ON THE PROPERTIES OF A MEMBRANE-ASSOCIATED PROTEIN KINASE FROM CHINESE HAMSTER OVARY CELLS

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1. Introduction

Chinese hamsters ovary (CHO) cells are known to undergo significant modifications in their growth properties as a result of exposure to cyclic AMP derivatives [1, 2]. As there is increasing evidence [3, 4] that selective protein phosphorylation may be involved in the processes mediated by cyclic AMP and related compounds, we have been concerned with the study of protein kinase activities in CHO cells. In a recent study [5], we observed that exposure of CHO cells to [^{32}P] phosphate and [^{3}H]leucine in the presence of dibutyryl cyclic AMP and testosterone [1, 2] revealed a modification in the nature of the protein phosphorylation seen in membranes from control cells [5]. However, such studies with whole CHO cells did not elucidate whether the phosphorylation of membrane proteins is carried out by cytoplasmic protein kinases prior to membrane assembly or whether CHO cells contain an endogenous membrane-bound protein kinase capable of in situ phosphorylation. We now wish to describe some properties of the protein phosphorylation which can be detected in vitro and which is catalyzed by a membrane-associated protein kinase. The results to be presented show that plasma membrane preparations from CHO cells in culture exhibit endogenous protein kinase activity and phosphate acceptor proteins. The kinase activity shows a definite dependence on magnesium ions and is not stimulated by exogenous phosphate acceptor proteins or by added cyclic AMP or dibutyryl cyclic AMP.

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2. Methods

CHO cells [1, 2] were propagated as monolayers in F-12 medium [6], supplemented with 10% foetal calf serum. Cells were detached from the surface with 0.01% trypsin in phosphate-buffered saline and used for the preparation of plasma membranes following the Tris procedure of Warren and Glick [7]. Protein kinase assays were carried out using 100 μg membrane protein in 0.05 M Tris–HCl (pH 7.5) containing 0.01 M sodium fluoride, 1 μM [γ^{32}P]ATP (10^5 cpm) and magnesium acetate (0.02 M), in a volume of 0.5 ml, unless otherwise indicated. Following the appropriate incubation period, the reaction was stopped by addition of trichloroacetic acid to a concentration of 20% and KH_2PO_4 to a concentration of 0.001 M. Bovine serum albumin (100 μg) was added as a carrier and the mixture heated to 90°C for 15 min. After cooling for 20 min at 4°C the suspension was centrifuged at 2000 g for 10 min. The supernatants were removed and, in order to release any adventitiously-bound labeled ATP [8, 9] the remaining sediments were rapidly dissolved in 0.2 ml 1 N NaOH at 4°C and subsequently reprecipitated with 2 ml of 20% trichloroacetic acid. The fractions insoluble in trichloroacetic acid were collected on glass fibre GF/A Whatman discs, washed sequentially in 10% trichloroacetic acid, ethanol-ether (2:1) and ether and counted in 5 ml of Aquasol scintillator (New England Nuclear Corp., Waltham, Mass. U.S.A.). Analysis of the reaction product by polyacrylamide gel electrophoresis and counting of the gels was carried out as described previously [5].

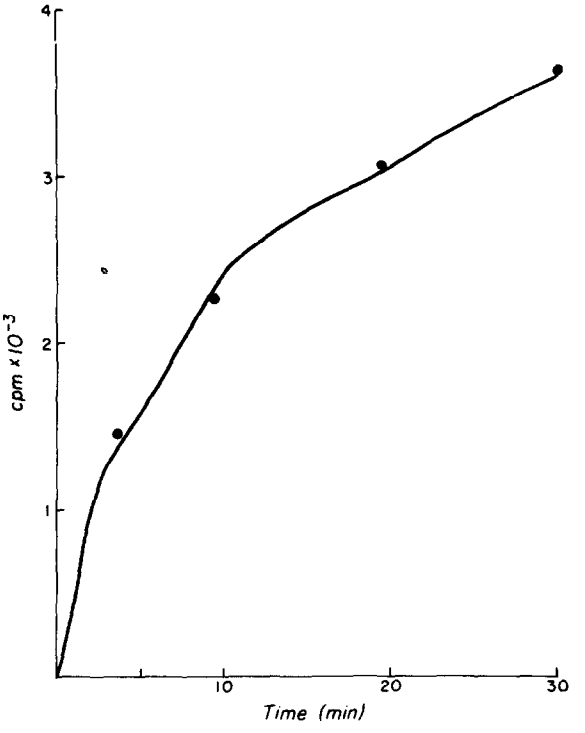

Fig. 1. Time course of incorporation of [γ^{32}P] ATP into protein. The reaction mixture and experimental conditions were as described under Methods, using duplicate samples for each point shown.

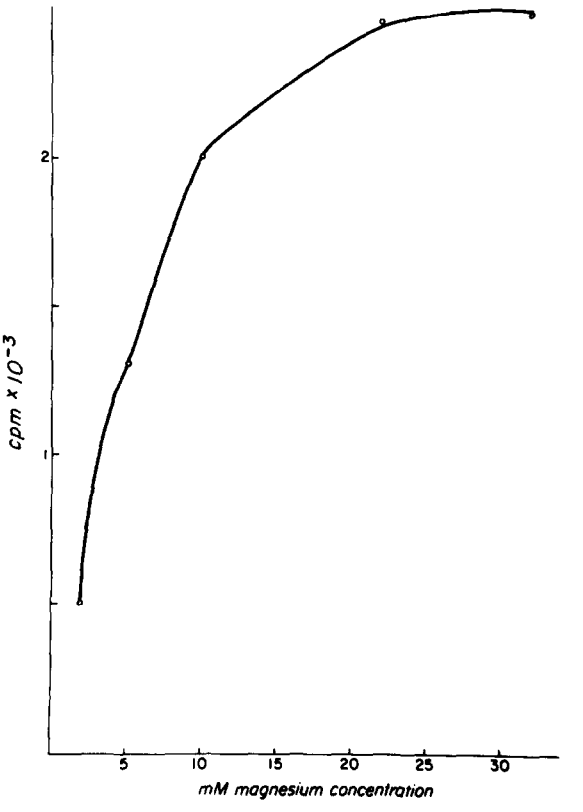

Fig. 2. Effect of magnesium ions on the relative protein kinase activity. The reactions were carried out in duplicate for 10 min at 37°C at the various magnesium ion concentrations indicated. The results presented correspond to points determined in duplicate from which zero time backgrounds of 180 to 200 cpm were subtracted.

3. Results and discussion

Incubation of plasma membrane fractions from CHO cells obtained by the Tris procedure [7] in the presence of [γ^{32}P] ATP resulted in the incorporation of ^{32}P into protein as shown in fig. 1. A complete lack of incorporation of radioactivity into protein from [^{14}C] ATP (not shown) demonstrated that the process being measured was not due to adsorption of ATP to the membrane preparation. As shown in fig. 2, ^{32}P incorporation into acid-insoluble material was strongly dependent on the availability of magnesium ions, reaching a plateau at about 20 mM. A significantly lower incorporation (about 25%) was observed when calcium ions were tested in the range 5–20 mM, for their ability to substitute for magnesium ions. Table 1 shows that the amount of ^{32}P incorporation into protein was completely unaffected by addition of either cyclic AMP or its more lipophilic derivative dibutyryl cyclic AMP, in the range 10^{-9} M–10^{-3} M. That this lack of effect of cyclic AMP, and the dibutyryl derivative is unlikely to be due to their degradation by a cyclic AMP phosphodiesterase was shown by the fact that no additional stimulation by the cyclic nucleotides was observed when the phosphodiesterase inhibitor aminophylline was added to the system. As shown in table 1, addition of aminophylline either in the presence or absence of the cyclic AMP led to a decreased activity, in agreement with a similar effect reported to occur when the protein kinase associated with the envelope of Rauscher murine leukemia virus is assayed in the presence of caffeine [10]. The possibility of stimulating the membrane protein kinase activity by adding exogenous

Table 1
Effect of various additions on the protein kinase activity.

Additions	Relative incorporation (cpm)
None	2139
Cyclic AMP	2286
Dibutyryl cyclic AMP	2275
Cyclic AMP and aminophylline	550
Aminophylline	534
Lysine-rich histone	2205
Arginine-rich histone	2220

The assay system was as described in Methods, with the addition of the components above indicated. The reaction mixtures were incubated for 10 min at 37°C, containing where indicated, histones (50 μg) aminophylline (0.005 M) and cyclic AMP or the dibutyryl derivative in the range (10^{-9} – 10^{-3}M). All the data above shown, correspond to duplicate experiments from which zero time backgrounds of 180 to 200 cpm were substracted.

substrates was tested as indicated in table 1, but no difference was observed in the system following the addition of either lysine-rich or arginine-rich histones.

Some properties of the product of the reaction were examined as described in table 2. This shows that the product is not a nucleic acid because of its resistance to nucleases and, unlike the phosphopeptide intermediate that is formed in the reaction of membrane ATPases, is not labile to succinic acid–hydroxylamine treatment [11].

Susceptibility to pronase and hot alkali (table 2) is consistent with the product being a phosphoprotein [10]. In order to identify the membrane polypeptides that acted as acceptors in the phosphorylation reaction, the product was subjected to fractionation by sodium dodecyl sulphate-polyacrylamide gel electrophoresis. Although identical experimental conditions have been found to separate CHO plasma membranes into about twenty well-defined membrane polypeptide components [5], fig. 3 shows that most of the radioactivity appeared to be associated with one region which exhibited a relative electrophoretic mobility of about 0.26.

It may be relevant that, although experiments involving membrane protein phosphorylation by whole CHO cells also revealed a selective region of phosphorylation, the latter possessed an electrophoretic mobility within the 0.1 range [5]. The significance of the

Table 2
Properties of the phosphorylated product.

Samples	Treatment	% Acid-insoluble ^{32}P
	None	100*
1	Heated with 0.1 N NaOH for 15 min at 90°C	8
2	Treated with pronase (100 μg) for 30 min at 37°C prior assay	5
3	Treated with RNAase (100 μg) for 30 min at 37°C prior assay	98
4	Treated with DNAase (100 μg) for 30 min at 37°C prior assay	97
5	Exposed for 30 min at 37°C to 1 M succinic acid–1 M hydroxylamine, pH 5.5	91

For the above experiments, duplicate incubations were carried out as described under Methods for 10 min at 37°C. The reactions were stopped by addition of trichloroacetic acid 10%. Samples 1–4 were suspended in 1 ml of (1 M) Tris, pH 8.0 and the corresponding additions were carried out as above indicated. Sample 5 was suspended directly in: succinic acid (1 M); hydroxylamine (1 M), pH 5.5. With all the samples the reactions were terminated by adding Trichloroacetic acid to 25%. Radioactivity remaining associated with the precipitate was then determined as described in Methods.

*Control experiments gave an incorporation of about 2600 cpm after subtraction of zero time samples.

different electrophoretic migration of the phosphorylated protein product as measured on whole cells [5] or in vitro is not clear. It is possible that proteolytic degradation of the in vitro product is responsible for its greater electrophoretic mobility. This, however, seems unlikely because: a) the electrophoretic conditions employed [5] using 1% sodium dodecyl sulphate are the same as those which have been found to prevent proteolytic degradation in erythrocyte membranes [12]; b) no different pattern was observed when the sample was dissociated in the presence of the protease inhibitor phenyl methyl sulphonyl fluoride (1 mM) prior to electrophoresis; c) the product was found to migrate electrophoretically essentially as one peak. (fig. 3). It thus seems more likely that the electrophoretic difference between the in vitro product and that obtained with whole cells due to the different steric and ionic environment for the reaction of the acceptor protein and the enzyme between the in vitro and whole cell conditions, which may lead to the different labelling

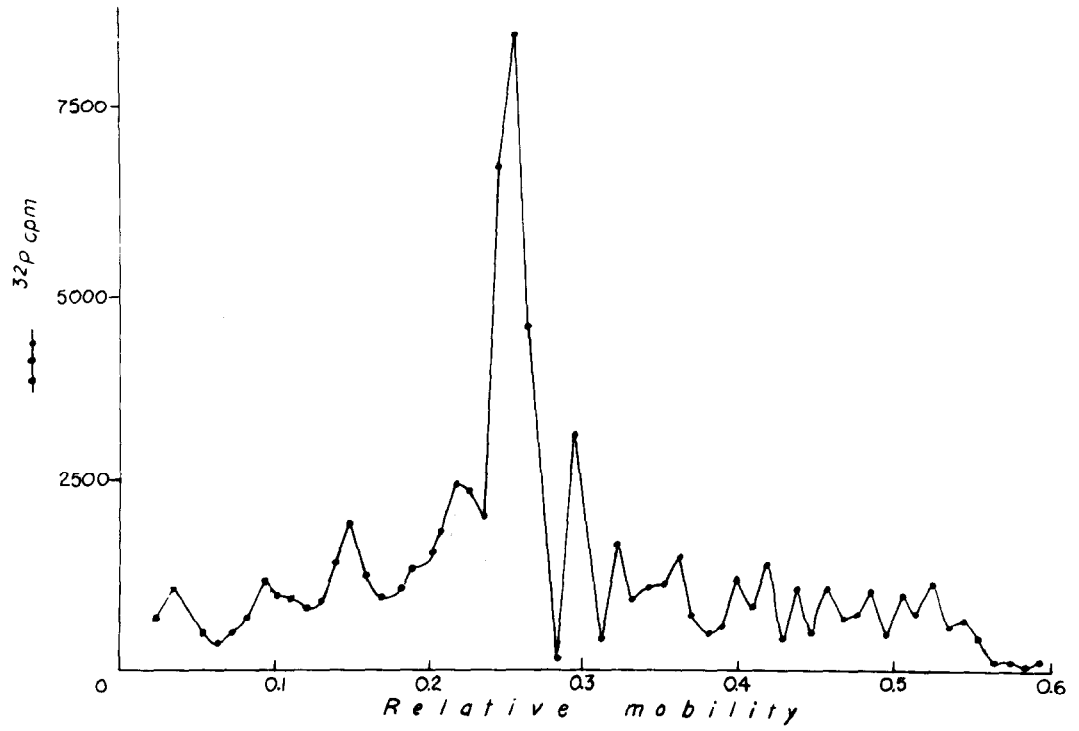

Fig. 3. Electrophoretogram of the reaction product of the membrane protein kinase. Incubations of 100 μg membrane protein with (1 μM) [γ^{32}P] ATP (10^6 cpm) and 0.02 M Mg^{2+} were carried out as described under Methods for 60 min at 37°C. The reaction was stopped with 10% trichloroacetic acid and the precipitate obtained was heated in another aliquot of the same acid for 15 min to 90°C. Following cooling and centrifugation the remaining residue was subjected to dissociation in: 20% sucrose; 2% (w/v) sodium dodecyl sulphate; 1% (v/v) β-mercaptoethanol; 0.01 M EDTA and 0.05 M Tris–HCl buffer, pH 7.5. Subsequent fractionation in 5.6% polyacrylamide gel and counting of the radioactivity was carried out as described elsewhere [5, 12].

now observed. Support for this assumption comes from the recent work of Majumdar and Turkington [13] who have reported significant differences between the patterns of ribosomal protein phosphorylation obtained either by in vitro reaction with cellular protein kinases or when culturing intact lactating mammary tissue with [^{32}P] phosphate. Also, Johnson and Allfrey [14] have observed that the electrophoretic pattern of acidic proteins phosphorylated in isolated rat liver nuclei is different from that obtained when the acidic proteins are phosphorylated in vivo. Hence, the findings of different phosphorylation of proteins between the in vivo and in vitro conditions emphasize the necessity of a thorough search for the natural substrates of the various cellular protein kinases and for the factors that may allow protein phosphorylation to be carried out specifically.

References

[1] Hsie, A.W. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. U.S. 68, 358.
[2] Hsie, A.W., Jones, C. and Puck, T.T. (1971) Proc. Natl. Acad. Sci. U.S. 68, 1648.
[3] Kuo, J.F. and Greengard, P.G. (1969) J. Biol. Chem. 244, 3417.
[4] Kuo, J.F. and Greengard, P.G. (1969) Proc. Natl. Acad. Sci. U.S. 64, 1349.
[5] Rieber, M. and Bacalao, J. (1973) Biochem. J. 131, 641.
[6] Ham, R.G. (1965) Proc. Natl. Acad. Sci. U.S. 53, 288.
[7] Warren, L. and Glick, M.C. (1969) in: Fundamental Techniques in Virology (Habel, K. and Salzman, N.P. eds), p. 66, Academic Press, New York.
[8] Greenway, P.J. (1972) Biochem. Biophys. Res. Commun. 47, 639.
[9] Lake, R.S. and Salzman, N.P. (1972) Biochemistry 11, 4817.

[10] Strand, M. and August, T. (1971) Nature New Biol. 233, 137.
[11] Avruch, J. and Fairbanks, G. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1216.
[12] Fairbanks, G., Steck, T.L. and Wallach, D.F. (1971) Biochemistry 10, 2606.
[13] Majumdar, G.C. and Turkington, R.W. (1972) J. Biol. Chem. 247, 7207.
[14] Johnson, E.M. and Allfrey, V.G. (1972) Arch. Biochem. Biophys. 152, 781