Evidence that insulin and guanosine triphosphate regulate dephosphorylation of the β -subunit of the insulin receptor in sarcolemma membranes isolated from skeletal muscle

Robert S. HORN,* Einar LYSTAD,* Arnfrid ADLER* and Otto WALAAS*††

*Institute of Medical Biochemistry, University of Oslo, P.O. 1112, Blindern, Oslo 3, Norway, and †California Metabolic Research Foundation, La Jolla, CA 92038, U.S.A.

When sarcolemma membranes isolated from rat skeletal muscle were incubated with [γ - 32 P]ATP, a membrane protein of apparent M_r 95000 was rapidly phosphorylated, with the 32 P content reaching a maximum within 2 s. On the basis of immunoprecipitation with anti-insulin-receptor antiserum, phosphoamino acid analysis and M_r , this protein probably represents the β -subunit of the insulin receptor. Similarly, on incubation of the membrane with adenosine 5'-[γ - 35 S]thiotriphosphate the 95 kDa protein was thiophosphorylated, indicating thiophosphorylation of the β -subunit of the insulin receptor on the basis of immunoprecipitation studies. The effect of insulin on the phosphorylation of this protein in the membrane was studied. Insulin induced a 20% decrease in the 32 P labelling of the protein when the membranes were phosphorylated for 10 s. This insulin effect was dose-dependent, with half-maximal effect obtained at 2–3 nM-insulin. Addition of GTP, but not GDP or guanosine 5'-[β , γ -imido]triphosphate, enhanced the effect to 35% inhibition, with half-maximal effect of GTP obtained at 0.5 μ M. GTP had no effect on the phosphorylation of the protein in the absence of insulin. Analysis of this insulin effect showed that insulin increased the rate of dephosphorylation of the 95 kDa protein in the membrane. In contrast, insulin had no effect on thiophosphorylation of the 95 kDa membrane protein after incubation with adenosine 5'-[γ - 35 S]thiotriphosphate. Since thiophosphorylated proteins are less sensitive to phosphatase action, these investigations suggest that insulin stimulated a protein phosphatase activity in a GTP-dependent manner. The possibility that GTP-regulatory proteins are involved in the action of insulin on the phosphorylation of the insulin receptor and other membrane proteins is discussed.

INTRODUCTION

It has become evident that rapid effects of insulin on metabolism may be mediated by alterations in the extent of phosphorylation of important intracellular enzymes, in some cases promoting net dephosphorylation of the proteins, whereas other proteins exhibit increased phosphorylation in response to insulin [for reviews see Cohen (1982) and Horn & Walaas (1984)]. Kasuga *et al.* (1982a) demonstrated that insulin stimulated the incorporation of 32 P into the β -subunit of the insulin receptor in cultured human lymphocytes and rat hepatoma cells.

This has subsequently been shown in rat hepatocytes (Van Obberghen & Kowalski, 1982), human placenta (Petruzzelli *et al.*, 1982; Kasuga *et al.*, 1983), 3T3-L1 adipocytes (Petruzzelli *et al.*, 1982), rat adipocytes (Håring *et al.*, 1982) and human erythrocytes (Grigorescu *et al.*, 1983). In cell-free systems, interaction of insulin with detergent-solubilized and partly purified insulin receptors resulted in increased autophosphorylation of the insulin receptor itself (Kasuga *et al.*, 1982b). In intact cells under basal conditions, insulin receptors were mainly phosphorylated on serine residues and to some extent on threonine residues. The increased phosphorylation in insulin-treated cells was predominantly due to the

increase in phosphoserine and to the appearance of phosphotyrosine (Kasuga *et al.*, 1982c; Gazzano *et al.*, 1983). In contrast, the increased phosphorylation caused by addition of insulin to solubilized and purified receptors was primarily due to increase in phosphotyrosine (Kasuga *et al.*, 1982b). Further work has indicated that the tyrosine-phosphorylated insulin receptor itself acts as a tyrosine-specific protein kinase (Petruzzelli *et al.*, 1982; Van Obberghen *et al.*, 1983; Kasuga *et al.*, 1983; Yu & Czech, 1984), which can phosphorylate the receptor by an intramolecular reaction. This receptor protein kinase has also been shown to promote phosphorylation of tyrosine residues of exogenous substrates such as histones and casein (Kasuga *et al.*, 1983; Zick *et al.*, 1983).

The aim of the present work was to examine the influence of insulin on the phosphorylation of the insulin receptor in sarcolemma membranes isolated from skeletal muscle. Previously it has been demonstrated that insulin slightly increased the phosphorylation of the 95 kDa β -subunit of the insulin receptor in human placental membranes (Machicao *et al.*, 1982) and membranes from rat liver (Blackshear *et al.*, 1984). These insulin effects were markedly increased after detergent solubilization of the receptor. We have previously reported that insulin increases the phosphorylation of a 15 kDa proteolipid in

Abbreviations used: ATP[S], adenosine 5'-[γ -thio]triphosphate; p[NH]ppG, guanosine 5'-[β , γ -imido]triphosphate.

† To whom reprint requests should be sent, at the Oslo address.

sarcolemma membranes (Walaas *et al.*, 1977, 1981a) through a mechanism which was enhanced by guanine nucleotides (Walaas *et al.*, 1979). Furthermore, guanine nucleotides also increased insulin stimulation of the phosphorylation of exogenous histones by sarcolemma membranes. This suggested that regulatory GTP-dependent protein(s) could be involved in the insulin effects on protein phosphorylation in these membranes.

In the present work we report extremely rapid phosphorylation of a 95 kDa sarcolemma protein. It is indicated that this protein probably represents the β -subunit of the insulin receptor, since it is phosphorylated on tyrosine as well as serine residues and is precipitated with anti-insulin-receptor antiserum. The phosphorylation of this protein was decreased in the presence of insulin. This insulin effect was enhanced by GTP, and appears to be due to an increased rate of dephosphorylation.

EXPERIMENTAL

Materials

Highly purified pig insulin was given by Nordic Insulin Laboratory, Gentofte, Denmark. [γ - 32 P]ATP (triethylammonium salt) in aqueous solution (3000 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. ATP [35 S] in ethanol/20 mM-Tricine, pH 7.6 (1:1, v/v), containing 20 mM-dithiothreitol (1000 Ci/mmol) was from New England Nuclear, Boston, MA, U.S.A. The ATP [35 S] was dried in a stream of N_2 immediately before use to remove ethanol. Phosphoserine, phosphothreonine, phosphotyrosine, phenylmethanesulphonyl fluoride and soya-bean trypsin inhibitor were from Sigma Chemical Co., St. Louis, MO, U.S.A. GTP, GDP and p[NH]ppG were from Boehringer, Mannheim, Germany. *Staphylococcus aureus* cells (Pansorbin) were from Calbiochem, San Diego, CA, U.S.A. Wheat-germ agglutinin-agarose was from Miles, Rehovot, Israel. Sera from patients with autoantibodies to the insulin receptor were generously given by Dr. Simeon I. Taylor and Dr. Phillip Gorden, National Institutes of Health, Bethesda, MD, U.S.A. The equipment for gel electrophoresis, Protean Slab Cell and Gel Slab Dryer model 224, and reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad Laboratories, Richmond, CA, U.S.A. Pre-coated silica-gel 60 t.l.c. plates were from Merck, Darmstadt, Germany. Autoradiography was done with Dupont Cronex 4 film.

Preparation and characterization of sarcolemma membrane fractions

The procedure used was that of Kidwai (1974), with some modifications. Hind-limb muscles (10 g) from rats were removed and kept on ice. Homogenization was done in 0.25 M-sucrose, for 4×5 s with a high-speed Ultra-Turrax homogenizer (type TP 18-19). In some experiments the proteinase inhibitors phenylmethanesulphonyl fluoride (1 mM) and soya-bean trypsin inhibitor (100 units/ml) were included in the solutions throughout the procedure. This did not change the results obtained. The homogenate was filtered with a device containing stainless-steel wire cloths of six different mesh sizes. The filtrate was centrifuged at 120000 g for 60 min. The pellet obtained was suspended in 0.25 M-sucrose and centrifuged in a linear 20–50% sucrose gradient. Centrifugation was performed at 110000 g for 120 min. Three different protein bands were separated on the gradient and characterized by morphology and enzyme markers. A light fraction (F_1) isolated at density 1.10–1.115 g/ml consisted of membrane vesicles, as demonstrated by electron microscopy. A second fraction (F_2) at density 1.12–1.14 g/ml showed vesicles contaminated with mitochondria. A fraction (F_3) at density 1.16–1.19 g/ml was a mitochondria-enriched fraction. The different fractions were characterized by enzyme markers (Table 1). The light fraction (F_1) showed high adenylate cyclase, 5'-nucleotidase and ATPase activities, consistent with a sarcolemma-enriched membrane fraction. Portions (3 ml) of this fraction were suspended in plastic tubes and kept in liquid N_2 until use.

Assay of protein phosphorylation

Membrane suspensions (3 ml) were diluted to 10 ml with 50 mM-Tris/HCl, pH 7.4, containing 0.1 mM-EDTA, and centrifuged at 60000 g for 45 min. The pellet was resuspended in the same buffer. Samples containing 50–100 μ g of protein were incubated in a standard assay system containing 50 μ M-[γ - 32 P]ATP (5×10^7 c.p.m.), 10 mM-MgCl₂ and 0.1 mM-EDTA in 50 mM-Tris/HCl buffer, pH 7.4. The membranes were preincubated with or without 5 nM-insulin for 3 min at 30 °C, and the reaction was started by addition of [γ - 32 P]ATP. Total volume was 50 μ l. Incubation usually was done for 10 s at 30 °C. The reaction was terminated by addition of 25 μ l of a stop solution containing 9% (v/v) SDS in 30 mM-Tris/HCl (pH 7.8)/15 mM-EDTA/20% (v/v) gly-

Table 1. Marker-enzyme activities of fractions isolated from rat skeletal muscle

The fractions were separated by sucrose-density-gradient centrifugation as described in the text. F_1 , fraction isolated at density 1.10–1.115 g/ml (sarcolemma-enriched fraction); F_2 , fraction isolated at density 1.12–1.14 g/ml (sarcoplasmic-reticulum-enriched fraction); F_3 , fraction isolated at density 1.16–1.19 g/ml (mitochondrial fraction). The data are mean values from three different experiments.

Fraction	Mg ²⁺ -dependent ATPase (μ mol/h per mg)	(Na ⁺ + K ⁺)-dependent ATPase (nmol/h per mg)	Ca ²⁺ -dependent ATPase (μ mol/h per mg)	Adenylate cyclase (pmol/min per mg)	5'-Nucleotidase (μ mol/min per mg)	Cytochrome oxidase (μ mol/min per mg)	NADH oxidase (ΔA_{546} /min per mg)
Homogenate	62	02	16	110	0.18	0.11	0.95
F_1	218	155	13	1380	1.60	0.15	0.90
F_2	222	210	56	0	1.35	0.26	0.95
F_3	169	45	25	0	0.35	1.61	2.04

cerol. After addition of dithiothreitol (100 mM) and pyronine as the tracking dye, the mixture was boiled for 3 min. After centrifugation for 30 min at 10000 *g*, the sample was subjected to gel electrophoresis. In some experiments the membranes were thiophosphorylated by incubation with ATP[³⁵S] under the same conditions.

Short-term incubation experiments (200 ms–2 s) for determination of protein phosphorylation were performed by using a rapid-mixing apparatus similar to that described by Strittmatter (1964). The apparatus was constructed with three syringes and two mixing devices. In a typical experiment syringe I contained 750 μ l of 100 μ M-[γ -³²P]ATP/20 mM-MgCl₂/0.2 mM EDTA in 50 mM-imidazole buffer, pH 7.4. Syringe II contained 750 μ l of the membrane suspension (3 mg of protein) in 50 mM-imidazole buffer, pH 7.4. Syringe III contained the SDS quenching solution. The syringes were filled and fixed in the apparatus by clamps. Mixing was accomplished by driving the syringes at a constant rate with a Harvard infusion pump. The reaction time was a function of the calibrated length of a polyethylene tube connecting the two mixing chambers. The contents of syringes I and II were mixed in the first chamber, and the quenching solution was added in the second chamber. Finally, the quenched reaction mixture was collected in a micro-vessel.

Polyacrylamide-gel electrophoresis

One-dimensional SDS/polyacrylamide-gel electrophoresis was done as described by Laemmli (1970), with 7.5% acrylamide as the resolving gel. Short-time incubation experiments were analysed by SDS/polyacrylamide-gel electrophoresis as described by Fairbanks *et al.* (1971). Samples of the solubilized phosphorylated membranes were electrophoresed for 18 h at 36 V. After electrophoresis the gels were stained, destained, dried and exposed to Dupont Cronex 4 film and enhancing screens. *M_r* values of standards used were: phosphorylase *a*, 93000; bovine serum albumin, 67000; ovalbumin, 45000; cytochrome *c*, 13500. The phosphorylated gel band of *M_r* 95000 was cut out, dissolved and decolorized by adding 0.5 ml of H₂O₂ (30%, v/v)+1 ml of Soluene 350, and kept for 3 h at 50 °C. Scintillation fluid (Insta-Gel II; 10 ml) was added to the vials, which were kept overnight before counting for radioactivity in a Packard liquid-scintillation spectrometer.

Identification of phosphoamino acids

The phosphoamino acids were analysed by a slight modification of the methods of Hunter & Sefton (1980) and of Blackshear *et al.* (1984). After autoradiography, the *M_r*-95000 phosphoprotein was cut from the dried gels. The gel slices were rehydrated in 50 mM-NH₄HCO₃ containing 1% SDS and 250 mM-dithiothreitol, and boiled for 30 min, followed by homogenization in a glass homogenizer. Extraction of the phosphoprotein from the gel was continued by incubation in a shaking bath at 37 °C for 18 h. After centrifugation for 30 min at 10000 *g*, the supernatant was removed and the precipitated gel suspended in 50 mM-NH₄HCO₃ and incubated at 37 °C for 1 h. After re-centrifugation at 10000 *g* for 30 min the supernatants were combined, 500 μ g of bovine serum albumin was added, and proteins were precipitated by adding 5 vol. of acetone at –20 °C overnight. After centrifugation at 10000 *g* for 30 min, the precipitate was washed with acetone at –20 °C, dried and subjected to

acid hydrolysis in 6 M-HCl under vacuum at 110 °C for 90 min. The hydrolysate was dried over NaOH under vacuum and dissolved in 100 μ l of electrophoresis buffer containing phosphoserine, phosphothreonine and phosphotyrosine (1 mg/ml each). The samples were spotted on silica-gel-precoated plastic sheets, and electrophoresis was performed at pH 3.5 (in pyridine/acetic acid/water, 1:10:189, by vol.) at 1000 V for 90 min at 10 °C. The position of phosphoamino acids was located by staining with ninhydrin, and radioactivity was determined by scintillation counting of the excised spots.

Immunoprecipitation of the insulin receptor

Insulin receptors were immunoprecipitated with antisera containing antibodies against the insulin receptor (Kasuga *et al.*, 1982a). Sarcolemma membranes (12 mg of protein) were extracted with 3 ml of 50 mM-Hepes, pH 7.4, containing 1% Triton X-100, 1 mM-phenylmethanesulphonyl fluoride and trypsin inhibitor (1000 units/ml) by shaking for 90 min at 4 °C. After centrifugation for 60 min at 40000 *g*, the supernatant was removed and phosphorylated with 50 μ M-[γ -³²P]ATP, 10 mM-MgCl₂, 5 mM-MnCl₂, 1 μ M-GTP and 5 nM-insulin in 50 mM-Hepes, pH 7.4, for 5 min at 30 °C. The reaction was terminated by adding 600 μ l of ice-cold stop solution, containing 50 mM-Hepes, pH 7.4, 600 mM-NaF, 100 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride and 0.01% trypsin inhibitor. Insoluble material was removed by centrifugation for 45 min at 40000 rev./min (150000 *g*). The supernatant was applied on to a wheat-germ agglutinin-agarose column. After sample application the column was washed with 50 ml of 50 mM-Hepes, pH 7.4, containing 10 mM-Na₂HPO₄, 100 mM-NaF, 10 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride, 0.01% trypsin inhibitor and 0.1% Triton X-100. Finally, the column was eluted with 4 ml of this solution supplemented with 300 mM-N-acetylglucosamine. Antiserum containing antibodies against the insulin receptor was added to samples of the eluate from the wheat-germ agglutinin column. The control experiment was performed by addition of normal human serum. Incubation with antiserum at 1:100 dilution was done for 18 h at 4 °C. Immunoprecipitation with protein A was performed by addition of 400 μ l of *Staph. aureus* cells (Pansorbin). After 4 h at 4 °C the immunoprecipitates were collected by centrifugation for 10 min at 10000 *g*, and the pellet was washed three times with 50 mM-Hepes buffer containing 0.1% Triton X-100. The precipitate was dissolved in 200 μ l of 30 mM-Tris/HCl buffer, pH 7.8, containing 10% glycerol and 3% SDS. After centrifugation for 10 min at 10000 *g*, the supernatant was subjected to SDS/polyacrylamide-gel electrophoresis by the standard procedure under reducing conditions.

Immunoprecipitation experiments were done with B-10 antiserum provided by Dr. S. I. Taylor. This antiserum has been shown to inhibit insulin binding, but does not stimulate tyrosine phosphorylation of the insulin receptor (Zick *et al.*, 1984).

Other methods

The following analytical methods were used: protein (Cleland & Slater, 1953); Mg²⁺-dependent ATPase and (Na⁺ + K⁺)-dependent ATPase (Kidwai *et al.*, 1971); Ca²⁺-dependent ATPase (Katz *et al.*, 1970); adenylate cyclase (Steiner *et al.*, 1972); 5'-nucleotidase (Avruch & Wallach, 1971); cytochrome *c* oxidase (Wharton &

Tzagaloff, 1967); NADH oxidase (Avruch & Wallach, 1971). [γ - ^{32}P]ATP hydrolysis was determined as described by Walaas *et al.* (1979) by separation of [γ - ^{32}P]ATP and [^{32}P]P_i by chromatography on Whatman no. 3 paper. The solvent was butan-1-ol/acetic acid/conc. NH₃ (sp.gr. 0.880)/water (14:6:1:9, by vol.).

RESULTS

Characteristics of phosphorylation of the 95 kDa membrane protein

Incubation of sarcolemma preparations in the presence of [γ - ^{32}P]ATP or ATP[^{35}S] under standard conditions for short incubation times led to phosphorylation of a

Fig. 1. Effect of insulin on phosphorylation of 95 kDa sarcolemma membrane protein

Sarcolemma membranes were preincubated with or without 7 nM-insulin for 3 min at 30 °C. They were then phosphorylated in a system (50 μl) containing 50 μM -[γ - ^{32}P]ATP or -ATP[^{35}S], 0.1 mM-EDTA, 10 mM-MgCl₂, 1 μM -GTP in 50 mM-Tris/HCl buffer, pH 7.4, at 30 °C for 10 s in the continued presence or absence of insulin. The reaction was terminated by addition of 25 μl of a stop solution containing SDS; dithiothreitol was added, and the sample was boiled for 3 min. The phosphoproteins were separated by SDS/polyacrylamide-gel electrophoresis on a 7.5%-acrylamide slab gel. After fixing, staining and destaining, the gel was dried and exposed to Dupont Cronex film for 2 days. Details are given in the Experimental section. Key: ^{32}P , incubation of the membrane with [γ - ^{32}P]ATP; ^{35}S , incubation of the membrane with ATP[^{35}S].

95 kDa protein. This became apparent when the reaction mixture was subjected to gel electrophoresis in the presence of SDS, followed by autoradiography (Fig. 1). The phosphorylation of this protein was extremely rapid, being much faster than that of other membrane proteins (Fig. 2), and it was totally dependent on the presence of a bivalent cation such as Mg²⁺ (Fig. 2). Phosphorylation of the protein was also stimulated by Mn²⁺, but the effect of Mg²⁺ was usually slightly greater than that of Mn²⁺ (Table 3). There was no additive effect of Mg²⁺ and Mn²⁺ on the phosphorylation of the 95 kDa protein (results not shown). With [γ - ^{32}P]ATP as phosphate donor and using a rapid-mixing procedure, we found that phosphorylation of the protein reached a maximum within 2 s. Continued incubation of the membrane slightly decreased the phosphate content of the protein, which reached a steady

Fig. 2. Time course of phosphorylation of sarcolemma membrane proteins

(a) The experiments were done by incubation of sarcolemma membranes with 50 μM -[γ - ^{32}P]ATP, 0.1 mM-EDTA and 10 mM-MgCl₂ in 50 mM-Tris/HCl buffer, pH 7.4, at 30 °C as described in the legend to Fig. 1 and in the Experimental section. The radioactivity was determined on the bands excised from the gel and processed as described in the Experimental section. [^{32}P]phosphorylation of: M_r -95000 protein (●); M_r -15000 protein (○); M_r -50000 protein (△). (b) Phosphorylation of the M_r -95000 membrane protein during short incubation times. The experiments were done by the rapid mixing technique described in the Experimental section. Incubation at 30 °C (○, $n = 3$); incubation at 4 °C (●, $n = 6$); incubation at 4 °C in the absence of Mg²⁺ (■). ATP hydrolysis was determined at 30 °C (▼) and at 4 °C (△).

state at 10 s (Fig. 2). The extent of phosphorylation under these conditions was slightly higher at 30 °C compared with incubation at 4 °C (Fig. 2). The rapid time course of phosphorylation could not be attributed to decreasing ATP concentrations owing to hydrolysis, since other membrane proteins were phosphorylated on a much slower time scale (Fig. 2). In addition, the concentration of ATP in the reaction mixture was only slightly decreased during the incubation, both at 4 °C and at 30 °C (Fig. 2).

Identification of the 95 kDa membrane protein as the β -subunit of the insulin receptor

The β -subunit of the insulin receptor is known to be a phosphoprotein of M_r 95000 (Kasuga *et al.*, 1982a). We therefore studied whether the 95 kDa phosphoprotein in skeletal-muscle sarcolemma might be related to this subunit of the insulin receptor. To investigate this question, we analysed the immunological properties and the phosphoamino acids in the sarcolemma protein. Immunoprecipitation with antisera directed against the insulin receptor was done after detergent solubilization of the phosphorylated membrane, followed by chromatography on wheat-germ agglutinin-agarose. As shown in Fig. 3, the 95 kDa phosphorylated protein was specifically precipitated by antisera used in this study. This antiserum also precipitated two membrane proteins, of 50 kDa and 15 kDa. In contrast, normal control serum did not precipitate the 95 kDa protein or any other membrane proteins (results not shown). The results are compatible with previous work which has shown that sera from patients containing antibodies against the insulin receptor also precipitate other membrane proteins (Kasuga *et al.*, 1982c). Furthermore, analysis of [32 P]phosphoamino acids in the 95 kDa protein showed that the protein was mainly phosphorylated on tyrosine residues, with threonine and serine residues being phosphorylated to a lesser extent. It is known that tyrosine phosphorylation is a specific characteristic of phosphorylated insulin receptors in other types of membranes (Häring *et al.*, 1984). The results therefore indicate that the 95 kDa sarcolemma phosphoprotein represents the β -subunit of the insulin receptor.

Further support for this has been obtained by isolation of the phosphorylated membrane proteins by a two-dimensional electrophoretic technique (S. I. Walaas, R. S. Horn & O. Walaas, unpublished work). The 95 kDa protein band appeared as a single component, whereas the 50 kDa band was resolved into four different species. The latter may include proteolytic degradation products of the 95 kDa protein. The 15 kDa protein has previously been identified as a proteolipid (Walaas *et al.*, 1981a). This insulin-dependent phosphorylated compound has now been subjected to extensive purification, and it has been shown to be firmly associated with the insulin receptor (R. S. Horn & O. Walaas, unpublished work).

Effect of insulin and of guanine nucleotides on phosphorylation of the 95 kDa membrane protein

After preincubation of the membrane suspension with insulin, phosphorylation of the 95 kDa protein band was slightly decreased (Fig. 1). The effect of insulin on protein phosphorylation was quantified by excising the 95 kDa protein band from the gel and measuring the 32 P radioactivity (Table 2). At an incubation time of 10 s, insulin decreased 32 P incorporation from [γ - 32 P]ATP into

Fig. 3. Immunoprecipitation of the 95 kDa membrane protein

Triton X-100 extracts of sarcolemma membranes were phosphorylated with [γ - 32 P]ATP for 5 min at 30 °C. The reaction was stopped by dilution in the presence of NaF and EDTA, and the mixture was partially purified on a wheat-germ-agglutinin-agarose column. After elution with *N*-acetylglucosamine, antiserum B-10 against insulin receptor was added, followed by precipitation with protein A. The precipitate was subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions, followed by autoradiography. Details of the procedure are given in the Experimental section. Key: P, immunoprecipitate; S, supernatant analysed after precipitation with antiserum.

the protein to an extent of 19% (mean value, $n = 10$). In the presence of 0.5 μ M-GTP and insulin, [32 P]phosphorylation of the 95 kDa protein was decreased by 35% (mean value, $n = 6$). In the presence of insulin and GTP all three phosphoamino acids in the protein were decreased. The data in the absence and the presence of insulin respectively were: phosphotyrosine 182 and 104 c.p.m.; phosphoserine 164 and 98 c.p.m.; phosphothreonine 102 and 56 c.p.m. (mean values from three different experiments).

The effect of insulin was dose-dependent, with half-maximal effect at 2–3 nM-insulin (Table 3), and with a similar response at various concentrations of both Mg^{2+} and Mn^{2+} (Table 3). The enhancement by GTP of the

Table 2. Effect of insulin on phosphorylation of M_r -95000 membrane protein in the absence and in the presence of GTP

Sarcolemma membranes were preincubated without or with 5 nM-insulin for 3 min at 30 °C, followed by phosphorylation with 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 10 mM- MgCl_2 in 50 mM-Tris/HCl, pH 7.4, for 10 s at 30 °C. The reaction was stopped and the mixture subjected to SDS/polyacrylamide-gel electrophoresis under reducing conditions. The 95 kDa phosphorylated protein band was excised from the gel and processed for determination of ^{32}P radioactivity as described in the legend to Fig. 1 and in the Experimental section. Results are expressed as means $\pm$ S.E.M. for n experiments.

Addition	n	^{32}P Phosphorylation (c.p.m.)		P (paired data)
		Control	+ Insulin	
None	10	401 $\pm$ 26	336 $\pm$ 22	< 0.01
1 μ M-GTP	6	409 $\pm$ 30	265 $\pm$ 40	< 0.01

Table 3. Effect of insulin in the presence of Mg^{2+} and Mn^{2+} on phosphorylation of M_r -95000 membrane protein

(a) Sarcolemma membranes were incubated with 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and 10 mM- MgCl_2 in 50 mM-Tris/HCl, pH 7.4, at various concentrations of insulin for 10 s at 30 °C. (b) The membranes were incubated with 50 μ M- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and MgCl_2 or MnCl_2 , without or with 8 nM-insulin, for 10 s at 30 °C. Details of the procedure are given in the legends to Fig. 1 and Table 2 and in the Experimental section. Results represent means of three experiments.

(a)

Insulin concn. (nM)	^{32}P Phosphorylation	
	(c.p.m.)	(insulin effect)
0	471	
2	435	-8%
4	388	-18%
8	368	-22%
12	365	-22%

(b)

Cation concn.	^{32}P Phosphorylation (c.p.m.)	
	Control	+ Insulin
2 mM- Mg^{2+}	152	95
5 mM- Mg^{2+}	398	280
10 mM- Mg^{2+}	490	405
5 mM- Mn^{2+}	334	224
10 mM- Mn^{2+}	415	339

insulin effect on the phosphorylation of the 95 kDa protein was dose-dependent, and with half-maximal effect at 0.5 μ M-GTP (Fig. 4). In contrast, GDP or p[NH]ppG did not significantly modify the insulin effect, and GTP had no effect on phosphorylation of the protein in the absence of insulin (Fig. 4).

Fig. 4. Effect of insulin on phosphorylation of the 95 kDa membrane protein in the presence of GTP

Sarcolemma membranes were incubated for 10 s with $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ in the presence and the absence of insulin as described in the legend to Fig. 1 and in the Experimental section. ●, GTP in the absence of insulin ($n = 4$); ▲, insulin in the presence of GTP ($n = 6$); ■, insulin in the presence of p[NH]ppG ($n = 4$); △, insulin in the presence of GDP ($n = 4$); □, insulin in the absence of nucleotide.

The decreased phosphorylation of the 95 kDa protein promoted by insulin could be due to inhibition of a protein kinase and/or to activation of protein phosphatase activity. The latter possibility was investigated in the experiments reported in Fig. 5. The membranes were ^{32}P phosphorylated by $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ for 10 s in the absence and in the presence of insulin plus GTP. Thereupon the rate of dephosphorylation was investigated by injection of 2 mM-ATP into the incubation mixture. The results show that the rate of dephosphorylation was increased in the presence of insulin, since $t_{1/2}$ for dephosphorylation under these conditions was 28 s in the absence of insulin and 17 s in its presence (Fig. 5). Indirect evidence for an insulin effect on protein phosphatase activity was also obtained by analysis of the membrane protein after incubation with $\text{ATP}^{[35}\text{S}]$ in the absence or presence of insulin, since thiophosphorylated proteins usually are resistant to protein phosphatase. Under these conditions, insulin had no apparent effect on the thiophosphorylation of the 95 kDa protein. These results suggest that insulin decreases phosphorylation of the 95 kDa protein by activating a protein phosphatase.

DISCUSSION

The present work has shown a rapid and guanine-nucleotide-potentiated inhibitory effect of insulin on the state of phosphorylation of a 95 kDa protein in skeletal-muscle sarcolemma preparations. This protein has the same M_r as the β -subunit of the insulin receptor, it is specifically precipitated by antiserum directed against the insulin receptor, and it is phosphorylated on tyrosine, serine and threonine residues. These properties strongly suggest that the protein represents the β -subunit of the insulin receptor (Kasuga *et al.*, 1982a; Van Obberghen & Kowalski, 1982; Petruzzelli *et al.*, 1982).

Fig. 5. Dephosphorylation of the 95 kDa membrane protein in the presence of insulin

(a) Sarcolemma membranes were incubated with $50 \mu\text{M}$ - $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ and $1 \mu\text{M}$ -GTP in the presence (○) and the absence (●) of insulin for 10 s at 30°C as described in the legend to Fig. 1 and in the Experimental section. Dephosphorylation was initiated by injection of 2 mM-ATP as indicated by the arrow. (b) Semilogarithmic plots of the data on dephosphorylation presented in (a), used to determine the $t_{1/2}$ for dephosphorylation of the M_r -95000 protein.

When these studies were initiated, it was considered important to analyse a membrane preparation which retained a number of morphological and physiological properties. Sarcolemma prepared by the present mild method (Kidwai, 1974) appears to give such a preparation.

On addition of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ to these membranes, the 95 kDa receptor subunit is endogenously phosphorylated within milliseconds on tyrosine, serine and threonine residues, without the addition of any exogenous stimulatory factor. The membranes must therefore contain insulin-receptor-directed protein kinases. The most obvious candidates for these protein kinases include the receptor-associated tyrosine and serine protein kinases, which, in the presence of insulin, are known to stimulate the phosphorylation of the insulin receptor (Petrucelli *et al.*, 1982; Van Obberghen *et al.*, 1983; Kasuga *et al.*, 1983; Gazzano *et al.*, 1983; Zick *et al.*, 1983; Yu & Czech, 1984). Unexpectedly, addition of insulin to our preparation did not further stimulate the incorporation of phosphate into the 95 kDa receptor subunit. This suggests that the insulin-regulated protein kinases may exist in an unresponsive form in the membranes. The reasons for this are not clear. They may, however, relate to the state of phosphorylation of specific sites on the insulin receptor in the membranes, and the short incubation time that we have used. This is commensurate with the rapid autophosphorylation of specific sites on the insulin receptor (sites 4 and 5), which reached a steady state after only 20 s (White *et al.*, 1984), and is comparable with the 15–30 s 'lag period' of insulin-induced cellular effects (Häring *et al.*, 1978). It is known that pre-phosphorylation of the insulin-receptor protein kinase makes it insensitive to insulin (Rosen *et al.*, 1983). Furthermore, the extent of phosphate incorporation into the insulin receptor greatly increases after solubilization of receptor-containing membranes (Machicao *et al.*, 1982), suggesting that several phosphorylation sites,

which are phosphorylated at widely different rates in purified receptor preparations (White *et al.*, 1984), may be partly excluded in intact membranes.

The main result of the present study is that insulin decreases the ^{32}P incorporation into the 95 kDa subunit of the insulin receptor during endogenous phosphorylation of the sarcolemma preparation. This effect occurs in a time-dependent and dose-dependent manner, is seen at physiological concentrations of insulin, and is caused by an accelerated dephosphorylation of the 95 kDa protein. In contrast, insulin has no effect during thiophosphorylation of the 95 kDa receptor subunit with $\text{ATP}^{[35}\text{S}]$. Since thiophosphorylated proteins are relatively resistant to protein phosphatase activity, these results indicate that the insulin effect is caused by an activation of an insulin-receptor-directed protein phosphatase. Previous work has indicated that membrane-bound phosphatases directed towards the insulin receptor exist, but has not shown any effect of insulin on the rate of dephosphorylation (Machicao *et al.*, 1982; Kowalski *et al.*, 1983; Häring *et al.*, 1984). Our results indicate that such an effect exists, and suggest that insulin may regulate both the phosphorylation and the dephosphorylation of its own receptor in a dynamic fashion. Indeed, insulin has been found to decrease the phosphorylation of other membrane proteins (Belsham *et al.*, 1980; Marchmont & Houslay, 1980), and it has also been shown to decrease the phosphorylation of insulin receptors purified from placental membranes when the receptors have been preincubated and phosphorylated with ATP (Rosen *et al.*, 1983). Our results are similar to these, in that we find that insulin decreases the phosphate content of the receptor when it is phosphorylated. The observation that this decrease occurs within seconds suggests that this could represent a 'feed-back' control in the very early events of insulin action.

The present study also demonstrates that the effect of insulin on the dephosphorylation of the 95 kDa receptor

subunit is specifically enhanced by GTP at nanomolar concentrations. This suggests that a GTP-binding protein may be involved in this insulin effect. We have previously reported that GTP-binding regulatory proteins may be involved in the stimulatory action of insulin on a sarcolemma protein kinase, since GTP enhanced the stimulatory effect of insulin on the phosphorylation of a membrane protein of 15 kDa as well as on the insulin-stimulated phosphorylation of exogenous histones by these membranes (Walaas *et al.*, 1977, 1979). This effect was probably mediated by a guanine-nucleotide-binding regulatory protein, since treatment of the membranes with cholera toxin, which is known to cause ADP-ribosylation of guanine-nucleotide-binding regulatory proteins (Cassel & Pfeuffer, 1978), abolished the stimulatory effect of insulin on this protein kinase activity (Walaas *et al.*, 1981b). More recently, other studies have also suggested that GTP may be of physiological importance in insulin action. Both insulin stimulation of cyclic AMP phosphodiesterase in hepatocyte membranes, which probably involves a protein kinase, and insulin inhibition of adenylate cyclase in these membranes were found to be dependent on GTP (Heyworth & Houslay, 1983; Heyworth *et al.*, 1983), and to be absent after treatment of the liver cells with cholera toxin. Furthermore, pertussis toxin, which is known to ADP-ribosylate another GTP-binding regulatory protein (Murayama & Ui, 1982), inhibited insulin stimulation of cyclic AMP phosphodiesterase in adipocytes (Elks *et al.*, 1983).

In the present work, GTP enhanced the effect of insulin on the phosphorylation of the 95 kDa protein, whereas p[NH]ppG had no influence. Similarly, p[NH]ppG only slightly enhanced insulin stimulation of phosphorylation of 15 kDa membrane protein (Walaas *et al.*, 1979). Furthermore, it has been shown that inhibition of adenylate cyclase by insulin is dependent on GTP, whereas p[NH]ppG had no effect (Heyworth & Houslay, 1983). This unusual type of activation by guanine nucleotides may be due to low affinity of a specific guanine-nucleotide regulatory protein for p[NH]ppG. Further work indicates that a distinct 25 kDa species may be a component of the guanine-nucleotide regulatory system for insulin action (Heyworth *et al.*, 1985).

Lastly, studies on the striated muscle from barnacle have shown that stimulation of sugar transport by insulin in these cells also was potentiated by GTP (Baker & Carruthers, 1983). These data indicate that guanine-nucleotide-binding regulatory proteins may be importantly involved in the mechanism of insulin action. Our present results indicate that this influence of guanine nucleotides may be, at least partly, mediated through the regulation of the state of phosphorylation of the insulin receptor itself.

The generous gift of antisera against insulin receptor from Dr. S. I. Taylor and Dr. P. Gorden is greatly appreciated. We also thank Nordic Insulin Laboratory for a gift of pig insulin. We are indebted to Mr. Jon Holten for valuable technical assistance. This work was supported by a Grant from Nordic Insulin Foundation and The Norwegian Diabetes Association.

REFERENCES

- Avruch, J. & Wallach, D. F. H. (1971) *Biochim. Biophys. Acta* **233**, 334–347.
- Baker, P. F. & Carruthers, A. (1983) *J. Physiol. (London)* **336**, 397–431.
- Belsham, G. J., Denton, R. M. & Tanner, M. J. A. (1980) *Biochem. J.* **192**, 457–467.
- Blackshear, P. J., Nemenoff, R. A. & Avruch, J. (1984) *Endocrinology (Baltimore)* **114**, 141–152.
- Cassel, D. & Pfeuffer, T. (1978) *Proc. Natl. Acad. Sci. U.S.A.* **75**, 2669–2673.
- Cleland, K. W. & Slater, E. C. (1953) *Biochem. J.* **53**, 547–556.
- Cohen, P. (1982) *Nature (London)* **296**, 613–620.
- Elks, M. L., Watkins, P. A., Manganiello, V. C., Moss, J., Hewlett, E. & Vaughan, M. (1983) *Biochem. Biophys. Res. Commun.* **116**, 593–598.
- Fairbanks, G., Steck, T. L. & Wallach, D. F. H. (1971) *Biochemistry* **10**, 2602–2617.
- Gazzano, H., Kowalski, A., Fehlmann, M. & Van Obberghen, E. (1983) *Biochem. J.* **216**, 575–583.
- Grigorescu, F., White, M. F. & Kahn, C. R. (1983) *J. Biol. Chem.* **258**, 13708–13716.
- Häring, H.-U., Kemmler, W., Renner, R. & Hepp, K. D. (1978) *FEBS Lett.* **132**, 235–238.
- Häring, H.-U., Kasuga, M. & Kahn, C. R. (1982) *Biochem. Biophys. Res. Commun.* **108**, 1538–1545.
- Häring, H.-U., Kasuga, M., White, M. F., Crettaz, M. & Kahn, C. R. (1984) *Biochemistry* **23**, 3298–3306.
- Heyworth, C. M. & Houslay, M. D. (1983) *Biochem. J.* **214**, 547–552.
- Heyworth, C. M., Rawal, S. & Houslay, M. D. (1983) *FEBS Lett.* **154**, 87–91.
- Heyworth, C. M., Whetton, A. D., Wong, S., Martin, B. R. & Houslay, M. D. (1985) *Biochem. J.* **228**, 593–603.
- Horn, R. S. & Walaas, O. (1984) in *Regulation of Target Cell Responsiveness* (McKerns, K. W., Aakvaag, A. & Hansson, V., eds.), vol. 1, pp. 161–182, Plenum, New York.
- Hunter, T. & Sefton, B. M. (1980) *Proc. Natl. Acad. Sci. U.S.A.* **77**, 1311–1315.
- Kasuga, M., Karlsson, F. A. & Kahn, C. R. (1982a) *Science* **215**, 185–187.
- Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M. & Kahn, C. R. (1982b) *Nature (London)* **298**, 667–669.
- Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Häring, H. U. & Kahn, C. R. (1982c) *J. Biol. Chem.* **257**, 9891–9894.
- Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M. F. & Kahn, C. R. (1983) *J. Biol. Chem.* **258**, 10973–10980.
- Katz, A. M., Repke, D. I., Upshan, J. E. & Polascik, M. A. (1970) *Biochim. Biophys. Acta* **205**, 473–490.
- Kidwai, A. M. (1974) *Methods Enzymol.* **31**, 134–144.
- Kidwai, A. M., Radcliffe, M. A., Duchou, G. & Daniel, E. E. (1971) *Biochem. Biophys. Res. Commun.* **45**, 901–910.
- Kowalski, A., Gazzano, H., Fehlmann, M. & Van Obberghen, E. (1983) *Biochem. Biophys. Res. Commun.* **117**, 885–893.
- Laemmli, U. K. (1970) *Nature (London)* **227**, 680–685.
- Machicao, F., Uromow, T. & Wieland, O. H. (1982) *FEBS Lett.* **149**, 96–100.
- Marchmont, R. J. & Houslay, M. D. (1980) *FEBS Lett.* **118**, 18–24.
- Murayama, T. & Ui, M. (1982) *J. Biol. Chem.* **258**, 3319–3326.
- Petrucelli, L. M., Ganguly, S., Smith, C. J., Cobb, M. H., Rubin, C. R. & Rosen, O. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6792–6796.
- Rosen, O. M., Herrera, R., Olowe, Y., Petrucci, L. M. & Cobb, M. H. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 3237–3240.
- Steiner, A. L., Pagliara, A. S., Chase, L. R. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1114–1120.
- Strittmatter, P. (1964) in *Rapid Mixing and Sampling Techniques in Biochemistry* (Chance, B., Eisenhardt, R. H., Gibson, Q. H. & Longbergholm, K. K., eds.) pp. 71–84, Academic Press, New York.
- Van Obberghen, E. & Kowalski, A. (1982) *FEBS Lett.* **143**, 179–182.
- Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A.* **80**, 945–949.
- Walaas, O., Walaas, E., Lystad, E., Rye-Alertsen, A., Horn, R. S. & Fossum, S. (1977) *FEBS Lett.* **80**, 417–422.

- Walaas, O., Walaas, E., Rye-Alertsen, Aa. & Horn, R. S. (1979) *Mol. Cell. Endocrinol.* **16**, 45–55
- Walaas, O., Sletten, K., Horn, R. S., Lystad, E., Adler, A. & Rye-Alertsen, Aa. (1981*a*) *FEBS Lett.* **128**, 137–141
- Walaas, O., Horn, R. S., Lystad, E. & Adler, A. (1981*b*) *FEBS Lett.* **128**, 133–136
- Wharton, D. C. & Tzagoloff, A. (1967) *Methods Enzymol.* **10**, 245–250
- White, M. F., Häring, H. U., Kasuga, M. & Kahn, C. R. (1984) *J. Biol. Chem.* **259**, 255–264
- Yu, K.-T. & Czech, M. (1984) *J. Biol. Chem.* **259**, 5277–5286
- Zick, Y., Grunberger, G., Podskalny, J. M., Moncada, V., Taylor, S. I., Gorden, P. & Roth, J. (1983) *Biochem. Biophys. Res. Commun.* **116**, 1129–1135
- Zick, Y., Rees-Jones, R. W., Taylor, S. I., Gorden, P. & Roth, J. (1984) *J. Biol. Chem.* **259**, 4396–4400

Received 15 July 1985/28 October 1985; accepted 7 November 1985