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Robert E. Lewis
University of Massachusetts Medical School

Michael P. Czech
University of Massachusetts Medical School, Michael.Czech@UMassmed.edu

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Phospholipid environment alters hormone-sensitivity of the purified insulin receptor kinase

Robert E. LEWIS and Michael P. CZECH
Department of Biochemistry, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01605, U.S.A.

Insulin receptor kinase, affinity-purified by adsorption and elution from immobilized insulin, is stimulated 2–3-fold by insulin in detergent solution. Reconstitution of the receptor kinase into leaky vesicles containing phosphatidylcholine and phosphatidylethanolamine (1:1, w/w) by detergent removal on Sephadex G-50 results in the complete loss of receptor kinase sensitivity to activation by insulin. Insulin receptors in these vesicles also exhibit an increase in their apparent affinity for ^125^I-insulin ($K_a = 0.12$ nM versus 0.76 nM). Inclusion of 8.3–16.7% phosphatidylserine into the reconstituted vesicles restores 40–50% of the insulin-sensitivity to the receptor kinase. An elevated apparent affinity for ^125^I-insulin of insulin receptors in vesicles containing phosphatidylcholine and phosphatidylethanolamine is also restored to the value observed in detergent solution by the inclusion of phosphatidylserine in the reconstituted system. The effect of phosphatidylserine on insulin receptor kinase appears specific, because cholesterol, phosphatidylinositol and phosphatidic acid are all unable to restore insulin-sensitivity to the receptor kinase. Autophosphorylation sites on the insulin receptor as analysed by h.p.l.c. of tryptic ^32^P-labelled receptor phosphopeptides are not different for insulin receptors autophosphorylated in detergent solution or for the reconstituted vesicles in the presence or absence of phosphatidylserine. These data indicate that the phospholipid environment of insulin receptors can modulate its binding and kinase activity, and phosphatidylserine acts to restore insulin-sensitivity to the receptor kinase incorporated into phosphatidylcholine/phosphatidylethanolamine vesicles.

INTRODUCTION

The insulin receptor is a transmembrane protein, composed of two $\alpha$ and two $\beta$ subunits, responsible for transducing information from the hormone insulin to the cell interior (Czech, 1985; Jacobs & Cuatrecasas, 1983). Examination of the deduced sequence of the human placental insulin receptor from the isolated cDNA demonstrated regions of marked homology between the insulin-receptor $\beta$ subunit and the family of src-related tyrosine kinases (Ulrich et al., 1985; Ebina et al., 1985). The $\beta$ subunits span the membrane, and have been shown to be phosphorylated on serine and tyrosine residues in intact cells (Kasuga et al., 1982a,b; Haring et al., 1982), and to undergo insulin-dependent phosphorylation (Petruzzelli et al., 1982; Avruch et al., 1982; Roth & Cassel, 1983; Zick et al., 1983). Phosphorylation of the receptor activates the insulin-sensitive kinase (Rosen et al., 1983; Yu & Czech, 1984). Kinetic studies with different peptide substrates have suggested that the specificity of the insulin-receptor-associated kinase is similar to the src-related kinases and to the epidermal-growth-factor receptor kinase (Stadtmueller & Rosen, 1983; Pike et al., 1984).

Factors which regulate the insulin receptor kinase activity are of major interest because the kinase may be intimately involved in one or more receptor functions. The lipid environment has been suggested as a modulator of insulin receptor activity. Insulin receptors reconstituted in crude form from turkey erythrocytes into liposomes were found to have a higher affinity for insulin in a more saturated lipid environment (Gould et al., 1982). Additionally, increasing the degree of unsaturation of membrane phospholipids resulted in both an increase in the available number of binding sites and a decrease in the affinity of these sites in Friend erythroleukaemia cells (Ginsberg et al., 1981). Effects of lipid composition on insulin receptor kinase activity have not been reported. Sweet et al. (1985) previously reconstituted purified insulin receptors in permanent phospholipid vesicles, with the binding site exposed on the outer surface of the liposomes. The aim of the present investigation was to evaluate the effects of lipids on the insulin receptor kinase by using a highly purified receptor preparation and a well-defined lipid environment. We have utilized an affinity-purification protocol to purify kinase-active insulin receptor from human placenta. The purified receptor has been reconstituted into liposomes of defined composition. We demonstrate in this paper that: (1) insulin receptors reconstituted into phosphatidylcholine/phosphatidylethanolamine vesicles lose insulin-sensitive kinase activity; (2) inclusion of phosphatidylserine in phosphatidylcholine/phosphatidylethanolamine vesicles restores insulin-sensitivity to the reconstituted insulin receptor; (3) phosphatidylinositol, phosphatidic acid and cholesterol do not restore insulin-sensitivity to reconstituted insulin receptors; (4) receptor reconstitution into phosphatidylcholine/phosphatidylethanolamine vesicles correlates with an increased high-affinity binding of insulin which is not observed in phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine vesicles.

EXPERIMENTAL

Materials

Wheat-germ-agglutinin–Sepharose was from Pharmacia (Piscataway, NJ, U.S.A.). Affigel and Enzymobeads were from Bio-Rad (Rockville Centre,
NY, U.S.A.). All phospholipids were from Supelco (Bellefonte, PA, U.S.A.). Cholesterol, N-acetyl-d-glucosamine, 4-phosphothreonine, phosphothreonine and phosphoserine were from Sigma (St. Louis, MO, U.S.A.). Histone 2B was obtained from Worthington (Melvern, PA, U.S.A.). [3H]phosphatidylcholine was purchased from New England Nuclear (Boston, MA, U.S.A.). Na131I and [γ-32P]ATP were purchased from Amersham (Arlington Heights, IL, U.S.A.). Trifluoroacetic acid was purchased from Pierce (Rockford, IL, U.S.A.). Propan-ol was from Burdick and Jackson Laboratories (Muskegon, MI, U.S.A.). Polygram gel 300 chromatography plates were purchased from Brinkmann Instruments (Westbury, NY, U.S.A.). Pig insulin was a gift from Dr. R. Chance, Eli Lilly Co. (Indianapolis, IN, U.S.A.).

Solubilization and purification of insulin receptors

Crude plasma membranes were prepared from human placenta by the method of Harrison & Itin (1980). Insulin receptor was purified by the method of Fujita-Yamaguchi et al. (1983). Membranes were solubilized in 50 mM-Tris/1 mM-phenylmethanesulphon fluoride/10 μM-leupeptin/1% Triton X-100, pH 7.6, by end-over-end mixing for 1 h at 4 °C. The membrane concentration at solubilization was 8–10 mg/ml. Solubilized membranes were centrifuged for 1 h at 100000 g in a Beckman 50.2 rotor. The resulting supernatant was diluted 10-fold with 50 mM-Tris/0.1 mM-phenylmethanesulphon fluoride/10 μM-leupeptin/0.1% Triton X-100 (buffer A) containing 10 mM-MgCl2, pH 7.6. The diluted supernatant (250–500 ml) was passed over 20 ml of wheat-germ-agglutinin-Sepharose and recycled over the column at 4 °C overnight. After washing the column with 10 column vol. of buffer A plus 10 mM-MgCl2, pH 7.6, the glycoproteins bound to the matrix were eluted with 0.3 M-N-acetyl-d-glucosamine. The resulting eluate was adjusted to 1 M with NaCl before being applied to 10 ml of insulin-Sepharose cross-linked at a ratio of approx. 0.8 mg of insulin/ml of Affigel. The wheat-germ eluate was passed twice over the insulin-Affigel at room temperature. The insulin-Affigel affinity column was then washed with 10 column vol. of 50 mM-Tris/1 M-NaCl/0.1% Triton X-100, pH 7.6. The column was eluted with 50 mM-sodium acetate/0.5 M-NaCl/0.1% Triton X-100, pH 5.5. Eluted receptor was collected in 10 ml fractions in tubes containing 5 ml of 0.5 M-Tris/HCl, pH 7.6. The peak binding fraction of purified receptor (fraction 2) was concentrated 3–6-fold on an Amicon concentrator with a YM30 membrane.

Insulin receptor reconstitution

The purified receptor was reconstituted by the method of Brunner et al. (1976) as modified by Shanahan & Czech (1977) for the glucose transporter. The concentrated purified receptor (0.5 ml) was added to a total of 12 mg of the indicated phospholipids, which had been previously evaporated to dryness under N2 and freeze-dried overnight. Then 10 mg of sodium cholate was added to the receptor/lipid mixture to make the final cholate concentration 2%. The mixture was vortex-mixed and sonicated in a bath sonicator until clear. After sonication, the solution was applied to an 18 cm x 0.7 cm Sephadex G-50 column pre-equilibrated with 10 mM-Tris/100 mM-NaCl/2 mM-MgSO4, pH 7.6, at 4 °C, and 0.5 ml fractions were collected. Vesicles were collected in the column void volume. Peak vesicle-containing fractions were monitored by the A280 and the addition of tracer [3H]phosphatidylcholine (60 Ci/nmol) to some preparations. Vesicle size was calculated from measurement of vesicle diffusion coefficients as determined by photon-correlation spectroscopy in a Coulter model N4 Submicron Particle Analyser with size-distribution processor.

Binding analysis

[125I]-insulin was prepared to a specific radioactivity of 130–200 Ci/g by the lactoperoxidase method using Enzymobeads. Specific binding to solubile and reconstituted insulin receptor was assessed by poly(ethylene glycol) precipitation and filtration. Briefly, insulin receptor was incubated with [125I]-insulin in a total volume of 0.5 ml of 50 mM-Tris/1% bovine serum albumin, pH 7.6, at 22 °C. Non-specific binding was determined by the addition of 100 nm unlabelled pig insulin to identical tubes. Precipitation of the labelled ligand–receptor complex was facilitated by rapid addition of 200 μl of ice-cold γ-globulin (2.5 mg/ml) in 50 mM-Tris, pH 7.6, and 0.5 ml of ice-cold 25% (v/v) poly(ethylene glycol) in 50 mM-Tris, pH 7.6. The mixture was quickly vortex-mixed and placed in an ice bath for 10 min. Filtration was performed on a Gilman filtration apparatus with 0.45 μm Millipore HA filters presoaked in 1% bovine serum albumin. Each filter was rapidly washed with 2 × 2.5 ml of ice-cold 8% poly(ethylene glycol)/0.025% Triton X-100 in 50 mM-Tris, pH 7.6. Radioactivity bound to filters was determined in a Beckman Gamma 5500 counter.

Receptor kinase assays

Soluble and reconstituted insulin receptors were incubated with 50 mM-Tris, pH 7.6, containing 10 mM-MgCl2, 3 mM-MnCl2, 1 mM-dithithreitol and in the presence or absence of 100 nm-insulin. Phosphorylation was initiated by addition of [γ-32P]ATP (final conc. 5 μM). In some reactions, histone 2B (0.3 mg/ml) was added to the reaction mixture. The final volume of each reaction was 100 μl. The reaction was terminated by adding an equal volume of electrophoresis sample buffer containing 62.5 mM-Tris, 20% (v/v) glycerol, 10% (w/v) SDS and 100 mM-dithithreitol, pH 6.8. All samples were boiled for 5 min. Samples containing histone were electrophoresed on 6–16%-polyacrylamide gels. After electrophoresis the gels were stained, destained, dried and autoradiographed with Kodak X-Omat film and enhancing screens.

Tryptic-peptide mapping by h.p.l.c.

Adequate identification of phosphorylated peptide fragments required increased radioactive labelling of the insulin receptor. Consequently, the specific radioactivity of [γ-32P]ATP used for these experiments was raised to 1 mCi/nmol. After phosphorylation, the sample was alkylated and reduced as follows: 100 μl of phosphorylated insulin receptor was combined with 110 μl of 28 mM-dithithreitol in 0% SDS and incubated at 80 °C for 15 min. The samples were cooled to 22 °C, and 54 μl of 800 mM-iodoacetamide in 0.25 M-Tris, pH 8.8, was added for 10 min. Next 68 μl of 25% (v/v) mercaptoethanol in 75% glycerol was added, and the mixture was incubated at 60 °C for a further 5 min. After reduction, 6 μl of 0.025% Bromophenol Blue was added
to each sample, and the samples were electrophoresed on a 7\%-polyacrylamide gel (two lanes/sample). The $^{32}$P-labelled $\beta$ subunit of the insulin receptor was localized after electrophoresis by autoradiography of the unfixed gel. Gel pieces corresponding to the $^{32}$P-labelled bands (1 cm x 2.5 cm) were excised and washed twice at 22 °C for 45 min with 50 ml of 50 mM-NH$_4$HCO$_3$ buffer. Each gel piece was then cut into five equal pieces and incubated with 4 ml of NH$_4$HCO$_3$ containing 50 $\mu$g of tosophenylalanychloromethane-treated trypsin/ml and incubated at 37 °C. A second addition of trypsin was made 15 h later, and incubation at 37 °C was continued for a further 10 h. The digestion product was removed and freeze-dried overnight. The dried digest was resuspended in 0.5 ml of 1\% trifluoroacetic acid and applied to a Waters h.p.l.c. reverse-phase $\mu$Bondapak C-18 column pre-equilibrated in 0.1\% trifluoroacetic acid. The $^{32}$P-labelled tryptic peptides were eluted from the column with a linear 0–40\% propan-1-ol gradient at 0.5 ml/min. Radioactivity was collected in 1 min fractions and quantified by Cerenkov counting.

RESULTS

Characterization of affinity-purified insulin receptor

The two-step affinity-purification method yields highly purified insulin receptor essentially identical with that prepared by Fujita-Yamaguchi et al. (1983). Electrophoresis of the receptor under non-reduced conditions yields 350 kDa, 320 kDa and 290 kDa silver-stained bands, whereas electrophoresis in the presence of reductant yields 125 kDa and 90 kDa bands (results not shown). The preparation appears homogeneous by silver staining, as no other silver-stained constituents are observed. The wheat-germ-agglutinin–agarose affinity column (15 ml) typically provides 90–100\% recovery of insulin receptor (assessed by $^{125}$I-insulin binding) from detergent-solubilized placental membranes. Some 20–40\% of the insulin-receptor band was recovered after insulin–Affigel affinity chromatography and elution of the wheat-germ-agglutinin–agarose eluate.

Characterization of reconstituted receptors

The purified insulin receptor preparation was reconstituted into membrane vesicles by passage of a detergent-soluble receptor/phospholipid mixture through Sephadex G-50 to remove the detergents Triton X-100 and sodium cholate. Insulin receptor binding and phospholipid were eluted simultaneously in the void volume of the column (Fig. 1a). Photon-correlation spectroscopy indicated the presence of a uniform population of phospholipid vesicles. Pearson V distribution-function analysis of correlation coefficients resulted in polydispersity factors of less than 0.3, indicating a highly uniform vesicle population (Fig. 1b) (McCully & Bargeron, 1977). The best angle of measurement was 60–90°, indicating that the vesicles behaved as spherical particles. Vesicle size did not vary appreciably when phospholipid composition was altered, except when high concentrations of phosphatidyserine (33\%) were used. Vesicles containing one-third phosphatidyserine by weight had vesicle diameters approx. twice that observed with all other vesicles in an experiment. The diameter of all other vesicle preparations ranged in different experiments from 32 to 51 nm, but were constant for reconstitutions performed within an experiment.

The transmembrane orientation of the insulin receptor was examined by testing the latency of receptor-catalysed histone phosphorylation in intact reconstituted vesicles compared with vesicles solubilized with 0.5\% Triton X-100 before the phosphorylation assay (Fig. 2). Insulin receptors oriented right-side-out in the reconstituted vesicles should have their insulin-binding domain oriented outside the vesicles. If the insulin receptor kinase is transmembrane to the binding site or sites (Ullrich et al., 1985) and the reconstituted vesicles are relatively impermeable to histone, then the rate of
Insulin receptor reconstituted in phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (11:1:2, by wt.) was incubated at 22 °C with end-over-end mixing in the absence or presence of 0.5% Triton X-100. Insulin was added to both groups after 40 min (final concn. 100 nM); 30 min later, each tube was adjusted to 10 mM-MgCl₂, 3 mM-MnCl₂, 0.3 mg of histone 2B/ml and 5 mM-[γ-³²P]ATP (50 μCi). Final Triton X-100 concentration was 0.4% in a final reaction volume of 100 μl. The phosphorylation reaction was terminated at various times by the rapid addition of 25 μl of 2.5 mg of γ-globulin/ml and 1 ml of 20% trichloroacetic acid. Samples were vortex-mixed and stored at 4 °C overnight. After precipitation the samples were centrifuged (27000 g for 5 min) and washed once with diethyl ether/ethanol (1:1, v/v; –20 °C). The samples were boiled in electrophoresis sample buffer, electrophoresed on a 6–16% acrylamide gradient gel, and the gel was stained with Coomassie Blue, destained, dried and autoradiographed. (a) Phosphorylation of reconstituted insulin receptor and histone in the absence or presence of 0.5% Triton X-100 at 10 min and 60 min incubation with [γ-³²P]ATP. (b) Histone phosphorylation at various times of vesicle incubation with (●) or without (○) Triton X-100 was assessed by scanning densitometry of the autoradiograph.

Histone phosphorylation should be decreased in intact vesicles relative to solubilized vesicles. Fig. 2(b) demonstrates that intact vesicles containing insulin receptor exhibit a rate of histone phosphorylation approximately half that of the detergent-solubilized vesicles, suggesting that the vesicular structure inhibits the ability of the kinase to phosphorylate histone. The β subunit of the insulin receptor also exhibits latency of phosphorylation in the intact vesicles (Fig. 2a). The latency of β-subunit phosphorylation is probably due to the rate of ATP diffusion to insulin receptor kinase oriented inside the liposome. The latency of histone phosphorylation is determined by the rate of diffusion of histone and ATP into the liposome. Since histone is larger, its rate of diffusion should be slower, accounting for the greater latency of phosphorylation relative to the insulin–receptor β subunit (Fig. 2a). Scatchard analysis of reconstituted insulin receptor with or without Triton X-100 to solubilize the vesicles yielded identical binding curves (results not shown).

**Phospholipid effects on reconstituted insulin receptor kinase**

Insulin-sensitive autophosphorylation of insulin receptors is present throughout purification (Fig. 3). However, reconstitution of kinase-active insulin receptor into phosphatidylcholine/phosphatidylethanolamine vesicles (1:1, w/w) results in a loss of insulin-stimulated β-subunit autophosphorylation. The loss of sensitivity of reconstituted insulin receptor kinase to activation by insulin is not due to sequestration of insulin-binding sites or the inability of ATP to penetrate the vesicles (Fig. 2, and results not shown). In fact, phosphorylation of the β subunit is observed in phosphatidylcholine/phos-
Insulin receptor reconstitution

Insulin receptor was reconstituted into liposomes containing equal amounts of phosphatidylcholine and phosphatidylethanolamine and increasing amounts of phosphatidyserine. Total lipid was kept constant at 12 mg. Phosphorylation was performed for 30 min as described in the Experimental section. (a) Autoradiograph of insulin receptor phosphorylated after reconstitution in phosphatidylcholine/phosphatidylethanolamine vesicles or phosphatidylcholine/phosphatidylethanolamine vesicles containing 8.3%, 16.7% or 33% phosphatidyserine. The autoradiograph was scanned by densitometry and the resulting peaks were cut out and weighed. The difference between control and insulin stimulation in phosphatidylcholine/phosphatidylethanolamine vesicles and phosphatidylcholine/phosphatidylethanolamine vesicles containing phosphatidyserine is illustrated in (b). Control values were 33.0, 34.2, 28.0 and 15.6 mg for 0%, 8.3%, 16.7% and 33.3% phosphatidyserine respectively.

Inclusion of phosphatidyserine into phosphatidylcholine/phosphatidylethanolamine vesicles restored insulin-sensitivity to reconstituted insulin receptor when the phosphatidyserine was added as either 8.3% or 16.7% of the total lipid by wt. (Fig. 4). Whereas insulin stimulated phosphorylation of the receptor β subunit by only 10% in phosphatidylcholine/phosphatidylethanolamine vesicles, the stimulation was 48% and 66% in vesicles containing 8.3% and 16.7% phosphatidyserine respectively. Restoration of insulin-sensitivity by phosphatidyserine was 40–50% of the insulin stimulation of soluble insulin receptor kinase. Although phosphatidyserine conferred insulin-sensitivity to the reconstituted insulin receptor kinase in all five experiments performed, it elevated basal kinase activity in only two of five experiments. Phosphorylation of insulin receptor reconstituted into vesicles containing 33% phosphatidyserine (by wt.) showed markedly decreased kinase activity and no insulin-sensitivity. Reconstituted vesicles prepared with 33% phosphatidyserine tended to have twice the diameter (70–100 nm) of other preparations, and samples of the vesicles had lower receptor content, as assessed by silver stain. This suggests that vesicles containing 33% phosphatidyserine (by wt.) are not suitable for reconstitution of insulin receptor.

**H.p.l.c. tryptic-peptide mapping of the reconstituted insulin receptor β subunit**

In order to determine whether changing the phospholipid environment affected phosphorylation of different or identical sites within the receptor β subunit, soluble insulin receptor or insulin receptor reconstituted in phosphatidylcholine/phosphatidylethanolamine (1:1, w/w) or phosphatidylcholine/phosphatidylethanolamine/phosphatidyserine (11:11:2, by wt.) was incubated with 5 μM-[γ-32P]ATP of high specific radioactivity (1 mCi/nmol). The tryptic phosphopeptides were resolved on a C-18 reverse-phase h.p.l.c. column with a linear propan-1-ol gradient (Yu & Czech, 1984). Analysis of the eluted peptides demonstrated no obvious differences in the phosphopeptide maps for each receptor preparation. No new peptide peaks were observed with receptor reconstitution into phosphatidylcholine/phosphatidylethanolamine or phosphatidylcholine/phosphatidylethanolamine/phosphatidyserine vesicles. Insulin receptor incorporated into phosphatidylcholine/phosphatidylethanolamine vesicles (Fig. 5b, 60 ng of insulin receptor) demonstrated the expected overall decrease in 32P labelling relative to insulin receptor incorporated into phosphatidylcholine/phosphatidylethanolamine/phosphatidyserine vesicles (Fig. 5c, 60 ng of insulin receptor) in the presence of insulin. Some 360 ng of soluble unreconstituted insulin receptor was labelled for the peptide map of Fig. 5(a), accounting for the overall increase in 32P incorporated into soluble...
Fig. 5. H.p.l.c. tryptic-peptide mapping of the \(^{32}\)P-labelled insulin receptor \(\beta\) subunit

Soluble insulin receptors (a), or insulin receptors reconstituted into phosphatidylcholine/phosphatidylethanolamine (1:1, w/w) vesicles (b) or phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (11:11:2, by wt.) vesicles (c) were phosphorylated for 60 min at 22 °C with 5 \(\mu\)M-[\(\gamma\)-\(^{32}\)P]ATP (1 mCi) in the presence of 100 nM-insulin. The samples were washed, reduced, alkylated and electrophoresed as described in the Experimental section. Gel pieces containing the phosphorylated insulin receptor were washed and treated with trypsin (tosylphenylalanylchloromethane-treated) at 37 °C. The tryptic digests were eluted from an h.p.l.c. reverse-phase C-18 column with a linear 0–40 % propan-1-ol gradient. Radioactivity in each fraction was determined by Cerenkov counting.

Fig. 6. Insulin receptor reconstituted into phosphatidylcholine/phosphatidylethanolamine vesicles demonstrate a pool of high-affinity binding sites not observed with the inclusion of phosphatidylserine into vesicles

A 50 \(\mu\)l sample of soluble insulin receptor (■), or insulin receptor reconstituted into phosphatidylcholine/phosphatidylethanolamine (1:1, w/w) vesicles (●), or phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (11:11:2, by wt.) vesicles (○) were incubated insulin receptor. The initial peak in each map was found to contain only \(^{32}\)P. Only phosphotyrosine was observed in peak 1 (22–24 min) and peak 2 (40–41 min) for each of the peptide maps in Fig. 5 (results not shown). These peptide maps indicate that similar sites on the insulin receptor were phosphorylated, regardless of whether the receptor is phosphorylated in a soluble state or when reconstituted into a lipid bilayer.

Reconstitution of insulin receptor into phosphatidylcholine/phosphatidylethanolamine vesicles increases high-affinity binding of insulin

In order to determine if the lack of insulin-sensitivity of insulin receptor reconstituted into phosphatidylcholine/phosphatidylethanolamine vesicles was due to an alteration of receptor affinity by the lipid environment, we performed equilibrium-binding experiments and analysed them by the method of Scatchard (1949) (Fig. 6). Reconstitution into phosphatidylcholine/phosphatidylethanolamine (1:1, w/w) vesicles resulted in a marked increase in apparent affinity of insulin receptors as compared with solubilized receptors (\(K_d = 0.12\) nM versus 0.76 nM respectively), and compared with insulin receptor reconstituted into phosphatidylcholine/phosphatidylethanolamine/phosphatidylserine (11:11:2, by wt.) vesicles (\(K_d = 0.12\) nM versus 0.44 nM respectively). The affinity constants given in parentheses are estimates with 0.05–22.3 nM-\(^{125}\)I-insulin in the absence or the presence of 100 nM-insulin. The experiment was performed in duplicate with similar results.
from linear regressions of the values from the higher-affinity regions of the Scatchard plots.

**DISCUSSION**

The studies reported here demonstrate that highly purified kinase-active insulin receptor can be reconstituted into vesicles of defined composition by combining receptor protein with phospholipid and passing the solubilized mixture over a molecular-sieving column to remove excess detergent. Particle sizing indicates that the vesicles are spherical in nature and have a uniform distribution (Fig. 1b). From experiments on the phosphorylation of reconstituted insulin receptors in the presence and absence of detergent, we have determined that the insulin receptor kinase in the vesicle preparation exhibits 50% latency, indicating a 50% right-side-out orientation (Fig. 2). Scatchard analysis of vesicles in the presence and absence of detergent indicated no difference in the maximum number of binding sites. This contrasts with the latency of phosphorylation observed with reconstituted receptor, and indicated the vesicles are leaky to small molecules such as insulin. Potential vesicle leakiness makes strict assessment of receptor orientation difficult, but does not impair the observations of phospholipid effects on the insulin receptor kinase activity. In fact, leakiness of the vesicles to ATP is required in order to assess receptor kinase activity.

A major finding of these studies is the loss of insulin-sensitive kinase activity in affinity-purified insulin receptors which have been reconstituted into phosphatidylincholinesphatidylethanolamine vesicles (Fig. 3). This is not due to inhibition of the kinase itself or to a changed receptor orientation, because basal auto-phosphorylation still occurs in such vesicles. Furthermore, the loss of responsiveness to insulin correlates with an increase in the high-affinity binding of $^{125}$I-insulin to reconstituted receptors (Fig. 6). The insensitivity of the receptor kinase to insulin and the increase in high-affinity binding are reversed by the inclusion of phosphatidylserine into receptor-containing phosphatidylincholinesphatidylethanolamine vesicles (Figs. 4 and 6). Addition of phosphatidylserine in the range 8.3–16.7% (by wt.) appears sufficient to restore insulin-responsiveness to the reconstituted receptor. However, the insulin-responsiveness is only 40–50% of that seen in the receptor before reconstitution. The loss of insulin-sensitivity of the receptor reconstituted into phosphatidylincholinesphatidylethanolamine vesicles suggests that a specific environment is required for maximal hormone-responsiveness of the insulin receptor kinase.

All five experiments with insulin receptor reconstituted into phosphatidylincholine/phosphatidylethanolamine/phosphatidylserine vesicles demonstrated enhanced kinase activity upon insulin addition. In two of those five experiments, the basal kinase activity was less than the basal activity in similar preparations made without phosphatidylserine. Small differences in the elution profile of different vesicle preparations, as well as small differences in the amount of receptor incorporated, may account for these differences. Nevertheless, we cannot exclude completely the possibility that phosphatidylserine has some negative effect on basal kinase activity as well as a positive effect on the coupling of the hormone-binding domain to the kinase.

Gould et al. (1982) reported that a saturated lipid environment increased insulin receptor affinity relative to an unsaturated environment. A change in saturation is not likely to affect insulin receptor kinase activity or binding activity in our present experiments, because the small amounts of phosphatidylserine added to phosphatidylcholine/phosphatidylethanolamine vesicles in these experiments should not significantly alter the overall saturated/unsaturated fatty acid composition. A more likely explanation for the effect of phosphatidylserine on insulin-sensitive kinase activity and binding is the effect of the phosphoserine head group on the reconstituted receptor protein. Phosphatidylcholine and phosphatidylethanolamine have a net charge of zero. Phosphatidylserine, however, is negatively charged. Phosphatidylserine has been shown to enhance preferentially the incorporation of cytochrome oxidase into lipid vesicles (Etyan & Racker, 1977). It is also known that phosphatidylserine has a marked ability to enhance the activity of reconstituted Na$^+$/K$^+$-ATPase. However, the effect on Na$^+$/K$^+$-ATPase was not specific for phosphatidylserine, because phosphatidylinositol and phosphatidylglycerol also had enhancing effects (Palatini et al., 1977; Mandersloot et al., 1978; Roelofson & Van Deenen, 1973). Ochoa et al. (1983) determined that cholesterol and negatively charged lipids enhanced agonist-stimulated ion flux of reconstituted acetylcholine receptor, although a portion of these effects could be ascribed to changes in vesicle size. In the present studies, changing phospholipid content did not affect vesicle size. Furthermore, the anionic phospholipids phosphatidylinositol and phosphatidic acid as well as cholesterol were ineffective in altering reconstituted insulin receptor kinase activity in amounts equal to the amount of phosphatidylserine used in these studies. Interestingly, phosphatidylserine has been shown to be a potent activator of protein kinase C (Nishizuka, 1984) and src kinase (Ito et al., 1982), the latter also being activated by other acidic phospholipids (Ito et al., 1982).

Phosphorylation of phosphatidylcholine/phosphatidylethanolamine vesicles containing insulin receptors in the presence of insulin demonstrates a general loss of $^{32}$P in both phosphopeptide peaks after trypic digestion, compared with receptors in detergent solution (Fig. 5b). Restoration of the insulin sensitivity by inclusion of phosphatidylserine into receptor-containing phosphatidylcholine/phosphatidylethanolamine vesicles elevates phosphorylation in both resolved phosphopeptide peaks (Fig. 5c). No newly formed peaks are evident with the addition of phosphatidylserine to vesicles, indicating that the enhanced phosphorylation is not due to the phosphorylation of previously unexposed sites.

The tryptic-peptide mapping system used here is identical with that used by Yu & Czech (1984) to map the immobilized phosphorylated insulin receptor. Phosphopeptide peaks 1 and 2 in Fig. 5 appear identical with peaks 2 and 3 in the peptide maps of Yu & Czech (1984). Interestingly, the insulin receptor kinase is activated by phosphorylation on the $\beta$ subunit (Yu & Czech, 1984; Rosen et al., 1983), and a tyrosine residue within peak 1 of Fig. 5 (peak 2 of Yu & Czech (1984)) is believed to be responsible for the activation. The addition of phosphatidylserine to reconstituted insulin receptor does not significantly enhance the phosphorylation of peak 1 over peak 2 (Fig. 5), suggesting that phosphatidylserine does not alter the relative phosphorylation of the site that activates the kinase. However, because each h.p.l.c. peak
may contain more than one peptide, the change in phosphorylation state of one peptide may be marked relative to the total.

Interestingly, phosphorylation of a band that co-migrates with the α subunit is prominent with insulin receptors reconstituted into vesicles containing phosphatidylinositol and phosphatidylethanolamine, but is not evident with soluble insulin receptors. If this phosphorylated protein is indeed the α subunit, it may reflect an intermolecular event, since deduction of the insulin-receptor amino acid sequence (Ulrich et al., 1985; Ebina et al., 1985) has indicated that the binding domain within the α subunit and the kinase region within the β subunit are on opposite sides of the lipid bilayer and the α subunit resides completely above the lipid bilayer. Thus, in our reconstituted system, phosphorylation of the α subunit might occur if two receptors were inserted within the bilayer with opposite orientations.

Reconstituted systems have been useful in a variety of schemes to assess the function and interaction of various membrane-associated proteins (Cerione et al., 1983; Radnay et al., 1985). Such reconstitution systems may be useful in determining the relevance of the tyrosine kinase activity endogenous to growth-factor receptors. The observation that affinity-purified insulin receptor can be inserted into an artificial lipid environment while still retaining insulin-sensitive kinase activity makes possible investigations to study the functional coupling of the insulin receptor with other cellular proteins. Combination of purified components in liposomes or in vivo by fusion of the purified insulin receptor kinase with target cells holds promise as important approaches to determining the role of tyrosine kinase activity in insulin receptor function.

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