Skeletal muscle rapidly develops severe insulin resistance following denervation, although insulin binding is unimpaired. Insulin-stimulated receptor tyrosyl kinase activity was studied in intact and 24-h denervated rat hind limb muscles using three preparations: (a) solubilized insulin receptors incubated with insulin in the presence of phosphoprotein phosphatase and kinase prelabeled with \([^{32}\text{P}]\)ATP and histone H\(_2\text{B}\); (b) soleus muscles prelabeled \textit{in vitro} with \([^{32}\text{P}]\)phosphate with subsequent insulin-stimulated phosphorylation of the receptor in \textit{situ}; (c) assessment of \textit{in vivo} activation of muscle receptor tyrosyl kinase by insulin. The latter was achieved by solubilizing muscle insulin receptors in the presence of phosphoprotein phosphatase and kinase inhibitors and measuring receptor-catalyzed histone H\(_2\text{B}\) phosphorylation in the presence of limiting (5 \(\mu\text{M}\)) \(\gamma\)-[\(^{32}\text{P}\)]ATP. Receptors isolated 5 and 30 min after intravenous insulin injection catalyzed \(^{32}\text{P}\) incorporation into histone H\(_2\text{B}\) twice as fast as those from saline-treated controls; insulin stimulated histone H\(_2\text{B}\) labeling exclusively on tyrosine. \textit{In vitro} activation was demonstrated using solubilized and insulin-agarose-bound receptors. Autophosphorylation of the \(\beta\)-subunit and receptor tyrosyl kinase activity toward histone H\(_2\text{B}\) was stimulated by insulin in denervated muscles as in controls, although the biological response to insulin, \textit{in vitro} and \textit{in vivo}, was markedly impaired after denervation, suggesting a postreceptor defect. The method developed to assess insulin-stimulated receptor activation \textit{in vivo} seems useful in characterizing mechanisms of insulin resistance.

Antecedent activity modulates the insulin response of skeletal muscle. Following exercise the muscle's sensitivity to insulin is increased as assessed by insulin-stimulated glucose transport (1), glycogen synthase activation (2), and amino acid transport (3). Basal glucose transport into muscle cells is also stimulated following vigorous exercise (4). The converse is also true, e.g. muscles which have been denervated (5-9) or immobilized (10) develop within 6-24 h decreased sensitivity and response to insulin. The insulin resistance encompasses multiple parameters of insulin action, e.g. glucose transport (5-9), glycogen synthesis (7-9), glycogen synthesis (7-9), and amino acid transport (7). Baseline glucose transport and glucose incorporation into glycogen are also decreased in denervated muscles. Several changes occur in the membrane of skeletal muscle after denervation. Extra junctional acetylcholine receptors appear (11) as well as sodium channels that are insensitive to tetrodotoxin (12). The resting membrane potential is decreased (13) and phospholipid turnover accelerates (14). However, no significant change in the binding of insulin to its receptor has been observed shortly after denervation to account for the severe insulin resistance (7,9,15), and thus the defect in denervated skeletal muscle may be classified as a postbinding defect. It has recently become evident that in some insulin-resistant states there is little or no change in insulin binding yet there is impaired insulin-stimulated autophosphorylation of the insulin receptor \(\beta\)-subunit (16-20). In the intact cell and in the solubilized insulin receptor system the initial insulin-stimulated autophosphorylation occurs on tyrosine residues of the \(\alpha\)-subunit, and this intramolecular event activates an intrinsic tyrosyl phosphotransferase to exogenous substrates. The latter may initiate the cascade of phosphorylation-dephosphorylation which ensues upon insulin binding to the cell surface (for review see Refs. 21 and 22).

Since the insulin resistance in denervated muscles encompasses multiple parameters, we postulated that the defect involves an early step in insulin's action (9); autophosphorylation of the \(\beta\)-subunit is the earliest recognized event after insulin binds to its receptor. In this report we investigated the effect of 24-h denervation on the insulin-stimulated autophosphorylation and tyrosyl kinase activity toward exogenous substrate of skeletal muscle insulin receptors using three systems: (a) receptors solubilized from hind limb muscles and exposed to insulin \textit{in vitro}; (b) receptor phosphorylation in incubated solei; (c) insulin activation of the muscle receptor tyrosyl kinase \textit{in vivo}. The latter approach required the development of methods to assess the activation state of the skeletal muscle insulin receptor kinase in the intact animal. Our data indicate that insulin-mediated receptor kinase activation is impaired in muscle 24 h after denervation, suggesting that in this model impaired receptor-function coupling is a true postreceptor defect.

**EXPERIMENTAL PROCEDURES**

**Materials**

Monoiodinated A14 \(^{125}\text{I}\) insulin was kindly provided by Dr. B. H. Frank (Lilly). Monocomponent crystalline pork insulin was a gift of Dr. Ronald Chance (Lilly). Polyclonal anti-insulin receptor antibody (A410) was a gift of Dr. Steve Jacobs (Wellcome Research Laboratories). Wheat germ agglutinin was from Vector, aprotinin was from FBA Pharmaceuticals, and bacitracin was from Pfizer. Electrophoresis reagents were from Serva and apparatus from LKB Instruments, Inc. Protein A bearing \textit{Staphylococcus aureus} was from Calbiochem.

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1 Supported by the Medical Scientist Training Program of the Medical University of South Carolina.

2 To whom correspondence should be sent.
Behring. CNBr-activated Sepharose 4B was from Pharmacia. Precoated cellulose TLC plates were from Brinkmann Instruments. Autoradiography film, X-Omat AR, was from Kodak and Lightning plus intensifying screens from DuPont. γ-[32P]ATP was prepared from [32P]orthophosphate (ICN) in Gammaprep vials (Promega) as described by the manufacturer. [3H]Glucose was from New England Nuclear. All other reagents and chemicals were from Sigma unless otherwise specified.

**Methods**

**Animals**—Male rats of the Wistar strain weighing 60-110 g were used; food was removed 24 h before experiments. Rats were unilaterally denervated 22-26 h before experiments by cutting the sciatic nerve through a small incision in the posterior aspect of the thigh as previously described (9).

**Insulin Receptor Solubilization**—The muscle groups innervated by the sciatic nerve (lower leg muscles) were rapidly removed and quickly frozen in liquid nitrogen, powdered, and the insulin receptors solubilized by homogenization in 25 mM Hepes, pH 7.4, 0.1% Triton X-100, 1 trypsin inhibitory unit/ml aprotinin, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. After 10 min of centrifugation at 10,000 g the supernatant was immediately centrifuged at 150,000 × g for 90 min (Method 1). The supernatant was then mixed with a 150,000 × g pellet in 5 ml of Buffer B and the insulin receptors eluted with buffer B solubilized by homogenization in 25 mM Hepes, pH 7.4, 1% Triton X-100, 1 trypsin inhibitory unit/ml aprotinin, 5 mM EDTA, and 2 mM phenylmethylsulfonyl fluoride. After 10 min of centrifugation at 100,000 × g the supernatant was stirred for 30 min at 22°C to complete solubilization and then centrifuged at 150,000 × g for 90 min (Method 2). The supernatant was then mixed with a 150,000 × g pellet in 5 ml of Buffer B and the insulin receptors eluted with buffer B. The resulting supernatant was applied to a WGA column pre-equilibrated with buffer A supplemented with 10 mM sodium pyrophosphate, 1.5 mg/ml bacitracin, 100 μM Na3V04, and 2 mM phenylmethylsulfonyl fluoride. After centrifugation at 10,000 × g for 10 min the supernatant was immediately centrifuged at 150,000 × g for 1 h (Method 2). The resulting supernatant was applied to a WGA column pre-equilibrated with buffer A supplemented with 10 mM sodium pyrophosphate, 5 mM EDTA, 1 trypsin inhibitory unit/ml aprotinin, and 2 mM phenylmethylsulfonyl fluoride (buffer B) and recycled three times. The column was then washed with 25 bed volumes of Buffer B and the insulin receptors eluted with buffer B supplemented with 0.3 M N-acetylglucosamine.

**Insulin Binding to the Solubilized Insulin Receptors**—This was performed as described above. The binding data were normalized to the protein content of the WGA eluate as determined by the Bradford reaction using bovine γ-globulin standards (24). All studies of receptor kinase activities were carried out using equal insulin-binding activities from WGA eluates prepared as described above.

**In vitro and in Vivo Activation of Insulin Receptor Kinase**—This was carried out as previously described (25). Briefly, hemi-soles (groups of 5-6 muscles) were incubated in Dulbecco’s modified Eagle’s medium (DMEM) containing 0.1 mM sodium phosphate for 1 h at 25 °C to lower endogenous phosphate pools. The muscles were then transferred to DMEM with 0.2 μCi/ml (0.1 mM) sodium [32P]phosphate and incubated at 37°C for an additional 3 h (preliminary experiments showed this was sufficient time to reach steady state labeling of nucleotide pools prior to the transfer to the insulin-containing medium). After freezing, the individual muscles were powdered with a glass rod in a microcentrifuge vial and the nucleotides extracted with 65 μl of 2 M dithiothreitol-heat-inactivated exogenous substrate kinase. The kinase reaction was initiated by adding 10 μl of γ-[32P]ATP (5 μM final concentration, 5 μCi/vial) and continued for 4 min when 4 × concentrated Laemmli’s sample buffer was added and the solution placed in boiling water for 3 min. After separation of the proteins on sodium dodecyl sulfate-polyacrylamide gel electrophoresis the histones were localized by autoradiography and the radioactivity incorporated determined as described under “Electrophoresis.”

**Electrophoresis and Quantitation of 32P Incorporated into Insulin Receptor in the Intact Soleus**—This was performed in an identical fashion in the modified DMEM medium described above, with or without 10 μmlits/ml of insulin, and the incubation continued for 1/2 h. The individual hemi-soles were then frozen with metal tongs precooled in liquid nitrogen and the insulin receptors solubilized and partially purified by Method 2 described above except that 40 mM unlabeled ATP was added to the homogenization buffer. Immunoprecipitation of the phosphorylated insulin receptor was achieved by incubating the WGA eluate overnight at 4°C with protein A and anti-insulin receptor antibody (transfected by protein A-bearing formalin-fixed Staphylococcus for an additional 2 h. After centrifugation, the precipitated complex was washed 3 times in Buffer B, and the pellet boiled in Laemmli’s sample buffer. Electrophoresis and quantitation of 32P incorporated into the β-subunit was as described above.

A parallel set of control and denervated hemi-soles was incubated in an identical fashion in the modified DMEM with [3H]glucose to determine the specific activity of the nucleotide pools prior to the transfer to the insulin-containing medium. After freezing, the individual muscles were powdered with a glass rod in a microcentrifuge vial and the nucleotides extracted with 60 μl of 3 M perchloric acid for 20-30 min at -10°C. Cold distilled water (200 μl) was added and incubation continued for an additional 10 min at 4°C (30). The protein precipitate was removed by centrifugation for 15 min in a microcentrifuge at 4°C and the supernatant neutralized with 65 μl of 2 N KHCO3 and clarified by centrifugation. The supernatant was filtered and 20 μl injected onto a 5-cm C18 reverse-phase column on a Perkin-Elmer Series 4 high pressure liquid chromatograph. The nucleotides (ATP, ADP, and GTP) were eluted isocratically using a buffer composed of 55 mM phosphoric acid brought to pH 6.5 with triethylamine (98%) and methanol (2%) (61). The location of the eluted nucleotides was determined by the detection of appropriate standards monitored by absorbance at 259 nm. Each peak was collected in duplicate determinations and counted in a liquid scintillation counter. The concentration of nucleotides was estimated by the peak area as determined by a Perkin-Elmer LCI-100 integrator. The specific activity was calculated as cpm/mmol.
nucleotide, and nucleotide content of the tissue was normalized to the protein content, measured by the Lowry reaction using bovine serum albumin standards (32).

**In Vivo Activation of the Exogenous Kinase Activity of the Insulin Receptor**—Unilaterally denervated rats were anesthetized with methoxyflurane (Metofane, Pitman-Moore, Inc.) and divided into two groups. One group received an intravenous injection of 5 units of regular insulin (Humulin, recombinant DNA origin, Lilly) via the femoral vein, the other received an equal volume of saline. Five or 30 min later the hind leg muscles were exposed, and the soleus muscles were rapidly removed, and mounted on wire frames, and incubated for \( \frac{1}{2} \) h in \(^{14} \text{C}\)glucose-containing buffer to estimate glycogen synthesis. The remaining leg muscles (primarily gastrocnemius and anterior compartment muscles) were removed, frozen in liquid nitrogen, homogenized, and the insulin receptors solubilized by Method 2 above. After elution from the WGA column, the exogenous kinase activity of the receptors was estimated by incubation in medium containing 56 mM MgSO\(_4\) (which was added first to precipitate inorganic phosphate and EDTA), 5 mM MnCl\(_2\), 0.05% BSA, 1 mg/ml histone H\(_2\)b, with or without \( 10^{-7} \) M insulin for 10 min at 25°C. \( \gamma^{32} \text{P} \)ATP (5\( \mu \)M final concentration, 7.5-10 \( \mu \)Ci/vial) was then added (5 or 50 \( \mu \)M final concentration, 7.5-10 \( \mu \)Ci/vial) to a final reaction volume of 75 \( \mu \)l. After 4 min the reaction was stopped by the addition of 4 \( \times \) concentrated Laemmli’s sample buffer and the solution placed in boiling water for 3 min. The proteins were separated by electrophoresis, autoradiographed, and \( 3^2 \text{P} \)incorporated into histone H\(_2\)b measured as described under “Electrophoresis.”

In a second assay WGA-eluted receptors were incubated with insulin-agarose (50 \( \mu \)/150 \( \mu \)l of WGA eluate, the latter diluted with 10 volumes of buffer A) for 15 min at 22°C after which the resin was washed, as described above (28). The insulin-agarose-bound receptors were resuspended in 70 \( \mu \)l of phosphorylation buffer supplemented with 1 mg/ml histone H\(_2\)b. The kinase reaction was initiated with 10 \( \mu \)l of \( \gamma^{32} \text{P} \)ATP (5 \( \mu \)M final concentration, 7.5 \( \mu \)Ci/vial). In a parallel reaction the receptor kinase was maximally activated by first incubating the washed insulin-agarose-bound receptors in phosphorylation buffer (without histone) with 750 \( \mu \)M unlabeled ATP for 20 min at 25°C. After washing twice in 1 ml of Buffer A the kinase activity against histone H\(_2\)b was measured as described above.

**Plasma Glucose**—This was measured with a Beckman Glucose Analyzer 2, using blood obtained by cardiac puncture. Only those animals whose plasma glucose fell below 65 mg/100 ml were considered “treated” in the in vivo activation experiments. The mean plasma glucose of insulin-treated rats was 39 ± 4 mg/dl after 5 min and 33 ± 11 mg/dl 30 min after insulin injection (n = 4 and 10, respectively); the mean plasma glucose of saline-treated controls was 55 ± 6 mg/dl.

**Glycogen Synthesis by Control and Denervated Solei**—This was determined as previously described (9).

**Phosphoamino Acid Analysis**—The position of histone H\(_2\)b for phosphoamino acid analysis was determined by autoradiography of unfixed undried gels aligned with fluorescent dye marks (Ultemit, New York Nuclear). The desired portion of the gel was excised and placed in a dialysis bag with 2 ml of electrophoresis buffer. The bag was closed with dialysis clips and the gel slice trapped between the plates of the vertical electrophoresis unit, one end plugged with a nucleotide, and nucleotide content of the tissue was normalized to the protein content, measured by the Lowry reaction using bovine serum albumin standards (32).

**Equilibrium Binding of \(^{125}\text{I}\)-Insulin to Insulin Receptors in the WGA Eluate Solubilized from Control and Denervated Hind Limb Muscles**—This yielded typical curvilinear binding kinetics (Fig. 1). When expressed per mg of protein in the WGA eluate, there was no significant difference in the number of insulin receptors isolated from control and denervated muscles (although the latter was slightly lower) nor was there a difference in the apparent insulin-binding affinity or in the amount of glycoprotein eluted from the WGA column/tissue weight.

**Solubilized Insulin Receptor Kinase Activities**—Upon binding insulin, the receptor, both in the intact cell and in the solubilized system, undergoes rapid autophosphorylation (for review see Refs. 21 and 22). This property of the solubilized receptor was tested by incubating WGA eluates, prepared from control and denervated muscles, with increasing concentrations of insulin (\( 10^{-6} \)–\( 10^{-7} \) M) using equal insulin-binding activities from each preparation. Insulin induced a dose-dependent increase in \( ^{32} \text{P} \)incorporation into the \( \beta \)-subunit of the receptor (Fig. 2). The latter was identified as the \( \beta \)-subunit by its apparent molecular weight (~97,000), its insulin-dependent labeling with \( ^{32} \text{P} \), and its precipitability with specific antibodies (25) Fig. 2A). The polyclonal antibody used (A410) has been reported to have relative specificity toward the insulin receptor as compared to its interaction with the IGF-I receptor (34). There was no apparent difference in

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**FIG. 1. Equilibrium binding of A14 \(^{125}\text{I}\)-insulin to solubilized WGA-purified insulin receptors. Aliquots of WGA eluate (7.5 \( \mu \)g of protein) from control and denervated skeletal muscle were incubated with tracer A14 \(^{125}\text{I}\)-insulin (specific activity, 100–140 \( \mu \)Ci/\( \mu \)g) and increasing concentrations of unlabeled insulin in a total volume of 200 \( \mu \)l of buffer containing 90 mM Hepes, 100 mM NaCl, 0.05 mg/ml bovine serum albumin, 0.1% Triton X-100 and 0.075 M N-acetylglucosamine, pH 7.8, for 16 h at 4°C. The receptor and bound insulin were precipitated by the sequential addition of 100 \( \mu \)l of 0.3% bovine \( \gamma \)-globulin and 300 \( \mu \)l of 25% polyethylene glycol. Nonspecific binding was considered that which occurred in the presence of 10 \( \mu \)g/ml unlabeled insulin and was subtracted from each point. The main figure shows a Scatchard plot of the data from control (V-V) or denervated muscle (0-0)-derived receptors. The inset shows displacement curve analysis of the same data before transformation. Each point is the mean ± S.E. of three receptor preparations; each point assayed in triplicate.
autophosphorylation between receptors derived from control and denervated skeletal muscles, respectively, each assayed in triplicate. To investigate whether or not denervation affects the insulin-stimulated autophosphorylation of the receptor in the intact cell, soleus muscles from control and denervated hind limbs were incubated in $^{[32P]}$ATP ($50 \mu M$ final concentration) and incubated at 25°C for 4 min. After electrophoresis and autoradiography, the excised $\beta$-subunit was digested and the radioactivity quantitated by scintillation counting. Histone phosphorylation by control or denervated derived receptors is shown. Each point is the mean ± S.E. of 6 determinations from 2 separate receptor preparations. B, phosphorylation of histone H$_{36}$ by solubilized insulin receptor activated on insulin-agarose. Equal insulin-binding activities from control and denervated derived wheat germ eluates (approximately 150 $\mu l$) were preincubated with 50 $\mu l$ of detergent-heat-inactivated insulin agarose at 25°C for 1 h and subsequently with 50 $\mu l$ of active insulin-agarose for 1 h at 25°C as described under "Methods." The suspension was washed and then incubated in the presence of 5 mm MnCl$_2$ and 750 $\mu M$ unlabeled ATP for 20 min at 25°C. After extensive washing the beads were resuspended in phosphorylation buffer with 1 mg/ml histone H$_{36}$. The kinase reaction was initiated by the addition of $^{[32P]}$ATP (5 $\mu M$ final concentration) and incubated at 25°C for 4 min. The samples were electrophoresed and the histone bands localized by autoradiography, excised, and the radioactivity determined by scintillation counting. $\square$, histone phosphorylation by control or $\square$, denervated derived receptors ($n = 3$/group).

Fig. 2. A, immunoprecipitation of the autophosphorylated insulin receptor. Equal insulin-binding activities of control and denervated derived WGA eluates were incubated in phosphorylation buffer without or with $10^{-3}$ M insulin for 1 h as described under "Methods." $\gamma$-$[^{32P}]$ATP (50 $\mu M$ final concentration, 7.5 $\mu Ci$/vial) was added and the autophosphorylation allowed to proceed for 4 min at 25°C; then anti-insulin receptor antibody (1:400 dilution) was added and incubated for 16 h at 4°C and for an additional 2 h with protein A-bearing formalin-fixed S. aureus (final 1% solution). After washing, the solution was electrophoresed under reducing conditions and autoradiographed. Lanes a and b, control; c and d, denervated derived receptors, without (a and c) or with $10^{-3}$ M insulin (b and d). B, insulin induced autophosphorylation of solubilized WGA-purified insulin receptors. WGA eluates were incubated without or with $10^{-3}$ or $10^{-5}$ M insulin as described in A. After 4 min of autophosphorylation with $\gamma$-$[^{32P}]$ATP (as in A) the reaction was terminated by adding Laemmli's sample buffer and boiling. After electrophoresis and autoradiography the excised $\beta$-subunit was digested and the radioactivity quantitated by scintillation counting. $\square$, control-derived receptors; $\square$, denervated muscle-derived receptors. Each value is the mean ± S.E. of 4 receptor preparations from control and denervated skeletal muscles, respectively, each assayed in triplicate.
were preincubated for 1 h in low phosphate DMEM, then for the protein content of individual muscles. Means under "Methods." Solei were immediately frozen and insulin receptors solubilized and partially purified by Method 2 and immunoprecipitated and processed as described in the legend to Fig. 2A. Values represent the mean ± S.E. for 5 experiments. Counts/min incorporated into the β-subunit were normalized to the wet weight of the muscles prior to incubation. B, solei from control and denervated hind limbs were incubated exactly as described for A except the specific activity of the [35S]phosphate was decreased to 1.5-2.5 Ci/ml. The muscles were frozen immediately following the 3-h incubation in DMEM and nucleotides extracted and separated as described under "Methods." The concentration of the nucleotides was normalized to the protein content of individual muscles. Means ± S.E. of 5 different experiments are shown; 6 control and denervated semi-solei were assayed individually in each experiment.

### Table I

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Control</th>
<th>Denervated</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>2.21 ± 0.28</td>
<td>2.23 ± 1.9</td>
</tr>
<tr>
<td>ADP</td>
<td>3.9 ± 0.6</td>
<td>4.2 ± 0.6</td>
</tr>
<tr>
<td>GTP</td>
<td>4.4 ± 0.8</td>
<td>4.2 ± 0.6</td>
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</tbody>
</table>

*p < 0.05 versus no insulin, paired analysis.

[Image of Fig. 4]

**FIG. 4. In vivo activation of the insulin receptor tyrosyl kinase.** Unilaterally denervated rats were anesthetized and injected intravenously with regular insulin (5 units) or with normal saline. After 5 min the hind limb muscles innervated by the sciatic nerve were removed and immediately frozen in liquid nitrogen. The insulin receptors were solubilized and partially purified on WGA by Method 2 described under "Methods." Equal insulin-binding activities of WGA eluates were incubated without or with 2 × 10⁻⁷ M insulin in phosphorylation buffer which was made 50 mM in MgSO₄, to precipitate the phosphatase inhibitors. The kinase reaction was initiated by adding either 5 or 50 μM γ⁻³²P]ATP and terminated after 4 min. After electrophoresis and autoradiography, the histone bands were excised, digested, and the radioactivity determined. The values plotted are the mean ± S.E. of duplicate determinations from four different control and denervated muscle receptor preparations. The schemes for the different treatments are indicated below the histogram and above the histone H₂B-containing area of a representative autoradiogram.
The differences in kinase activity between insulin receptors derived from insulin- and saline-treated rats may have been due to insulin carried through the receptor purification procedure or to a co-purified histone kinase activity that was independent of the insulin receptor. To test this, a second assay was performed. The WGA-purified receptors were incubated with insulin-bound agarose beads, and after centrifugation and washing, the kinase activity toward histone H₂b was assessed in a reaction using 5 μM γ-[^32P]ATP, a concentration which, as shown above, does not allow the further activation of the insulin receptor kinase. As in the previous assay, receptors isolated from insulin-treated rats showed increased histone kinase activity, and in vivo activation by insulin was not affected by denervation (Fig. 5). When these insulin-agarose-bound receptors were first allowed to autoprophosphorylate with 750 μM unlabeled ATP, the histone kinase activity increased to identical levels in all groups (Fig. 5). Histone phosphorylation after activation with 750 μM ATP was slightly lower than that observed after in vivo activation with insulin; this difference, although not significant, likely represents small losses of receptors during washing and/or slight dilution of the labeled ATP, resulting in decreased specific radioactivity. When WGA eluates were incubated with dithiothreitol-heat-inactivated insulin-agarose beads, the histone kinase activity observed was less than 10% of that seen in any of the experiments using fully activated beads, suggesting that the bulk of the kinase activity pertained to insulin receptors bound to the insulin-agarose. Phosphoamino acid analysis of the histones phosphorylated in the presence of insulin-agarose-bound receptors revealed considerable ^32P incorporation into serine residues, which, however, was not affected by pretreating the rats with insulin in vivo or by preincubating receptors from saline-treated controls with 750 μM ATP, while both maneuvers approximately doubled ^32P incorporation into tyrosine residues (Fig. 6). Identical increments were observed using receptors prepared from denervated muscles (not shown).

The intravenous insulin bolus stimulated the metabolism of innervated muscles, as assessed by the in vitro incorporation of [14C]glucose into glycogen. Denervated muscles were resistant to intravenously administered insulin in the same way as they are resistant to insulin in vivo (7–9). Innervated solei from nontreated animals had a low basal rate of glycogen synthesis (Fig. 7) which was stimulated by adding insulin in vitro. The 24-h denervated solei from saline-treated controls demonstrated a markedly reduced basal rate of glycogen synthesis, and this did not increase significantly upon addition of insulin to the incubation medium. On the other hand, solei from the innervated legs of insulin-treated animals synthesized twice as much glycogen than those from saline-treated controls when incubated without insulin, and this rate was not increased further by adding insulin in vitro. Stimulation of glycogen synthesis in nondenervated solei was apparently complete within 5 min after the intravenous bolus of insulin (Fig. 7) which correlates with the increase in the exogenous kinase activity of the receptors isolated from the ipsilateral muscle groups at this time. Attempts at investigating the activation after shorter time periods proved difficult in that at least 1 min was required to remove both solei and excise and freeze the remaining muscles. We could demonstrate nearly complete activation of both glucose incorporation into glycogen and exogenous kinase activity within 30–45 s after the intravenous injection of insulin; this result was variable, however (data not shown).

In these studies soleus muscles, consisting mainly of slow twitch oxidative fibers, were used to test the biological effects of insulin, while the receptors were purified from muscles with denervation.

**Fig. 5.** In vivo activated insulin receptor kinase assayed on insulin-agarose. Treatment of rats and insulin receptor solubilization is described in the legend to Fig. 4. WGA eluates prepared from control and denervated skeletal muscles (approximately 150 μl, with equal insulin-binding activity) were incubated with 50 μl of insulin-agarose and the resin washed as described under “Methods.” Part of the samples were allowed to autophosphorylate in the presence of 750 μM unlabeled ATP at 25 °C for 20 min. After washing, the insulin-agarose-bound receptors were resuspended in phosphorylation buffer with 1 mg/ml histone H₂b and the kinase reaction initiated by the addition of 10 μl of γ-[^32P]ATP (5 μM final concentration), stopped after 4 min, and the samples processed as described in the legend to Fig. 4. Panel A, samples obtained after 5 min in vivo treatment with either saline or insulin. Panel B, 30 min after treatment. The values plotted are the mean ± S.E. of duplicate determinations from 4–6 different control and denervated muscle receptor preparations. The schemes for the different treatments are indicated below the histograms and above the histone H₂b-containing area of a representative autoradiogram (5-min treatment experiment). The slightly lower histone phosphorylation in panel A was due to a lower concentration of insulin-binding activity used in these experiments.

**Fig. 6.** Phosphoamino acid analysis of ^32P-labeled histone. Histone H₂b was phosphorylated with 5 μM γ-[^32P]ATP as described in the legend to Fig. 5. The histones were eluted from the gel, precipitated, and hydrolyzed as described under “Methods.” The hydrolysate was dissolved in 5 μl of water containing 0.33 mg/ml each unlabeled phosphoserine, phosphothreonine, and phosphotyrosine, spotted on precoated cellulose plates, and electrophoresed for 40 min at 1000 V. The position of the phosphoamino acids was located by ninhydrin staining. Histones were phosphorylated by insulin receptors obtained from innervated muscles from rats treated with saline (lane A); saline controls with receptor kinase activated in vitro by preincubation of the insulin-agarose-bound receptors with 750 μM unlabeled ATP (lane B); or muscles from animals treated in vivo for 5 min with insulin to activate receptor kinase (lane C). Identical results were obtained from histone samples phosphorylated with denervated muscle-derived receptor preparations.
In Vitro and in Vivo Activation of Insulin Receptor Kinase

**FIG. 7. Glycogen synthesis by control and denervated solei.** Rats were injected with saline or insulin as described in the legend to Fig. 4, and after 5 or 30 min the soleus muscles were removed and incubated for 30 min in a balanced salt solution containing 5 mM [14C]glucose (0.3 μCi/ml) without or with 10 milliunits/ml insulin. The muscles were digested in 30% KOH and the glycogen precipitated and washed as described in Ref. 9. The radioactivity in glycogen was determined by scintillation counting and the nmol of glucose incorporated into glycogen was related to the media specific activity and normalized to the tissue wet weight. Panel A, 5-min in vivo treatment. Panel B, 30-min in vivo treatment. The schemes for the different treatments are indicated below the histograms.

In the course of these studies we developed techniques to assess the in vivo activation state of the insulin receptor in skeletal muscle. The methods are based on the apparent quantitative relationship between the phosphorylation state of the insulin receptor β subunit and its kinase activity toward exogenous substrate (27, 28), the observation that in vitro receptor tyrosyl kinase activation is critically dependent on the ATP concentration even in the presence of saturating insulin concentrations (28, 37), and the fact that under steady state conditions insulin binding is not affected by the phosphorylation state of the receptor (27). We reasoned that if in vivo the phosphorylation state of the receptor depends on the ambient insulin concentration, this should be reflected in its tyrosyl kinase activity in vitro, provided that the in vivo phosphorylation state of the receptor is not altered during extraction, purification, and assay. Rapid freezing of the excised muscles in liquid nitrogen, solubilizing, and purifying the insulin receptors in the presence of phosphoprotein-phosphatase and kinase inhibitors (NaF, Na4P207, Na2VO4, and EDTA) allowed the demonstration of increased histone H2B phosphorylation by WGA eluates from animals which had been treated in vivo with insulin. Two methods were used to clear the solution of EDTA and inorganic phosphate (which would chelate the Mn2+ ions required for activation). In the first assay, MgSO4 was added in sufficient quantities to remove the chelators. Using 5 μM γ-[32P]ATP no stimulation of histone kinase activity was seen when insulin was added to the incubation medium prior to the initiation of the kinase reaction. The inability to activate the exogenous substrate kinase of the receptor with insulin at this low ATP concentration has been attributed to the decreased phosphorylation of a critical site on the insulin receptor β-subunit (38, 37). By raising the ATP concentration to 50 μM an increase in the histone kinase activity could be demonstrated in the WGA eluates in response to maximally stimulating insulin concentrations in vitro; as expected the increment caused by insulin in vitro was much greater using receptors prepared from controls than from insulin-treated rats. It is noteworthy that while the base-line histone phosphorylation occurred predominantly on serine residues, the increment in histone phosphorylation elicited by insulin in vivo or in vitro occurred exclusively on tyrosine.

In the second method the insulin receptors were further purified by binding them to insulin-agarose, thus removing them from the WGA eluate containing Mn2+-chelating anions as well as enzymes which may modify receptor-induced histone phosphorylation. Preliminary reports described a similar approach for assessing the activation state of the receptor in isolated cell systems (40, 41). The results obtained with this method (Fig. 5) confirmed the preceding ones (Fig. 4). Insulin-
agarse-bound receptors prepared from insulin-treated rats demonstrated ~2-fold greater histone Hb phosphorylation in the presence of 5 μM γ-[32P]ATP than identically prepared receptors from saline-treated controls. When both sets of insulin-agarse-bound receptors were preincubated with unlabeled 750 μM ATP, a concentration which fully activates the receptor tyrosyl kinase (28), histone phosphorylation by receptors from saline-treated controls was activated to the same level as that by receptors from rats treated in vivo with insulin. The insulin-binding activity of the WGA eluates from insulin- and saline-treated rats was determined before incubation with insulin-agarse, and the beads were incubated with equal binding activities. In preliminary experiments insulin-binding curves prepared from WGA eluates derived from insulin- and saline-treated rats revealed no difference in binding kinetics between the groups, confirming the observations of Rosen et al. (27) with in vitro phosphorylated receptors. Furthermore, addition of Mn2+ and ATP to binding assays did not alter the apparent affinity of the insulin receptor (not shown).

It is unlikely that our data were compromised by insulin carried over through the receptor purification procedure because (a) when [35S]-insulin was included in muscle homogenates, with or without unlabeled insulin (10 μg/ml), less than 0.1% of the radioactivity was associated with the WGA eluate and this was nonspecifically bound; (b) when 0.5 unit/ml (20 μg/ml) insulin was included in the buffer in which muscles from saline-treated controls were homogenized, no activation of the receptor tyrosyl kinase was detected in assays of the WGA eluate. Similarly, inclusion of 40 mM ATP in the homogenization buffer did not affect the subsequent measurements of histone kinase activity in any group. WGA eluates prepared from skeletal muscles were devoid of detectable phosphoprotein phosphatase activity (not shown). ATPase activity was assayed under a number of experimental conditions by measuring the radioactivity associated with γ-[32P]ATP and with inorganic phosphate, separated by high pressure liquid chromatography (as described under "Methods") after incubation of the receptor preparations with γ-[32P]ATP. Less than 10% of γ-[32P]ATP was degraded after 4 min of incubation in any histone kinase assay (or in assays of receptor autophosphorylation), and this measurement was not affected by previous treatments, e.g., insulin or denervation. While significant serine-kinase activity was associated with the receptor preparations, even in the assay using insulin-agarse-bound receptors, receptor-induced histone phosphorylation on serine residues was unchanged after treating rats with insulin in vivo or after activating the receptors in vitro. On the other hand, both maneuvers markedly stimulated the phosphorylation of histone Hb on tyrosine residues (Fig. 6).

Since denervated muscles demonstrate decreased sensitivity as well as a decreased response to insulin (5–9) we injected high concentrations of the hormone to elicit maximal responses in both the denervated and innervated muscles. In innervated muscles both receptor activation and the biological response (assessed as glucose incorporation into glycogen) were essentially fully stimulated within 5 min of such injections. In denervated muscles, identical activation of the receptor tyrosine kinase by insulin was not accompanied by the biological response. In experiments where receptor histone-kinase activity and stimulation of glycogen synthesis were measured in parallel 5 and 30 min after the intravenous injection of insulin, the responses were essentially identical at the two time points, suggesting that phosphorylation of the tyrosine, which is critical for activation of the receptor tyrosyl kinase, occurs rapidly and persists in vivo. Dose-response curves injecting lower doses of insulin have not yet been tested; they may be required for the quantitative assessment of receptor activation in different physiological and pathological conditions. While skeletal muscle is a major site of insulin action, similar measurements should be feasible in other tissues as well. The method for assessing the activation state of the insulin receptor in skeletal muscle in vivo is relatively rapid, easily applicable to various animal models of insulin resistance, and in contrast to cell culture systems, the availability of tissue is not limiting. It should be useful in characterizing insulin-resistant states by identifying conditions which may be associated with defective activation of the β-subunit tyrosyl kinase upon insulin binding in vivo.

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