Alteration of Intramolecular Disulfides in Insulin Receptor/Kinase by Insulin and Dithiothreitol: Insulin Potentiates the Apparent Dithiothreitol-Dependent Subunit Reduction of Insulin Receptor†

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ABSTRACT: Dithiothreitol (DTT) was observed to increase both β-subunit autophosphorylation and exogenous substrate phosphorylation of the insulin receptor in the absence of insulin. The natural protein reducing agent thioredoxin was also observed to increase the insulin receptor β-subunit autophosphorylation. The activation of the insulin receptor/kinase by both DTT and thioredoxin was found to be additive with that of insulin. Further, the increase in the insulin receptor β-subunit autophosphorylation in the presence of DTT and insulin was demonstrated to be due to an increase in the initial rate of autophosphorylation without alteration in the extent of phosphorylation. Similarly, the increase in the exogenous substrate phosphorylation was due to an increase in the V_{max} of phosphorylation without significant effect on the apparent K_m of substrate binding. In the presence of relatively low concentrations of DTT, insulin was found to potentiate the apparent insulin receptor subunit reduction of the native $\alpha_2\beta_2$ heterotetrameric complex into $\alpha\beta$ heterodimers, when observed by silver staining of sodium dodecyl sulfate–polyacrylamide gels. *N*-[^{3}H]Ethylmaleimide ([^{3}H]NEM) labeling in the absence of DTT pretreatment demonstrated that only the β subunit had accessible sulfhydryl group(s). However, treatment of insulin receptors with DTT increased the amount of [^{3}H]NEM labeling in the β subunit as well as exposing sites on the α subunit. Further, incubation of the insulin receptors with the combination of DTT and insulin also demonstrated the apparent insulin-potentiated subunit reduction without any increase in the total amount of [^{3}H]NEM labeling. These results suggest that the insulin activation of the insulin receptor/kinase involves an increased sensitivity of the insulin receptor to reducing agents without any alteration in the total number of accessible sulfhydryl groups.

It is generally accepted that the initial event of insulin action is the binding of insulin to its receptor on the cell surface of target cells. The insulin receptor has been proposed to be an integral membrane glycoprotein composed of two M_r 130 000 (α) and two M_r 95 000 (β) subunits, linked by disulfide bonds into a M_r 350 000–400 000 heterotetrameric complex (Hedo et al., 1981; Massague et al., 1981; Van Obberghen et al., 1981; Fujita-Yamaguchi, 1984; Boyle et al., 1985). Photoaffinity labeling (Yip et al., 1978, 1980; Yeung et al., 1980) and affinity cross-linking studies (Jacobs et al., 1979; Pilch & Czech, 1980a,b) have suggested that the α subunit contains the high-affinity insulin binding site. The β subunit has been well documented to exhibit insulin-stimulated tyrosine-specific protein kinase activity and undergoes autophosphorylation predominantly on tyrosine residues in cell-free systems (Avruch et al., 1982; Kasuga et al., 1982a,b, 1983a,b; Petruzzelli et al., 1982, 1984; Shia & Pilch, 1983; Tamura et al., 1983; Zick et al., 1983). However, insulin receptor β-subunit autophosphorylation in vivo occurs not only on tyrosine residues but also on threonine and serine residues in response to insulin binding (Kasuga et al., 1982a; White et al., 1985). Direct affinity labeling studies with adenosine 5′-triphosphate (ATP)[1] analogues have further demonstrated that the β subunit contains an ATP binding site (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen et al., 1983), consistent with the β subunit being the catalytic protein kinase subunit and the α subunit being the regulatory subunit.

While the biological role of tyrosine phosphorylation in insulin receptor function is not known, the molecular events responsible for the activation of the intracellular β-subunit kinase domain upon insulin binding to the extracellular α-subunit binding domain are of fundamental importance to our understanding of the transmembrane signaling process. Recently, the entire amino acid sequence of the human placental insulin receptor precursor has been independently deduced from two complementary DNA clones (Ebina et al., 1985; Ullrich et al., 1985); however, the molecular mechanism of transmembrane signal transduction still remains unresolved.

Over the past several years, there has been increasing evidence for the role of biological disulfides and disulfide–sulfhydryl exchange reactions in a variety of metabolic events (Ernest & Kim, 1974; Freedman, 1979; Vauquelin & Maquire, 1980; Mukherjee & Mukherjee, 1981; Gilbert, 1982). In particular, sulfhydryl reagents have been reported to inhibit insulin biological responsiveness (Cadenas et al., 1961; Carter & Martin, 1969; Czech et al., 1974; Clark & Harrison, 1983), and reducing agents have been observed to act as insulin-omimetic agents (Goko et al., 1981; Crettaz et al., 1984). Directly related to these observations, the insulin receptor/kinase activity has recently been shown to be inhibited by the

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[1] Abbreviations: SDS, sodium dodecyl sulfate; NEM, *N*-ethylmaleimide; DTT, dithiothreitol; ATP, adenosine 5′-triphosphate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; Cl_3CCOOH, trichloroacetic acid.

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highly specific sulfhydryl alkylating reagent *N*-ethylmaleimide (NEM) and to be activated by the reducing agent DTT (Shia et al., 1983; Zick et al., 1983; Pike et al., 1984; Fujita-Yamaguchi & Kathuria, 1985). In this paper, we provide evidence demonstrating that the insulin stimulation of the insulin receptor/kinase occurs via an insulin-induced conformational change of the insulin receptor resulting in an increased sensitivity to apparent reduction by DTT.

Experimental Procedures

Materials

Affi-Gel 10, silver staining kits, Triton X-100, and reagents for SDS–polyacrylamide gel electrophoresis were all obtained from Bio-Rad. Centricons, *n*-octyl β-D-glucopyranoside, and Sephacryl S-400 were purchased from Amicon, Cal Biochem, and Pharmacia, respectively. NCS tissue solubilizer and 3MM filter paper were purchased from Amersham and Whatman, respectively. Molecular weight standards, protease inhibitors, ATP, dithiothreitol, *N*-ethylmaleimide, and the synthetic polymer Glu-Tyr (4:1) were purchased from Sigma. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{3}H]NEM (80 Ci/mmol) were from New England Nuclear. XAR-5 film and Cronex lightning plus enhancing screens were obtained from Eastman Kodak. Porcine insulin was a gift from Dr. R. Chance, Eli Lilly Co. Thioredoxin was graciously provided by Dr. E. Stellwagen, The University of Iowa, and monoiodinated [A^{14}]iodoinsulin was kindly provided by the Diabetes and Endocrinology Research Center, The University of Iowa.

Methods

Purification of Insulin Receptor. Insulin receptors were purified from freshly obtained full-term human placentas as previously described (Boyle et al., 1985; Sweet et al., 1985). Briefly, placental membranes were prepared as described by Harrison and Itin (1980). The membranes (10 mg/mL) were solubilized in 2.0% Triton X-100 for 1 h at 4 °C in 10 mM Tris-HCl, pH 8.0, 250 mM sucrose, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 25 mM benzamidine hydrochloride, 10 μM leupeptin, 0.05 trypsin inhibitor unit/mL aprotinin, 1 mM 1,10-phenanthroline, and 1 μM pepstatin A. After sedimentation at 100000*g* for 1 h, the supernatant was directly applied to a Sephacryl S-400 column (5.0 × 100 cm) equilibrated with 50 mM Tris-HCl, pH 8.0, 0.1% Triton X-100, and 0.02% NaN_3. The peak of insulin binding was pooled and applied to an insulin–agarose (0.8 mg of insulin/mL of Affi-Gel 10) column (1.6 × 10 cm). The column was extensively washed with 50 mM Hepes, pH 7.4, 1.0 M NaCl, and 0.6% *n*-octyl β-D-glucopyranoside in order to exchange the Triton X-100. The insulin receptors were eluted from the insulin–agarose column with 50 mM sodium acetate, pH 5.0, 1.0 M NaCl, 10% glycerol, and 0.6% *n*-octyl β-D-glucopyranoside and immediately neutralized with 1.0 M Hepes, pH 8.0. The eluent was concentrated on Centricon 30 microconcentrators, washed with 50 mM Hepes, pH 7.4, 10% glycerol, and 0.6% *n*-octyl β-D-glucopyranoside, and stored at 4 °C until used.

Insulin Receptor Autophosphorylation. Insulin receptors were incubated for 1 h in the presence or absence of 200 nM insulin in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, and 5 mM $MnCl_2$. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1.0 mM ATP, 3.0 μCi/nmol) to obtain a final concentration of 100 μM ATP. The reaction was allowed to continue for various times at 23 °C and was terminated by the addition of Laemmli sample buffer (50 mM Tris-HCl, pH 6.9, 10% glycerol, 0.05% bromophenol blue, and 1.0% SDS) containing 100 mM DTT and subjected to SDS–polyacrylamide gel electrophoresis.

Insulin Receptor Exogenous Substrate Phosphorylation. Insulin receptors were incubated with various concentrations of Glu-Tyr polymer (4:1) for 1 h in the presence or absence of 200 nM insulin and 1 mM DTT or of 200 nM insulin and 1 mM DTT in 50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, and 5 mM $MnCl_2$. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1.0 mM ATP, 3.0 μCi/nmol) to obtain a final concentration of 100 μM ATP. The reaction was allowed to continue for 15 min at 23 °C and was terminated by the addition of the sample to 3MM Whatman filter paper with immersion in ice-cold 10% Cl_3CCOOH and 10 mM NaH_2PO_4. Filters were washed with 2 L of the above buffer and dried; scintillation fluid was added and counted for ^{32}P.

SDS–Polyacrylamide Gel Electrophoresis. One-dimensional gel electrophoresis was performed by using either a 3–10% gradient or a 7% separating gel and a 3% stacking gel [acrylamide:bis(acrylamide) ratio of 37.5:1] as described by Laemmli (1970). Insulin receptor samples were treated under various conditions as described in the figure legends. The gels were fixed followed by staining with silver or Coomassie Brillant Blue R250 and dried. Coomassie-stained gels were then autoradiographed on Kodak XAR-5 film with Cronex lightning plus enhancing screens. The M_r 95 000 β-subunit bands were excised, rehydrated, solubilized with NCS tissue solubilizer, mixed with scintillation solution, and counted for ^{32}P.

[^{3}H]NEM Labeling. Insulin receptors in Krebs–Ringer–phosphate buffer (10 mM NaH_2PO_4, 5.1 mM KCl, 1.3 mM $CaCl_2$, and 1.3 mM $MgSO_4$, pH 7.4) were incubated with various concentrations of DTT in the presence or absence of 200 nM insulin as described in the figure legends. The samples were extensively dialyzed and subsequently incubated with 10 μM [^{3}H]NEM (50 Ci/mmol) for 1 h at 23 °C. The reaction was stopped by the addition of 10 mM unlabeled NEM, and the samples were mixed with Laemmli sample buffer with or without 100 mM DTT prior to SDS–polyacrylamide gel electrophoresis. The gels were then sliced into 1-mm sections, solubilized with NCS, mixed with scintillation solution, and counted for the covalent incorporation of [^{3}H]NEM.

Results

Apparent Insulin-Potentiated DTT-Dependent Reduction of the Insulin Receptor. We have previously observed that the purified human placental insulin receptor can readily undergo intramolecular disulfide–sulfhydryl exchange when heated prior to SDS–polyacrylamide gel electrophoresis (Boyle et al., 1985). This process was found to result in subunit dissociation of the $\alpha_2\beta_2$ heterotetrameric complex into $\alpha_2\beta$, α_2, and monomeric β species. However, insulin receptor preparations which were not heated or treated with sulfhydryl and/or oxidizing agents before SDS–polyacrylamide gel electrophoresis preserved the native $\alpha_2\beta_2$ heterotetrameric subunit structure. In order to further characterize this phenomenon, we initially examined the effect of dithiothreitol (DTT) and insulin treatment on the subunit structure of the purified insulin receptor without heating prior to SDS–polyacrylamide gel electrophoresis (Figure 1). In the absence of reductant, the insulin receptor primarily exists as a heterotetrameric disulfide-linked complex with M_r ~400 000 (lanes 1 and 2). This complex has been well documented to have the subunit stoichiometry of $\alpha_2\beta_2$ (Jacobs & Cuatrecasas, 1983; Pessin et al., 1985). The other two high molecular weight bands with M_r ~350 000 and ~300 000 correspond to small amounts of proteolytic degradation products of the $\alpha_2\beta_2$ complex into $\alpha_2\beta\beta_1$ and $\alpha_2(\beta_1)_2$ forms, respectively

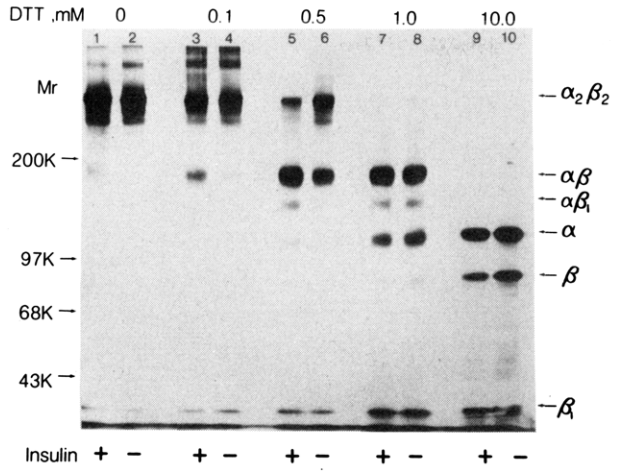

FIGURE 1: Effect of DTT and insulin on the silver-stained gel profile of purified human placental insulin receptors. Insulin receptors (2.0 μg) were diluted into Krebs–Ringer–phosphate buffer and incubated with various concentrations of DTT listed in the figure for 1 h at 23 °C. The samples were then incubated for an additional 1 h in the presence (lanes 1, 3, 5, 7, and 9) or absence (lanes 2, 4, 6, 8, and 10) of 200 nM insulin. The samples were then prepared for electrophoresis by the addition of Laemmli sample buffer and electrophoresed in a 3–10% SDS–polyacrylamide gradient gel under nonreducing conditions without heating as described under Experimental Procedures. The gel was then fixed and processed for silver staining.

(Massague et al., 1981; Fujita-Yamaguchi, 1984). The addition of DTT results in a concentration-dependent reduction of the $\alpha_2\beta_2$ heterotetrameric complex, as observed by silver staining of the SDS–polyacrylamide gels (Figure 1) similar to that reported for [^{125}I]insulin affinity-labeled receptors (Massague & Czech, 1982). Treatment of the insulin receptors with 1.0 mM DTT (lanes 5 and 6) generates mostly $\alpha\beta$ heterodimers, and after treatment with 10 mM DTT (lanes 7 and 8), the insulin receptors were found to be almost completely dissociated into monomeric α and β subunits. In contrast, insulin treatment in the presence of low DTT concentrations markedly enhanced the relative amount of subunit reduction into $\alpha\beta$ heterodimers compared to the insulin receptors treated with DTT alone. This apparent insulin-potentiated DTT-dependent reduction of the insulin receptor is clearly observed at 0.1 and 0.5 mM DTT which results in an increase in the relative amount of $\alpha\beta$ heterodimers with a concomitant decrease in the amount of the $\alpha_2\beta_2$ heterotetrameric complex (compare lane 3 to lane 4 and lane 5 to lane 6). At higher DTT concentrations, in which $\alpha\beta$ heterodimeric subunit structures began to predominate, insulin was unable to potentiate any further reduction by DTT.

This apparent insulin-potentiated reduction of the insulin receptor was also observed when the insulin receptors were ^{32}P labeled by autophosphorylation with [γ-^{32}P]ATP (data not shown). To examine whether this effect of insulin to potentiate the apparent insulin receptor reduction by DTT occurred prior to or during SDS–polyacrylamide gel electrophoresis, NEM was added to the samples immediately before the electrophoresis process in order to alkylate all accessible sulfhydryl groups. Under these conditions, results similar to those shown in Figure 1 (data not shown) were observed.

Insulin, DTT, and Thioredoxin Stimulation of the Insulin Receptor/Kinase. Since insulin is known to stimulate the insulin receptor/kinase activity and insulin potentiates DTT-dependent subunit reduction of the insulin receptor, we next examined the effect of insulin and DTT on β-subunit autophosphorylation (Table I). Under these conditions of phosphorylation, 100 μM [γ-^{32}P]ATP for 5 min at 23 °C, insulin typically stimulates β-subunit autophosphorylation 1.5–2.0-fold. The addition of DTT (0.2 mM) was found to stimulate β-subunit autophosphorylation approximately 3.0-fold and is apparently additive with that observed for the insulin stimulation of β-subunit autophosphorylation (Table I). On the basis of the ability of DTT to stimulate the insulin receptor/kinase, we also examined the effect of thioredoxin, a naturally occurring protein-reducing agent, on insulin receptor β-subunit autophosphorylation (Table I). Thioredoxin was found to be capable of maximally stimulating insulin receptor β-subunit autophosphorylation to approximately the same extent as does insulin (1.5–2.0-fold), unlike DTT which maximally stimulates insulin receptor β-subunit autophosphorylation 3–4-fold. Under these assay conditions, the maximum activation of β-subunit autophosphorylation occurred at approximately 1.0 μM thioredoxin (data not shown). Similar to that observed for the DTT stimulation, the thioredoxin stimulation is apparently additive with that of insulin (Table I).

Table I: Effects of Insulin, Dithiothreitol (DTT), and Thioredoxin on Insulin Receptor β-Subunit Autophosphorylation[a]

treatment	rel β-subunit autophosphorylation	treatment	rel β-subunit autophosphorylation
untreated	1.0	untreated	1.0
insulin (200 nM)	1.6 ± 0.1	insulin (200 nM)	1.7 ± 0.1
DTT (0.2 mM)	3.0 ± 0.2	thioredoxin (1.0 μM)	1.6 ± 0.1
insulin + DTT	4.3 ± 0.4	insulin + thioredoxin	2.7 ± 0.2

[a] Purified insulin receptors (1.0 μg) were incubated for 30 min at 23 °C under each condition prior to the addition of 100 μM [γ-^{32}P]ATP for 5 min. The ^{32}P-autophosphorylated β subunit was separated by SDS–polyacrylamide gel electrophoresis under fully reducing conditions. The values represent four independent determinations with their respective standard errors of the mean.

Time Course of Insulin Receptor β-Subunit Autophosphorylation. To determine if insulin and DTT stimulation of the insulin receptor β-subunit autophosphorylation occurs due to a change in the initial rate and/or extent of phosphorylation, a time dependence of [γ-^{32}P]ATP autophosphorylation was performed (Figure 2). At short times of incubation with [γ-^{32}P]ATP, there was a significant change in the initial rate of β-subunit autophosphorylation. The initial autophosphorylation rates were found to be 0.21, 0.32, 0.78, and 1.32 pmol of ^{32}P/min for control, insulin, DTT, and insulin plus DTT, respectively. However, at relatively long incubation times with [γ-^{32}P]ATP, the extent of insulin receptor β-subunit autophosphorylation was found to be identical, saturating at approximately 1.8 mol of ^{32}P incorporated/mol of β subunit. Thus, both insulin and DTT increased the efficiency of the insulin receptor/kinase to autophosphorylate and apparently do not expose any additional phosphorylation sites on the β subunits.

Exogenous Substrate Phosphorylation. Since the insulin receptor/kinase contains both the phosphotransferase activity and also the phosphoacceptor sites, we next examined the effects of insulin and DTT on the phosphotransferase activity using an exogenous substrate. The synthetic polymer Glu-Tyr (4:1) is an excellent substrate for the insulin receptor/kinase (Braun et al., 1985) and should not be affected by reducing agents due to the absence of cysteine residues. A concentration dependence of Glu-Tyr polymer phosphorylation in the absence and presence of insulin and DTT is shown in Figure 3. The apparent K_m of substrate binding under these various conditions was found not to be significantly different, ranging from 2.1 to 3.0 μM. Consistent with the alterations observed in the

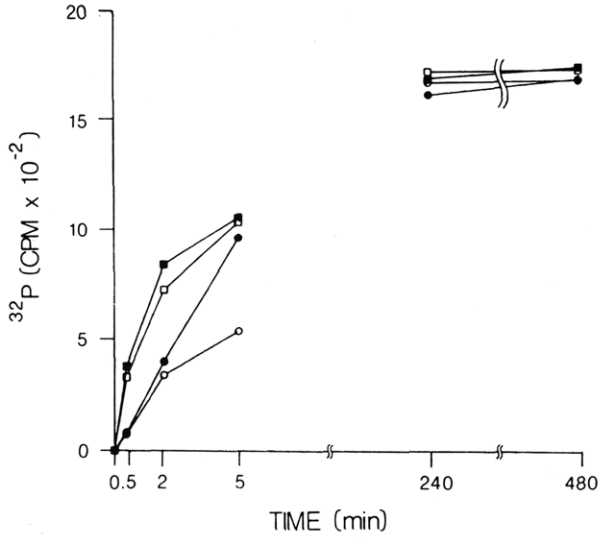

FIGURE 2: Time course of insulin receptor [γ-^{32}P]ATP β-subunit autophosphorylation in the presence and absence of insulin and DTT. Insulin receptors (0.6 μg) were incubated in the absence of insulin (○), in the presence of 200 nM insulin (●), in the presence of 1.0 mM DTT (□), or in the presence of 200 nM insulin plus 1.0 mM DTT (■) for 1 h at 23 °C. The samples were subsequently autophosphorylated with 100 μM [γ-^{32}P]ATP for the times shown at 23 °C as described under Experimental Procedures. The reaction was stopped by the addition of Laemmli sample buffer containing 100 mM DTT and electrophoresed in a 7.0% SDS–polyacrylamide gel. The gel was processed for autoradiography. The β subunit was excised, hydrated, solubilized, and prepared for scintillation counting as described under Experimental Procedures.

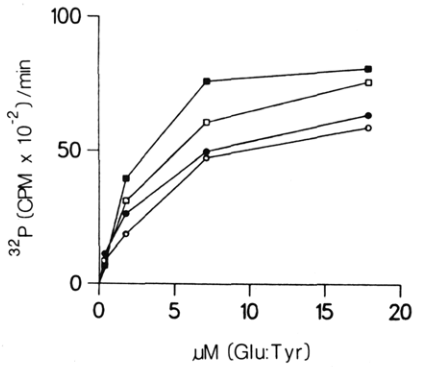

FIGURE 3: Exogenous substrate concentration dependence of insulin receptor/kinase. Insulin receptors (0.25 μg) were incubated in the absence of insulin (○), in the presence of 200 nM insulin (●), in the presence of 1.0 mM DTT (□), or in the presence of 200 nM insulin plus 1.0 mM DTT (■) for 1 h at 23 °C. The kinase reaction was initiated with 100 μM [γ-^{32}P]ATP for 15 min at 23 °C. The samples were then processed as described under Experimental Procedures.

initial rates of autophosphorylation (Figure 2), insulin and DTT both stimulated the V_{max} of Glu-Tyr polymer phosphorylation, with values of 1.95, 2.13, 2.52, and 2.70 pmol of ^{32}P/min for control, insulin, DTT, and DTT plus insulin, respectively.

NEM Inhibition of Insulin Receptor/Kinase Activity. NEM has been reported to inhibit the insulin-stimulated insulin receptor/kinase activity (Shia et al., 1983; Pike et al., 1984). We have observed that 10 mM NEM will completely abolish the insulin receptor β-subunit autophosphorylation not only in the insulin-stimulated state but also in the control and DTT-stimulated states as well (Figure 4). Further, we have repeatedly observed that the DTT stimulation of the insulin receptor/kinase also results in the autophosphorylation of the α subunit, although to a relatively small degree compared to the β subunit. However, in the presence of insulin, the amount

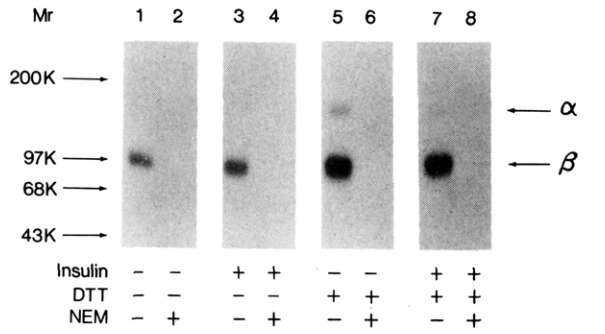

FIGURE 4: *N*-Ethylmaleimide inhibition of insulin receptor [γ-^{32}P]ATP β-subunit autophosphorylation. Insulin receptors (1.0 μg) were incubated in the absence of insulin (lanes 1 and 2), in the presence of 200 nM insulin (lanes 3 and 4), in the presence of 0.2 mM DTT (lanes 5 and 6), or in the presence of 200 nM insulin plus 0.2 mM DTT (lanes 7 and 8) for 1 h at 23 °C. The samples were then incubated for an additional 1 h at 23 °C in the absence (lanes 1, 3, 5, and 7) or presence (lanes 2, 4, 6, and 8) of 10 mM NEM. The samples were subsequently autophosphorylated with 100 μM [γ-^{32}P]ATP for 5 min at 23 °C as described under Experimental Procedures. The reaction was stopped by the addition of Laemmli sample buffer containing 100 mM DTT and heated at 100 °C for 30 s before electrophoresis in a 7.0% SDS–polyacrylamide gel. The gel was then processed for autoradiography.

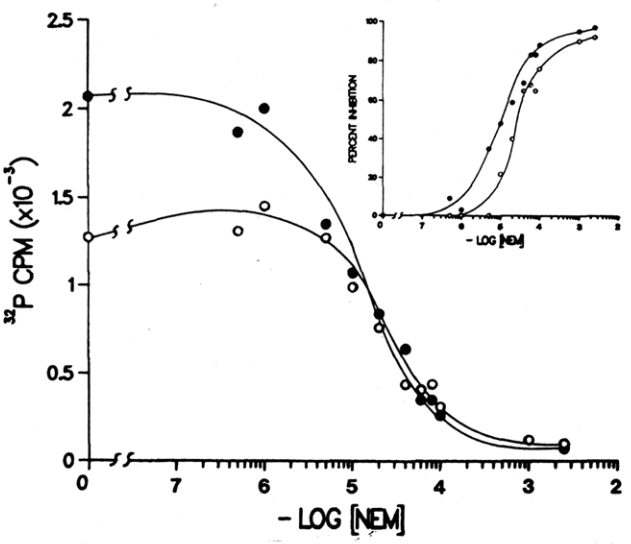

FIGURE 5: NEM concentration dependence of the inhibition of [γ-^{32}P]ATP β-subunit autophosphorylation of insulin receptor/kinase. Insulin receptors (0.5 μg) were incubated in the absence (○) or presence (●) of 200 nM insulin for 1 h at 23 °C. Samples were then incubated with various concentrations of NEM for 1 h at 23 °C. Autophosphorylation was carried out for 5 min at 23 °C as described under Experimental Procedures. Laemmli sample buffer containing 100 mM DTT was added to the samples which were then heated at 100 °C for 30 s before electrophoresis in a 7% SDS–polyacrylamide gel. The gels were processed for autoradiography. The β subunit was excised, hydrated, solubilized, and prepared for scintillation counting as described under Experimental Procedures.

of α-subunit autophosphorylation was consistently found to be greatly diminished (Figure 4).

The concentration dependence of NEM on the control and insulin-stimulated β-subunit autophosphorylation is shown in Figure 5. NEM inhibition of autophosphorylation was found to be slightly greater in the presence (IC_{50} = 15 μM) than in the absence (IC_{50} = 50 μM) of insulin. The 3-fold difference in NEM sensitivity is more readily apparent when plotted as a percentage of the β-subunit autophosphorylation in the absence of NEM (Figure 5, inset). This small alteration in sensitivity was also observed when a time course of NEM inhibition of the insulin receptor β-subunit autophosphorylation was examined (data not shown).

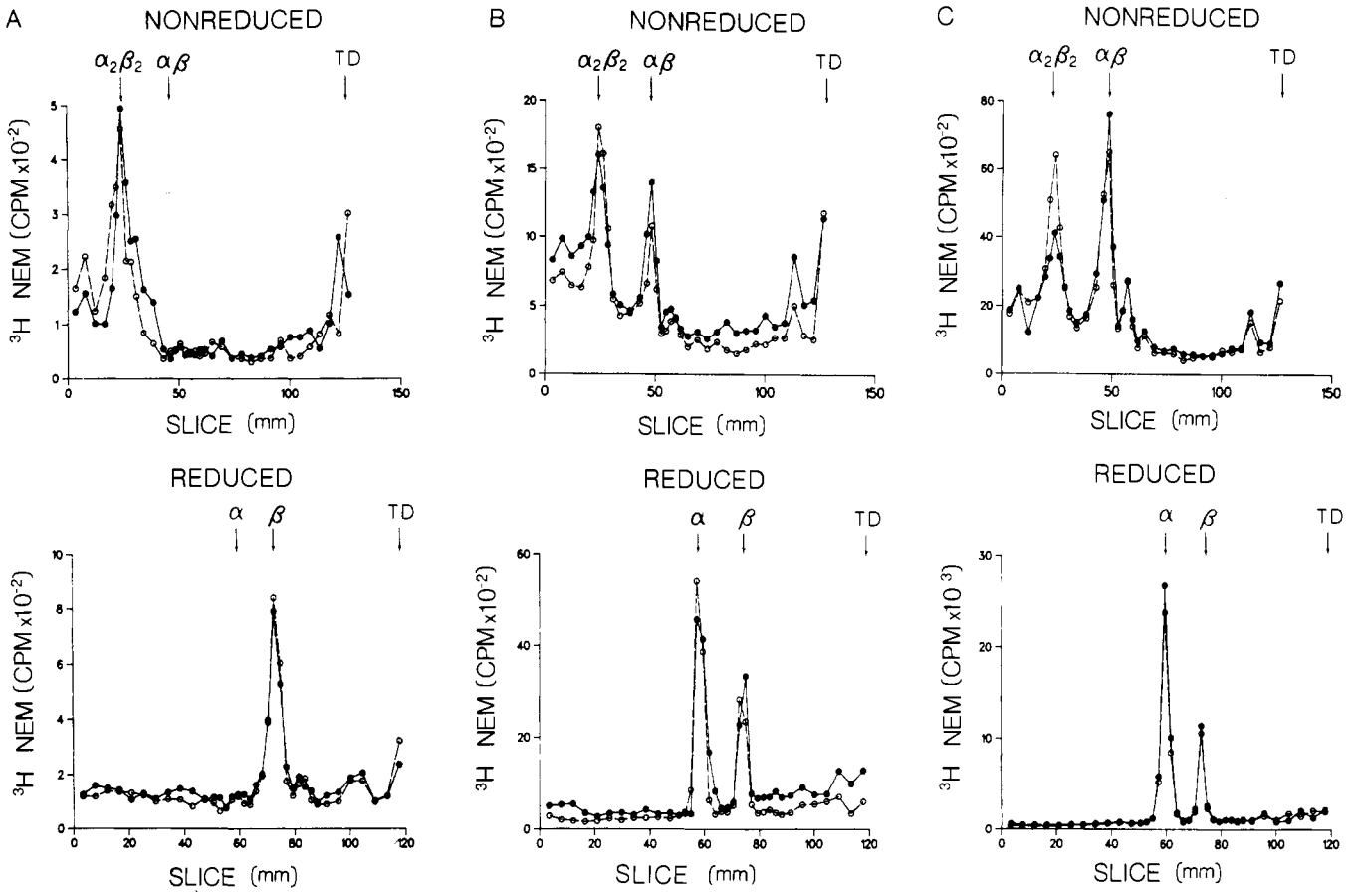

FIGURE 6: Effects of insulin and DTT on [^{3}H]NEM labeling of insulin receptors. Insulin receptors (2.0 μg) were incubated in the absence (O) or presence (●) of 200 nM insulin for 1 h at 23 °C. A second 1-h incubation was performed at 23 °C in the absence (A) or presence of 0.1 mM DTT (B) or 0.5 mM DTT (C). The samples were extensively dialyzed to remove DTT and then incubated with 10 μM [^{3}H]NEM (50 Ci/mmol) for 1 h as described under Experimental Procedures. Equal amounts of samples (panels A–C) were mixed either with Laemmli sample buffer (nonreduced, upper panels) or with Laemmli sample buffer containing 100 mM DTT (reduced, lower panels). The samples were electrophoresed in a 3–10% SDS–polyacrylamide gradient gel as described under Experimental Procedures. The gels were fixed, Coomassie stained, destained, cut into 2-mm slices, and prepared for scintillation counting. TD represents the position of the tracking dye gel front.

[^{3}H]NEM Labeling of the Insulin Receptor. The ability of NEM to inhibit the insulin receptor β-subunit autophosphorylation (Figure 4) coupled with the stimulation of the insulin receptor/kinase by reductants (Table I, Figures 2 and 3) and the insulin-potentiated DTT-dependent subunit reduction of the insulin receptor heterotetrameric structure (Figure 1) strongly suggests the involvement of sulfhydryl group(s) in the regulation of insulin receptor function. We therefore examined the susceptibility of the insulin receptor to [^{3}H]NEM labeling under a variety of conditions (Figure 6). Insulin receptors were incubated with 10 μM [^{3}H]NEM in the presence and absence of insulin and subjected to nonreduced and reduced SDS gel electrophoresis (Figure 6A). The amount of [^{3}H]NEM labeling was essentially identical whether or not the insulin receptors were first preincubated with insulin prior to the addition of [^{3}H]NEM. In the nonreduced SDS–polyacrylamide gel, all the [^{3}H]NEM label was found to be completely located in the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex. Surprisingly, complete reduction of the insulin receptors into monomeric α and β subunits, subsequent to [^{3}H]NEM labeling, demonstrated that only the β subunit had accessible sulfhydryl group(s) despite the fact that the α subunit contains nearly 3 times the number of cysteine residues (Ebina et al., 1985; Ullrich et al., 1985). In a second set of experiments, the insulin receptors were incubated with 0.1 (Figure 6B) or 0.5 mM DTT (Figure 6C) in the presence and absence of insulin, dialyzed to remove the DTT, and then subjected to [^{3}H]NEM labeling. Separation of the insulin receptors in nonreducing SDS–polyacrylamide gels demonstrated a relative decrease in the amount of [^{3}H]NEM-labeled $\alpha_2\beta_2$ heterotetrameric complex concomitant with a relative increase in the amount of [^{3}H]NEM-labeled $\alpha\beta$ heterodimeric species after insulin treatment. This was found to occur without any alteration in the total amount of [^{3}H]NEM-labeled insulin receptors. Upon full reduction, the α subunits were now observed to be labeled to a greater extent than the β subunits, consistent with an increased number of accessible cysteine residues in the α subunits after DTT pretreatment. Although there was an increase of the amount of [^{3}H]NEM labeling in both the α and β subunits, we have found that at these concentrations of NEM and DTT no significant alteration of insulin binding was detectable (data not shown).

Discussion

The propensity of the insulin receptor to autodissociate (Boyle et al., 1985), presumably as a result of disulfide–sulfhydryl rearrangement and the involvement of reducing and sulfhydryl agents on insulin action in vivo (Cadenas et al., 1961; Carter & Martin, 1969; Czech et al., 1974, 1976; Goke et al., 1981; Clark & Harrison, 1982, 1983, 1985; Crettaz et al., 1984), has suggested to us a possible role for disulfide–sulfhydryl event(s) in the activation of insulin receptor function. In support of this concept, we and others (Shia et al., 1983; Petruzzelli et al., 1984; Fujita-Yamaguchi & Kathuria, 1985) have observed that reductants will directly activate the

purified insulin receptor/kinase (Table I and Figures 2 and 3). The effects of reducing agents such as DTT and the naturally occurring reductant thioredoxin were found to activate the insulin receptor/kinase, and this stimulation was observed to be approximately additive with the insulin receptor/kinase activation by insulin (Table I). Thioredoxin is a small heat-stable ubiquitous protein which has been reported to be an endogenous activator of glucocorticoid receptor binding in rat liver (Grippo et al., 1985) and is also thought to supply reducing equivalents for the activation of chloroplast coupling factor 1 ATPase in intact chloroplasts (Mills et al., 1980). Similar to the effects of DTT (Shia et al., 1983; Fugita-Yamaguchi & Kathuria, 1985), thioredoxin was found to activate the insulin receptor/kinase in a concentration-dependent manner and to the same extent as does insulin. However, the magnitude of the thioredoxin-stimulated β-subunit autophosphorylation was found to be significantly less than that observed for the DTT-stimulated autophosphorylation. Since the cytoplasm from tissues such as liver has high levels of endogenous reducing activity, it is tempting to speculate that thioredoxin may be directly involved in insulin receptor function in vivo.

Activation of the insulin receptor autophosphorylation by reducing agents could occur either by an increase in the phosphotransferase activity of the insulin receptor/kinase and/or by induction of a change in the substrate phosphoacceptor sites. Since the insulin receptor is both the enzyme and the substrate, we examined the time course of β-subunit autophosphorylation (Figure 2) and the concentration dependence of exogenous substrate phosphorylation (Figure 3) in order to distinguish between these possibilities. At short times in the presence of [γ-^{32}P]ATP, there is marked activation of the initial rate of autophosphorylation by insulin and DTT. In contrast, no significant change in the overall extent of β-subunit autophosphorylation after relatively long incubation times was detectable (Figure 2). These results suggest that the total number of substrate acceptor sites is unchanged after insulin and DTT treatment. The apparent K_m values for the phosphorylation of the synthetic polymer Glu-Tyr (4:1) ranged from 2.1 to 3.0 μM under all the conditions examined. This is in excellent agreement with previous reports for the insulin receptor/kinase in the control and insulin-stimulated states (Braun et al., 1984; Pike et al., 1984). Consistent with an increase in the insulin receptor phosphotransferase activity, the phosphorylation of Glu-Tyr (4:1) polymer was observed to be stimulated by both insulin and DTT, with turnover numbers of 97, 106, 126, and 135 pmol of ^{32}P min^{-1} (mg of insulin receptors)$^{-1}$ for control, insulin, DTT, and DTT plus insulin, respectively (Figure 3). Thus, the activation of insulin receptor/kinase by DTT is similar to that observed for insulin activation, and both result in an increase in the phosphotransferase activity of the insulin receptor/kinase.

Incubation of the $\alpha_2\beta_2$ heterotetrameric insulin receptor complex at relatively low DTT concentrations in the presence of insulin was also found to result in a greater degree of reduction into $\alpha\beta$ heterodimers compared to the insulin receptors treated with DTT alone (Figure 1). In these experiments, the insulin receptor preparations were mixed with Laemmli sample buffer containing 1.0% SDS and directly loaded onto the polyacrylamide gels without heating in order to prevent the heat/SDS-dependent subunit dissociation reported previously (Boyle et al., 1985). To rule out the possibility that the various incubation conditions in Figure 1 resulted in differential oxidation of DTT, the absolute number of reducing equivalents was determined by using Ellman's reagent [5,5'-dithiobis(2-nitrobenzoic acid)]. Under all the above incubation conditions, there was no measurable change in the amount of reducing equivalents.[2] It should be noted that this apparent insulin-potentiated DTT-dependent reduction of the insulin receptor heterotetrameric form ($\alpha_2\beta_2$) into the $\alpha\beta$ heterodimeric form has been examined only under conditions involving SDS–polyacrylamide gel electrophoresis.

Nevertheless, these results document an insulin-induced conformational change within the $\alpha_2\beta_2$ heterotetrameric complex that results in a greater degree of sensitivity to reduction by DTT. Several laboratories have reported insulin receptor conformational changes in response to insulin binding. These have included an increase in sensitivity of the α subunit to exogenously added proteases (Pilch & Czech, 1980; Donner & Yonkers, 1983) and changes in chromatographic gel profiles in detergent solution (Krupp & Livingston, 1978; Donner & Yonkers, 1983; Maturo et al., 1983). This apparent insulin-potentiated DTT-dependent reduction of the $\alpha_2\beta_2$ heterotetrameric complex is consistent with these other reports of insulin-induced conformational changes and suggests that these conformational changes may be related to the activation of the insulin receptor/kinase.

We (Figure 4) and others (Shia et al., 1983; Zick et al., 1983; Pike et al., 1984) have observed that NEM is also able to inhibit the insulin receptor/kinase activity directed toward itself or exogenous substrates. To examine the possible involvement of sulfhydryl groups in the insulin and DTT activation of the insulin receptor/kinase activity, the insulin receptors were labeled with [^{3}H]NEM (Figure 6). These studies indicated that only the β subunit of the insulin receptor has accessible sulfhydryl group(s) despite the fact that the α subunit contains more than 3 times as many cysteine residues in the primary amino acid sequence (Ebina et al., 1985; Ullrich et al., 1985). Insulin had no apparent effect on the total amount of [^{3}H]NEM labeling of the β subunit (Figure 6). In contrast, a previously reported study (Maturo et al., 1983) indicated that insulin can enhance the amount of [^{3}H]NEM labeling of the intact insulin receptor. Although we cannot directly account for these differences, our preparation is significantly different primarily due to the purification of the insulin receptor by insulin–agarose affinity chromatography and use of *n*-octyl β-D-glucopyranoside instead of Triton X-100. Treatment of the insulin receptors with low concentrations of DTT was found to dramatically increase the amount of [^{3}H]NEM labeling in the α subunit as well as exposing new sulfhydryl groups in the β subunit. These studies further demonstrated the ability of insulin to potentiate the apparent DTT-dependent subunit reduction into $\alpha\beta$ heterodimers from the native $\alpha_2\beta_2$ heterotetrameric insulin receptor complex. It should also be noted that at 0.1 mM DTT the proportion of $\alpha\beta$ heterodimers relative to the $\alpha_2\beta_2$ complex is relatively small (Figure 1) yet the extent of [^{3}H]NEM labeling into the $\alpha\beta$ halves is equal to the amount in the $\alpha_2\beta_2$ complex (Figure 6). This indicates a high degree of intramolecular disulfide bonds within the α subunit, consistent with a highly cysteine-rich region in the α subunit (Ebina et al., 1985; Ullrich et al., 1985).

The stimulation of the insulin receptor/kinase by reductants and the inhibition by sulfhydryl alkylation, coupled with the insulin-potentiated DTT-dependent subunit reduction of the native $\alpha_2\beta_2$ heterotetrameric insulin receptor complex, strongly suggest the involvement of disulfide–sulfhydryl events in the insulin activation of the insulin receptor/kinase. We would speculate that insulin binding to the α subunit of the insulin

[2] P. A. Wilden, T. R. Boyle, and J. E. Pessin, unpublished results.

receptor results in a conformational change which increases the accessibility of the disulfide bonds between the α subunits to reduction and may be critical to the kinase activation process. Alternatively, since insulin does not contain any reducing equivalents, it is possible that insulin binding results in a disulfide–sulfhydryl rearrangement such that the number of disulfide and sulfhydryl groups remains constant. This disulfide–sulfhydryl rearrangement could then account for the increased sensitivity of the insulin receptor to reducing agents. These models are currently being examined with respect to the mechanisms of insulin-induced transmembrane activation of the insulin receptor/kinase.

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REFERENCES

Avruch, J., Nemenoff, R. A., Blackshear, P. J., Pierce, M. W., & Osathanondh, R. (1982) *J. Biol. Chem. 257*, 15162–15166.

Boyle, T. R., Campana, J., Sweet, L. J., & Pessin, J. E. (1985) *J. Biol. Chem. 260*, 8593–8600.

Braun, S., Raymond, W. E., & Racker, E. (1984) *J. Biol. Chem. 259*, 2051–2054.

Cadenas, E., Kaji, H., Park, C. R., & Rasmussen, H. (1961) *J. Biol. Chem. 236*, PC63–PC64.

Carter, J. R., & Martin, D. B. (1969) *Biochim. Biophys. Acta 177*, 521–526.

Clark, S., & Harrison, L. C. (1982) *J. Biol. Chem. 257*, 12239–12244.

Clark, S., & Harrison, L. C. (1983) *J. Biol. Chem. 258*, 11434–11437.

Clark, S., & Harrison, L. C. (1985) *Biochem. J. 229*, 513–519.

Crettaz, M., Jialal, I., Kassaga, M., & Kahn, C. R. (1984) *J. Biol. Chem. 259*, 11543–11549.

Czech, M. P. (1976) *J. Cell. Physiol. 89*, 661–668.

Czech, M. P. Lawrence, J. C., & Lynn, W. S. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 4173–4177.

Donner, D. B., & Yonkers, K. (1983) *J. Biol. Chem. 258*, 9413–9418.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E. Ou, J.-H., Masiarz, F., Kan, Y. W., Goldfine, I. D., Roth, R. A., & Rutter, W. J. (1985) *Cell (Cambridge, Mass.) 40*, 747–758.

Ernest, J. J., & Kim, K.-H. (1974) *J. Biol. Chem. 249*, 5011–5018.

Freedman, R. B. (1979) *FEBS Lett. 97*, 201–210.

Fujita-Yamaguchi, Y. (1984) *J. Biol. Chem. 259*, 1206–1211.

Fujita-Yamaguchi, Y., & Kathuria, S. (1985) *Proc. Natl. Acad. Sci. U.S.A. 82*, 6095–6099.

Gilbert, H. F. (1982) *J. Biol. Chem. 257*, 12086–12091.

Goko, H., Takashima, S., Kawamuro, A., & Matsuoka, A. (1981) *Biochem. J. 200*, 425–428.

Grippo, J. F., Holmgren, A., & Pratt, W. B. (1985) *J. Biol. Chem. 260*, 93–97.

Harrison, L. C., & Itin, A. (1980) *J. Biol. Chem. 255*, 12066–12072.

Hedo, J. A., Kasuga, M., Van Obberghen, E., Roth, J., & Kahn, C. R. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 4791–4795.

Jacobs, S., & Cuatrecasas, P. (1983) *Annu. Rev. Pharmacol. Toxicol. 23*, 461–479.

Jacobs, S., Hazum, E., Shechter, Y., & Cuatrecasas, P. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 4918–4921.

Kasuga, M., Zick, Y., Blithe, D. L., Karlsson, F. A., Haring, H. U., & Kahn, C. R. (1982a) *J. Biol. Chem. 257*, 9891–9894.

Kasuga, M., Karlsson, F. A., & Kahn, C. R. (1982b) *Science (Washington, D.C.) 215*, 185–187.

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., White, M. F., & Kahn, C. R. (1983a) *J. Biol. Chem. 258*, 10973–10980.

Kasuga, M., Fujita-Yamaguchi, Y., Blithe, D. L., & Kahn, C. R. (1983b) *Proc. Natl. Acad. Sci. U.S.A. 80*, 2137–2141.

Krupp, M. N., & Livingston, J. N. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 2593–2597.

Laemmli, U. K. (1970) *Nature (London) 227*, 680–685.

Massague, J., & Czech, M. P. (1982) *J. Biol. Chem. 257*, 6729–6738.

Massague, J., Pilch, P. F., & Czech, M. P. (1981) *J. Biol. Chem. 256*, 3182–3190.

Maturo, J. M., Hollenberg, M. D., & Aglio, L. S. (1983) *Biochemistry 22*, 2579–2586.

Mills, J. D., Mitchell, P., & Schurmann, D. (1980) *FEBS Lett. 112*, 173–177.

Mukherjee, S. P., & Mukherjee, C. (1981) *Biochim. Biophys. Acta 677*, 339–349.

Pessin, J. E., Mottola, C., Yu, K.-T., & Czech, M. P. (1985) in *Molecular Basis of Insulin Action* (Czech, M. P., Ed.) pp 3–29, Plenum Press, New York.

Petruzzelli, L. M., Ganguly, S., Smith, C. J., Cobb, M. H., Rubin, C. S., & Rosen, O. M. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 6792–6796.

Petruzzelli, L. M., Herrera, R., & Rosen, O. M. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 3327–3331.

Pike, L. J., Kuenzel, E. A., Casnellie, J. E., & Krebs, E. G. (1984) *J. Biol. Chem. 259*, 9913–9921.

Pilch, P. F., & Czech, M. P. (1980a) *Science (Washington, D.C.) 210*, 1152–1153.

Pilch, P. F., & Czech, M. P. (1980b) *J. Biol. Chem. 255*, 1722–1731.

Roth, R. A., & Cassell, D. J. (1983) *Science (Washington, D.C.) 219*, 299–301.

Shia, M. A., & Pilch, P. F. (1983) *Biochemistry 22*, 717–721.

Shia, M. A., Rubin, J. B., & Pilch, P. F. (1983) *J. Biol. Chem. 258*, 14450–14455.

Sweet, L. J. Wilden, P. A., Spector, A. A., & Pessin, J. E. (1985) *Biochemistry 24*, 6571–6580.

Tamura, S., Fujita-Yamaguchi, Y., & Larner, J. (1983) *J. Biol. Chem. 258*, 14749–14752.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzzelli, L. M., Dull, T. J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P. H., Grunfeld, C., Rosen, O. M., & Ramachandran, J. (1985) *Nature (London) 313*, 756–761.

Van Obberghen, E., Kasuga, M., LeCam, A., Hedo, J. A., Itin, A., & Harrison, L. C. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1052–1056.

Van Obberghen, E., Rossi, B., Kowalski, A., Gazzano, H., & Ponzio, G. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 945–949.

Vauquelin, G., & Maquire, M. E. (1980) *Mol. Pharmacol. 18*, 362–369.

White, M. F., Takayama, S., & Kahn, C. R. (1985) *J. Biol. Chem. 260*, 9470–9478.

Yeung, C. W. T., Moule, M. L., & Yip, C. C. (1980) *Biochemistry 19*, 2196-2203.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1978) *J. Biol. Chem. 253*, 1743-1745.
Yip, C. C., Yeung, C. W. T., & Moule, M. L. (1980) *Biochemistry 19*, 70-76.
Zick, Y., Kasuga, M., Kahn, C. R., & Roth, J. (1983) *J. Biol. Chem. 258*, 75-80.

Biochemical Signal Transmitted by Fc Receptor for Immunoglobulin G_{2a} of a Murine Macrophage-like Cell Line, $P388D_1$: Mode of Activation of Adenylate Cyclase Mediated by Immunoglobulin G_{2a} Binding Proteins†

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ABSTRACT: The effects of immunoglobulin G_{2a} binding proteins isolated from $P388D_1$ cells on adenylate cyclase of cyc^- cells were investigated to explore a potential role of $Fc\gamma_{2a}$ receptor in the activation of the adenylate cyclase system. Immunoglobulin G (IgG) binding proteins obtained from the detergent lysate of $P388D_1$ cells by affinity chromatography on IgG–Sepharose were separated into two fractions (denoted as IgG-B1 and IgG-B2) by Sephadex G-100 gel filtration in the presence of 6 M urea. Polyacrylamide gel electrophoretic analysis in the presence of sodium dodecyl sulfate revealed that the major component in the IgG-B1 fraction was a protein of molecular weight near 50000, whereas the IgG-B2 fraction contained two major components of molecular weight near 25000 and 17000. Both IgG-B1 and -B2 proteins can be inserted into liposome consisting of phosphatidylcholine and phosphatidylethanolamine. Liposomes containing IgG-B1 proteins effectively inhibited EA_{2a}, but not EA_{2b}, rosetting by either S49 or $P388D_1$ cells, suggesting their proper orientation within liposome, whereas IgG-B2-containing liposome failed to do so. Simultaneous fusion of the liposomes containing IgG-B1 and -B2 proteins with guanine nucleotide binding stimulatory (G/F) protein/$Fc\gamma_{2a}$R-deficient cyc^- cells resulted in the formation of the hybrid membrane whose adenylate cyclase responds to immune complex formed with IgG_{2a}-subclass antibody (IC_{2a}) by about a 2.7-fold increase in the activity over the control (hybrid membrane between cyc^- cells and liposome containing no protein). The response appeared to be specific, since IC_{2b} failed to stimulate the enzymatic activity of this hybrid membrane. Furthermore, IgG-B1 and -B2 proteins were able to confer their activating effects on the enzyme only in concert, since the fusion of liposomes containing either type of protein alone with cyc^- cells did not result in the activation of adenylate cyclase of cyc^- membrane. IgG-B1 and -B2 proteins could also confer their activating effects in concert to the enzyme in cholate-solubilized forms. Such activation was dependent on the concentration of IC_{2a}, suppressed by the chelating agent ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid, and significantly inhibited by trifluoperazine, suggesting potential involvement of Ca^{2+} and calmodulin in the activating process. Since the activation of adenylate cyclase of cyc^- cells mediated by IgG-B1 and -B2 proteins was not augmented in vitro by either addition of NaF or deletion of GTP and since IgG-B1 and -B2 proteins inserted into liposome or in cholate-solubilized form had neither detectable GTPase activity nor a cholera toxin sensitive ADP-ribosylation site, the mechanism by which liposome-inserted or cholate-solubilized IgG-binding proteins confer their activating effect on cyc^- cell adenylate cyclase may be different from that mediated by G/F protein in a classical manner.

Murine macrophages and macrophage-like cell lines such as $P388D_1$ carry on their surface at least two biochemically distinct Fc receptors, one specific for IgG_{2a} ($Fc\gamma_{2a}R$)[1] and another for IgG_{2b} ($Fc\gamma_{2b}R$) (Walker, 1976; Heusser et al., 1977; Unkeless, 1977; Anderson & Grey, 1978; Suzuki et al., 1982). $Fc\gamma R$ plays an essential role in antibody-dependent cell-mediated cytotoxicity (Perlman et al., 1972), suppression of humoral immune response (Uhr & Moller, 1968) or B cell differentiation (Kolsch et al., 1980) by circulating immune complexes, or triggering of prostaglandin synthesis by macrophages (Passwell et al., 1979, 1980; Bonney et al., 1979). Two types of $Fc\gamma R$ present on the surface of macrophages then should transmit, upon binding of specific ligands, signals unique to each type that trigger and maintain a complex program of biochemical events, involving the generation, amplification, and propagation of a series of signals, leading to

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[1] Abbreviations: EA, SRBC coated with IgG-class monoclonal anti-SRBC antibody; EDTA, ethylenediaminetetraacetate; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid; $Fc\gamma R$, receptor for the Fc portion of IgG; G/F protein, guanine nucleotide binding stimulatory protein; Gpp(NH)p, 5′-guanylyl imidodiphosphate; HBSS, Hank's balanced salt solution without Mg^{2+} and Ca^{2+}; IC_{2a} and IC_{2b}, immune complexes formed with IgG_{2a}- and IgG_{2b}-subclass monoclonal antibodies, respectively; IgG, immunoglobulin G; Ni, guanine nucleotide binding inhibitory protein; PBS, phosphate-buffered saline (0.15 M, pH 7.4); PC, *rac*-1-(9-carboxynonyl)-2-hexadecylglycero-3-phosphocholine; PEG, poly(ethylene glycol); PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; SRBC, sheep erythrocytes; TBS, Tris-HCl-buffered saline (0.15 M, pH 8); TCA, trichloroacetic acid; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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