Volume 176, number 1 FEBS 1890 October 1984

Cyclic AMP-dependent protein kinase and Ca^{2+}-calmodulin stimulate the formation of polyphosphoinositides in a sarcoplasmic reticulum preparation of rabbit heart

Ágnes Enyedi, Anna Faragó*, B. Sarkadi and G. Gárdos

National Institute of Haematology and Blood Transfusion, 1113 Budapest and **1st Institute of Biochemistry, Semmelweis University Medical School, 1088 Budapest, Hungary*

Received 9 July 1984; revised version received 21 August 1984

A rabbit heart membrane fraction enriched in sarcoplasmic reticulum was incubated in a reaction mixture containing [γ-^{32}P]ATP. The catalytic subunit of cyclic AMP-dependent protein kinase enhanced the ^{32}P-labelling of both phosphatidylinositol-4-phosphate and phosphatidylinositol-4,5-bisphosphate. Ca^{2+}-calmodulin also increased the ^{32}P-incorporation into both polyphosphoinositides. Upon SDS gel-electrophoretic analysis of the membrane proteins, phospholamban was found to be concurrently phosphorylated by the exogenous catalytic subunit as well as by an endogenous Ca^{2+}-calmodulin-dependent protein kinase.

Cyclic AMP *Protein kinase* *Calmodulin* *Heart sarcoplasmic reticulum* *Polyphosphoinositide*

1. INTRODUCTION

The modulation of cytosolic Ca^{2+} levels by cyclic AMP is an important factor in the intimate interrelation of the two intracellular messenger systems [1]. The effects of cyclic AMP are generally mediated by the phosphorylation of specific target proteins [2] but the exact mechanism by which cyclic AMP exerts its effect on the Ca^{2+} transport is not known. We have reported that cyclic AMP-dependent protein kinase stimulates the formation of polyphosphoinositides in the plasma membranes of human lymphocytes [3], platelets and red cells [4]. Polyphosphoinositides are powerful activators of a purified reconstituted Ca^{2+}-ATPase [5], hence the stimulation of polyphosphoinositide formation is a possible way to regulate the Ca^{2+} transport by cyclic AMP. We have postulated that in the plasma membranes of lymphocytes, red cells and platelets, the phosphorylation of a 24 kDa protein is responsible for the stimulation of polyphosphoinositide formation [4]. In platelets, a 22 or 24 kDa membrane protein which is phosphorylated by cyclic AMP-dependent protein kinase has been extensively studied by other groups, and it has been suggested that this protein plays an essential role in the regulation of the intracellular Ca^{2+} level [6–8]. This protein has been regarded as being similar to phospholamban [7], a 22 kDa phosphoprotein which regulates the Ca^{2+}-pumping activity of cardiac sarcoplasmic reticulum [9]. The phosphorylation of phospholamban by cyclic AMP-dependent protein kinase or by a Ca^{2+}-calmodulin-dependent protein kinase enhances the activity of Ca^{2+}-ATPase of the cardiac sarcoplasmic reticulum membrane [10–13]. Presuming that the stimulation of polyphosphoinositide formation is related to the regulation of calcium pump activity in the membrane [5], the present experiments were undertaken to demonstrate whether cyclic AMP-dependent protein kinase stimulates the polyphosphoinositide formation in a membrane fraction enriched in sarcoplasmic reticulum of the heart.

Published by Elsevier Science Publishers B.V.
00145793/84/$3.00

2. EXPERIMENTAL

A membrane fraction enriched in sarcoplasmic reticulum was isolated from rabbit heart as in [14]. The preparation was stored at −20°C for several days. The catalytic subunit of cyclic AMP-dependent protein kinase was prepared as in [3]. Calmodulin was purified from red blood cells [15]. The standard reaction mixture for membrane phosphorylation contained 55 μM [γ-^{32}P]ATP (spec. act., 70 GBq/mmol), 5 mM $MgCl_2$, 20 mM KF and a membrane preparation containing about 0.25 mg protein. To this mixture we added 1 mM EGTA and the catalytic subunit preparation which catalyzed the transfer of 1–2 nmol/min phosphate to H2b histone (1 mg/ml) or, alternatively, 50 μM $CaCl_2$ and 500 nM calmodulin. The final volume was 500 μl. The incubation was carried out as in [3]. The precipitated membrane pellet was dissolved in 5 ml of a chloroform–methanol–HCl (100: 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 1 N HCl. The organic phase containing lipids was separated, and ^{32}P-radioactivity was measured as in [3]. The high-performance thin-layer chromatography (HPTLC) was carried out on Silica Gel 60 F-254 (Merck) HPTLC plates in a solvent system of chlorofom–methanol–2 M NH_4OH (45:35:10). Authentic reference compounds, phosphatidylinositol (PhI), phosphatidic acid (PhA), phosphatidylinositol-4-phosphate (PhIP) and phosphatidylinositol-4,5-bisphosphate ($PhIP_2$) were run in parallel. The measurement of ^{32}P-incorporation into lipids was carried out as in [4]. Membrane proteins were separated on 10% polyacrylamide gels as in [4].

3. RESULTS AND DISCUSSION

A rabbit heart membrane fraction enriched in sarcoplasmic reticulum was incubated in the presence of [γ-^{32}P-]ATP. The reaction mixture contained potassium fluoride to inhibit high phosphoprotein phosphatase activity of the membrane. We investigated the effects of the catalytic subunit of cyclic AMP-dependent protein kinase of Ca^{2+}-calmodulin on the ^{32}P-incorporation into the protein and lipid fractions of the membrane. Results obtained from one of five similar experiments are shown in table 1. By adding the catalytic subunit, the ^{32}P-labelling of both protein and lipid fractions was increased by 50–100% after 5 min incubation. In the presence of heat-stable inhibitor protein of the protein kinase [16], the phosphorylation was not stimulated by the catalytic subunit (not shown). When Ca^{2+} was added to the reaction mixture the ^{32}P-incorporation into the protein and lipid fractions was slightly lower than that observed in the presence of EGTA. However, when this reaction mixture was supplemented with calmodulin, a 30–60% increase of^{32}P-incorporation into both protein and lipid fractions was demonstrated.

Table 1

Effect of the catalytic subunit of cyclic AMP-dependent protein kinase or Ca^{2+}-calmodulin on the phosphorylation of membrane preparations enriched in sarcoplasmic reticulum from rabbit heart

Addition	^{32}P-incorporation into membrane	
	Lipid	Protein
EGTA	60	75
EGTA, catalytic subunit	105	170
Ca^{2+}	56	71
Ca^{2+}, calmodulin	79	101

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

The radioactive membrane lipids were then analyzed by thin-layer chromatography (TLC). In the TLC system employed for our previous experiments [3,4], a small amount of radioactivity was detected in phosphatidic acid, but the increase of ^{32}P-labelling that was found upon the addition of the catalytic subunit or Ca^{2+}-calmodulin was exclusively localized in polyphosphoinositides (not shown). The results obtained in a HPTLC system (fig.1) indicated that the formation of PhIP from PhI was stimulated, and that the ^{32}P-incorporation into $PhIP_2$ was also significantly increased in the presence of the catalytic subunit or Ca^{2+}-calmodulin. Further investigations are necessary to decide whether the stimulated formation of $PhIP_2$ was a result of the increase in the formation of PhIP, or whether the cyclic AMP-dependent pro-

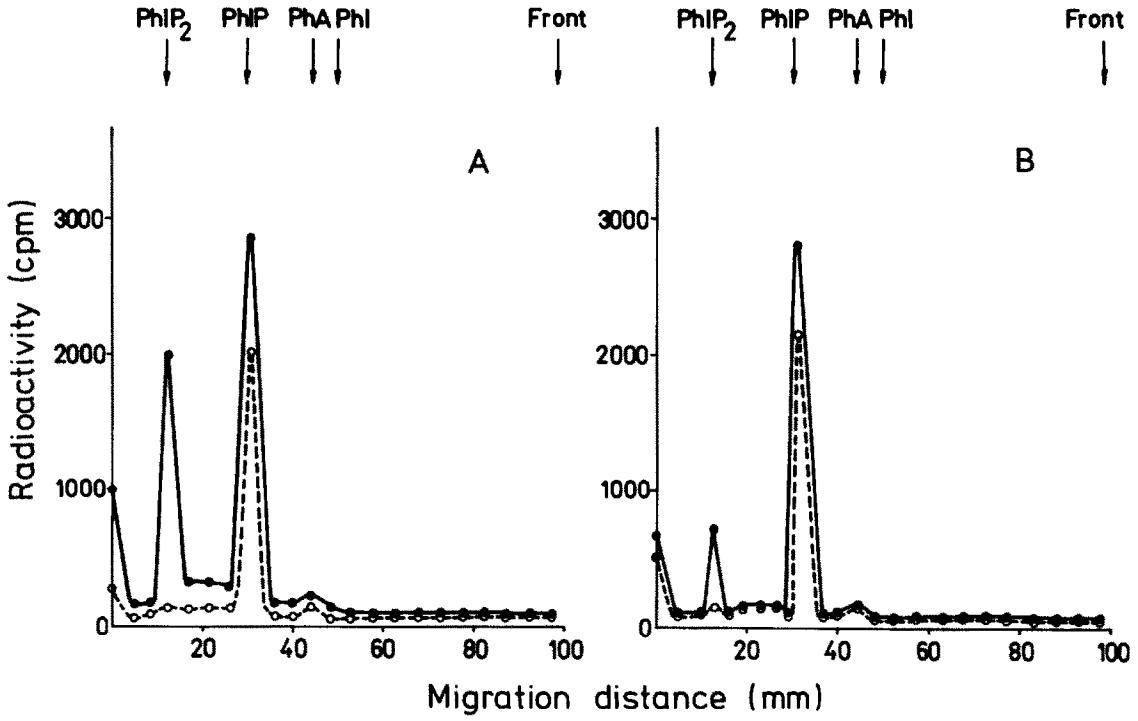

Fig. 1. High-performance thin-layer chromatographic patterns of the ^{32}P-labelled lipids extracted from membrane preparations enriched in sarcoplasmic reticulum. The membrane preparation was phosphorylated (A) in the absence (○---○) or presence (●——●) of the catalytic subunit of cyclic AMP-dependent protein kinase, or (B) in the presence of 50 μM Ca^{2+} alone (○---○) or 50 μM Ca^{2+} and 500 nM calmodulin (●——●). The figure shows data from 1 of 3 similar experiments. PhA, phosphatidic acid; PhI, phosphatidylinositol; PhIP, phosphatidylinositol-4-phosphate; $PhIP_2$, phosphatidylinositol-4,5-bisphosphate.

tein kinase could also stimulate the conversion of PhIP into $PhIP_2$.

The protein components of the membrane were investigated by SDS gel electrophoresis on 10% polyacrylamide gels. It is known that the 22–24 kDa phospholamban is composed of tightly associated subunits, and its 11 kDa component carries the phosphorylatable site [12,17]. These subunits are known to be dissociated by boiling the protein in SDS prior to gel electrophoresis [17,18]. When the membrane was phosphorylated in the presence of the catalytic subunit, and was boiled in SDS before being subjected to electrophoresis, a 11 kDa phosphoprotein band appeared on the gel (fig.2A,C). When the membrane sample was not boiled in SDS, a small 22 kDa phosphoprotein band was observed in addition to the 11 kDa band. Nevertheless, in rabbit heart a major part of phospholamban was dissociated into subunits even when the samples were held at room temperature (fig.2B). The phosphorylation of the membrane by an endogenous protein kinase activated by Ca^{2+}-calmodulin resulted also in the increased labelling of an 11 kDa protein (fig.2D). In each case, on the gel a ^{32}P-labelled band was also found which migrated slightly faster than the 11 kDa protein. Under our experimental conditions this ^{32}P-labelled membrane component was not clearly separated from the 11 kDa phosphoprotein.

Apart from this fast moving ^{32}P-labelled membrane component the protein band corresponding to phospholamban was the only protein of our membrane preparation which was phosphorylated by the exogenous catalytic subunit or by an endogenous Ca^{2+}-calmodulin-dependent protein kinase. The formation of polyphosphoinositides appeared concurrently with this protein phosphorylation. Further investigations are necessary to

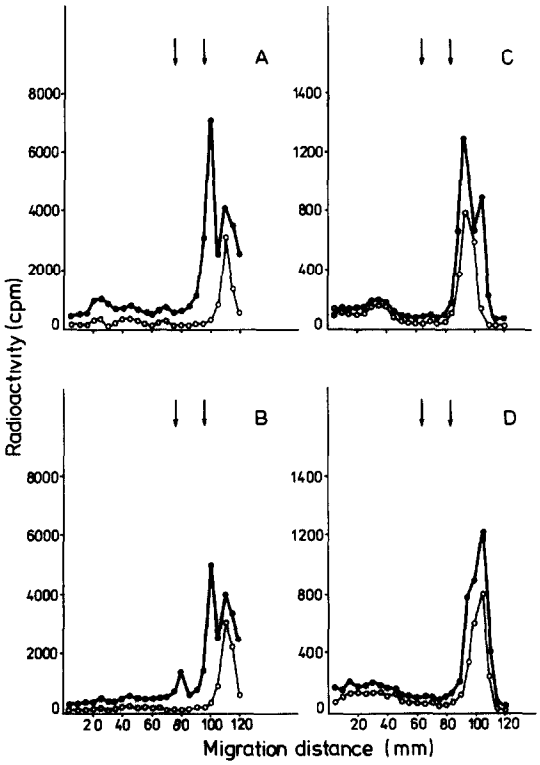

Fig. 2. SDS gel-electrophoretic patterns of the ^{32}P-labelled proteins of membrane preparations enriched in sarcoplasmic reticulum. (A,B) A membrane preparation was phosphorylated in the absence (o---o) or presence (●—●) of the catalytic subunit of cyclic AMP-dependent protein kinase. The samples were boiled (A) or not boiled (B) before electrophoresis. (C,D) A membrane preparation was phosphorylated (C) in the absence (o---o) or presence (●—●) of the catalytic subunit or (D) in the presence of 50 μM Ca^{2+} alone (o---o) or 50 μM Ca^{2+} and 500 nM calmodulin (●—●). The arrows indicate the positions of proteins of 24 kDa (trypsinogen) and 14 kDa (lysozyme).

show what is the exact mechanism and role of the stimulation of polyphosphoinositide synthesis in the sarcoplasmic reticulum of the heart.

After the submission of the present manuscript for publication, authors in [20] reported that the catalytic subunit of cyclic AMP-dependent protein kinase directly catalyzed the phosphorylation of PhIP to $PhIP_2$. Any similar effect of the catalytic subunit in our membrane preparation has yet to be examined.

ACKNOWLEDGEMENTS

The authors wish to thank Mrs M. Berényi, Mrs M. Sarkadi and Mrs A. Bart for their skilful technical assistance.

REFERENCES

[1] Rasmussen, H. (1981) Calcium and cAMP as Synarchic Messengers, Wiley, New York.
[2] Krebs, E.G. and Beavo, J.A. (1979) Annu. Rev. Biochem. 48, 923–959.
[3] Sarkadi, B., Enyedi, Á., Faragó, A., Mészáros, G., Kremer, T. and Gárdos, G. (1983) FEBS Lett. 152, 195–198.
[4] Enyedi, Á., Faragó, A., Sarkadi, B., Szás, I. and Gárdos, G. (1983) FEBS Lett. 161, 158–162.
[5] Carafoli, E. and Zurini, M. (1982) Biochim. Biophys. Acta 683, 279–301.
[6] Kaser-Glanzmann, R., Gerber, E. and Lüscher, E.F. (1979) Biochim. Biophys. Acta 558, 344–347.
[7] Fox, J.E.B., Say, A.K. and Haslam, R.J. (1979) Biochem. J. 184, 651–661.
[8] Le Peuch, C.J., Le Peuch, D.A.M., Katz, S., Demaille, J.G., Hincke, M.T., Bredoux, R., Enouf, J., Levy-Toledano, S. and Caen, J. (1983) Biochim. Biophys. Acta 731, 456–464.
[9] Tada, M., Ohncori, F., Kinoshita, N. and Abe, H. (1978) Adv. Cyclic Nucl. Res. 9, 335–369.
[10] Katz, A.M. (1979) Adv. Cyclic Nucl. Res. 11, 303–343.
[11] Kirchberger, M.A., Tada, M. and Katz, A.M. (1974) J. Biol. Chem. 249, 6166–6173.
[12] Le Peuch, C.J., Haiech, J. and Demaille, J.C. (1979) Biochemistry 18, 5150–5157.
[13] Katz, S. and Remtulla, M.A. (1978) Biochem. Biophys. Res. Commun. 83, 1373–1379.
[14] Harigaya, S. and Schwartz, A. (1969) Circulation Res. 25, 781–794.
[15] Sarkadi, B., Szász, I. and Gárdos, G. (1980) Biochim. Biophys. Acta 598, 326–338.
[16] Ashley, C.D. and Walsh, D.A. (1972) J. Biol. Chem. 247, 6637–6642.
[17] Lammers, J.M.H. and Stinis, H.T. (1980) Biochim. Biophys. Acta 624, 443–459.
[18] Kirchberger, M.A. and Antonetz, T. (1982) Biochem. Biophys. Res. Commun. 105, 152–156.
[19] Chiesi, M., Gasser, J. and Carafoli, E. (1983) FEBS Lett. 160, 61–66.
[20] Sugimoto, Y., Whitman, M., Cantley, L.C. and Erikson, R.L. (1984) Proc. Natl. Acad. Sci. USA 81, 2117–2121.