CYCLIC AMP-DEPENDENT PROTEIN PHOSPHORYLATION ON THE SURFACE OF RAT HEPATOCYTES

Marianne SOMMARIN, Thomas HENRIKSSON and Bengt JERGIL

Biochemistry, Chemical Centre, POB 740, S-220 07 Lund, Sweden

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1. Introduction

Proteins of isolated plasma membranes from rat liver cells can be phosphorylated either by endogenous [1–3] or exogenously added [4] protein kinases. Protein phosphorylation in plasma membranes presumably serves as a general regulatory mechanism as is the case in other subcellular fractions. An example is the phosphorylation and thereby activation of a cyclic nucleotide phosphodiesterase associated as a peripheral protein with rat liver plasma membranes [5]. The phosphorylation of other, as yet unidentified, plasma membrane proteins appears to be modulated by insulin [3].

No studies have been performed so far on the phosphorylation of proteins on the external surface of isolated rat hepatocytes. The phosphorylation of surface proteins in other kinds of cells has been described, however [6–8]. An examination of surface protein phosphorylation may yield information about regulatory mechanisms on the plasma membrane level. Here we report on cyclic AMP-dependent and independent phosphorylation of proteins on the outer surface of rat hepatocytes.

2. Experimental

Hepatocytes were prepared [9,10] from male Sprague-Dawley rats weighing 200–300 g. Bicarbonate-free Hanks' medium [11], buffered to pH 7.4 with 20 mM HEPES, also containing 0.05% collagenase, 0.1% hyaluronidase and 0.2% bovine serum albumin (all from Sigma) was used for the perfusion. The viability of the cells, measured as exclusion of 0.1% trypan blue, was above 90%.

In control experiments to examine the influence of leakage of protein kinase and cyclic adenosine 3′, 5′-monophosphate (cyclic AMP) binding proteins on hepatocyte surface phosphorylation 1 g of hydroxyapatite-agarose gel (HA-Ultrogel, LKB) was added per g liver, when the perfused and collagenase-treated liver was dispersed in medium. The gel was present during the entire handling of the cells before phosphorylation analyses. The recovery of cells dropped to approx. 50% by this treatment due to adsorption of cells to the gel, but the viability of the remaining cells increased to above 95%.

Protein kinase analyses were performed (usually in triplicate) at 37°C in Ca^{2+}-free Hanks' medium buffered with 20 mM HEPES, pH 7.4. 1 ml of incubation mixture contained 2 μmol [γ-^{32}P]ATP (100–150 dpm/pmol; prepared as in [12]), 3 mg histones (Sigma, type II A), 10–15 × 10^6 hepatocytes, and (when indicated) 5 nmol cyclic AMP. At indicated times 50 μl of the mixture was spotted on a Whatman 3 MM filter paper disc, which was washed and counted as in [13]. Protein phosphatase activity was followed after the addition of hexokinase (Sigma, 180 U/ml) to remove unreacted ATP (glucose is present in the incubation medium). In some experiments 360 μg/ml rabbit muscle protein kinase inhibitor (Sigma) was added.

Endogenous phosphorylation of hepatocytes was performed as above but omitting histones. The filter paper discs were immersed immediately in 10% trichloroacetic acid. Before counting they were washed in the following way to remove unreacted ATP and radioactively labelled phospholipids (particularly phosphoinositides, cf. [14]): twice for 10 min in 10% trichloroacetic acid and 1 M H_3PO_4, boiled once for 15 min in 5% trichloroacetic acid, washed once each for 5 min in 10% trichloroacetic acid and 1 M H_3PO_4,

ethanol, ethanol/ether (1:1, by vol.), chloroform/methanol 12 M HCl (200:100:1, by vol.), and ether. The discs were dried and counted in 0.4% Omnifluor (New England Nuclear) in toluene in a liquid scintillation counter.

Samples for electrophoresis were incubated for 5 min in 50 μl medium also containing 0.1 μmol [γ-^{32}P]ATP (800–1000 dpm/pmol), 200 000 hepatocytes, and 0.25 nmol cyclic AMP (when indicated). SDS-polyacrylamide gel electrophoresis and autoradiography of the gels were as in [15,16].

3. Results

3.1. *Phosphorylation of exogenous substrate*

To test whether hepatocytes possess protein kinase activity on their external surface, isolated cells were incubated with [γ-^{32}P]ATP and histones as exogenous substrate (fig.1). There was an incorporation of radioactively labelled phosphate, and cyclic AMP stimulated the reaction by approx. 50%. The cyclic AMP-dependent incorporation was partly inhibited by rabbit muscle protein kinase inhibitor [17], which acts specifically on cyclic AMP-dependent protein kinases. When hexokinase was added to remove unreacted ATP, a time-dependent decrease of bound phosphate was observed (– – –). This indicates the presence of phosphoprotein phosphatase activity as well on the cell surface.

The rate of phosphate incorporation was fast initially, and then slower and close to linear for a few minutes. The rate of incorporation was then approx. 40 and 25 pmol of phosphate/10^6 cells per min in presence and absence of cyclic AMP, respectively. The rate of dephosphorylation was also higher in presence of cyclic AMP, presumably due to a higher concentration of phosphate groups on the substrate in this case as compared to incubations without cyclic AMP.

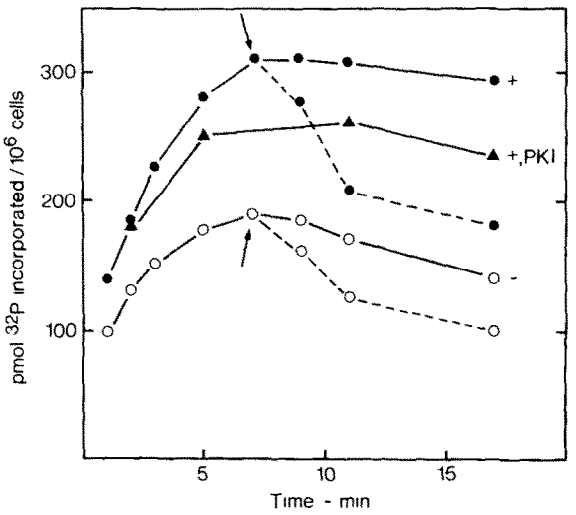

Fig.1. Phosphorylation and dephosphorylation of histones by hepatocytes. Analyses were performed as in section 2 in presence (+) or absence (–) of cyclic AMP. Arrows indicate addition of hexokinase to follow the dephosphorylation (– – –). PKI, protein kinase inhibitor.

The possibility that the protein kinase activity was due to an association of intracellular enzymes released

Table 1
Effect of adsorption of released protein kinase on hepatocyte surface phosphorylation

Treatment during cell preparation	Protein kinase in cell washes (nmol/min per g liver)		Cyclic AMP binding in cell washes (pmol/g liver)	Hepatocyte protein kinase (pmol/min per 10^6 cells)		Endogenous phosphorylation (pmol/min per 10^6 cells)	
	–[a]	+[a]		–	+	–	+
Untreated	4.36	6.22	6.23	23.1	30.8	2.87	3.62
Hydroxyapatite agarose gel	1.12	1.21	1.34	36.0	44.1	3.52	3.95

After dispersion of the collagenase-treated liver in the absence or presence of hydroxyapatite the cells were incubated for 15 min at 37°C and washed twice as in [10]. The combined supernatants were analyzed for protein kinase (see section 2) and cyclic AMP binding (by a slight modification of [20]), and the cells for protein kinase and endogenous phosphorylation

[a] – and +, in the absence or presence of cyclic amp, respectively

from leaking or broken cells during the preparation was considered. Approx. 80% of the released protein kinase and cyclic AMP-binding proteins were adsorbed by including hydroxyapatite in the cell preparation medium (table 1). The concentration of protein kinase on the hepatocyte surface did not decrease by this treatment (it rather increased in this particular, but not in other, control experiments), which would have been expected if released enzyme components associate with the cell surface. The hepatocytes could also be incubated with liver cytosol without an increase in the surface protein kinase activity (not shown).

3.2. *Endogenous phosphorylation*

The incorporation of phosphate into endogenous substrates was examined next (fig.2). The rate of this reaction was approx. 15 times lower than with histones as substrates (3 and 2 pmol incorporated/10^6 cells per min in presence and absence of cyclic AMP, respectively). A dephosphorylation of endogenously phosphorylated material occurred after addition of hexokinase to remove unreacted ATP. The rate and extent of this dephosphorylation was less than that observed with exogenous substrate. The endogenous phosphorylation was not affected when cells were prepared in the presence of hydroxyapatite to adsorb material released from leaking or broken cells (table 1).

The protein kinase inhibitor from rabbit skeletal muscle inhibited endogenous phosphate incorporation (fig.2). The rate of incorporation in presence of both inhibitor and cyclic AMP was similar to that obtained without these additions. The inhibitor did not affect the cyclic AMP-independent incorporation appreciably (not shown).

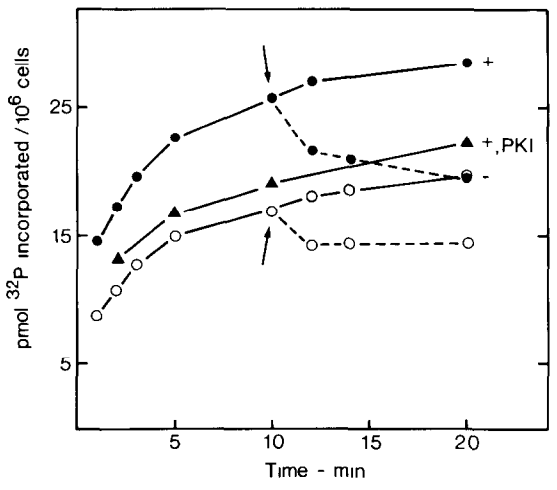

Fig.2. Endogenous phosphorylation and dephosphorylation of hepatocyte surface proteins. See legend to fig.1.

Table 2
Subcellular location of phosphorylated components

Preparation	Total incorporation	Supernatant	Pellet	% recovery
Intact cells	21.9	0.1	9.9	46
Homogenized cells	73.2	6.8	35.6	58

Hepatocytes or homogenized cells (30 strokes of a tight-fitting Dounce homogenizer) were incubated with [γ-^{32}P]ATP for 5 min at 37°C. A 10-fold excess of unlabelled ATP and 0.05 M sodium fluoride were added at 0°C to stop further labelling and to inhibit phosphatase activity. The cells were homogenized as above and centrifuged at 110 000 × *g* for 90 min. Samples from supernatants and pellets were spotted on filter paper discs and washed as for endogenous phosphorylation before counting. The incorporation is given as pmol/10^6 cells

To verify that the endogenous phosphorylation occurred on the hepatocyte surface, and was not due to leakage of radioactively labelled ATP into the cells or of intracellular material to the medium, we examined the incorporation of phosphate into soluble and particulate fractions of hepatocytes (table 2). The radioactivity was recovered almost exclusively in the particulate fraction after incubation of intact cells with radioactively labelled ATP. If, however, the cells were homogenized before the incubation, an appreciable incorporation was into the soluble fraction, i.e. cytosolic material. The incorporation into particulate material was also higher in homogenized cells, presumably due to the phosphorylation of components in the exposed intracellular membranes. These findings strongly indicate that the incorporation into intact cells was localized to the cell surface.

3.3. *Phosphoprotein patterns*

Phosphoprotein patterns were examined by SDS-polyacrylamide gel electrophoresis and autoradiography after incubation of hepatocytes with radioactively labelled ATP (fig.3). A few proteins became labelled, and the incorporation into 5 of these (indicated by dots) was dependent on cyclic AMP. Presumably the incorporation was into surface proteins, since the pattern did not change by including hydroxy-

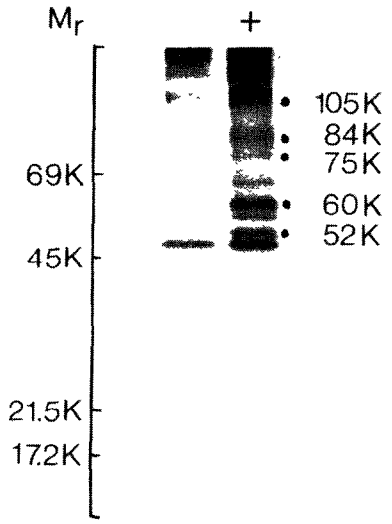

Fig.3. Phosphoprotein patterns. Hepatocytes were incubated with [γ-^{32}P]ATP in presence (+) or absence (−) of cyclic AMP. Proteins were separated by SDS-polyacrylamide gel electrophoresis and autoradiographed. Dots indicate components with a cyclic AMP-stimulated phosphorylation. The M_r of reference proteins is indicated to the left.

apatite in the cell preparation medium, and since no [^{32}P]phosphate was found in the soluble fraction after homogenization of the cells (see above). We have also found in other experiments that the phosphoprotein patterns obtained with homogenized cells or with rat liver cytosol are quite different from the patterns presented here.

4. Discussion

The results provide evidence for a surface location of protein kinase, phosphoprotein phosphatase, and substrates for these enzymes in isolated rat liver hepatocytes. At least a part of the exposed protein kinase activity is cyclic AMP dependent. The evidence for a surface location is based on the phosphorylation/dephosphorylation of histones (a substrate which presumably does not enter intact cells) and the incorporation of phosphate into particulate, but not into soluble, material in the cell. In addition, there was no lag phase in the phosphorylation of endogenous proteins. This would have been expected if labelled ATP or phosphate had to enter the cells before phosphorylation, since these would have to equilibrate with intracellular pools to yield a maximum rate of incorporation. Thus, a lag phase of at least 10 min is seen in the phosphorylation of proteins when hepatocytes are incubated with [^{32}P]phosphate (unpublished).

The possibility that intracellular enzymes catalyzed the cell surface phosphorylation after leakage to the medium and association with the plasma membrane appears less likely, since 80% of the protein kinase and cyclic AMP-binding proteins set free during the cell preparation could be trapped without decreasing the protein kinase activity of the cells. The possible leakage of enzymes during collagenase treatment could not be checked, however. Since the liver was continuously perfused during this stage released enzymes were likely to be carried away before they could associate extensively with the cell surface. Cyclic AMP-dependent protein kinases at low concentrations do not bind extensively to microsomal membranes [18], and we did not observe any increase in the protein kinase activity of hepatocytes after incubation of the cells with liver cytosol. The leakage of protein kinase from the cells during analysis was also low.

The functional importance of a location of cyclic AMP-dependent protein kinase and substrates for this enzyme on the hepatocyte surface is a matter for speculation. It is in line, however, with the recent finding [19], that a fraction of the cellular cyclic AMP synthesis may take place on the outer surface of hepatocytes. Since a surface location of cyclic AMP-dependent protein kinase activity has been reported for several kinds of cells [7,8], this might be a property of most cells. It is of interest now to identify the phosphoproteins located on the hepatocyte surface, and to examine the function of these phosphorylations.

Acknowledgement

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