PROTEIN PHOSPHORYLATION IN RAT LIVER PLASMA MEMBRANES

In vitro and in vivo inhibition by insulin

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1. Introduction

Insulin affect the phosphorylation of specific proteins in the plasma membrane of target cells. Thus, direct addition of the hormone to plasma membranes of rat adipocytes results in a decrease in the labeling of a protein of mol. wt 120 000 by [γ-^{32}P]ATP, [1,2]. A protein of mol. wt 15 000, the phosphorylation of which is stimulated by insulin in vitro, has also been identified in a sarcolemna preparation of rat skeletal muscle [3]. In addition, treatment of intact adipocytes with insulin has been shown to alter the incorporation of [^{32}P]phosphate into several proteins, some of which were associated with particulate structures [4,5]. It has been proposed that these membranes phosphoproteins, as well as other membrane phosphoproteins which were not directly affected by insulin [6], may be functionally related to a primary action of the hormone [1].

Here, insulin has been examined for its ability to alter the phosphorylation of specific proteins in the plasma membrane of liver cells. The phosphorylation of two proteins of molecular weights 120 000 and 60 000, respectively, are inhibited by the hormone in vitro and in vivo.

2. Experimental

Experiments were performed on normal and insulin-pretreated male Sprague Dawley rats (body. wt 180–200 g); the latter received a single injection of porcine insulin (10 nmol) at various times prior to sacrifice. Plasma membranes were prepared according to [7] up to step 11 and stored in liquid nitrogen until used. The phosphorylation reaction was carried out in vitro using [γ-^{32}P]ATP as a phosphate donor. The incubation mixture contained, in 60 μl, 50 mM Tris–HCl (pH 7.0), 0.1 mM ATP (1000–1500 cpm/pmol), 5 mM $MgCl_2$, 0.3 mM EGTA, 10 mM NaF, 2 mM aminophylline and 0.01% bovine serum albumin. Insulin, when present, was preincubated with the plasma membranes at 4°C prior to addition of the ATP. After 3 min at 30°C, 1 ml of 10% (w/v) trichloroacetic acid containing 0.1 M phosphoric acid and 5 mM ATP were added, and the incubation mixtures were filtered on Whatmann glass fiber GF/B filters under vacuum. The filters were washed 3 times with 5% trichloroacetic acid containing 0.1 M phosphoric acid. They were dried and counted using Biofluor (New England Nuclear) as a scintillation medium. In those experiments where membrane proteins were submitted to electrophoresis, the incubation mixtures were diluted in 0.5 ml 50 mM Tris–HCl (pH 7.0) containing 10 mM ATP, and centrifuged at 10 000 × g for 10 min; the pellet was solubilized in sodium dodecylsulfate (SDS) and the proteins resolved by polyacrylamide gel electrophoresis according to [8]. In some experiments, gradient gels from 5–15% (w/v) acrylamide were used. Gels were stained with Coomassie blue, destained, fried and exposed to Kodak X-Ray films (Kodirex). The ^{32}P-labeled components resolved were quantified by densitometric scanning at 660 nm.

Membrane protein was measured according to [9] using bovine serum albumin as a standard.

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3. Results

3.1. *Characteristics of membrane labeling by [γ-^{32}P]ATP*

As observed previously [10,11], the incubation of liver plasma membranes with [γ-^{32}P] ATP results in the incorporation of radioactivity into trichloroacetic acid-precipitable material. Under the standard assay conditions, phosphorylation of the membranes occurs rapidly, reaching a maximal level by 3 min. About 30–40% of the ATP originally present in the incubation medium is hydrolyzed within this time (not shown).

Analysis of the plasma membrane preparation by polyacrylamide gel electrophoresis reveals the presence of many Coomassie blue-stainable bands with mobilities corresponding to mol. wt 19 000–200 000. This pattern is similar to that observed in [7,12]. On autoradiography, multiple phosphorylated components with mobilities corresponding to mol. wt 30 000–190 000 are observed. One prominent component has mol. wt 110 000.

3.2. *Effect of in vitro additions of insulin, cAMP and cGMP on membrane phosphorylation*

Insulin, when preincubated with the plasma membranes at 4°C for 45 min prior to addition of the ATP, causes a 20% decrease in the incorporation of radioactivity into total trichloroacetic acid-precipitable proteins (table 1). A similar decrease, albeit less pronounced, occurs when the preincubation time is shortened to 15 min or extended to 90 min. The effect of insulin on membrane labeling does not result from an increase in the hydrolysis of the substrate (not shown). The effect of insulin is dependent on the concentration of the hormone in the incubation medium; it is maximal at 100 nM, and half-maximal at ~0.5 nM (table 1). This range of concentration is similar to the one in which the binding of insulin to its receptor occurs [13].

Table 1
Effect of insulin, cAMP and cGMP on the phosphorylation of liver plasma membranes

Additions	*n*	[^{32}P]Phosphate incorporated (% of control[a])
Insulin, 0.01 nM	3	105 ± 9
Insulin, 0.1 nM	3	97 ± 18
Insulin, 1 nM	3	85 ± 7
Insulin, 10 nM	3	84 ± 9
Insulin, 100 nM	10	77 ± 9
cAMP, 10 μM	4	69 ± 8
cGMP, 10 μM	3	76 ± 13

[a] 52 + 5 pmol . mg^{-1} . min^{-1}

The phosphorylation reaction was carried out as in section 2. Insulin was preincubated with plasma membranes in 50 μl medium for 45 min at 4°C prior to addition of ATP; cAMP and cGMP were added at zero time of the reaction. The results are expressed as the % (mean ± SD) of the ^{32}P incorporated into control membranes. The changes resulting from the addition of insulin 100 nM are statistically significant when analyzed by Student's *t*-test ($p < 0.005$)

In agreement with [10,11], cAMP decreases the incorporation of ^{32}P into total membrane protein, and so does cGMP (table 1). This decrease slightly exceeds that observed with insulin. The effect of either nucleotide are not additive with that of insulin (not shown).

Electrophoretic analysis of the membranes that have been exposed either to insulin, cAMP or cGMP does not reveal any change in the pattern of Coomassie blue-stainable bands, but show a decrease in the intensity of the radioactive components as resolved by autoradiography. These changes, however, were not quantified.

3.3. *Effects of in vivo administration of insulin on membrane phosphorylation*

Injection of 10 nmol insulin, a dose which ensures nearly full occupancy of the receptors in vivo [14,15], causes a marked decrease in the phosphorylation of total membrane proteins (table 2). These

Table 2
Phosphorylation of liver plasma membranes obtained at various times after in vivo administration of insulin

Time of sacrifice after injection (min)	*n*	[^{32}P]Phosphate incorporated (% of control[a])
1	1	83
4	1	57
10	3	62 ± 9
30	3	60 ± 8
60	3	61 ± 5
120	3	70 ± 8

[a] 50.3 ± 4 pmol . mg^{-1} . min^{-1}

Insulin pretreatment and phosphorylation reaction were performed as in section 2. Each determination was performed on membranes obtained from pooled liver of two rats. Routinely, 1 pool of livers from control rats and 5 pools of livers from insulin-injected rats were processed on the same day. The results are expressed as the % (mean ± SD) of the ^{32}P incorporated in control membranes

changes occur as early as 1 min after injection, are maximal from 10–60 min, and persists up to 2 h. At maximum, the decrease in the incorporation of ^{32}P is ~40%, a value which exceed by 2-fold that observed in the in vitro studies.

As membranes exposed to insulin in vitro, membranes of insulin-pretreated rats display a decrease in the intensity of the labeled components visible on the autoradiogramms (fig.1). On the densitometric scans two phosphoproteins, of mol. wt 110 000 and 60 000, respectively, are predominantly affected by the hormone (fig.2). The former phosphoprotein is more affected than the latter (table 3). Other phosphoproteins are relatively unchanged.

Addition of cAMP to membranes of insulin-pretreated animals does not cause a further decrease in labeling of total membrane proteins with [γ-^{32}P]-ATP (not shown).

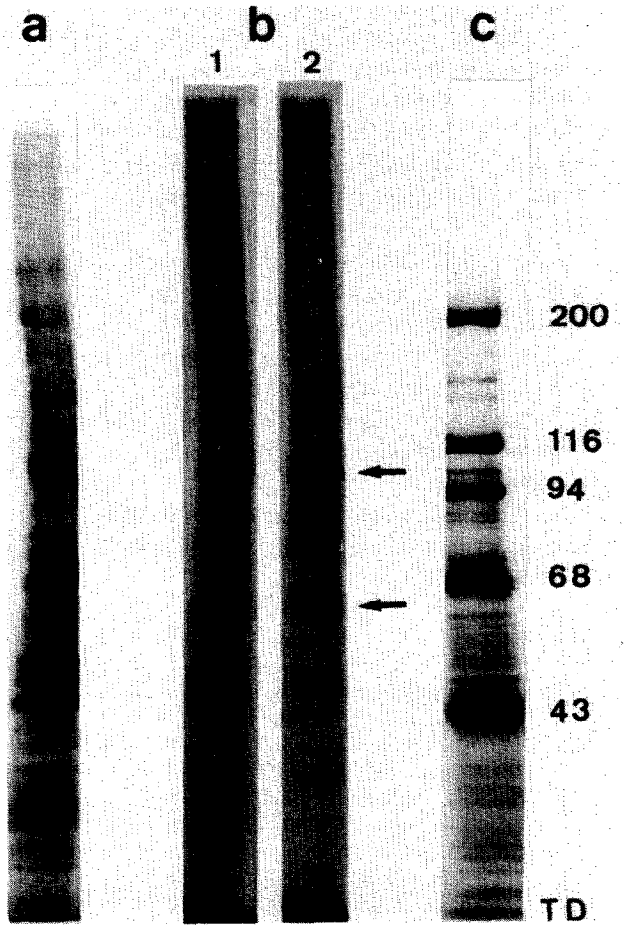

Fig.1. Electrophoretic pattern of the plasma membrane proteins; a, Coomassie blue staining of control membranes; b (1), autoradiographic pattern of control membranes; b (2), autoradiographic pattern of insulin-treated membrane (time of sacrifice of the animal: 10 min after administration of insulin). The arrows indicate the two labelled bands which have mol. wt 110 000 and 60 000, respectively. The final slot contains standard molecular weight markers as: myosin (200 000), β-galactosidase (130 000), phosphorylase *b* (94 000), bovine serum albumin (68 000) and ovalbumin (45 000). The electrophoresis was carried out on a 5–15% gradient slab gel at 70 V over 5 h. TD, tracking dye.

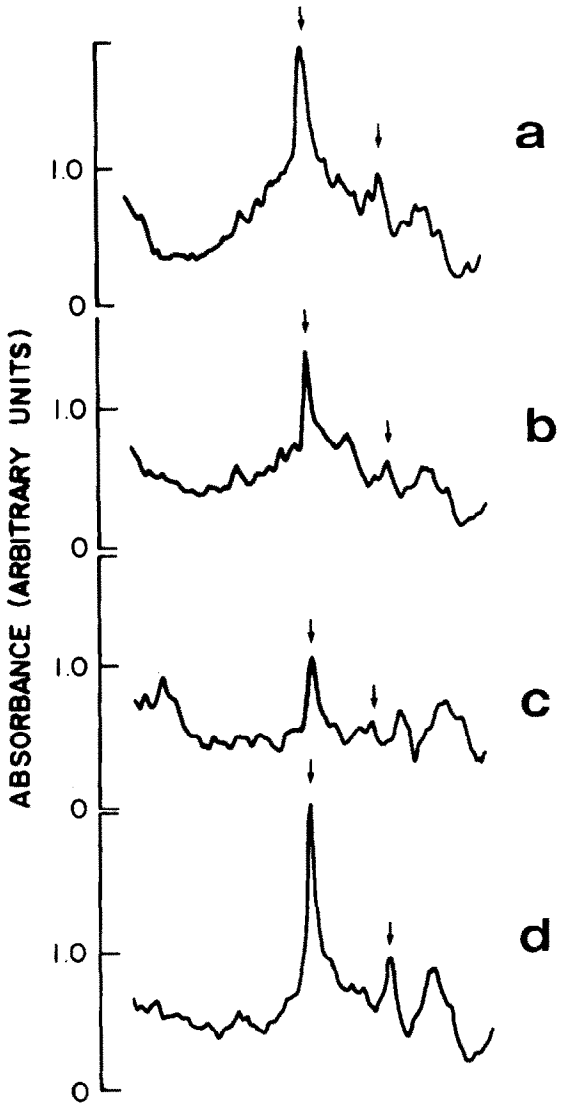

Fig.2. Densitometric traces of autoradiographs locating ^{32}P-labeled proteins separated by SDS–polyacrylamide gel electrophoresis from equivalent amounts of: (a) control; (b) 10 min after insulin injection; (c) 30 min after insulin injection; (d) 60 min after insulin injection. The arrows indicate the two labelled bands which have mol. wt 110 000 and 60 000, respectively.

Table 3
Decrease of two endogenous membrane phosphoproteins after injection of insulin (% of control)

Mol. wt	Time of sacrifice after injection (min)			
	10	30	60	120
110 000	54.0	46.2	46.0	84.6
60 000	76.5	88.2	70.5	82.3

The values were determined by measuring the areas of each band. The results are expressed as the % of the area corresponding to the respective bands of the control experiment

4. Discussion

The results described here indicate that insulin decreases the incorporation of ^{32}P into protein of isolated liver plasma membranes. Such effects occur both when the hormone is incubated with membranes in vitro or when it is injected prior to membrane isolation; however, they are more pronounced in the latter conditions. The insulin-sensitive components appear to consist of phosphoproteins; two such phosphoproteins, of mol. wt 110 000 and 60 000, respectively, are predominantly affected.

Unlike in skeletal muscle [3], but like in adipocytes plasma membranes [1,2], there is an inhibitory effect of insulin on liver membrane autophosphorylation. Furthermore, one of the insulin-sensitive phosphoproteins identified here has a molecular weight similar to the one identified in adipocyte plasma membranes [1]. It has been suggested that the latter phosphoprotein may represent either the insulin receptor itself, the monomer of which is comparable in size [17], or other membrane proteins which might have some role in the insulin action. The same considerations apply to the 110 000 mol. wt phosphoprotein identified in liver membranes.

The mechanism by which insulin affects the phosphorylation of the plasma membrane proteins is at present unknown. The ability of the hormone to exert effects in broken cell preparations suggests that it may act by inhibiting (a) membrane-associated protein kinase(s) and/or stimulating (a) membrane-associated phosphatase(s). On the other hand, the results of the in vivo studies suggest that insulin could act by stimulating membrane phosphorylation, thus decreasing the number of remaining sites available for the ulterior phosphorylation in a broken cell preparation. Studies on the effect of insulin on the incorporation of $^{32}P_i$ into membrane phosphoproteins in vivo should help to clarify this point.

The ability of insulin to dephosphorylate (and activate) the enzymes, pyruvate dehydrogenase and glycogen synthase, in intact cells, results from the generation of a second messenger, the chemical structure of which is unknown [17–19]. Whether the altered phosphorylation of the liver, as well as adipocyte, plasma membrane proteins is also mediated by this messenger will require further investigations.

Studies using subcellular fractionation [14,15] have shown that, following its interaction with plasma membrane in intact liver, insulin is internalized into light density vesicular structures, which have been assigned to the Golgi apparatus [15]. Furthermore, biochemical evidence has been presented that internalization of the receptor accompanies, and presumably mediates that of its ligand [14]. We have found that insulin given in vivo affects the phosphorylation of specific proteins in these light density subfractions, the molecular weight of which appear to differ from that of the plasma membrane proteins.

Studies are in progress to characterize the insulin-sensitive phosphoproteins with respect to pH dependence, ion requirements and nature of the phosphorylated moieties, and to determine whether those in the Golgi apparatus are affected by internalized hormone.

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