Protein kinase activity of the insulin receptor from muscle

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The insulin receptor is associated with a protein kinase activity. This has been shown for the receptor of liver, fat, and some other tissues which are not primary targets of insulin action. Here kinase activity is demonstrated for the insulin receptor of rat skeletal and cardiac muscle with similar characteristics. Insulin (10^{-7} mol/l) stimulates phosphorylation of the 95-kDa receptor subunit 3- to 18-fold. The effect is detectable at 10^{-10} mol/l insulin; the ED_{50} is approx. 3×10^{-9} mol/l. The kinase phosphorylates exogenous substrate as well, and it is recovered after immunoprecipitation of the receptor with antireceptor antibody suggesting that kinase activity is intrinsic to the muscle receptor.

Insulin *Receptor* *Protein kinase* *Muscle*

1. INTRODUCTION

Insulin stimulates phosphorylation of the 95-kDa β-subunit of the insulin receptor. This was shown first in intact hepatoma cells and IM[9] lymphocytes [1]. Subsequently, insulin-stimulated phosphorylation was shown in other intact cell systems [2–6], and in partially purified receptor preparations from several tissues [3–5, 7–10]. This phosphorylation appears to be due to the activation of a tyrosine specific protein kinase and this kinase phosphorylates exogenous substrates as well [8]. In two of the main target tissues of the metabolic effects of insulin, the liver [4] and the fat [3], receptor kinase activity was already demonstrated and characterized. We show here receptor kinase activity in another main target tissue, the muscle.

2. MATERIALS AND METHODS

2.1. *Materials*

Porcine insulin was purchased from Novo Industrie (Denmark), [^{32}P]ATP, 2900 Ci/mmol was from NEN (FRG), Triton X-100 was from New England Nuclear, aprotinin, phenylmethylsulfonyl fluoride (PMSF) and *N*-acetylglucosamide were obtained from Sigma (Munich, FRG). Wheat germ agglutinin coupled to agarose was from Miles, all reagents for SDS–polyacrylamide gel electrophoresis were from Bio-Rad, all other reagents were of the best grade commercially available. Male Sprague–Dawley rats fed ad libitum were used (180–220 g body weight).

2.2. *Methods*

Muscle receptor was prepared from skeletal muscle, cardiac muscle or freshly isolated cardiomyocytes. Isolated cardiomyocytes were prepared as in [11]. The cells were lysed by repeated freezing and thawing in the presence of protease inhibitors (PMSF, 1 mmol/l; aprotinin, 1 trypsin inhibiting unit/ml; leupeptin, 2 μmol/l and pepstatin, 2 μmol/l). Cardiac or skeletal muscle tissue were homogenized in the presence of the same protease inhibitors in a buffer containing Hepes, 50 mmol/l (pH 7.4). The homogenates were centrifuged at 20000 × *g* for 40 min, the pellet was then solubilized with Triton X-100 at a final concentration of 1% (by vol.). Insoluble material was removed by centrifugation at 200000 × *g* for 60 min and the supernatant was applied to columns

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containing wheat germ agglutinin coupled to agarose. After extensive washing with 25 mmol/l, Hepes buffer (pH 7.4) containing 0.1% Triton X-100, the bound material was eluted with the same buffer supplemented with 0.3 mol/l *N*-acetylglucosamine. Phosphorylation was studied in the solubilized material before wheat germ purification, after wheat germ purification and after further purification by immunoprecipitation with antireceptor antibody. Aliquots of each fraction (protein amount is given in the legends to the figures) were incubated with or without insulin at room temperature for 1/2 h. Phosphorylation was studied by incubation with [^{32}P]ATP (40 μmol/l) and in Hepes buffer (100 mmol/l, pH 7.4) containing 10 mmol/l $MnCl_2$ and 2 mmol/l vanadate at 25°C for 10 min. The incubation was stopped by addition of Laemmli buffer and boiling for 15 min. To study substrate phosphorylation, 10 μg actin were added. Actin was prepared as in [12]. Subsequently, phosphoproteins were separated by polyacrylamide gel electrophoresis and identified by autoradiography. The phosphoproteins identified by the autoradiography were cut from the gel and counted in a scintillation counter.

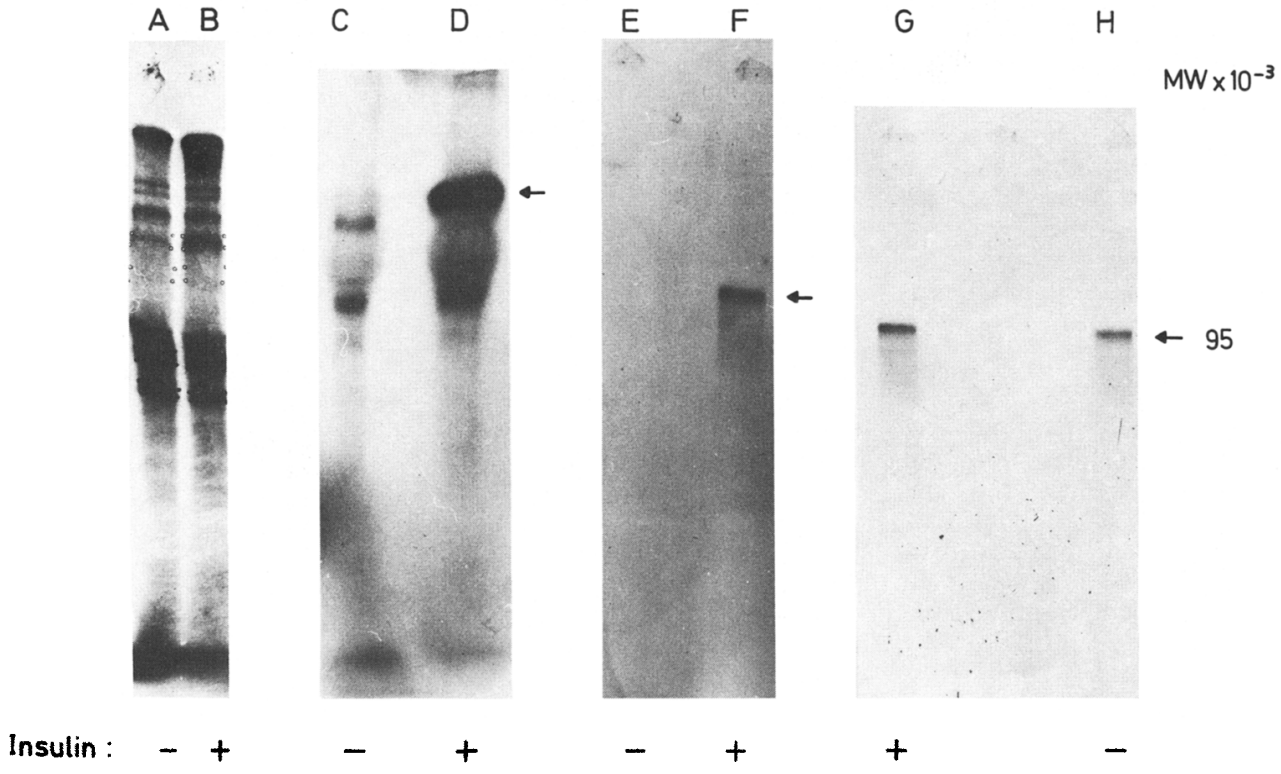

Fig.1. Autoradiogram of the phosphorylation of the insulin receptor from skeletal muscle. Phosphorylation was studied at different stages of receptor purification, shown from left to right: phosphorylation of solubilized proteins before wheat germ purification (lane A,B); after wheat germ purification without subsequent immunoprecipitation (lane C,D); and with subsequent immunoprecipitation (lane E,F) as well as phosphorylation after further purification of wheat germ purified proteins by immunoprecipitation (lane G,H). Phosphorylation was studied at 25°C with [γ-^{32}P]ATP (40 μM, 0.01 mCi) for 10 min. The reaction was stopped by boiling except in the experiments shown in lane E and F where stopping solution (NaF 100 mmol/l, sodium pyrophosphate 10 mmol/l, EDTA 5 mmol/l, ATP 5 mmol/l and vanadate 2 mmol/l) was added before immunoprecipitation. Phosphoproteins were analyzed with SDS–10% polyacrylamide gel electrophoresis. The 95-kDa band was identified by autoradiography and the band was cut from the gel and counted in a scintillation counter. A control band of equal size was counted to determine background radioactivity and the value was subtracted from the value found in the 95-kDa band.

3. RESULTS

When Triton X-100-solubilized proteins from skeletal muscle or from cardiomyocytes are preincubated with insulin for 30 min followed by a 10 min phosphorylation period, several phosphoproteins appear on the autoradiogram (fig.1, lane A). Increased ^{32}P incorporation after addition of insulin is seen in a phosphoprotein of approx. 95 kDa (arrow) corresponding to the known molecular mass of the β-subunit of the insulin receptor (fig.1, lane B). If the Triton-solubilized proteins were passed over a wheat germ affinity column prior to phosphorylation, most of the other phosphoproteins are eliminated and the insulin-stimulated phosphorylation of the 95-kDa band is increased (fig.1, lane C,D). If these phosphoproteins were subsequently immunoprecipitated with a serum, which contains anti-insulinreceptor antibody, the insulin-stimulated 95-kDa band was precipitated by the receptor antibody (fig.1, lane F) but not by normal control serum (not shown). This further indicates that the 95-kDa band contains insulin receptor protein.

In order to test if the insulin-stimulated kinase activity is intrinsic to the insulin receptor, wheat germ purified proteins were first immunoprecipitated with receptor antibody and the phosphorylation was then initiated by addition of [^{32}P]ATP. Phosphorylation of the insulin receptor is still detectable and is stimulated by insulin (fig.1, lane G,H). Insulin stimulates the phosphorylation of the immunopurified receptor about 2-fold. The coprecipitation of kinase activity with the muscle receptor suggests that the kinase is tightly associated or intrinsic to the receptor protein.

A strong phosphorylation of the 95-kDa band of the immunopurified receptor is also seen in the absence of insulin (fig.1, lane H). This is different from the picture which is seen when receptor is

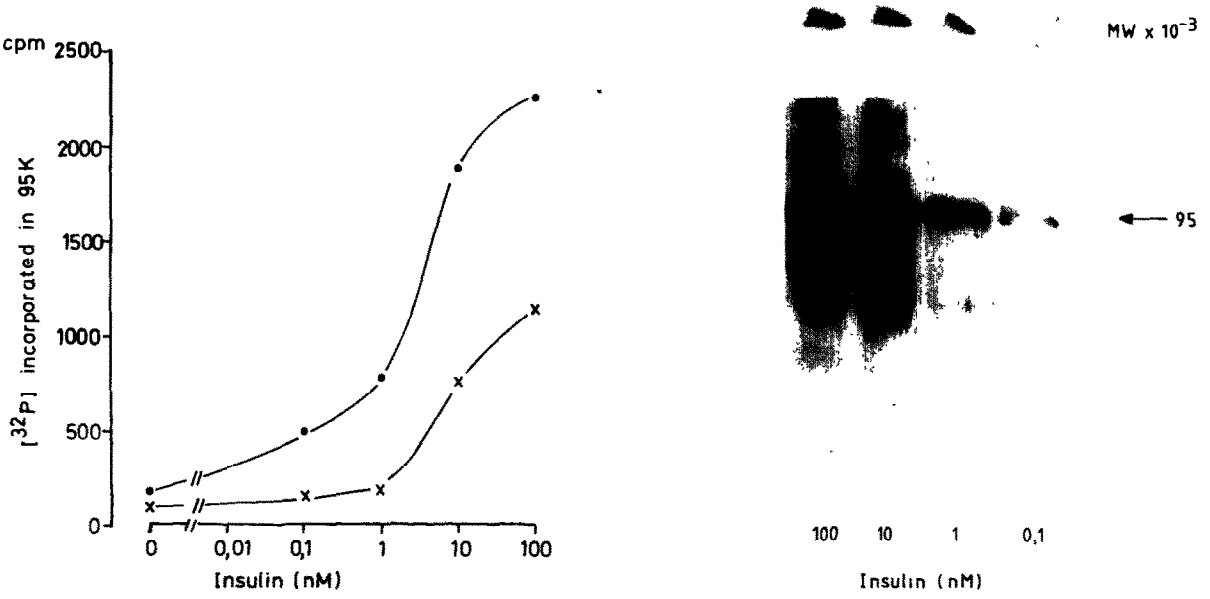

Fig.2. Dependence of insulin receptor phosphorylation on insulin concentration. Left: dose-response curve of insulin action on phosphorylation of the 95-kDa subunit of the insulin receptor. Wheat germ affinity purified receptor from skeletal muscle (●—●) or cardiomyocytes (×—×) was incubated for 0.5 h at room temperature with insulin (10^{-10} mol/l–10^{-7} mol/l). Phosphorylation was studied at 25°C with [γ^{32}P]ATP (40 μM, 0.01 mCi) for 10 min; 3–5 μg protein was used in each assay. The reaction was stopped by boiling. Phosphoproteins were analyzed with SDS–10% polyacrylamide gel electrophoresis. The 95-kDa band was identified by autoradiography and the band was cut from the gel and counted in a scintillation counter. A control band of equal size was counted to determine background radioactivity and the value was subtracted from the value found in the 95-kDa band. The values represent the mean of 4 experiments. Right: autoradiogram showing 1 of 4 experiments which were performed as described above.

used which was purified only by wheat germ affinity chromatography (fig.1, lane C,E). We have shown earlier [6] that this difference is due to the fact that the partially purified receptor fraction still contains phosphatase activity which is separated when the receptor is immunoprecipitated. Therefore, the basal kinase activity is visible if immunopurified receptor is used, while it is overcome by phosphatase activity if the partially purified receptor preparation is used.

Fig.2 shows the insulin sensitivity of receptor phosphorylation. Wheat germ purified receptor was used. Phosphorylation is stimulated about 2-fold by insulin concentrations of 10^{-10} mol/l, half maximal effect is seen at about 3×10^{-9} mol/l and the maximal effect is reached at 10^{-8} mol/l. The maximal insulin response and the insulin sensitivity were different in skeletal muscle and cardiomyocytes. The lower curve of fig.2 shows the insulin effect on phosphorylation of receptor isolated from cardiomyocytes; it is less sensitive than the receptor preparation from skeletal muscle (upper curve). Table 1 compares the maximal stimulation found in the different receptors. Receptor from intact skeletal muscle and intact cardiac muscle show an insulin responsiveness up to a 18-fold stimulation, while isolated cardiomyocytes show only a 3-fold stimulation.

Fig.3 shows the phosphorylation of an exogenous substrate by the receptor kinase. Actin was used as substrate [12]. The first two lanes show the phosphorylation of the 95-kDa receptor subunit in the absence and presence of insulin (10^{-7} mol/l). Actin was added in the experiments shown in lane C and D. A phosphoprotein of 43 kDa corresponding to the molecular mass of actin is detected. If actin was incubated with [^{32}P]ATP without insulin receptor, no phosphorylation was found (not shown). The phosphorylation of actin is increased by the addition of insulin (10^{-7} mol/l), shown in lane 4. The increase of ^{32}P incorporation into the 43-kDa band ranged, in 4 separate experiments, between 30 and 50%.

Table 1

Insulin effect on phosphorylation of the 95-kDa receptor subunit

Insulin 10^{-7} mol/l	Basal phosphorylation (%)
Isolated cardiomyocytes	318 ± 170 SD (n = 5)
Cardiac muscle	1841 ± 71 SD (n = 5)
Skeletal muscle	1096 ± 304 SD (n = 5)

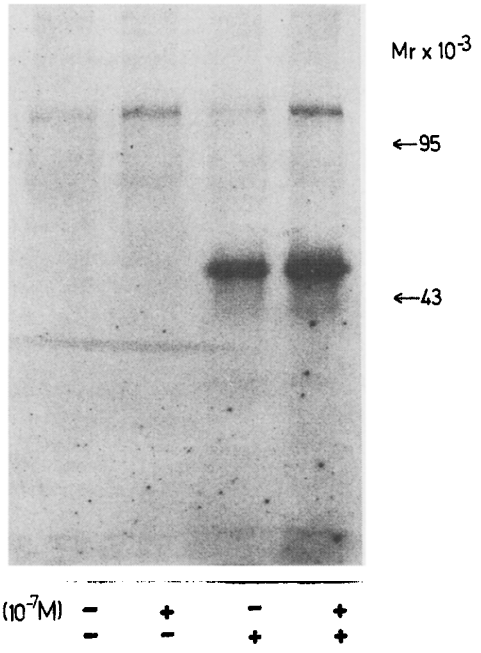

Fig.3. Autoradiogram of actin phosphorylation by the insulin receptor kinase. Lanes A and B show autophosphorylation of the 95-kDa insulin receptor subunit; lanes C and D show phosphorylation of actin (43 kDa). Phosphorylation was studied by 5 μg wheat germ-purified protein and 10 μg actin at 25°C with [γ-^{32}P]-ATP (40 μM, 0.01 mCi) for 10 min. The reaction was stopped by boiling. Phosphoproteins were analyzed with SDS-10% polyacrylamide gel electrophoresis. The 95-kDa and the 43-kDa bands were identified by autoradiography and the bands were cut from the gel and counted in a scintillation counter. A control band of equal size was counted to determine background radioactivity and the value was subtracted from the value found in the 95-kDa and the 43-kDa band.

4. DISCUSSION

The insulin receptor from muscle contains protein kinase activity and the characteristics of the kinase are virtually identical to those found for receptor kinase of the other target tissues of insulin.

(i) The kinase activity is preserved after immunoprecipitation of the receptor with antireceptor an-

tibody suggesting that the kinase is part of the receptor itself or is at least tightly associated with the receptor and therefore coprecipitated with the receptor protein. This confirms earlier studies with the receptor from other tissues which suggested that the insulin receptor is itself a protein kinase: the kinase activity of receptor from hepatoma cells and from human placenta was also retained after immunoprecipitation [6,13]. The kinase activity of receptor from human placenta was preserved after purification of the receptor protein to near homogeneity by insulin-affinity chromatography [13]. Furthermore, the 95-kDa subunit of receptor from liver was labelled with ATP-affinity reagents suggesting that the catalytic domain is located in this subunit [14].

(ii) Insulin stimulates the kinase at physiological concentrations and the ED_{50} of kinase from skeletal muscle is identical to the ED_{50} of receptor kinase from fat [3] and liver [4,10]. The kinase from cardiomyocytes appears to be less sensitive. It is not clear if this is an in vitro effect due to the isolation procedure of cardiomyocytes or if it reflects a true difference in sensitivity between skeletal and cardiac muscle.

(iii) The kinase from skeletal muscle phosphorylates an exogenous substrate. Phosphorylation of histone, synthetic peptides, caseine, angiotensine and some other peptides was shown with receptor from several tissues [8,13,15]. Recently, also phosphorylation of actin by receptor from human placenta was demonstrated [12]. While a physiological significance of insulin-stimulated phosphorylation of the upper substrates seems unlikely, the phosphorylation of actin is of great potential interest as the microtubular system appears to be involved in the activation of the glucose transport system by insulin [16]. The insulin stimulation of actin phosphorylation is very low if it is compared to the stimulation of receptor autophosphorylation. However, this also parallels the findings with receptor from other tissues and other substrates [8,13,15].

The mechanism of insulin action is still not well understood and it is at present unclear if receptor kinase activation is involved in the transmission of an insulin signal. It is not known if kinase activity might play a role in long-term growth-regulating effects of insulin [5] or if it is involved in the acute metabolic effects of insulin in the main target tissues. However, insulin has been shown to affect the state of phosphorylation for a variety of cellular proteins and enzymes [17,18] and this protein modification has been suggested to be at least part of the post-receptor transmission of the insulin signal. It seems possible that activation of the receptor kinase could be the first post-binding event in the transmission of an insulin signal, and the kinetic properties, specificity and insulin sensitivity of receptor kinase activation in the tissues, which have so far been studied, would be compatible with this model.

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REFERENCES

[1] Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982) Science 215, 185–187.
[2] Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Häring, H.U. and Kahn, C.R. (1982) J. Biol. Chem. 257, 9891–9894.
[3] Häring, H.U., Kasuga, M. and Kahn, C.R. (1982) Biochem. Biophys. Res. Commun. 108, 1538–1545.
[4] Van Obberghen, E. and Kowalski, A. (1982) FEBS Lett. 143, 179–182.
[5] Häring, H.U., Kasuga, M., Lauris, V., Kahn, C.R., Fleischmann, R., Murray, M. and Pawelek, J. (1984) J. Cell Biol., in press.
[6] Häring, H.U., Kasuga, M., White, M.F., Crettaz, M. and Kahn, C.R. (1984) Biochemistry 23/14, 3298–3305.
[7] Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982) Nature 298, 667–669.
[8] Petruzzelli, L.M., Ganguly, S., Smith, C.R., Cobb, M.H., Rubin, C.S. and Rosen, O. (1982) Proc. Natl. Acad. Sci. USA 79, 6792–6796.
[9] Machicao, F., Urumow, T. and Wieland, O.H. (1982) FEBS Lett. 149, 96–100.
[10] Zick, Y., Kasuga, M., Kahn, C.R. and Roth, J. (1983) J. Biol. Chem. 258, 75–80.
[11] Eckel, J. and Reinauer, H. (1984) Diabetes 33, 214–218.
[12] Machicao, F., Urumow, T. and Wieland, O.H. (1983) FEBS Lett. 163, 76–80.

[13] Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D.L. and Kahn, C.R. (1983) Proc. Natl. Acad. Sci. USA 80, 2137–2141.
[14] Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H. and Ponzio, G. (1983) Proc. Natl. Acad. Sci. USA 80, 945–949.
[15] Yu, K.T. and Czech, M.P. (1984) J. Biol. Chem. 255, 5277–5286.
[16] Häring, H.U., Kemmler, W. and Hepp, K.D. (1979) FEBS Lett. 105, 329–332.
[17] Alexander, M.C., Palmer, J.L., Pointer, R.H., Kowaloff, E.M., Koumjian, L.L. and Avruch, J. (1982) J. Biol. Chem. 257, 2049–2055.
[18] Brownsey, R.W. and Denton, R.M. (1982) Biochem. J. 202, 77–86.