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Effect of cyclic AMP-dependent protein kinase on insulin receptor tyrosine kinase activity

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To explain the insulin resistance induced by catecholamines, we studied the tyrosine kinase activity of insulin receptors in a state characterized by elevated noradrenaline concentrations *in vivo*, i.e. cold-acclimation. Insulin receptors were partially purified from brown adipose tissue of 3-week- or 48 h-cold-acclimated mice. Insulin-stimulated receptor autophosphorylation and tyrosine kinase activity of insulin receptors prepared from cold-acclimated mice were decreased. Since the effect of noradrenaline is mediated by cyclic AMP and cyclic AMP-dependent protein kinase, we tested the effect of the purified catalytic subunit of this enzyme on insulin receptors purified by wheat-germ agglutinin chromatography. The catalytic subunit had no effect on basal phosphorylation, but completely inhibited the insulin-stimulated receptor phosphorylation. Similarly, receptor kinase activity towards exogenous substrates such as histone or a tyrosine-containing copolymer was abolished. This inhibitory effect was observed with receptors prepared from brown adipose tissue, isolated hepatocytes and skeletal muscle. The same results were obtained on epidermal-growth-factor receptors. Further, the catalytic subunit exerted a comparable effect on the phosphorylation of highly purified insulin receptors. To explain this inhibition, we were able to rule out the following phenomena: a change in insulin binding, a change in the K_m of the enzyme for ATP, activation of a phosphatase activity present in the insulin-receptor preparation, depletion of ATP, and phosphorylation of a serine residue of the receptor. These results suggest that the alteration in the insulin-receptor tyrosine kinase activity induced by cyclic AMP-dependent protein kinase could contribute to the insulin resistance produced by catecholamines.

INTRODUCTION

It has been known for a long time that catecholamines induce insulin resistance *in vivo* (Rizza *et al.*, 1980; Deibert & DeFronzo, 1980). It is possible to reproduce this state of insulin resistance *in vitro* by incubation of isolated adipocytes (Kirsch *et al.*, 1983*a*,*b*) or skeletal muscle (Chiasson *et al.*, 1981) with catecholamines. The insulin-antagonistic effect of catecholamines has been attributed to different mechanisms: insulin binding is depressed in catecholamine-treated fat-cells by cyclic AMP-dependent mechanisms (Pessin *et al.*, 1983; Häring *et al.*, 1986) and tyrosine kinase activity of insulin receptor prepared from isoprenaline-treated adipocytes is altered (Häring *et al.*, 1986). Along the same line, agents which increase the cyclic AMP content of IM-9 lymphocytes alter the phosphorylation state and protein kinase activity of their insulin receptors (Stadtmauer & Rosen, 1986). All the previous results have been obtained in isolated cells or tissues incubated *in vitro* with catecholamines. In the present study, we have looked for the tyrosine kinase activity of insulin receptors prepared from brown adipose tissue of cold-acclimated animals. The main function of brown adipose tissue is heat production (Himms-Hagen, 1985; Ricquier & Mory, 1984). In this tissue, acute or chronic cold-exposure induces a large release of noradrenaline with subsequent activation of lipolysis and heat production (Himms-Hagen, 1985; Ricquier & Mory, 1984). This approach allowed us to study the tyrosine kinase activity of insulin receptors prepared from a tissue rich in functional insulin receptors (Tanti *et al.*, 1986) and which had been exposed *in vivo* to high concentrations of catecholamines. In the present paper we report a decrease in tyrosine kinase activity of receptors isolated from cold-acclimated animals. Thus we have searched for a direct effect of the cyclic AMP-dependent protein kinase on insulin-receptor tyrosine kinase activity in a cell-free system.

EXPERIMENTAL

Materials

The catalytic subunit of cyclic AMP-dependent protein kinase, purified to homogeneity as described by Demaille *et al.* (1977), and the specific protein kinase inhibitor were gifts from Dr. C. Le Peuch (Montpellier, France). Commercial preparation of catalytic subunit was obtained from Sigma. Anti-peptide serum against the EGF receptor was generously given by Dr. J. Schlessinger (Rehovot, Israel). Monocomponent insulin was a gift from Novo (Copenhagen, Denmark). Wheat-germ-agglutinin–agarose was from Miles or Sigma; [γ-^{32}P]ATP (triethylammonium salt; 3000 Ci/mmol) was from The Radiochemical Centre (Amersham, Bucks., U.K.). All reagents for SDS/polyacrylamide-gel electrophoresis were from Bio-Rad or Serva.

Abbreviation used: EGF, epidermal growth factor.

Preparation of partially purified insulin receptors from brown adipose tissue

Male Swiss Albino mice (7–8 weeks old) were given laboratory chow *ad libitum* and maintained at 23 °C on a 12 h-light/12 h-dark cycle. When indicated, mice were acclimated for a period of 2 days or 3 weeks at 4 °C. Interscapular brown adipose tissue, obtained from five to eight mice, was homogenized (15 s at speed 7; probe type 7T; Polytron, Kinematica, Luzern, Switzerland) in 10 vol. of Hepes buffer (50 mM, pH 7.6), containing NaCl (150 mM), bacitracin (1 mM), aprotinin (1000 trypsin-inhibitor units/ml), phenylmethanesulphonyl fluoride (1 mM) and Triton X-100 (1%) (Tanti *et al.*, 1986; Van Obberghen *et al.*, 1981). The homogenates were solubilized for 90 min at 4 °C by continuous stirring, and centrifuged at 150000 *g* at 4 °C for 90 min. The supernatants were applied on a wheat-germ-agglutinin–agarose column, and recycled three times. After washing, bound glycoproteins were desorbed with 0.3 M-*N*-acetylglucosamine. This preparation is termed 'partially purified insulin receptors'. The same procedure was used to prepare insulin receptors from skeletal muscle (Le Marchand-Brustel *et al.*, 1985) or from isolated hepatocytes (Van Obberghen & Kowalski, 1982). Insulin binding to solubilized insulin receptors was routinely measured at tracer insulin concentrations as previously described (Tanti *et al.*, 1986; Le Marchand-Brustel *et al.*, 1985), since full Scatchard analysis was performed and did not show any change in binding affinities between control and cold-acclimated mice (results not shown).

Preparation of highly purified insulin receptors

Insulin receptors were further purified by the following procedure (Roth *et al.*, 1983): insulin receptors partially purified on the wheat-germ-agglutinin column were passed over a 3 ml column of anti-receptor antibody (serum from patient B7) coupled to CNBr-activated Sepharose. The column was washed with 30 mM-Hepes/30 mM-NaCl/0.1% Triton X-100, and then with 0.5 M-NaCl/0.1% Triton X-100. The receptor was eluted with 1.5 M-$MgCl_2$ in 120 mM-borate buffer (pH 7.2)/0.1% Triton X-100. The eluted receptor was immediately diluted 6-fold with 30 mM-Hepes/30 mM-NaCl/0.1% Triton X-100 and further purified on a wheat-germ-agglutinin affinity column by the procedure described above. These preparations are termed 'highly purified insulin receptors'.

Cell-free phosphorylation of insulin receptors

Partially or highly purified insulin receptors (50 μl of wheat-germ-agglutinin-purified preparations containing approx. 25 ng of receptor) were incubated in a reaction mixture containing Hepes buffer (50 mM, pH 7.6) and NaCl (150 mM) without or with insulin (0.1 μM) at 15 °C for 90 min. Thereafter, the phosphorylation reaction was initiated by adding [γ-^{32}P]ATP (15 μM), $MnCl_2$ (4 mM) and $MgCl_2$ (8 mM). After 15 min at 20 °C, the samples were solubilized in boiling 3% (w/v) SDS containing 10% (v/v) glycerol, 2% (v/v) β-mercaptoethanol and 0.01% (w/v) Bromophenol Blue. When necessary, the catalytic subunit of cyclic AMP-dependent protein kinase was added 3–5 min before the phosphorylation reaction. Analysis of samples (volumes were adjusted in order to correspond to the same insulin-binding capacity) was performed by one-dimensional SDS/polyacrylamide-gel electrophoresis with a 7.5%-acrylamide resolving gel (Laemmli, 1970). The gels were stained, dried and autoradiographed by exposing them to Kodak X-Omat film. The M_r values of the standards used were: myosin, 200000; β-galactosidase, 116000; phosphorylase *b*, 94000; bovine serum albumin, 66000; ovalbumin, 45000; carbonic anhydrase, 30000; soya-bean trypsin inhibitor, 20000; lysozyme, 14400. At the end of the phosphorylation, ATP concentration was determined in the phosphorylation mixture with an ATP-monitoring kit (Sigma). This assay is based on the fluorimetric determination of NAD^+ generated in a phosphoglycerate kinase–glyceraldehyde phosphate dehydrogenase coupled reaction.

Cell-free phosphorylation and immunoprecipitation of partially purified EGF receptors

Wheat-germ-agglutinin–agarose eluates obtained from hepatocytes were preincubated without or with 0.1 μM-EGF for 90 min, and phosphorylation was performed as described above. The reaction was stopped by adding an ice-cold stopping solution containing NaF (80 mM) and EDTA (30 mM). Samples were immunoprecipitated with anti-peptide serum directed against EGF receptor (serum RK2) or with the corresponding preimmune serum (PI 2) at a 1/100 dilution (Kris *et al.*, 1985). After precipitation with Protein A, the pellets containing the immunoadsorbed proteins were washed with 3 × 1 ml of ice-cold Hepes buffer (30 mM, pH 7.6)/NaCl (30 mM)/0.1% Triton X-100 and subsequently analysed by SDS/polyacrylamide-gel electrophoresis.

Phosphorylation of exogenous substrates

Partially purified insulin receptors were preincubated without or with insulin as described above; substrates were added at the following final concentrations (histone 2b, 0.4 mg/ml; copolymer polyglutamate–tyrosine, 4:1 molar ratio, 0.25 mg/ml) for 30 min. The phosphorylation reaction was initiated and carried on 30 min; the samples were analysed by polyacrylamide-gel electrophoresis, with a 7.5–17.5%-acrylamide linear gradient as resolving gel, or the phosphate incorporation into trichloroacetic acid-precipitable proteins was measured as described previously (Braun *et al.*, 1984; Le Marchand-Brustel *et al.*, 1985) by a filter-paper assay.

RESULTS

Effect of cold-acclimation on insulin-receptor tyrosine kinase activity

We first studied autophosphorylation of insulin receptors prepared from brown adipose tissue of 3-weeks-cold-acclimated mice. After cold-acclimation the tissue developed markedly, but no major change in insulin binding was observed when expressed per μg of glycoprotein (results not shown). The kinase activity of the same amounts of insulin receptors from control and cold-acclimated animals, as defined by identical insulin-binding activity, was studied. Scatchard analysis of the binding data revealed no change in the affinity of insulin receptors from cold-acclimated mice compared with controls (results not shown). When a time course of insulin-stimulated autophosphorylation of insulin receptors derived from control and cold-acclimated mice

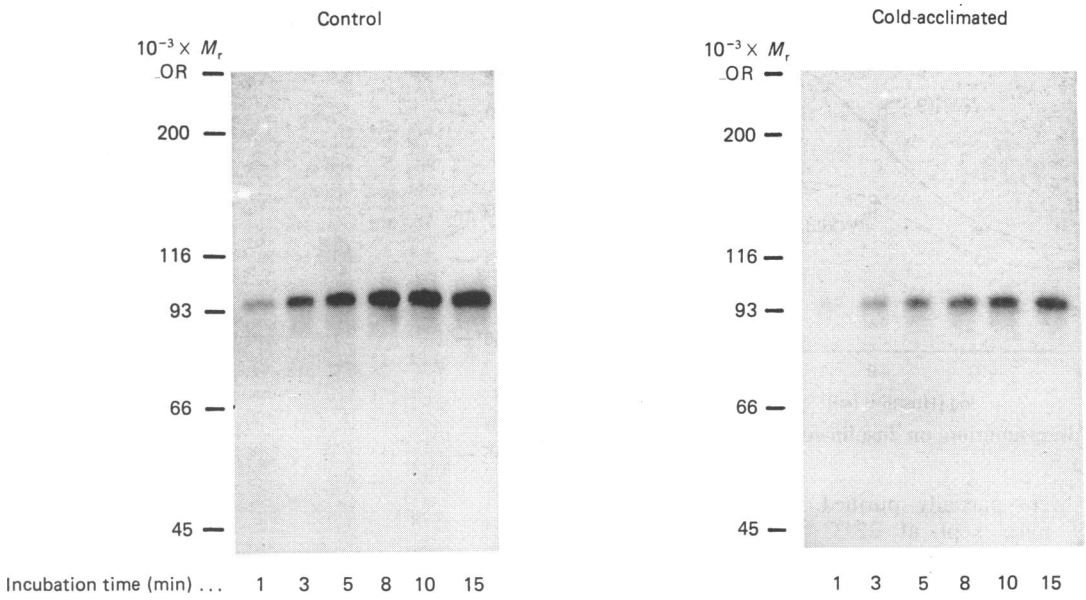

Fig. 1. Effects of 3-week cold-acclimation on phosphorylation of insulin receptors from brown adipose tissue

Insulin receptors were partially purified from the brown adipose tissue isolated from five mice kept at 22 °C (control) or maintained for 3 weeks at 4 °C (cold-acclimated) as described in the Experimental section. Amounts of insulin receptors (partially purified on wheat-germ-agglutinin columns) corresponding to the same binding capacity were preincubated for 90 min at 15 °C in the presence of 0.1 μM-insulin. Phosphorylation was then initiated by addition of [γ-^{32}P]ATP (15 μM; 20000 c.pm./pmol), $MnCl_2$ (4 mM) and $MgCl_2$ (8 mM). The reaction was stopped after different incubation periods by addition of a solution containing 3% SDS, 10% glycerol, 10 mM-sodium phosphate, 5% 2-mercaptoethanol and 0.05% Bromophenol Blue (final concns.). Samples were boiled for 5 min, and then analysed by SDS/polyacrylamide-gel electrophoresis in 7.5%-polyacrylamide gels (Laemmli, 1970). An autoradiogram of the gel is shown. Positions of M_r markers are shown to the left (in this and other Figures); OR, origin.

was performed (Fig. 1), the labelling of the 95 kDa phosphopeptide, corresponding to the receptor β-subunit, was lower at all time points in cold-acclimated mice than in controls. For both groups of mice, receptor phosphorylation reached a steady state within 8 min. In the absence of insulin, the insulin-receptor phosphorylation was similar in preparations from control and cold-acclimated animals. An identical alteration of tyrosine kinase activity of insulin receptors was observed in 48 h-cold-acclimated animals (results not shown), a short-term acclimation period which induces an increase in circulating catecholamines (Ricquier & Mory, 1984). As shown in Fig. 2, the capacity of insulin receptors from cold-acclimated mice to catalyse the phosphorylation of a tyrosine-containing synthetic polypeptide was markedly affected. However, the half-maximally effective insulin concentration (0.5 nM) was similar with both insulin-receptor preparations.

Effect of cyclic AMP-dependent protein kinase on insulin-receptor autophosphorylation and kinase activity towards exogenous substrates

Since the effects of noradrenaline are mediated by cyclic AMP and cyclic AMP-dependent protein kinase, we have searched for a direct effect of this enzyme on the insulin-receptor kinase activity in a cell-free system. Partially purified insulin receptors were preincubated in the absence or the presence of insulin; the purified catalytic subunit was added and the phosphorylation reaction was then performed. The autoradiogram in Fig. 3 shows that the catalytic subunit inhibits significantly the insulin-stimulated autophosphorylation of the insulin-receptor β-subunit (lane *d* compared with lane *b*). The action of the catalytic subunit was prevented by the addition of its specific protein kinase inhibitor (lane *e*). In the absence of insulin, the phosphorylation of the insulin-receptor β-subunit was negligible (lane *a*) and was not enhanced by the addition of protein kinase catalytic subunit (lane *c*). The decrease in insulin-receptor kinase activity induced by the catalytic subunit was very rapid, since it could be detected as early as 1–2 min after its addition (results not shown). A similar inhibition of insulin-stimulated autophosphorylation of insulin receptors was observed when a commercial preparation of catalytic subunit was used.

To exclude the possibility that ATP depletion was responsible for the observed effect of the catalytic subunit of the cyclic AMP-dependent protein kinase, ATP concentration was determined at the end of the phosphorylation. Values were identical when the phosphorylation was performed with or without the catalytic subunit, and corresponded to 85% of the initial ATP concentration (results not shown).

A similar inhibitory effect of the catalytic subunit was obtained when insulin receptors were prepared from liver (Fig. 4, lanes *a–d*) or from skeletal muscle (results not shown), indicating that this effect was found in all tissues examined. We then looked for the effect of the catalytic subunit on another tyrosine kinase activity. The glycoprotein preparation obtained from isolated hepatocytes by use of a wheat-germ-agglutinin affinity column also contains EGF receptors, which can be

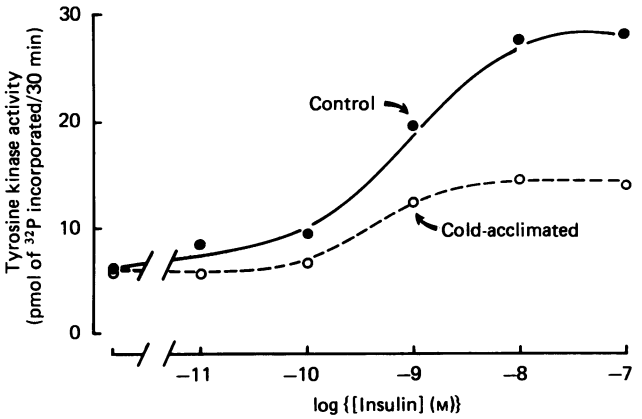

Fig. 2. Effect of cold-acclimation on insulin-receptor tyrosine kinase activity

Insulin receptors were partially purified from brown adipose tissue of mice kept at 22 °C (control) or maintained for 3 weeks at 4 °C (cold-acclimated). Amounts of wheat-germ-agglutinin-purified insulin receptors corresponding to the same binding capacity were preincubated in Hepes buffer (50 mM, pH 7.6)/NaCl (150 mM)/bovine serum albumin (0.2 mg/ml), final volume 140 μl, without or with various concentrations of insulin for 60 min at 24 °C. The substrate [poly(glutamate–tyrosine), final concn. 0.25 mg/ml] was then added for a further 30 min. The phosphorylation was initiated by adding 20 μl of a solution containing [γ-^{32}P]ATP, $MnCl_2$ and $MgCl_2$ [final concns. 20 μM (3000 c.p.m./pmol), 4 mM and 8 mM respectively]. After 30 min, 75 μl samples were applied to filter papers (Whatman ET 31) and immersed in 10% trichloroacetic acid containing 10 mM-pyrophosphate. After washing, the papers were counted for the Čerenkov radiation.

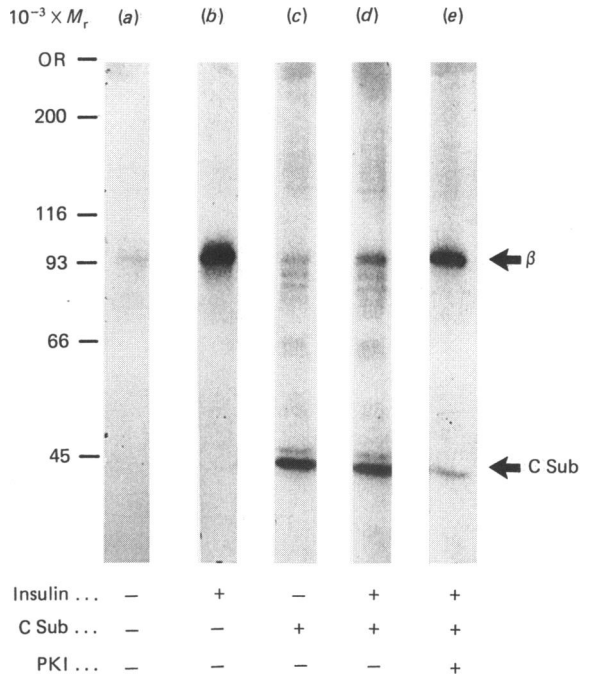

Fig. 3. Effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the autophosphorylation of insulin receptors prepared from brown adipose tissue

Insulin receptors were partially purified from brown adipose tissue from control mice. Samples (50 μl) were preincubated for 90 min at 15 °C without or with insulin (0.1 μM). The catalytic subunit (C Sub) of the cyclic AMP-dependent protein kinase (20 μg/ml) was added during the last 10 min of the preincubation. When indicated, the specific protein kinase inhibitor (PKI; 40 μg/ml) was added with the C Sub. Phosphorylation was then performed for 15 min as described in Fig. 1.

immunoprecipitated by a specific antipeptide serum. The catalytic subunit markedly inhibited EGF-stimulated phosphorylation of EGF receptors (Fig. 4, lanes *e–h*).

We next investigated the effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the insulin-receptor kinase activity towards exogenous substrates. Tyrosine kinase was measured with the tyrosine-containing synthetic polypeptide poly(glutamate–tyrosine) as substrate. Both the basal activity (measured in the absence of insulin) and the insulin-evoked stimulation of the receptor tyrosine kinase activity were inhibited by the catalytic subunit of the cyclic AMP-dependent protein kinase (Table 1). As expected, this substrate was not phosphorylated by the catalytic subunit itself, which is a serine-specific kinase. The dose–response of the catalytic-subunit effect on the insulin-receptor tyrosine kinase activity was measured (Fig. 5). Maximal and half-maximal inhibitory effects were obtained with 10 and 8 μg/ml respectively.

Possible mechanisms of the inhibition of insulin-receptor kinase activity by the cyclic AMP-dependent protein kinase

We varied the ATP concentration during the phosphorylation assay, to test if the decreased autophosphorylation of the receptor might reflect a decreased affinity of the receptor kinase for ATP. This appears not to be the case, since the degree of inhibition of the insulin-receptor autophosphorylation was identical when increasing ATP concentrations were used in the phosphorylation assay (results not shown).

To eliminate the possibility that the catalytic subunit of the cyclic AMP-dependent protein kinase could phosphorylate, and thereafter activate, a phosphatase activity that could be present in the partially purified insulin-receptor preparations, we performed the following two types of experiments. (1) We examined the effect of the catalytic subunit added at different times after the initiation of the insulin-stimulated phosphorylation of the tyrosine-containing polymer. As shown in Fig. 6, when the catalytic subunit was added after 6 or 15 min, its effect was maximal after 4 min. After this period of time, the phosphorylation of the substrate was nearly constant. (2) A time course of the dephosphorylation of the labelled receptor was studied. Insulin receptors were labelled in the presence of insulin and [γ-^{32}P]ATP; then a large excess of unlabelled ATP was added and the decrease in the labelling of the 95 kDa phosphorylated receptor was followed in the presence or in the absence of the catalytic subunit. No differences were observed between the two conditions at all time points studied (results not shown). Taken together, these data indicate that the decreased extent of phosphorylation of the insulin receptor observed with the catalytic subunit is not due to an increased phosphatase activity.

To support further a direct effect of the catalytic

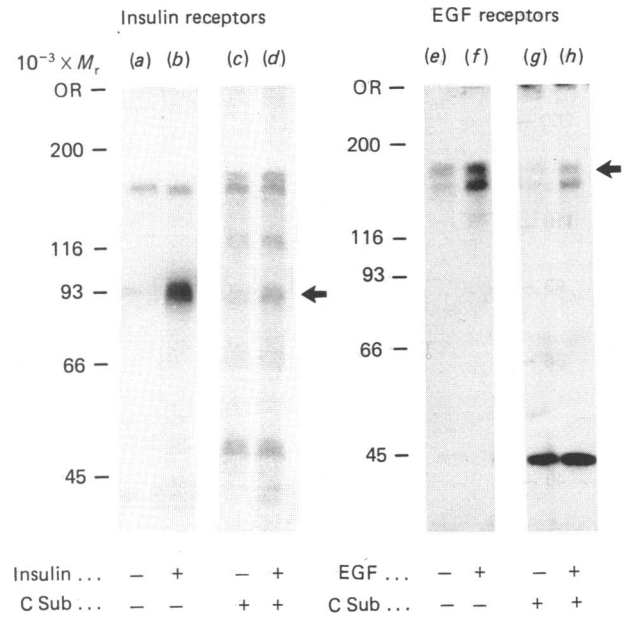

Fig. 4. Effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the autophosphorylation of insulin and EGF receptors prepared from rat hepatocytes

Insulin and EGF receptors were partially purified from rat hepatocytes by wheat-germ-agglutinin affinity chromatography as described in the Experimental section. Left panel: wheat-germ-agglutinin eluates were preincubated without or with insulin and the catalytic subunit of the cyclic AMP-dependent protein kinase as described in Fig. 3, and samples were analysed by SDS/polyacrylamide-gel electrophoresis. Right panel: eluates from wheat-germ-agglutinin columns were preincubated without or with EGF (0.1 μM) for 90 min at 15 °C; catalytic subunit (C Sub) was added for the last 10 min at the end of the phosphorylation, the reaction was stopped by NaF (80 mM) and EDTA (30 mM), and samples were immunoprecipitated with anti-peptide serum directed against EGF receptor. After precipitation with Protein A, the pellets were washed and analysed by SDS/polyacrylamide-gel electrophoresis.

subunit on the insulin receptor, we have extended our study to insulin receptors highly purified by the use of antibody affinity column (Roth *et al.*, 1983), and thus totally devoid of contaminating kinase or phosphatase activities. As shown in Fig. 7, the basal phosphorylation of the insulin-receptor β-subunit was similar in the absence or in the presence of the catalytic subunit. Further, the insulin-stimulated phosphorylation was markedly inhibited in the presence of the catalytic subunit.

DISCUSSION

The results presented in this paper show that insulin receptors partially purified from brown adipose tissue of cold-acclimated mice display a decreased tyrosine kinase activity both for autophosphorylation and for phosphorylation of exogenous substrates. This decrease in kinase activity was unlikely to be due to an increase in a phosphatase activity, since it was observed when initial rates of phosphorylation were measured. Acute exposure to cold induces a rapid increase in noradrenaline release in the brown adipose tissue, and this sympathetic

Table 1. Effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the tyrosine kinase activity of insulin receptors

Insulin receptors were partially purified from brown adipose tissue as described in the Experimental section. They were incubated without or with 0.1 μM-insulin for 60 min at 20 °C. Poly(glutamate–tyrosine) (0.25 mg/ml) was added for 30 min. The catalytic subunit (10 μg/ml) was added during the last 5 min of the preincubation. The ^{32}P incorporation into trichloroacetic acid-precipitable material after 30 min of phosphorylation was measured as detailed in the Experimental section. Results are expressed as percentages of basal activity measured in the absence of insulin and catalytic subunit, and are means ± S.E.M. for five experiments performed with five different insulin-receptor preparations.

	Tyrosine kinase activity (% of basal)	
Incubation conditions	Without insulin	With insulin
Without catalytic subunit	100	300 ± 50
With catalytic subunit	55 ± 8	54 ± 3

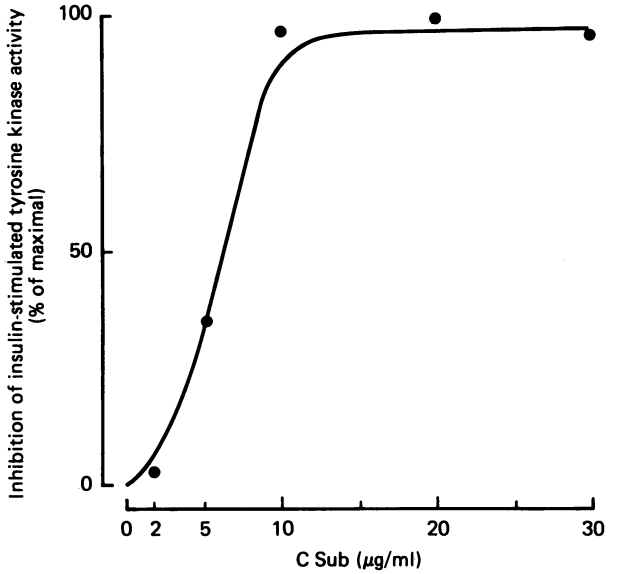

Fig. 5. Inhibitory effect of cyclic AMP-dependent protein kinase on insulin-receptor activity: dose-dependency

Insulin receptors partially purified from brown adipose tissue were preincubated in the presence of insulin (0.1 μM) for 60 min at 20 °C. Poly(glutamate–tyrosine) (0.25 mg/ml) was added for 30 min. Tyrosine kinase activity was measured as described in Fig. 2. Catalytic subunit (C Sub; at the concentration indicated) was added during the last 10 min of preincubation.

activation persists during chronic cold exposure (Himms-Hagen, 1985; Ricquier & Mory, 1984). The decrease in kinase activity found in insulin receptors isolated from a tissue which has been chronically exposed *in vivo* to increased concentrations of catecholamines is analogous to the decrease in insulin-receptor kinase activity in isolated adipocytes rendered insulin-resistant by cate-

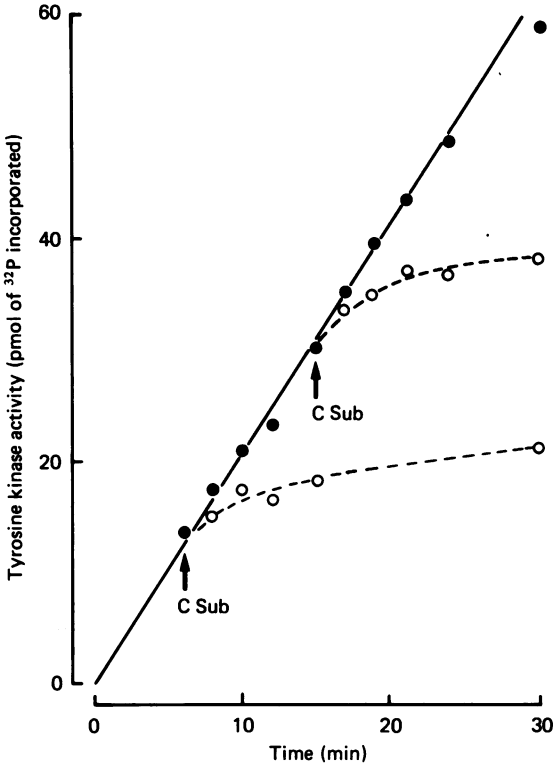

Fig. 6. Effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the phosphorylation of exogenous substrate

Insulin receptors partially purified from brown adipose tissue were preincubated in the presence of insulin (0.1 μM) for 60 min at 20 °C. Poly(glutamate–tyrosine) (0.25 mg/ml) was added for 30 min. Phosphorylation was initiated as described in Fig. 1. Catalytic subunit (C Sub; as indicated) was added after 6 or 15 min of phosphorylation. Samples were taken at various time intervals to measure ^{32}P incorporation into trichloroacetic acid-precipitable material as described in the Experimental section. ——, Control; – – – –, + catalytic subunit.

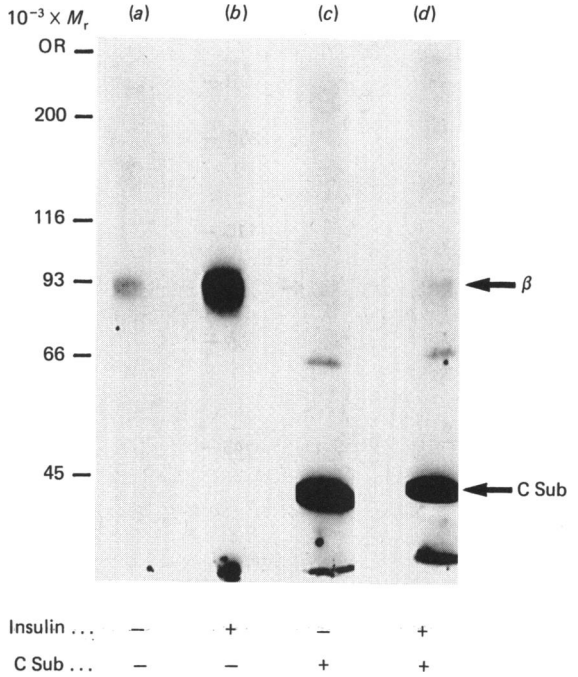

Fig. 7. Effect of the catalytic subunit of the cyclic AMP-dependent protein kinase on the phosphorylation of highly purified insulin receptors

Insulin receptors were purified by antibody affinity column as described in the Experimental section, and phosphorylated as described in Fig. 3. Abbreviation: C Sub, catalytic subunit.

cholamine treatment *in vitro* (Häring *et al.*, 1986). Insulin receptors prepared from skeletal muscle isolated from the same animals also displayed a decrease in enzyme activity, although to a much lesser degree. All these events are undoubtedly mediated by the activation of the sympathetic nerves in the brown adipose tissue and the subsequent release of noradrenaline. Indeed, denervation of this tissue prevents the tissue response to cold exposure (Girardier, 1983). Further, it is possible to induce hypertrophy of the brown adipose tissue and an increased content of thermogenin by chronic catecholamine infusion (Bouillaud *et al.*, 1984). Similarly, we could mimic the effects of cold-acclimation on insulin-receptor kinase activity in brown adipose tissue by a chronic infusion of catecholamine through an osmotic mini-pump (results not shown).

To elucidate the mechanisms of this decrease in insulin-receptor kinase activity, we have searched for an effect *in vitro* of the cyclic AMP-dependent protein kinase on insulin receptors. The results presented in this paper show that the catalytic subunit of the cyclic AMP-dependent protein kinase is able to inhibit the insulin-receptor tyrosine kinase activity. We obtained this effect of cyclic AMP-dependent protein kinase in a concentration range which has been used by other investigators in a variety of cell-free systems (Gunzburg, 1985). This effect was observed on insulin receptors prepared from different tissues, including brown adipose tissue, hepatocytes and skeletal muscle. Our results differ from those of Joost *et al.* (1986), who could not detect a functional interaction between cyclic AMP-dependent protein kinase and insulin receptors purified from human placenta. A similar lack of interaction was reported for EGF receptors purified from A431 cells (Gosh-Dastidar & Fox, 1984) or from livers after partial hepatectomy (Rackoff *et al.*, 1984). It is not known whether this discrepancy is due to difference in the type of tissues used (Gosh-Dastidar & Fox, 1984; Rackoff *et al.*, 1984), or in experimental protocols (phosphorylation of EGF receptors was performed directly on detergent-solubilized membranes in the work reported by Gosh-Dastidar & Fox, 1984).

Multiple mechanisms can be evoked to explain the effect of the cyclic AMP-dependent protein kinase; at least the following ones can be envisaged. (1) Insulin binding could be decreased. However, when the catalytic subunit was added to partially purified insulin-receptor preparations in the absence or in the presence of ATP, no modification of the binding properties of the receptors was observed (results not shown). It should be noted that, in accordance with previously reported results (Joost *et al.*, 1986), we could not detect an effect of the receptor phosphorylation on the insulin-binding characteristics. (2) An increase in the K_m for ATP of the insulin-receptor tyrosine kinase has been reported in

catecholamine-treated white adipocytes (Häring *et al.*, 1986). Such a mechanism does not seem to occur in the cell-free system used in our study, since the effect of the catalytic subunit of the cyclic AMP-dependent protein kinase was of the same order of magnitude at all ATP concentrations tested. (3) The insulin-receptor preparations used in this study contain phosphatase activity (Kowalski *et al.*, 1983; Häring *et al.*, 1984). It could be conceivable that the catalytic subunit phosphorylates and activates a phosphatase present in the preparations, which would subsequently induce a dephosphorylation of the labelled insulin-receptor β-subunit. However, such a sequence of events seems unlikely, since this inhibition persisted with highly purified insulin receptors devoid of contaminating phosphatase activity. This observation strongly suggests that the protein kinase catalytic subunit directly inhibits the insulin-receptor tyrosine kinase activity. (4) Some other studies have suggested that cyclic AMP-dependent protein kinase might phosphorylate insulin receptors on serine residues, resulting in a decreased receptor tyrosine kinase activity (Pessin *et al.*, 1985; Stadtmauer & Rosen, 1986). A similar mechanism could occur in our present study. However, we did not detect a direct phosphorylation of the partially or highly purified receptors by cyclic AMP-dependent protein kinase. This absence of demonstrable cyclic AMP-dependent phosphorylation of the insulin receptor is in accordance with the fact that the amino acid sequence of the receptor β-subunit does not contain amino acid consensus sequences classically recognized by the cyclic AMP-dependent protein kinase (Ebina *et al.*, 1985; Ullrich *et al.*, 1985). It should, however, be noted that the small amount of labelling remaining in the 95 kDa phosphorylated receptor could be in part due to phosphorylation on serine residues. The very low incorporation of phosphate in those conditions did not enable us to perform phospho-amino acid analysis with confidence.

The results presented here suggest that the catalytic subunit of the cyclic AMP-dependent protein kinase could modulate the insulin-receptor tyrosine kinase activity. The most plausible mechanism would have been phosphorylation of a serine residue of the receptor. However, at present our data do not favour the idea that the effect of the protein kinase catalytic subunit on the insulin receptor is due to a mechanism involving phosphorylation. Another possibility is a direct physical interaction between the catalytic subunit of the cyclic AMP-dependent protein kinase and the insulin-receptor β-subunit. This would lead to a masking of the receptor autophosphorylation site(s) involved in the tyrosine kinase activity.

We have shown here that both cold-acclimation *in vivo* and the exposure *in vitro* to cyclic AMP-dependent protein kinase produced an inhibitory effect on the insulin-receptor tyrosine kinase activity. Furthermore, catecholamine infusion induces a similar effect on the receptor enzyme function to that of cold-acclimation. We thus consider that these observations *in vitro* and *in vivo* are very likely to be related, and due to the same, or a similar, underlying mechanism. The receptor modifications induced by cold-acclimation *in vivo* are evident in receptors subsequently purified by lectin chromatography. This implies that the catecholamine-evoked alteration of the insulin receptor persists at least during the receptor isolation. Such a 'stable' change, observed with both catecholamines and cyclic AMP, could evidently result from a phosphorylation mechanism, a hypothesis which is, however, not supported by our data, as discussed above. It is thus suggested that the receptor can be the subject of a non-enzymic and 'stable' modification, the mechanism of which is as yet unidentified.

Catecholamine-induced insulin resistance probably results from the conjunction of a variety of phenomena. Catecholamines modulate the binding properties of the insulin receptors of treated cells (Häring *et al.*, 1986; Pessin *et al.*, 1983); they modify insulin action by altering the state of insulin-receptor phosphorylation in intact cells; they increase the phosphorylation of the insulin receptors on serine residues, and thereby alter in exposed cells the kinase activity of receptors by increasing their K_m for ATP (Häring *et al.*, 1986). Finally, the results presented here show that the catalytic subunit of the cyclic AMP-dependent protein kinase is able to inhibit the tyrosine kinase activity of the insulin receptors in a cell-free system. All those effects could contribute in concert to the well-known antagonistic effects of catecholamines and insulin.

Note added in proof (received 27 April 1987)

During the revision of this paper, a similar inhibition of insulin-receptor tyrosine kinase activity by cyclic AMP-dependent protein kinase was reported (Roth & Beaudoin, 1987). This inhibition was explained by a direct phosphorylation of insulin receptor by the cyclic AMP-dependent kinase.

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