act. 0.7 TBq/mmol), about 0.04 mg enzyme protein and 0.5–0.6 mg

*Published by Elsevier Biomedical Press*
00145793/83/0000–0000/$3.00  

membrane (0.25–0.3 mg membrane protein) per ml medium. Membrane phosphorylation was measured by precipitating and washing the incubated membranes in trichloroacetic acid + ATP + $P_i$ containing media and counting liquid scintillation as in [12].

Lipid extraction was performed as in [13]. Precipitated and washed membrane pellet was dissolved in 6 ml of chloroform–methanol–HCl (200:100:0.6) solution and a 1-ml aliquot was taken to measure total $^{32}P$-labelling. The remaining solution was mixed with 1 ml of 0.16 M NaCl shaken for 2 min at 25°C and then centrifuged for 10 min at 3000 × *g*. The upper phase and the inter-0.25phase precipitate were removed. $^{32}P$ activity was measured in a 1 -ml aliquot of the organic phase and after corrections for the volume changes this is referred to as activity in membrane lipids. The difference between the total activity and the activity of the lipid fraction is referred to as activity in membrane proteins.

Thin-layer chromatography of polar (phospho) lipids was performed on silica gel 60 (10 × 20 cm) precoated plates (Merck) in a solvent system of chloroform–aethylacetate–*n*-propanol–methanol –0.25% KCl (10:10:10:13:5.5). Authentic reference compounds (phosphatidic acid, phosphatidylinositol, diphosphoinositide and triphosphoinositide, extracted brain lipids) were run parallel. Chromatograms were scanned by a Bertold-Dünnschicht Scanner II (speed: 60 cm/h; sensitivity: 1000 Kcpm; slit: 1 mm) and visualised by phosphomolibdenic acid staining.

The heat-stable inhibitor protein of cyclic AMP-dependent protein kinase was purified as in [14].

## 3. RESULTS AND DISCUSSION

Inside-out vesicles from both red cell membranes and lymphocyte plasma membranes are rapidly phosphorylated in vitro by [$\gamma$-$^{32}P$]ATP. In lymphocyte membranes $^{32}P$ incorporation was linearly proportional to the concentration of ATP between 0.2–5 $\mu$M, Mg concentration dependence showed saturation with a $K_{Mg}$ of 5–8 mM. As shown in fig.1a, the addition of the dissociated catalytic subunit of cyclic AMP-dependent protein kinase almost doubled the amount of phosphate incorporation in lymphocyte IOVs, while in red cell membrane vesicles the effect of the catalytic subunit

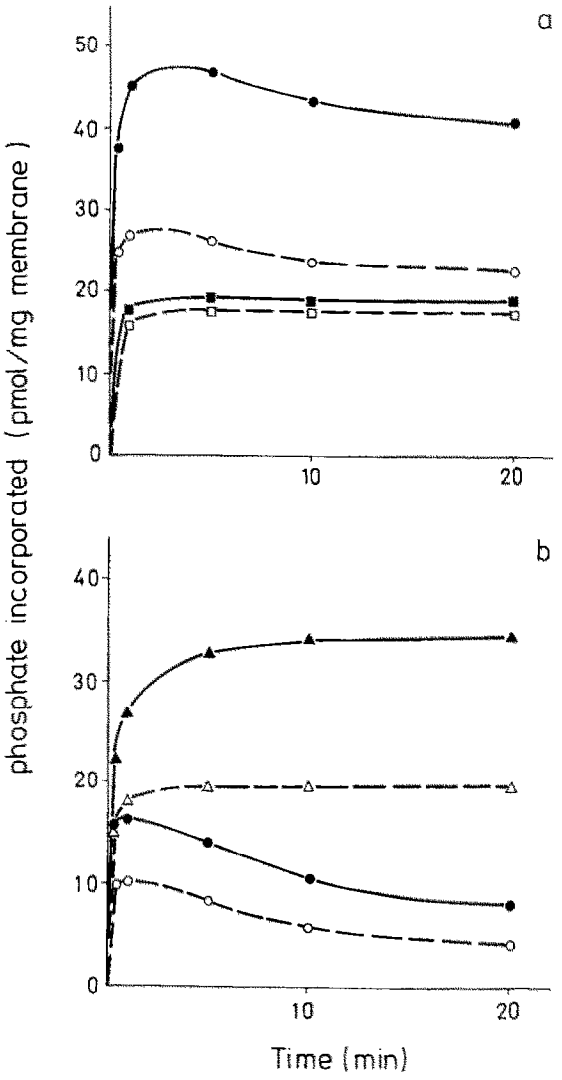


Fig.1. Effects of the catalytic subunit of cyclic AMP-dependent protein kinase on membrane phosphorylation. (a) $^{32}P$ incorporation into plasma membrane inside-out vesicles. Red cell membrane IOVs incubated in the absence (□---□) and presence (■——■) of the catalytic subunit; lymphocyte membrane IOVs incubated in the absence (○---○) and presence (●——●) of the catalytic subunit. Data from one of 3 similar experiments. (b) Protein and lipid phosphorylation in lymphocyte membrane inside-out vesicles. $^{32}P$ incorporation into membrane proteins in the absence (○---○) and presence (●——●) of the catalytic subunit; $^{32}P$ incorporation into membrane lipids in the absence (△---△) and presence (▲——▲) of the catalytic subunit. The figure shows data from one of 3 similar experiments.

was insignificant. In red cell membranes there was no measurable dephosphorylation in the 20 min incubation period, while in lymphocyte membranes $^{32}$P-labelling significantly decreased after the first 1–2 min of incubation at 37°C. The rate of dephosphorylation was greater if ATP concentration was smaller or lymphocyte membrane concentration was greater; most probably dephosphorylation became predominant after ATP had been consumed. Here we have to note that phosphorylation of lymphocyte membrane vesicles was not significantly stimulated either by the addition of cyclic AMP-independent protein (Ser-X-Lys) kinase [5,15], or by various casein kinases prepared from lymphoid tissues [6]. In lymphocyte IOVs the characteristics of membrane phosphorylation and its stimulation by the catalytic subunit of cyclic AMP-dependent protein kinase were not changed if the IOVs had been washed 3-times with 10 mM Tris–HCl (pH 7.5) or with a 10 mM Tris–HCl (pH 7.5) + 0.5 mM EDTA solution. This latter finding indicates the involvement of membrane-bound enzymes and substrates in this process.

Fig.1b shows the distribution of $^{32}$P label between lymphocyte membrane proteins and lipids, after phosphorylation either in the absence or presence of the catalytic subunit of cyclic AMP-dependent protein kinase. Protein phosphorylation was predominant at the beginning of the reaction, while the labelling of membrane lipids became more pronounced after 1–2 min of incubation. Significant protein dephosphorylation could be observed in these studies, while lipid dephosphorylation was insignificant. An impressive finding is that the catalytic subunit of cyclic AMP-dependent protein kinase stimulated both protein and lipid phosphorylation, although the action of this enzyme is specifically restricted to certain peptide regions of proteins [16]. Thus we have to suppose a specific phosphorylation and activation of membrane enzymes which in turn phosphorylate lipids in this preparation. As shown in table 1, the addition of purified heat-stable inhibitor protein [14] of the cyclic AMP-dependent protein kinase antagonized the stimulation of both membrane protein and lipid phosphorylation, while it did not alter the basal value.

SDS–polyacrylamide gel electrophoresis of the phosphorylated lymphocyte membrane proteins yielded several labelled bands, as in [7,8,17], and the addition of the catalytic subunit of cyclic AMP-dependent protein kinase increased $^{32}$P incorporation in at least 3 bands (not shown).

Table 1

Effects of the catalytic subunit of cyclic AMP-dependent protein kinase and its heat-stable protein inhibitor [14] on the phosphorylation of lymphocyte membrane inside-out vesicles

| Additions | $^{32}$P incorporation into membrane | |
|---|---|---|
| | Lipid | Protein |
| None | 18.4 | 8.0 |
| Inhibitor | 17.2 | 7.3 |
| Catalytic subunit | 38.8 | 14.2 |
| Catalytic subunit + inhibitor | 19.4 | 8.5 |

$^{32}$P incorporation into membrane is expressed as pmol phosphate/mg membrane lipid or protein. The incubation period was 5 min. Data from one of 3 similar expts

Extracted membrane lipids were analysed by thin-layer chromatography (fig.2). Some degradation products remained at the start in each case. In red cell membranes the label was found in the position of polyphosphoinositides as in [12] and it did not change in the presence of the catalytic subunit. In lymphocyte membranes one more labelled fraction was demonstrated in the position of phosphatidic acid, in addition to polyphosphoinositides. However, increase of $^{32}$P-labelling, stimulated by the catalytic subunit was found exclusively in the position of polyphosphoinositides; while the labelling of the precursor molecules was not affected. Identical pattern was obtained also in another thin-layer chromatography system as in [18]. Though on the basis of our present experiments we cannot tell whether the phosphatidylinositol kinase or the phosphatidylinositol-4-phosphate kinase, or both, were activated, the lymphocyte membrane lipid phosphorylation stimulated by cyclic AMP-dependent protein kinase is obviously due to the formation of polyphosphoinositides.

Phosphatidylinositol metabolism and polyphosphoinositides have been suggested to play some role in the transducing mechanism of several hormones using calcium as second messenger [19–21]. ACTH or cyclic AMP have been found to increase the diphospho- and triphosphoinositide content of

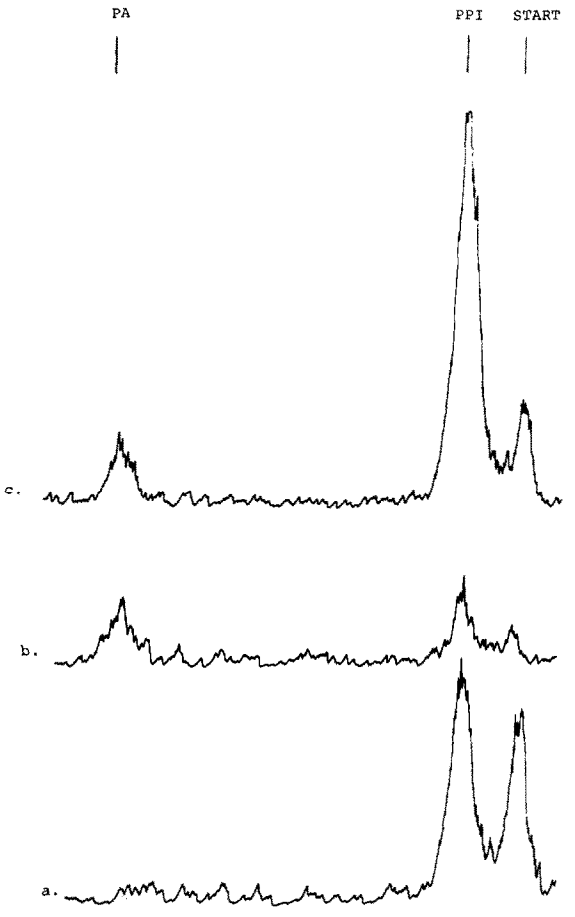


Fig.2. Radiodensitometric scanning of phosphorylated membrane lipids separated by thin-layer chromatography. Human erythrocyte membrane IOVs (a) were incubated in the absence, and lymphocyte membrane IOVs were incubated in the absence (b) and presence (c) of the dissociated catalytic subunit of cyclic AMP-dependent protein kinase. (PA: phosphatidic acid; PPI: diphosphoinositide and triphosphoinositide.) The figure shows the result of one of 3 similar experiments.

rat adrenal sections in the presence of $Ca^{2+}$ [22], but the increased synthesis of the precursor phosphatidylinositol has been considered to be responsible for this effect [23]. The lymphocyte membrane preparation used in our experiments is probably depleted from the enzymes and cofactors of phosphatidylinositol synthesis. The calcium and cyclic AMP messenger systems are intimately interrelated [24,25]. The formation of polyphosphoinositides may also represent a possible mechanism for this interaction.

## REFERENCES

[1] Wedner, H.J. and Parker, C.W. (1975) Biochem. Biophys. Res. Commun. 62, 808–815.
[2] Whitfield, J.F., Boyton, A.L., McManus, J.P., Sikorska, M. and Tsang, B.K. (1979) Mol. Cell. Biochem. 27, 155–179.
[3] Parker, Ch.W. (1978) in: Advances in Cyclic Nucl. Res. (George, W.J. and Ignarro, L.I. eds) vol.9, pp.647–660, Raven, New York.
[4] Walton, K.G. (1978) in: Receptor and Hormone Action (Birnbauer, L. and O'Malley, B.W. eds) vol.3, pp.579–614, Academic Press, New York.
[5] Faragó, A., Antoni, F., Takáts, A. and Fábián, F. (1973) Biochim. Biophys. Acta 297, 517–526.
[6] Kemp, B.E., Froscio, M., Rogers, A. and Murray, A.W. (1975) Biochem. J. 145, 241–249.
[7] Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1979) Biochem. J. 182, 525–536.
[8] Chaplin, D.D., Wedner, H.J. and Parker, C.W. (1979) Biochem. J. 182, 537–546.
[9] Sarkadi, B., Enyedi, Á., Szász, I. and Gárdos, G. (1982) Cell Calcium 3, 163–182.
[10] Sarkadi, B., Szász, I. and Gárdos, G. (1980) Biochim. Biophys. Acta 598, 326–338.
[11] Romhányi, T., Seprödi, J., Antoni, F., Nikolics, K., Mészáros, G. and Faragó, A. (1982) Biochim. Biophys. Acta 701, 57–62.
[12] Enyedi, Á., Sarkadi, B., Nyers, Á. and Gárdos, G. (1982) Biochim. Biophys. Acta 690, 41–49.
[13] Folch, J., Lees, M. and Sloane-Stanley, G.H. (1975) J. Biol. Chem. 226, 497–509.
[14] Ashley, C.D. and Walsh, D.A. (1972) J. Biol. Chem. 247, 6637–6642.
[15] Romhányi, T., Seprödi, J., Mészáros, Gy., Antoni, F. and Faragó, A. (1982) FEBS Lett. 144, 223–225.
[16] Krebs, E.G. and Beavo, J.A. (1979) Ann. Rev. Biochem. 48, 923–959.
[17] Johnstone, A.P., Dubois, J.H. and Crumpton, M.J. (1981) Biochem. J. 194, 309–318.
[18] Jolles, J., Schrama, L.H. and Gispen, W.H. (1981) Biochim. Biophys. Acta 666, 90–98.
[19] Michell, R.H. (1975) Biochim. Biophys. Acta 415, 81–148.
[20] Hawthorne, J.N. (1982) Nature 295, 281–282.
[21] Berridge, M.J. (1981) Molecular and Cellular Endocrinology 24, 115–140.
[22] Farese, R.V., Sabir, A.M., Vandor, S.L. and Larson, R.E. (1980) J. Biol. Chem. 255, 5728–5734.
[23] Farese, R.V., Sabir, M.A. and Larson, R.E. (1980) J. Biol. Chem. 255, 7232–7237.
[24] Rasmussen, H. (1981) Calcium and cAMP as Synarchic Messengers, Wiley, New York.
[25] Cohen, P. (1982) Nature 296, 613–620.