URIDINE PHOSPHATE BINDING PROTEINS IN THE PLASMA MEMBRANE OF RAT LIVER

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Received 29 March 1982

1. Introduction

Proteins and glycoproteins of the plasma membrane exert a key role mediating a variety of cell surface functions as adhesiveness, contact inhibition of growth, or binding of serum constituents. The regulation of these functions, however, is largely unknown. At least two mechanisms of regulation should be taken into account. One is regulation by turnover, which controls the concentration of functional constituents in the plasma membrane [1,2]. Another regulatory mechanism is provided by chemical modification of proteins, which has so far been mostly studied among enzymes (for review see [3]) and nuclear proteins (for review see [4,5]). However, studies on chemical modifications of plasma membrane proteins are few. A phosphorylation of several plasma membrane proteins from rat liver and erythrocytes has been described [6,7]. This report describes the binding of uridine phosphate to specific plasma membrane proteins suggesting a new type of plasma membrane modification.

2. Materials and methods

Male Buffalo rats (160–180 g each) were intraperitoneally injected with 50 μCi [2-^{14}C]uridine (55 mCi/mmol) or 330 μCi [5-^{3}H]uridine (25 Ci/mmol) per 100 g body wt. each. After 1 h livers were perfused with saline, containing 2 mM $CaCl_2$, removed and plasma membranes were prepared as in [2]. In vitro binding was studied incubating 500 μg of unlabelled plasma membrane protein at 25°C in 500 μl of reaction buffer containing 20 mM Hepes, 100 mM sodium acetate, 1 mM magnesium acetate, 2 mM $CaCl_2$ (pH 7.4). The reaction was started by the addition of [α-^{32}P]UTP (2000–3000 Ci/mmol), [4-^{14}C]UTP (48 mCi/mmol), or [5-^{3}H]UTP (25 Ci/mmol) respectively. In some experiments an ATP regenerating system was added to the binding assay containing 5 mM ATP, 10 mM phosphoenolpyruvate and 50 U/ml of pyruvate kinase (EC 2.7.1.40, Boehringer-Mannheim). Labelled membrane samples were extensively washed and dialyzed against ice-cold reaction buffer before further analysis. Treatment of radiolabelled plasma membranes (1 mg/ml) with ribonuclease A (200 μg/ml, Boehringer-Mannheim) or deoxyribonuclease I (400 μg/ml, Sigma) was carried out in a 0.1 M sodium acetate buffer, 50 mM $MgCl_2$, 50 mM $CaCl_2$ (pH 5.0) at 37°C for 12 h. Digestion with proteinase K (1 mg/ml, Boehringer-Mannheim) was done in reaction buffer at 37°C for 12 h. Contaminating RNA was removed from the plasma membrane fraction according to [8].

SDS–polyacrylamide gel electrophoresis was performed by the procedure of Laemmli [9] as described in [2]. For autoradiography both ^{14}C- and ^{32}P-labelled gels were processed as in [10] and put in contact with pre-exposed X-ray film (X Omat, Kodak) [11]. Protein-bound radioactivity was determined by a modified method [2] of [12]. [5-^{3}H]UTP metabolites in the binding assay were analyzed by descending paper chromatography. Aliquots of the incubation mixture were precipitated by addition of 0.5 vol. of 20% (w/v) of ice-cold trichloroacetic acid. After centrifugation the clear supernatant was chromatographed on Whatman No. 3 paper using ethanol/1 M ammonium acetate (pH 3.8) (5:2, v/v) as a solvent [13]. UTP metabolites were identified by use of ^{3}H-labelled UTP, UDP, UMP and uridine standards. Radioactivity was determined by counting 1.0 cm strips successively over the entire length of the chromatogram in a toluene-based scintillator. Radioactive substances were all obtained from the Radiochemical Center, Amersham. Protein content was determined as in [14] with bovine serum albumin as a standard.

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00145793/82/0000–0000/$02.75

3. Results and discussion

Injection of radiolabelled uridine into male Buffalo rats resulted in a significant binding of label to plasma membrane proteins (table 1). The label could not be removed by exhaustive washing and dialysis, and coprecipitated with protein after addition of trichloroacetic acid. Moreover, label was not removed by heating of plasma membranes in 3% SDS. Analysis of the plasma membranes by SDS–polyacrylamide gel electrophoresis revealed that mainly 5 polypeptide bands corresponding to M_r-values of 220 000, 180 000, 130 000, 100 000 and 70 000 were labelled (fig.1). The pattern of binding was completely reproducible in 4 independent experiments. Contamination of plasma membrane proteins by labelled nucleic acids was excluded by the following experiments. Extensive treatment of plasma membranes with RNase or DNase did not alter the fluorographic pattern (fig.1), and did not decrease trichloroacetic acid-precipitable radioactivity (table 2).

Table 1
Binding of uridine and uridine phosphate to plasma membranes in vivo and in vitro

Labelling precursor		Trichloroacetic acid-precipitable protein-bound radioactivity (cpm/mg of protein × 10^{-3})
In vivo	[5-^{3}H]uridine	6.4 ± 0.7
	[5-^{3}H]uridine + actinomycin	6.5 ± 1.3
In vitro	[5-^{3}H]UTP	20.7 ± 3.5
	[α-^{32}P]UTP	339.2 ± 5.4
	[α-^{32}P]UTP after heating	71.0 ± 0.2

Plasma membranes were labelled (a) in vivo with 330 μCi [5-^{3}H]uridine injected intraperitoneally to male Wistar rats, per 100 g body wt. each and (b) in vitro by incubation with 25 μCi [5-^{3}H]UTP or 50 μCi [α-^{32}P]UTP, as in section 2. Actinomycin D, 80 μg/100 g body wt., was administered intraperitoneally 20 min before injection of label. Heating of plasma membranes was done at 70°C for 1 h, before addition of [α-^{32}P]UTP. Protein-bound radioactivity was determined as in section 2. Values are means ± SEM, n = 4

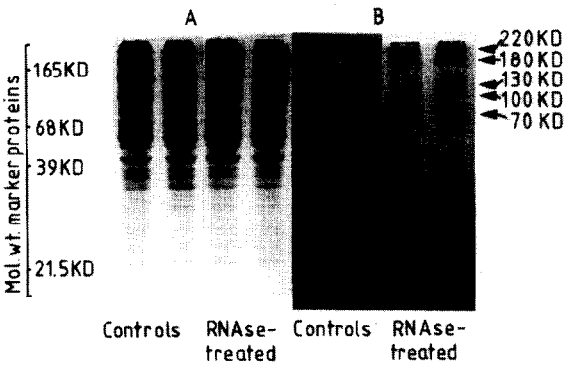

Fig.1. Polyacrylamide gel electrophoresis of plasma membrane polypeptides labelled in vivo with [2-^{14}C]uridine. Male Buffalo rats received 50 μCi of [2-^{14}C]uridine/100 g body wt. intraperitoneally 1 h before isolation of plasma membranes. Isolated plasma membranes were treated with RNase as described in section 2. Untreated controls and RNase-treated plasma membrane samples were separated on 10% SDS–polyacrylamide gels, applying 70 μg protein to each lane. Apparent M_r-values of the labelled bands, indicated by arrows, were calculated from the relative mobilities of calibration proteins [2]. (A), Coomassie staining; (B), fluorographic pattern.

Table 2
Enzyme treatment of labelled plasma membrane proteins

Enzyme treatment	Trichloroacetic acid-precipitable protein-bound radioactivity	
	Plasma membranes labelled with	
	[5-^{3}H]uridine, in vivo	[α-^{32}P]UTP, in vitro
	(cpm/mg of protein)	(cpm/mg of protein × 10^{-3})
Controls	6454 ± 832	336 ± 15
RNAse	6483 ± 1339	332 ± 17
DNAse	n.d.	338 ± 27
Proteinase K	n.d.	1.6 ± 0.04

Plasma membranes were labelled in vivo with [5-^{3}H]uridine or in vitro with [α-^{32}P]-UTP, as described in table 1. Radio-labelled plasma membranes were treated with RNAse, DNAse or proteinase K as described in section 2. Values are means ± SEM, n = 4

Furthermore, no ethidium bromide staining was found in the labelled bands. Treatment of rats with actinomycin D prior to injection of label did not inhibit the incorporation of [^{3}H]uridine into the plasma membrane (table 1).

Binding of uridine phosphate to plasma membrane proteins was also found in vitro. Incubation of isolated plasma membranes with UTP radiolabelled in the uracil or phosphate moiety resulted in the incorporation of radioactivity into the trichloroacetic acid-precipitable plasma membrane fraction (table 1). High concentrations of ATP, GTP or CTP in the incubation mixture did not inhibit the binding of [^{3}H]UTP to the plasma membrane, indicating a specificity of the binding sites for uridine nucleotides (table 3). Furthermore, binding of [^{3}H]UTP to plasma membranes was not decreased in the presence of unlabelled UDP, UMP or uridine, showing that the binding is specific for UTP (table 3).

In the binding assay UTP was rapidly hydrolyzed by nucleotidases of the plasma membrane (fig.2a) in accordance with [15,16]. Addition of 5 mM ATP and ATP regenerating system effectively inhibited hydrolysis of UTP and maintained the levels of UTP during incubation (fig.2b). Co-addition of ATP and ATP regenerating system did not interfere with UTP binding to the plasma membrane (table 3). The time course of [^{3}H]UTP binding to plasma membranes showed a rapid increase, followed by a plateau and a subsequent gradual decrease (fig.3). The extent of UTP binding was dependent on the concentration of plasma membrane proteins in the binding assay. Binding increased as the concentration of plasma membrane proteins was increased up to ~300 μg (fig.4). At higher membrane concentrations, little further

Table 3
Binding of [^{3}H]UTP to isolated plasma membranes in the presence of unlabelled nucleotides

Incubation in the presence of	Trichloroacetic acid-precipitable protein-bound radioactivity (dpm/mg of protein × 10^{-3})
(a) control	21.3 ± 2.1
GTP	25.8 ± 0.9
ATP	18.0 ± 3.2
CTP	23.3 ± 4.8
(b) control	45.1 ± 3.0
UDP	44.8 ± 2.0
UMP	39.9 ± 6.2
uridine	45.9 ± 11.6

Plasma membrane protein (500 μg) was incubated at 25°C for 30 min with (a) 25 μCi of [5-^{3}H]UTP (25 Ci/mmol) in the presence of 5 mM ATP, 0.5 mM GTP or 0.5 mM CTP, or (b) 50 μCi [5-^{3}H]UTP and 0.5 mM UDP, UMP or uridine. Protein-bound radioactivity was measured in the plasma membrane as described in section 2. Means ± SEM, n = 2

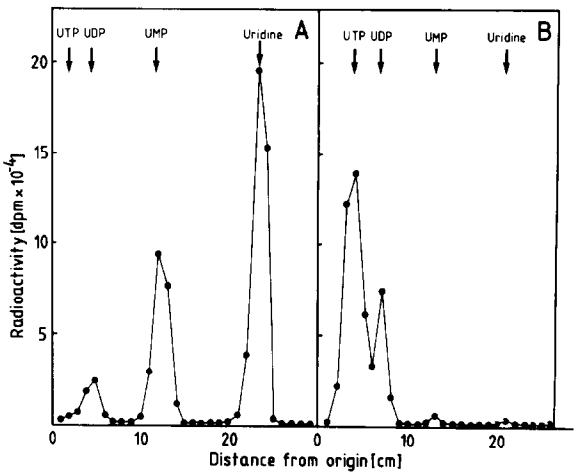

Fig.2. Hydrolysis of [^{3}H]UTP by isolated plasma membranes. Plasma membrane protein (500 μg) was incubated with 50 μCi of [5-^{3}H]UTP (25 Ci/mmol) in 500 μl of reaction-buffer at 25°C for 45 s; (A) controls, (B) in the presence of 5 mM ATP, 10 mM phosphoenolpyruvate and 25 U of pyruvate kinase. Incubation mixtures were precipitated with trichloroacetic acid, and 50 μl of the clear supernatant were chromatographed. Radioactivity was determined by counting of 1.0 cm strips, as in section 2.

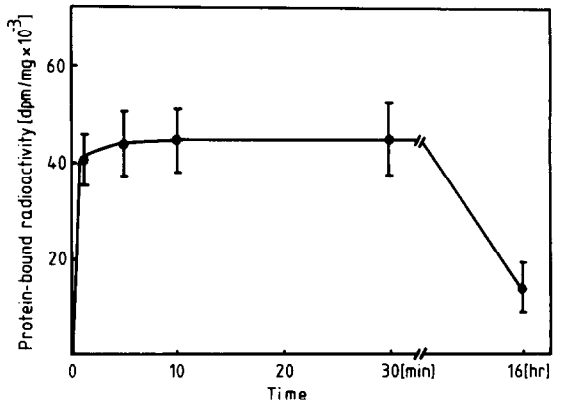

Fig.3. Time course of [^{3}H]UTP binding to isolated plasma membranes. Plasma membrane protein (500 μg) was incubated with 50 μCi of [5-^{3}H]UTP at 25°C in the presence of 5 mM ATP and ATP regenerating system (see section 2). At the times indicated protein-bound radioactivity was determined in the plasma membrane. Values are means ± SEM, n = 4.

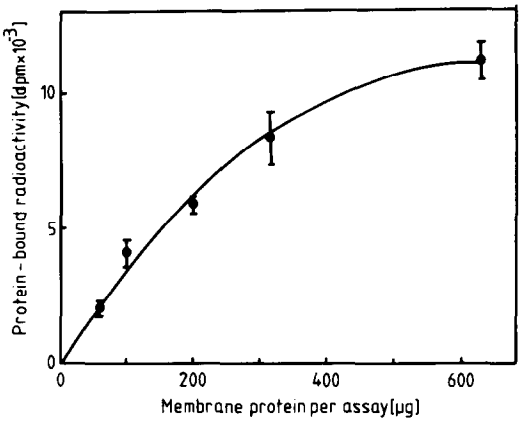

Fig.4. Effect of membrane protein concentration on the binding of [^{3}H]UTP. Increasing amounts of plasma membrane protein were incubated with 50 µCi of [5-^{3}H]UTP in the presence of 5 mM ATP, and ATP regenerating system at 25°C for 30 min. Protein-bound radioactivity was measured in the plasma membrane as described in section 2. Values are means ± SEM, n = 4.

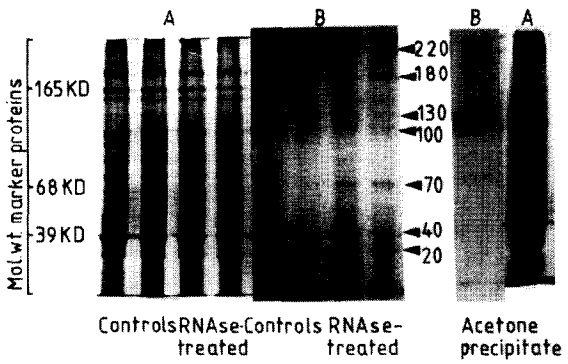

Fig.5. Polyacrylamide gel electrophoresis of plasma membrane polypeptides labelled in vitro by [α-^{32}P]UTP. Plasma membranes were labelled in vitro with 50 µCi [α-^{32}P]UTP (see section 2). RNase treatment, and precipitation by acetone of plasma membranes, which had been extracted with acetic acid according to [8] was performed as in section 2. Controls, RNase-treated plasma membranes, and the acetone-precipitated fraction were separated on 7.5% SDS–polyacrylamide gels as indicated in fig.1. (A), Coomassie staining; (B), autoradiographic pattern.

increase was found, probably as the hydrolysis of UTP exceeds the extent of binding.

Individual proteins with a binding affinity for uridine phosphate were revealed by SDS–polyacrylamide gel electrophoresis of plasma membranes labelled with [α-^{32}P]UTP. The pattern of labelled proteins was completely reproducible in twelve independent experiments, using separate plasma membrane preparations for incubation. Five polypeptide bands were labelled in vitro with apparent M_r-values of 220 000, 180 000, 130 000, 100 000 and 70 000 corresponding to the polypeptide bands with identical M_r labelled in vivo. Two polypeptide bands in the low M_r region with apparent M_r-values of 40 000 and 20 000 were found in vitro, but not in vivo (fig.5). As found in vivo, the radiolabelled components were completely digested by proteinase K, but were resistant to treatment with RNase or DNase (fig.5, table 2). Precipitation with acetone of in vitro labelled plasma membranes after solubilization and removal of RNA according to [8] significantly enriched the 130 000 M_r polypeptide. No label was found in the RNA fraction (fig.5). By contrast, incubation with [α-^{32}P]GTP mainly labelled polypeptide bands with apparent M_r-values of 250 000, 85 000 and 50 000 in accordance with [17]. Moreover, when plasma membranes were phosphorylated using [γ-^{32}P]ATP as precursor, the autoradiographic pattern after gel electrophoresis was completely different. Numerous polypeptide bands corresponding to M_r-values between 20 000 and 250 000 were phosphorylated (Tauber, R. and Reutter, W. unpublished results). From the present findings it is obvious, that specific plasma membrane proteins of rat liver bind uridine phosphate, both in vivo and in vitro. The high resistance of labelled protein bands to SDS treatment suggests covalent binding. Though heating of plasma membranes for 1 h at 70°C prior to incubation with UTP decreased labelling of plasma membranes by 80% (table 1), a non-enzymatic mechanism cannot be excluded. It may be assumed that the binding of uridine phosphate to plasma membrane proteins is involved in the regulation of plasma membrane function. In *Escherichia coli* it could be shown that the activity of glutamine synthetase is regulated by uridylylation [18]. Uridine binding in the plasma membrane may be correlated to the reported high activities of 5′-nucleotidase and nucleotide pyrophosphatase in the plasma membrane [15,19].

Acknowledgements

The work was partly supported by the Sonderforschungsbereich 29 (Differenzierung) of the Deutsche Forschungsgemeinschaft.

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