peptide chain is extended beyond Met[5], the N-terminus becomes progressively resistant to LAP, presumably through formation of some type of tertiary structure.

The presence of tertiary structure in the two relatively resistant peptides is further confirmed by the data in Figure 3. The analogues were first treated with thermolysin until a stable difference spectrum was established (1–2 h). If, after completion of the thermolysin reactions, LAP is added, all three undergo rapid blue shifts. Apparently, following thermolysin cleavage of the Phe[4]–Met[5] bond in the two longer peptides, the tertiary structure is lost, and removal of Tyr[1] by LAP becomes quite facile. If the LAP-induced increases in ΔE_M for the thermolysin-pretreated peptides are coplotted as first-order reactions, it is found that the three LAP reactions are now kinetically equivalent.

Registry No. β_h-EP-(1–9), 59481-79-7; β_h-EP-(1–17), 60893-02-9; β_h-EP-(1–5), 58569-55-4; LAP, 9001-61-0.

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Incorporation of the Purified Human Placental Insulin Receptor into Phospholipid Vesicles†

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ABSTRACT: Purified human placental insulin receptors were incorporated into small unilamellar phospholipid vesicles by the addition of *n*-octyl β-glucopyranoside solubilized phospholipids, followed by removal of the detergent on a Sephadex G-50 gel filtration column and extensive dialysis. The vesicles have an average diameter of 142 ± 24 nm by Sephacryl S-1000 gel filtration chromatography and 119 ± 20 nm by transmission electron microscopy. These vesicles are impermeant to small molecules as indicated by their ability to retain [γ-^{32}P]ATP, which could be released by the addition of 0.05% Triton X-100. Detergent permeabilization or freeze–thawing of the insulin receptor containing vesicles in the presence of ^{125}I-insulin indicated that approximately 75% of the insulin binding sites were oriented right side out (extravesicularly). Sucrose gradient centrifugation of insulin receptors incorporated at various protein to phospholipid mole ratios demonstrated that the insulin receptors were inserted into the phospholipid bilayer structure in a concentration-dependent manner. Addition of [γ-^{32}P]ATP to the insulin receptor containing vesicles was relatively ineffective in promoting the autophosphorylation of the β subunit in the absence or presence of insulin. Permeabilization of the vesicles with low detergent concentrations, however, stimulated the β-subunit autophosphorylation approximately 2-fold in the absence and 10-fold in the presence of insulin. Insulin-stimulated β-subunit autophosphorylation was also observed under conditions such that 94% of those vesicles containing insulin receptors had a single receptor per vesicle, suggesting that the initial β-subunit autophosphorylating activity is intramolecular. Phospho amino acid analysis of the vesicle-incorporated insulin receptors demonstrated that the basal and insulin-stimulated β-subunit autophosphorylation occurs exclusively on tyrosine residues. It is concluded that when purified insulin receptors are incorporated into a phospholipid bilayer, they insert into the vesicles primarily in the same orientation as occurs in the plasma membrane of intact cells and retain insulin binding as well as insulin-stimulated β-subunit autophosphorylating activities.

The insulin receptor is generally envisaged as an integral membrane glycoprotein composed of two M_r 135 000 (α) and two M_r 95 000 (β) subunits, linked by disulfide bonds into an M_r 350 000 heterotetrameric complex (Hedo et al., 1981; Van Obberghen et al., 1981; Massagué 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., 1980; Pilch & Czech, 1980) have suggested that the α subunit contains the insulin binding site. Insulin binding stimulates the phosphorylation of the β subunit in intact cells, detergent soluble, and purified preparations of

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the insulin receptor (Kasuga et al., 1982a, 1983a; Zick et al., 1983; Tamura et al., 1983; Petruzelli et al., 1984). The β subunit becomes phosphorylated in vivo on tyrosine, threonine, and serine residues in response to insulin binding (Kasuga et al., 1982c). In contrast, phosphorylation in vitro occurs exclusively on tyrosine residues (Avruch et al., 1982; Petruzzeli et al., 1982; Shia & Pilch, 1983). Affinity-labeling studies with ATP analogues have demonstrated that the β subunit has an ATP binding site (Roth & Cassell, 1983; Shia & Pilch, 1983; Van Obberghen, 1983), consistent with the β subunit being the catalytic protein kinase subunit and the α subunit being the regulatory subunit.

Recently, the entire 1370 amino acid sequence of the human placental insulin receptor precursor has been deduced from complementary DNA clones (Ebina et al., 1985; Ullrich et al., 1985). These data suggest that the β subunit contains a single hydrophobic sequence capable of acting as a transmembrane anchor and that the α subunit is attached to it by disulfide bonds. Although the full-length amino acid sequence is available, the molecular mechanism of transmembrane signal transduction still remains unresolved. Formulation of a mechanism is complicated by the structural constraints of only one β-subunit transmembrane spanning domain through which the flow of information can be propagated across the phospholipid bilayer. The availability of purified insulin receptor preparations that retain full biological activity (Fujita-Yamaguchi et al., 1983; Kasuga et al., 1983a) now makes it possible to directly address mechanistic questions concerning the molecular basis of the insulin-dependent transmembrane signaling process. With this objective, we now report a rapid and reproducible method for incorporating the purified human placental insulin receptor into small unilamellar phospholipid vesicles. Receptors inserted into phospholipid bilayers by this method retain both the insulin binding and β-subunit autophosphorylating activities in the same spatial orientation as is found for the native insulin receptors in the plasma membrane of intact cells.

Experimental Procedures

Materials

Molecular weight standards, phosphoserine, phosphothreonine, adenosine triphosphate (ATP),[1] protease inhibitors, L-α-phosphatidylcholine (soybean commercial grade, type II-S), Triton X-100, and ninhydrin were obtained from Sigma. β-OG and phosphotyrosine were obtained from Calbiochem. [γ-^{32}P]ATP (3000 Ci/mmol) and [^{14}C]DOPC (120 mCi/mmol) were from New England Nuclear. XAR-5 film, Cronex lightening plus enhancing screens, and 13255 chromagram sheets were from Eastman Kodak. SDS–polyacrylamide gel electrophoresis reagents were obtained from United States Biochemical Corp. Sephacryl S-400 and Sephacryl S-1000 were purchased from Pharmacia. Trypsin, NCS tissue solubilizer, polystyrene bead standards, and Centricon miniconcentrators were obtained from Worthington, Amersham, Polysciences, and Amicon, respectively. Affi-Gel 10 was from Bio-Rad. Porcine insulin was a gift from Dr. R. Chance, Eli Lilly Co. Monoiodinated [A^{14}]porcine insulin was kindly provided by the Diabetes Endocrinology Research Center, University of Iowa.

Methods

Purification of Insulin Receptor. Insulin receptors were purified from freshly obtained human placental membranes by modifications of the methods described by Fujita-Yamaguchi et al. (1983) and Pilch et al. (1985). Briefly, placental membranes were prepared as described by Harrison & Itin (1980). Membranes (30 mg/mL) were solubilized, with stirring, for 1 h at 4 °C in a buffer consisting of 0.25 M sucrose, 10 mM Tris, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 25 mM benzamidine hydrochloride, 10 μM leupeptin, 50 trypsin inhibiting units aprotinin, 1 mM 1,10-phenanthroline, and 1 μM pepstatin A, pH 8.0, plus 2% Triton X-100. After sedimentation at 104000g for 1 h at 4 °C, the supernatant was directly applied to a Sephacryl S-400 column (5.0 × 100 cm) equilibrated with 50 mM Tris, 0.1% Triton X-100, and 0.02% NaN_3, pH 8.0. The peak of insulin binding activity eluted from the Sephacryl S-400 column was concentrated and applied to a 1.6 × 10 cm column of insulin coupled to Affi-Gel 10 (0.8 mg of insulin/mL of resin). The insulin–agarose column was washed with 10–15 column volumes of 50 mM HEPES, 1.0 M NaCl, and 0.6% β-OG, pH 7.4, to exchange the Triton X-100 for β-OG. The amount of Triton X-100 was reduced to below detectable levels (<0.0002%) after washing with approximately 10 column volumes of this buffer. Insulin receptors were eluted from the insulin–agarose column with 50 mM sodium acetate, 1.0 M NaCl, 10% glycerol, and 0.6% β-OG, pH 5.0, and were immediately neutralized with 1.0 M HEPES, pH 8.0. The eluant was concentrated on Centricon miniconcentrators, resuspended in 50 mM HEPES, 10% glycerol, and 0.6% β-OG, pH 7.4, concentrated as before, and stored at 4 °C until use. The amount of protein present in the purified insulin receptor preparation was determined by using the Bio-Rad protein assay.

Incorporation of Purified Insulin Receptors into Phospholipid Vesicles. Purified insulin receptors were incorporated into phospholipid vesicles by using Sephadex G-50 gel filtration chromatography followed by extensive dialysis. Soybean phospholipid (3.2 mg) and tracer amounts of [^{14}C]DOPC were solubilized in β-OG at a detergent to phospholipid mole ratio of 10:1. Purified insulin receptors (10 μg), at a protein to phospholipid mole ratio of 1 to 1 × 10^5, were incubated with the soluble phospholipids for 20 min at 4 °C before applying the mixture to a Sephadex G-50 column (1.5 × 25 cm). The column was eluted with 25 mM HEPES, 150 mM NaCl, and 5 mM $MgCl_2$, pH 7.4 (buffer A). Column fractions were assayed for insulin binding activity, [^{14}C]DOPC, and β-OG. The coincident insulin binding peak and phospholipid peak was pooled and subjected to dialysis for 72 h at 4 °C against buffer A. Insulin receptors were incorporated into vesicles at a protein to phospholipid mole ratio of 1 to 1 × 10^6 as described for those at a mole ratio of 1 to 1 × 10^5, except for the addition of 32 mg of soybean phospholipid and gel filtration on a 2.5 × 35 cm Sephadex G-50 column. The insulin binding and autophosphorylating activities of the insulin receptor remained stable at 4 °C for at least 1 week after incorporation into phospholipid vesicles.

Vesicle Characterization. The average diameters of the receptor-containing vesicles were determined by using a 1.0 × 44 cm Sephacryl S-1000 gel filtration column (Reynolds et al., 1983), equilibrated at 4 °C with buffer A containing 1 mg/mL bovine serum albumin. Vesicles were untreated and treated with 0.05% or 0.5% Triton X-100 for 1 h before being

[1] Abbreviations: ATP, adenosine triphosphate; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; β-OG, n-octyl β-D-glucopyranoside; soybean phospholipid, L-α-phosphatidylcholine (type II-S); DOPC, dioleoylphosphatidylcholine; TCA, trichloroacetic acid.

applied to the column. The elution profiles were determined by assaying column fractions for [^{14}C]DOPC. The column was calibrated with defined diameter polystyrene beads by using absorbance at 440 nm to determine the elution profiles. Total column volume was determined by the elution of ^{125}I-insulin. Column calibration was performed in the presence of 2% Triton X-100 to prevent aggregation of the polystyrene beads.

[γ-^{32}P]ATP was trapped within the vesicles containing the purified insulin receptors by using a Sephadex G-50 column that had been equilibrated with buffer A containing 10 μCi/mL [γ-^{32}P]ATP. The resulting phospholipid vesicles were dialyzed against buffer A to remove the extravesicular [γ-^{32}P]ATP. The trapped ATP was released from the vesicles by the addition of Triton X-100 to a final concentration of 0.05%. The presence of [γ-^{32}P]ATP within the dialysis membrane was determined by counting aliquots of the retentate.

Purified insulin receptors (25 pmol) were combined with 2.5, 25, 250, or 2500 nmol of soybean phospholipids plus tracer amounts of [^{14}C]DOPC. Following Sephadex G-50 gel filtration chromatography to remove the β-OG, the resulting vesicles were overlayed onto 11-mL 5–35% linear sucrose gradients containing a 0.5-mL 60% sucrose cushion. Purified insulin receptors without added lipid and phospholipid vesicles prepared in the absence of insulin receptors were treated identically. The gradients were centrifuged for 34 h at 36 000 rpm in a Beckman SW41 rotor. Fractions were drawn from the bottoms of the gradients and assayed for [^{14}C]DOPC and insulin binding. The refractive index of each fraction was measured by using a Bausch & Lomb refractometer to determine the sucrose densities.

To obtain transmission electron micrographs of the phospholipid vesicles, they were fixed in solution with 1% osmium tetraoxide for 30 min at 23 °C, following a 1-h incubation with various concentrations of Triton X-100. The fixed vesicles were negatively stained with 2% phosphotungstic acid, pH 7.0, on grids coated with Formvar and carbon. Specimens were examined with a Hitachi H-600 electron microscope at an accelerating voltage of 75 kV.

Insulin Binding Activity. Soluble and purified insulin receptors and receptors incorporated into phospholipid vesicles were incubated with 0.25 nM ^{125}I-[A^{14}]insulin in a final volume of 0.2 mL of Krebs–Ringer–phosphate buffer (10 mM sodium phosphate, 5.1 mM KCl, 1.3 mM $CaCl_2$, 1.3 mM $MgSO_4$, pH 7.4, plus 0.5% bovine serum albumin) for 60 min at 23 °C. Free ^{125}I-insulin was separated from the bound hormone by two different methods. One involved precipitation of the bound hormone by the addition of 0.1% bovine γ-globulin with the subsequent addition of 12.5% polyethylene glycol followed by centrifugation at 12000g for 10 min in a Beckman Microfuge B. The other involved filtration on 0.45-μm cellulose acetate filters, as previously described for soluble receptors (Cuatrecasas, 1972) and for receptors incorporated into vesicles (Gould et al., 1982). Nonspecific binding in the presence of 1.0 μM unlabeled insulin was found to range from 3 to 5% of the total binding and was always subtracted to yield specific binding. The concentrations of β-OG or Triton X-100 in the assays of soluble insulin binding activity were routinely maintained below 0.05%.

β-OG Assay. To measure the amount of β-OG present, samples were brought to a final volume of 0.4 mL with the appropriate buffer. To each sample was added 10 μL of 80% phenol and 1 mL of concentrated H_2SO_4 with vortexing immediately after each addition. The reaction was allowed to develop for 30 min at 23 °C before measuring the absorbance at 490 nm (Ashwell, 1966).

Phosphorylation Assay. Samples to be assayed for β-subunit autophosphorylating activity were incubated for 30 min at 23 °C in the presence or absence of 200 nM porcine insulin in 50 mM HEPES, 0.2 mM dithiothreitol, and 2.0 mM $MnCl_2$, pH 7.4. The phosphorylation reaction was initiated by the addition of [γ-^{32}P]ATP (1.0 mM, 3.0 μCi/nmol) to obtain a final concentration of 100 μM ATP. Each phosphorylation reaction was terminated by the addition of Laemmli sample buffer containing 100 mM dithiothreitol, followed by heating at 100 °C for 1 min. The phosphorylated samples were then subjected to SDS–polyacrylamide (7%) gel electrophoresis according to the method of Laemmli (1970). The gels were then stained, destained, dried, and autoradiographed on Kodak XAR-5 film with Cronex lighting 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.

Phospho Amino Acid Analysis. The identities of phosphorylated amino acids were determined by a modification of the method of Yu & Czech (1984). Briefly, purified insulin receptors and receptors incorporated into phospholipid vesicles were subjected to the phosphorylation protocol as described above. The phosphorylation reaction was stopped by the addition of 50 μg of bovine serum albumin and TCA to a final concentration of 20%. After 15 min at 4 °C, the TCA precipitates were centrifuged for 15 min at 40000g. The pellets were washed with 10% TCA, centrifuged as before, washed with ethanol–ether (1:1 v/v), and resuspended in a solution containing equimolar amounts of phosphoserine, phosphotyrosine, and phosphothreonine. After lyophilization, the samples were hydrolyzed for 1 h in 6 N HCl at 110 °C and resuspended in H_2O. The hydrolysates were spotted on 13255 cellulose chromagram sheets and electrophoresed in pyridine–acetic acid–H_2O (1:25:225 v/v/v), pH 3.3. The phospho amino acid standards were visualized by spraying with ninhydrin, and the stained regions of the chromatogram were compared to the ^{32}P-labeled phospho amino acids observed by autoradiography.

Results

Purification of the Human Placental Insulin Receptor. Insulin receptors from human placental membranes were purified by using a combination of methods previously described by others (Fujita-Yamaguchi et al., 1983; Petruzzelli et al., 1984; Pilch, 1985) with the modification of exchanging Triton X-100 for β-OG on the insulin–agarose column. As shown in Figure 1, insulin receptors purified by gel filtration and insulin affinity chromatography, after the exchange of Triton X-100 for β-OG, can be resolved into two major protein bands of M_r 130 000 (α) and M_r 95 000 (β) on SDS–polyacrylamide gels under reducing conditions (lane A). Under nonreducing conditions (lane B), only the high molecular weight insulin receptor oligomers are seen, consistent with the published heterotetrameric structure of the purified insulin receptor. These results indicate that gel filtration chromatography is an effective purification step, resulting in highly purified insulin receptors when used in conjuction with insulin–agarose affinity chromatography.

Incorporation of Purified Insulin Receptors into Phospholipid Vesicles. The purified human placental insulin receptors were incorporated into soybean phospholipid vesicles containing tracer amounts of [^{14}C]DOPC by Sephadex G-50 column chromatography followed by extensive dialysis. Sephadex G-50 column chromatography allowed rapid and

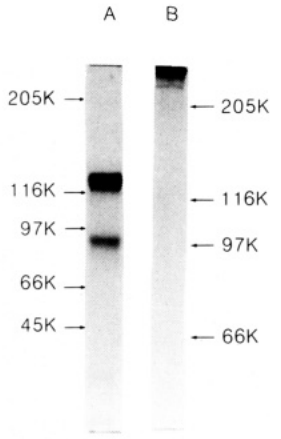

FIGURE 1: SDS–polyacrylamide gel electrophoresis of the purified insulin receptor isolated from human placenta. Insulin receptors were purified as described under Experimental Procedures and were resolved in a 6% SDS–polyacrylamide gel in the presence (lane A) or absence (lane B) of 25 mM dithiothreitol. The protein bands were visualized by silver staining. Arrows depict the position of molecular weight markers.

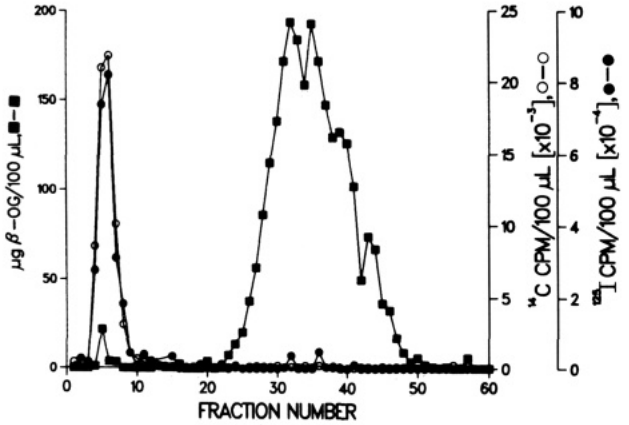

FIGURE 2: Incorporation of the purified insulin receptor into phospholipid vesicles by gel filtration on Sephadex G-50. Purified insulin receptors (10.5 μg) were mixed with 1.5 mg of soybean phospholipids containing 2 μCi of [^{14}C]DOPC (13 μg) and chromatographed on a Sephadex G-50 column as described under Experimental Procedures. Fractions (0.75 mL) were collected and assayed for insulin binding activity (●), [^{14}C]DOPC (○), and β-OG (■). Under these conditions the efficiency of protein insertion into the vesicles as measured by insulin binding is greater than 90%. Counting efficiencies were 90% for ^{14}C and 75% for ^{125}I.

effective removal of β-OG, with the concomitant formation of small unilamellar vesicles. As shown in Figure 2, insulin binding activity coeluted with the labeled phospholipid in a single excluded peak. The majority of the β-OG was eluted in a highly included peak, and only a small amount of the detergent was present in the peak containing phospholipid and insulin binding activity. The trace amount of β-OG in the phospholipid and insulin binding peak was consistently found to be at a detergent to phospholipid mole ratio of 0.19 ± 0.04 and was further reduced below the limits of detection (detergent to phospholipid mole ratio <0.004) by extensive dialysis against buffer A.

To demonstrate that the insulin receptors were inserted into the phospholipid bilayer structure, vesicles were formed at various mole ratios of insulin receptor protein to phospholipid and then subjected to sucrose gradient centrifugation. Gradient fractions, drawn from the bottom of the tubes, were analyzed for insulin binding, [^{14}C]DOPC, and sucrose density. The results shown in Figure 3 illustrate gradient profiles of

Table I: Effect of Trypsin on the Insulin Binding Activity of Insulin Receptors Incorporated into Phospholipid Vesicles[a]

treatment	fmol of insulin bound/μg of protein	% total binding
trypsin inhibitor	30.4 ± 0.3	70
trypsin inhibitor + 0.05% Triton X-100	43.4 ± 1.8	100
trypsin + trypsin inhibitor	ND[b]	ND
trypsin + trypsin inhibitor + 0.05% Triton X-100	4.1 ± 0.1	9.4

[a] Insulin receptors incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1 × 10^5 were incubated with trypsin (0.5 mg/mL) for 30 min at 23 °C followed by the addition of trypsin inhibitor (6 mg/mL) for 10 min at 23 °C. The vesicles were separated from the trypsin and trypsin inhibitor by Sephadex G-50 gel filtration chromatography and assayed for insulin binding activity (0.25 nM ^{125}I-insulin) in the presence or absence of 0.05% Triton X-100. Values represent the average of three determinations ± standard error of the mean. [b] ND, not detectale.

the vesicles prepared at insulin receptor to phospholipid mole ratios of 1 to 1 × 10^2 (A), 1 to 1 × 10^3 (B), 1 to 1 × 10^4 (C), and 1 to 1 × 10^5 (D), each having a constant amount of protein with increasing amounts of phospholipid. Under each of these conditions, the peak of insulin binding activity occurred in fractions containing phospholipids. At high concentrations of insulin receptor to phospholipid, however, a substantial amount of protein-free phospholipid was observed (Figure 3A). As the protein to phospholipid mole ratio decreased, the migration of the peak of insulin binding activity in the sucrose gradient also decreased in parallel. Thus, at insulin receptor to phospholipid mole ratios of 1 to 1 × 10^2, 1 to 1 × 10^3, 1 to 1 × 10^4, and 1 to 1 × 10^5, the insulin binding activities sedimented to positions within the gradients that had apparent average densities corresponding to 37%, 25%, 20%, and 16% sucrose, respectively. Protein-free vesicles and insulin receptors without added lipids sedimented to positions corresponding to 8% and 41% sucrose, respectively. The altered migration within the sucrose gradients suggests that different numbers of insulin receptors were inserted into the phospholipid vesicles when the vesicles were formed at different protein to phospholipid ratios, thus causing the vesicles to have different sedimentation properties.

Orientation of the Insulin Receptors in the Phospholipid Vesicles. Three methods were used to determine the orientation of the insulin binding activity incorporated into phospholipid vesicles. Insulin binding was determined for vesicles subjected to extravesicular trypsinization, incubated with increasing concentrations of detergent, and subjected to freeze–thawing. The trypsin susceptibility of the insulin binding activity of the vesicle-incorporated receptors was determined by incubation with 0.5 mg/mL trypsin (189 units/mg) for 30 min at 23 °C, followed by incubation with 6 mg/mL trypsin inhibitor for 10 min (Table I). Vesicles were separated from the trypsin and trypsin inhibitor by rapid Sephadex G-50 gel filtration chromatography. Following this step, trypsin-treated vesicles and vesicles treated with only trypsin inhibitor were assayed for insulin binding activity in the presence or absence of 0.05% Triton X-100. As shown in Table I, maximal insulin binding activity was observed for receptors in vesicles that were treated with trypsin inhibitor and 0.05% Triton X-100 to expose both intravesicular and extravesicular binding sites. Vesicles treated with trypsin inhibitor but no detergent showed 70% of the maximal insulin binding activity observed in the presence of Triton X-100. No insulin binding activity was detected in vesicles treated with trypsin and trypsin inhibitor, suggesting that the trypsin ef-

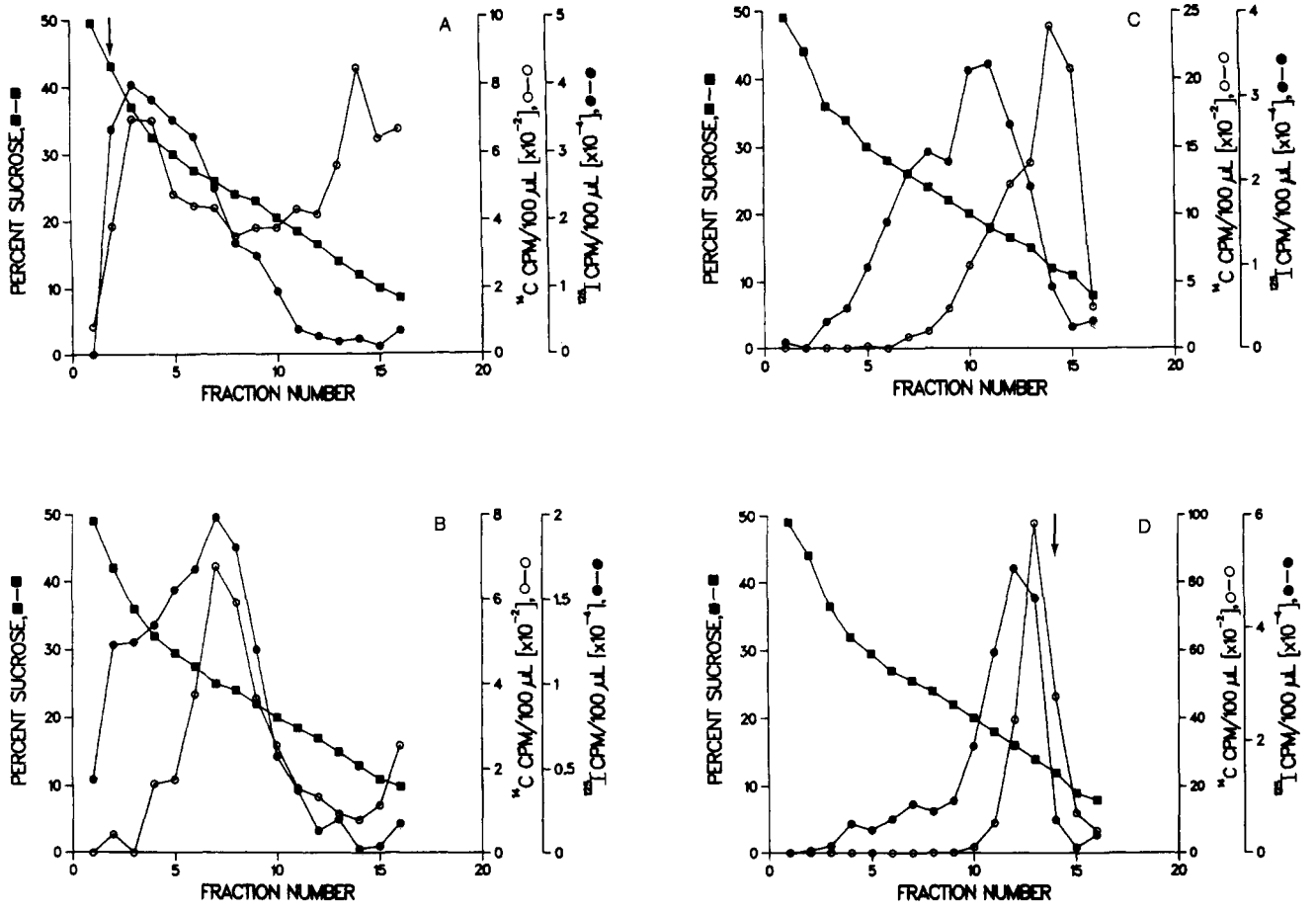

FIGURE 3: Sucrose gradient centrifugation of the receptor-containing vesicles. Purified insulin receptors were incorporated into vesicles at four different protein to phospholipid mole ratios and subjected to sucrose density centrifugation as described under Experimental Procedures. Fractions were drawn from the bottom of the gradients and assayed for insulin binding activity (●), [^{14}C]DOPC (○), and sucrose density (■). The protein to phospholipid mole ratios examined were (A) 1 to 1 × 10^2, (B) 1 to 1 × 10^3, (C) 1 to 1 × 10^4, and (D) 1 to 1 × 10^5. The sedimentation position of insulin receptors with no exogeneously added lipid and vesicles formed without insulin receptors are denoted by the arrows in (A) and (D), respectively. Counting efficiencies were 90% for ^{14}C and 75% for ^{125}I.

fectively proteolyzed the extravesicular insulin binding sites of the receptors incorporated into the vesicles. Vesicles that were treated with 0.05% Triton X-100 after the trypsinization procedure had 9.4% of the maximal insulin binding activity remaining.

The insulin binding activity of the receptor-containing vesicles permeabilized with increasing concentrations of Triton X-100 or β-OG is shown in Figure 4. In the absence of detergent as well as at low detergent concentrations, the amount of insulin binding remained relatively constant and presumably reflects only the binding sites oriented externally. At higher detergent concentrations, insulin binding increased, reflecting the permeabilization of the vesicles which then allows insulin access to the internally oriented binding sites. Maximum insulin binding occurred when the vesicles were incubated with either 0.05% Triton X-100 or 0.1% β-OG, suggesting that the vesicles were fully permeable to insulin at these detergent concentrations. At high detergent concentrations, there was a marked decrease in insulin binding, consistent with the detergent-induced inhibition of insulin binding reported previously by others (Cuatrecasas, 1972).

Table II contains values for the orientation of the insulin binding sites incorporated into vesicles as measured under conditions that gave maximum insulin binding compared to untreated vesicles. Results obtained with vesicles exposed to either Triton X-100 or β-OG are in agreement, indicating that 70–75% of the insulin binding sites are oriented extravesicularly and 25–30% are exposed to the internal face of the

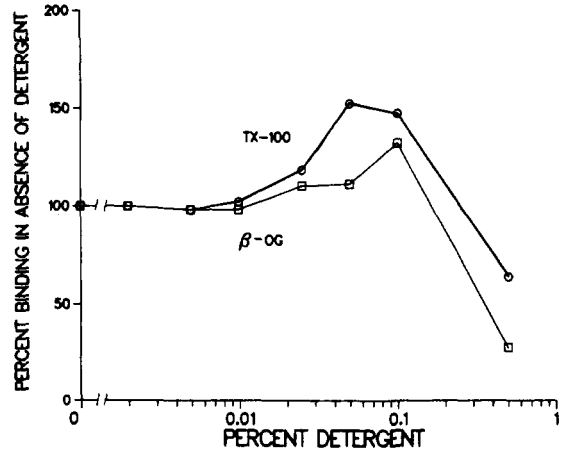

FIGURE 4: Relative orientation of the insulin receptor binding sites in phospholipid vesicles. Insulin receptors were incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1 × 10^5 and assayed for insulin binding activity in the presence of increasing concentrations of Triton X-100 [TX-100 (○)] or β-OG (□). Total insulin binding to receptors in the absence of detergent is represented as 100% binding after correction for nonspecific binding. The detergent to phospholipid mole ratios that gave maximal insulin binding (0.05% Triton X-100 and 0.1% β-OG) for the insulin receptor containing vesicles was 2.4 and 10.2, respectively.

vesicles. Similarly, receptors incorporated into vesicles that were incubated in the presence of ^{125}I-insulin and subsequently frozen at −70 °C for 15 min followed by rapid thawing in

Table II: Comparison of Methods Used To Determine the Relative Orientation of Insulin Binding Sites of Insulin Receptors Incorporated into Phospholipid Vesicles[a]

perturbation	orientation of insulin binding sites	
	% external	% internal
incubation with 0.05% Triton X-100	68.0 ± 5.6	32.0 ± 2.6
incubation with 0.1% octyl β-glucoside	76.0 ± 3.4	24.0 ± 1.0
freeze–thawing	76.0 ± 2.3	24.0 ± 0.7

[a] Insulin binding to receptors incorporated into vesicles was determined as described in Table I. Values represent the average of four independent determinations ± standard error of the mean. [b] ^{125}I-Insulin (0.25 nM) was added to the insulin receptor containing vesicles in the presence or absence of excess unlabeled insulin. Vesicles were frozen for 15 min at −70 °C, rapidly thawed, and incubated for 1 h at 23 °C followed by polyethylene glycol precipitation of the bound hormone. Control assays were conducted in the same manner but without freezing.

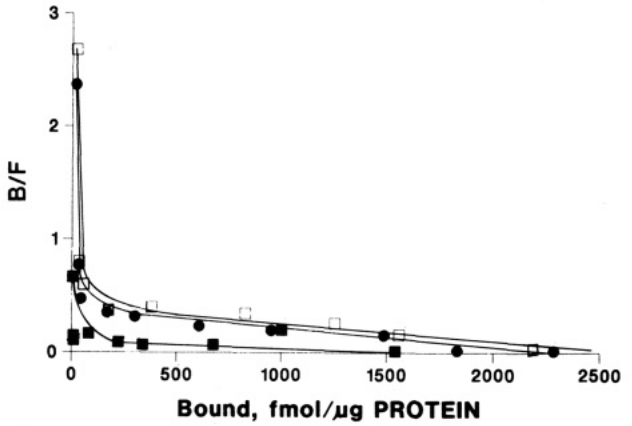

FIGURE 5: Scatchard plots of insulin binding to vesicle-incorporated insulin receptors. Insulin receptors were incorporated into vesicles as described in Figure 4 and incubated without (●) and with 0.05% Triton X-100 (□) or with 0.5% Triton X-100 (■). The samples were then incubated with 0.08–0.8 nM ^{125}I-insulin or with 0.1–100 nM unlabeled insulin plus 0.8 nM ^{125}I-insulin. The amount of specifically bound insulin was determined as described under Experimental Procedures.

warm water demonstrated about 25% more insulin binding activity as compared to vesicles not subjected to the freeze–thawing procedure (Table II).

Scatchard analysis (Scatchard, 1949) of ^{125}I-insulin binding to receptor-containing vesicles in the presence and absence of Triton X-100 is depicted in Figure 5. This type of data transformation has been well documented to produce curvilinear plots for ^{125}I-insulin binding to purified preparations of insulin receptors (Fujita-Yamaguchi, 1983; Pilch, 1985). It is apparent that the binding curves obtained in the presence and absence of 0.05% Triton X-100 are essentially parallel from 0.01 to 100 nM insulin (Figure 5). In this experiment the binding in the presence of 0.05% Triton X-100 was an average of 27% greater compared to the untreated receptor vesicles.

Vesicle Size and Integrity. Sephacryl S-1000 gel filtration chromatography was used to determine the average size of these receptor-containing phospholipid vesicles. As shown in Table III, the vesicles have an average diameter of 142 ± 24 nm. Vesicles that were treated with 0.05% Triton X-100 for 1 h before chromatography have an average diameter of 132 ± 27 nm. Due to the total peak widths of the elution profiles (30–38% total column volume), the difference between the untreated vesicles and those treated with 0.05% Triton X-100 is not statistically significant. Receptor-containing vesicles treated with 0.5% Triton X-100 have an apparent average diameter of 37 ± 4 nm, indicating the loss of vesicular structure.

Table III: Sephacryl S-1000 Gel Filtration Chromatography of the Insulin Receptors Incorporated into Phospholipid Vesicles[a]

sample	K_{av}	average diameter (nm)
vesicles	0.355 ± 0.06[b]	142 ± 24
vesicles + 0.05% Triton X-100	0.398 ± 0.08	132 ± 27
vesicles + 0.5% Triton X-100	0.785 ± 0.08	37 ± 4

[a] Insulin receptors were incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1 × 10^5. A total of 0.5 mL (0.8 mg of soybean phospholipid/mL) of vesicles was untreated or treated with 0.05% or 0.5% Triton X-100 for 1 h at 23 °C exactly as described in Figure 4, before being applied on a 1 × 44 cm Sephacryl S-1000 column at 4 °C. Fractions of 0.5 mL were collected and assayed for [^{14}C]DOPC. [b] The standard deviation represents 1 standard deviation of the total peak width.

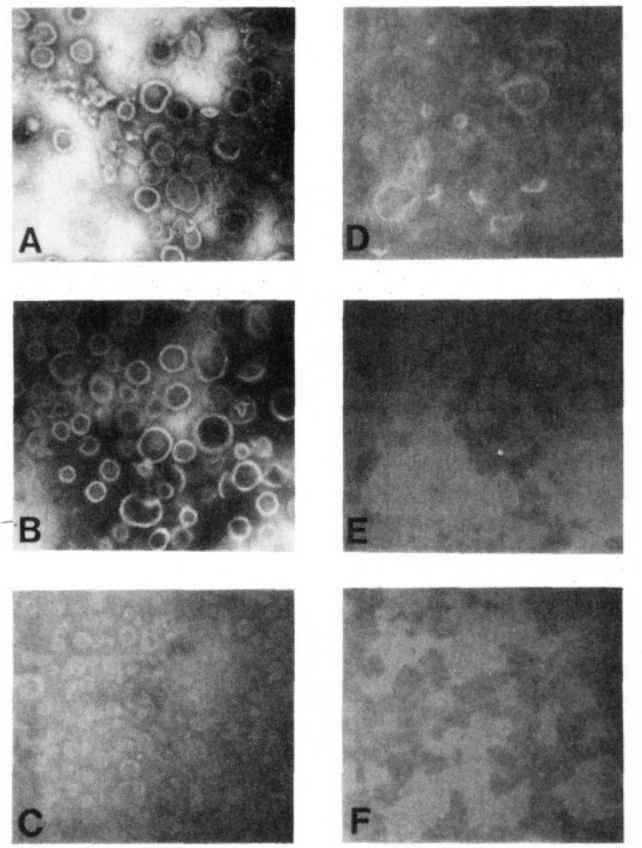

FIGURE 6: Transmission electron microscopy of vesicles treated with Triton X-100. Purified insulin receptors were incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1 × 10^5 and were incubated with increasing concentrations of Triton X-100 for 1 h before preparation for electron microscopy as described under Experimental Procedures. Representative electron micrographs of vesicles incubated with 0% (A), 0.01% (B), 0.05% (C), 0.1% (D), 0.2% (E), and 0.5% Triton X-100 (F) are shown. The micrographs were at 45000× magnification.

Vesicles containing insulin receptors were permeabilized with increasing concentrations of Triton X-100, fixed with osmium tetraoxide, negatively stained, and observed by transmission electron microscopy. Figure 6 shows the structure of the vesicles in the presence of several Triton X-100 concentrations. In the absence of detergent, the vesicles had a unilamellar structure with an average diameter of 119 ± 20 nm (Figure 6A). At Triton X-100 concentrations of 0.01% (Figure 6B) and 0.05% (Figure 6C), no change in the vesicle integrity was observed. However, at 0.1% Triton X-100 (Figure 6D), the vesicles became irregular in shape, and at

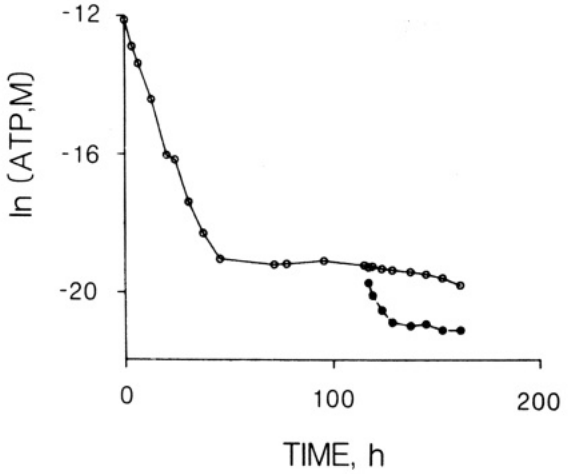

FIGURE 7: Permeability of the insulin receptor containing vesicles to ATP. Insulin receptors were incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1×10^5, on a Sephadex G-50 column preequilibrated with buffer A containing 10 μCi/mL [γ-^{32}P]ATP. At the times shown, aliquots of the retentate were withdrawn and counted for ^{32}P. The sample (2.5 mL) was dialyzed against 1 L of buffer A which was changed at each time point. Release of the trapped [γ-^{32}P]ATP by the addition of 0.05% Triton X-100 is indicated (●).

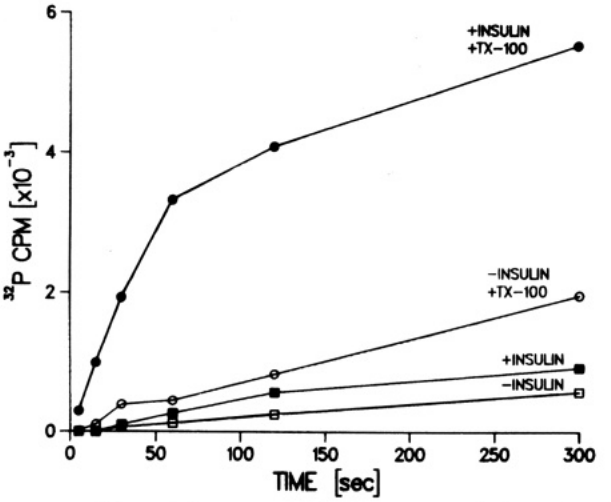

FIGURE 8: Effect of detergent on the time course of the β-subunit autophosphorylation of insulin receptors incorporated into phospholipid vesicles. Purified insulin receptors were incorporated into vesicles at a protein to phospholipid mole ratio of 1 to 1×10^5. The resulting phospholipid vesicles were incubated in the absence or presence of 0.05% Triton X-100 for 1 h before the addition of [γ-^{32}P]ATP for the indicated times. The phosphorylation reaction was terminated by the addition of Laemmli sample buffer. Samples were subjected to SDS–polyacrylamide gel electrophoresis, and the β subunit of the insulin receptor was localized by autoradiography, excised, and counted. The data are expressed as cpm of ^{32}P incorporated into the β subunit of 1.1 μg of total insulin receptor protein in the absence of insulin (□), in the presence of insulin (■), in the absence of insulin and the presence of Triton X-100 (○), and in the presence of both insulin and Triton X-100 (●).

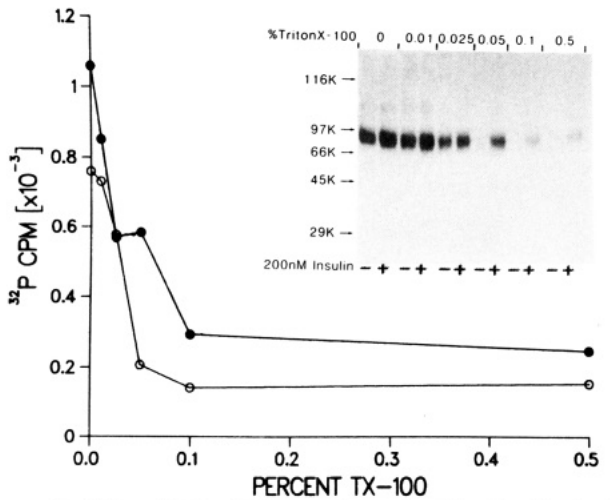

FIGURE 9: Effects of Triton X-100 on the basal and insulin-stimulated β-subunit autophosphorylation of the soluble insulin receptor. Increasing concentrations of Triton X-100 were added to purified insulin receptors (0.8 μg) in 0.6% β-OG in the absence (○) or presence (●) of 200 nM insulin. The phosphorylation was allowed to proceed for 1 min before the addition of Laemmli sample buffer and separation by 7% SDS–polyacrylamide gel electrophoresis. The insert shows the autoradiogram of the gel from which the β subunit was excised and counted.

0.2% (Figure 6E) and 0.5% (Figure 6F) Triton X-100, vesicular integrity was lost completely. Together, the Sephacryl S-1000 gel filtration profiles and the electron micrographs suggest that the vesicles into which insulin receptors have been incorporated can be permeabilized with up to 0.05% Triton X-100 without the loss of gross vesicular structure.

In order to demonstrate the relative permeability of these receptor-containing vesicles, the vesicles were formed in the presence of 10 μCi/mL [γ-^{32}P]ATP (Figure 7). There was a rapid release of [γ-^{32}P]ATP during the initial 48 h of dialysis, followed by the attainment of an equilibrium value which was unchanged by continuous dialysis. The initial release is consistent with the loss of extravesicular [γ-^{32}P]ATP, and the attainment of an equilibrium value suggests that the nondialyzable ATP was trapped intravesicularly. Addition of 0.05% Triton X-100 after 120 h of dialysis caused the release of the trapped [γ-^{32}P]ATP at a rate equivalent to that initially observed during the first 48 h of dialysis, demonstrating that exposure to Triton X-100 makes the vesicles permeable to ATP. Identical results were observed when the vesicles were formed in the absence of insulin receptors (data not shown).

Insulin Receptor Autophosphorylation. Figures 4–7 and Tables I–III establish that the receptor-containing vesicles are relatively impermeable to small molecules such as insulin and ATP but can be made permeable by the addition of 0.05% Triton X-100, without the loss of vesicular structure. The insulin receptor β-subunit autophosphorylation was examined under these conditions (Figure 8). In the absence of Triton X-100, the vesicle-incorporated receptors exhibited a maximal 2-fold insulin-stimulated β-subunit autophosphorylation after a 60-s incubation with [γ-^{32}P]ATP, and this fold stimulation of phosphorylation remained relatively constant for 5 min. In contrast, insulin stimulation in the presence of 0.05% Triton X-100 was maximal (9.4-fold) after 15 s, and the amount of ^{32}P incorporation was greater over the entire time course. The basal state autophosphorylation of the β subunit in the presence of Triton X-100 occurred at a slower constant rate than the corresponding insulin-stimulated ^{32}P incorporation. There was a decrease in the degree of insulin stimulation at the longer incubation times, apparently indicating saturation of the insulin-stimulated β-subunit autophosphorylation. These results suggest that permeabilization is required to produce maximal insulin-stimulated β-subunit autophosphorylation of the insulin receptors incorporated into the phospholipid vesicle structure.

To determine whether the increase in ^{32}P incorporation is due to Triton X-100 stimulation of the autophosphorylating activity, rather than due to permeabilization of the vesicles to [γ-^{32}P]ATP, the effect of Triton X-100 on ^{32}P incorporation was determined by using purified insulin receptors in 0.6% β-OG. As shown in Figure 9, the addition of Triton X-100 to soluble insulin receptors inhibited both the basal and in-

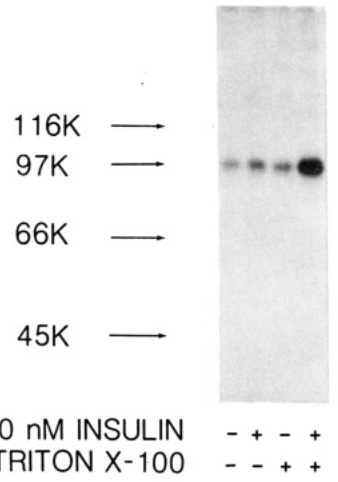

FIGURE 10: Intramolecular β-subunit autophosphorylation of insulin receptors incorporated into phospholipid vesicles. Insulin receptors were incorporated into vesicles at a protein to phospholipid mole ratio of 1 to 1×10^6 and then phosphorylated for 5 min in the presence or absence of 0.5% Triton X-100 as described under Experimental Procedures. Arrows depict the location of molecular weight markers.

sulin-stimulated autophosphorylation of the β subunit. Since Triton X-100 does not stimulate the autophosphorylating activity of the insulin receptors, the greater ^{32}P incorporation into the β subunit of the receptor-containing vesicles after 0.05% Triton X-100 treatment must therefore be due to permeabilization of the vesicles to ATP.

To investigate whether the β-subunit autophosphorylation occurs by an intra- or intermolecular mechanism, insulin receptors were incorporated into vesicles at an initial protein to phospholipid mole ratio of 1 to 1×10^6. Under these conditions, 94% of the insulin receptors exist as one per vesicle with 6% of the receptors as two or more per vesicle.[2] To permeabilize these vesicles, Triton X-100 was included at 0.5% to maintain the same phospholipid to detergent ratio as was included in the phosphorylation of receptors formed at a ratio of 1 to 1×10^5. Figure 10 shows the autoradiogram of these vesicle-incorporated receptors which have been phosphorylated for 5 min. In the absence of Triton X-100, insulin produces a 1.5-fold stimulation of the β-subunit autophosphorylation. In the presence of Triton X-100, the insulin stimulation was 2.8-fold. These results are consistent with the amount of insulin stimulation occurring after 5 min in the presence of [γ-^{32}P]ATP when receptors were incorporated at a protein to phospholipid mole ratio of 1 to 1×10^5 (Figure 8).

Phospho Amino Acid Analysis. Detergent-soluble receptors and receptors incorporated into phospholipid vesicles were phosphorylated as previously described and then subjected to acid hydrolysis. The phospho amino acids in each sample were then separated by one-dimensional electrophoresis at pH 3.3. Phosphotyrosine was the only phosphorylated amino acid detected in the insulin-receptor β subunit, either in the absence or in the presence of insulin (Figure 11). The presence of

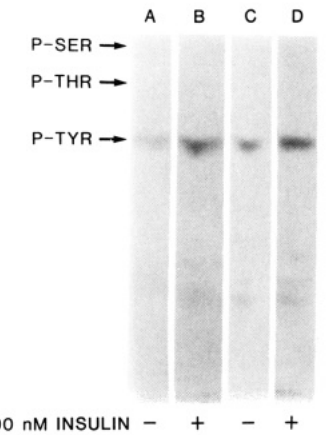

FIGURE 11: One-dimensional thin-layer electrophoresis of phospho amino acids of the purified, soluble receptor and receptor incorporated into phospholipid vesicles. Soluble (lanes A and B) insulin receptors were autophosphorylated in the presence or absence of 200 nM insulin, TCA precipitated, and hydrolyzed as described under Experimental Procedures. Receptors incorporated into vesicles at a protein to phospholipid mole ratio of 1 to 1×10^5 (lanes C and D) were treated identically to soluble receptors but with the inclusion of 0.05% Triton X-100 in the phosphorylation reaction. Similar amounts of labeled material were spotted in each lane, and after separation by thin-layer electrophoresis, the phospho amino acids were visualized by autoradiography. The phospho amino acid standards were identified by ninhydrin staining.

phosphotyrosine indicates that the amino acid specificity of the β-subunit autophosphorylation of the purified, detergent-soluble, insulin receptor is maintained when the receptor is incorporated into a phospholipid bilayer.

Discussion

Insulin receptor preparations solubilized from turkey erythrocytes, which contain both endogeneous membrane protein and lipid, can be incorporated into mixed phospholipid vesicles composed of soybean phosphatidylcholine plus bovine brain phosphatidylserine or dimyristoylphosphatidylcholine plus bovine brain phosphatidylserine (Gould et al., 1979). The degree of unsaturation of the phospholipids affected both the apparent number of insulin binding sites and the insulin binding affinities, suggesting that the membrane lipid environment may alter the activity of the insulin receptor (Gould et al., 1982). We now describe a method for incorporating purified human placental insulin receptors into small unilamellar phospholipid vesicles. These preparations retain both the insulin binding activity and the insulin-stimulated β-subunit autophosphorylating activities of the detergent-soluble insulin receptors. As a preliminary step to insulin–agarose affinity chromatography, we utilized a Sephacryl S-400 gel filtration step in place of the previously reported wheat germ agglutinin chromatography (Fujita-Yamaguchi et al., 1983). Once the insulin receptors were adsorbed onto the insulin–agarose affinity resin, complete exchange of Triton X-100 for β-OG was readily accomplished. Silver-stained SDS–polyacrylamide gels (Figure 1) and assays of insulin binding activity showed that insulin receptors purified by our procedure were comparable to those purified by the previously reported methods (Fujita-Yamaguchi et al., 1983; Petruzzelli et al., 1984; Pilch, 1985).

Removal of β-OG by extensive dialysis or by Sephadex G-50 chromatography has been reported for the incorporation of several integral membrane proteins into phospholipid vesicles, including (Na^+,K^+)ATPase (Chin & Forgac, 1984), β-adrenergic receptor (Cerione et al., 1983), vesicular stomatitis virus G protein (Eidelman et al., 1984; Petri & Wagner, 1979), glycophorin A (Mimms et al., 1981), and the acetylcholine receptor (Gonzales-Ros et al., 1980). Consistent with these

[2] The basis for this calculation is that the vesicles in the presence and absence of insulin receptors have a similar size distribution (approximately 150 nm) with an average surface area of 0.70 nm^2/phospholipid molecule (Mimms et al., 1980). The mean of the Poisson binomial distribution, ($\bar{X}$), assuming a random distribution, is calculated to be 0.120 (1.51×10^{13} insulin receptor molecules/1.25×10^{14} phospholipid vesicles, 10 μg of protein/32 mg of phospholipid). The probability of each vesicle containing zero receptors (P_0) is $e^{-\bar{X}} = 0.887$, one receptor (P_1) is $e^{-\bar{X}}\bar{X} = 0.106$, two receptors ($P_2$) is $e^{-\bar{X}}\bar{X}^2/2 = 0.005$, and three receptors ($P_3$) is $e^{-\bar{X}}\bar{X}^3/(3 \times 2) = 0.0003$. Thus, of all the vesicles that contain insulin receptors ($P_{IR} = 1.00 - P_0 = P_1 + P_2 + P_3 + \ldots P_n$) the fraction that has one receptor per vesicle is given by $P_1/P_{IR} = 0.94$.

studies, the phospholipid vesicles containing purified insulin receptors formed during β-OG removal by Sephadex G-50 chromatography were unilamellar and of fairly uniform size. The average diameter (142 ± 42 nm) of the receptor-containing vesicles as determined by Sephacryl S-1000 gel filtration (Table III) is in reasonable agreement with the estimated average diameter (119 ± 20 nm) obtained from transmission electron micrographs (Figure 6).

In contrast to the vesicular stomatitis virus G protein which inserts with a relatively fixed number of proteins per phospholipid vesicle (Eidelman et al., 1984), the number of insulin receptors incorporated per phospholipid vesicle is apparently dependent upon the available concentrations of phospholipids and insulin receptors (Figure 3). Thus, incorporation of the insulin receptors into phospholipid vesicles at high protein to phospholipid mole ratios (1 to 1×10^2) results in vesicles that sediment in a fairly broad zone within the sucrose gradient, having an average migration corresponding to 37% sucrose. It should be noted, however, that under these conditions not all the vesicles appear to contain insulin receptors since some protein-free phospholipid was found at the top of the sucrose gradient (Figure 3A). Currently, we have no explanation for this apparently anomalous behavior. Nevertheless, at very dilute protein to phospholipid mole ratios (1 to 1×10^5), the vesicles sediment in a much narrower range of sucrose densities, the average being 16% sucrose, suggesting that they are more uniform with respect to the number of insulin receptors per phospholipid vesicle.

Detergent permeabilization and freeze–thawing of receptors both indicate that approximately 75% of the insulin binding sites are extravesicular (Table II and Figure 4). The presence of insulin binding activity that is not susceptible to trypsin provides further evidence that there are cryptic intravesicular binding sites that become accessible to insulin when the vesicles are ruptured by freeze–thawing or detergent permeabilization (Table I). The difference between the apparent number of binding sites exposed by freeze–thawing or detergent permeabilization (25%) as compared to the apparent number protected from trypsinization (10%) is probably due to extensive proteolysis of non-insulin binding regions of the receptor, resulting in reduced insulin binding activity after detergent permeabilization of the phospholipid vesicles. This asymmetry of receptor insertion into the phospholipid vesicles is not surprising in light of the inherent asymmetric structure of the insulin receptor molecule (Hedo et al., 1981; Van Obberghen et al., 1981; Ebina et al., 1985; Ullrich et al., 1985).

A number of findings are consistent with our conclusion that the vesicles are impermeable to small molecules such as ATP and insulin. First, there was an increase in insulin binding activity when vesicles were exposed to low concentrations of detergent or ruptured by freeze–thawing (Figures 4 and 5 and Table II). Second, residual insulin binding activity was protected from trypsin when trypsin was applied extravesicularly in the absence of detergent (Table I). Third, [γ-^{32}P]ATP could be trapped intravesicularly (Figure 7), indicating that these vesicles are sealed to small molecules and could be made permeable by the subsequent addition of 0.05% Triton X-100. Finally, there was an increase in insulin-stimulated β-subunit autophosphorylation in the presence of 0.05% Triton X-100 (Figure 8).

Weinstein et al. (1977) have shown that incubation with 0.1% Triton X-100 makes phospholipid vesicles freely permeable to the small, water-soluble, fluorescent probe 6-carboxyfluorescein. These results agree with our finding that 0.05% Triton X-100 permeabilizes the receptor-containing vesicles to ATP. In addition all of the latent oxidase activity of vesicle-incorporated cytochrome *c* oxidase is released by incubation of vesicles with 0.03–0.06% Triton X-100 and 0.41–0.52% β-OG (Madden & Cullis, 1984). It is important to note that, under our conditions, the addition of 0.05% Triton X-100 to the vesicles does not significantly alter the gross vesicular structure as measured by transmission electron microscopy (Figure 6) and Sephacryl S-1000 gel filtration chromatography (Table III).

Insulin-stimulated autophosphorylation of the β subunit of the receptor has been reported for insulin receptors immunoprecipitated from whole cells and partially purified cell extracts (Kasuga et al., 1982a–c) and for solubilized, highly purified insulin receptors (Kasuga et al., 1983a; Petruzzelli et al., 1984). In vitro phosphorylation has been observed to occur exclusively on tyrosine residues in the β subunit of soluble insulin receptors (Kasuga et al., 1982a,b, 1983b; Petruzzelli et al., 1984; Tamura et al., 1983). However, in the intact cell, insulin stimulates the phosphorylation of serine and possibly threonine residues in addition to tyrosine residues (Kasuga et al., 1982c). We find that the basal and insulin-stimulated autophosphorylations of insulin receptors incorporated into phospholipid vesicles also occur exclusively on tyrosine residues of the β subunit, and no evidence was observed for a lipid-dependent alteration in amino acid specificity (Figure 11). These results suggest that other cellular protein kinases and/or phosphatases are responsible for the differences between in vivo and in vitro β-subunit autophosphorylation.

It has previously been observed that the intial rate of insulin receptor β-subunit autophosphorylation is concentration (dilution) independent (Shia et al., 1983; Petruzzelli et al., 1984), suggesting that the insulin-stimulated β-subunit autophosphorylation is an intramolecular as opposed to an intermolecular process. These studies have assumed that the insulin receptors exist as independent monomers within the detergent micelles. It can be calculated that a Poisson distribution of insulin receptors incorporated into phospholipid vesicles at a protein to phospholipid mole ratio of 1 to 1×10^6, assuming an average vesicle diameter of 150 nm (Figure 6 and Table III) and a random distribution (Figure 3), results in vesicle formation such that 94% of the receptor-containing vesicles have one receptor per vesicle.[2] Under these conditions, the β-subunit autophosphorylation was stimulated 3-fold by insulin (Figure 10), a finding consistent with an intramolecular autophosphorylation mechanism. Further, these results suggest that a single insulin receptor molecule is capable of transmitting an insulin-induced signal across the phospholipid bilayer to the β-subunit kinase domain.

The methodology described in this report provides a defined system well suited for looking at the effects of specific phospholipids on the insulin binding, autophosphorylation, and protein kinase activities of the purified insulin receptors incorporated into phospholipid vesicles. In addition, reinsertion of the purified protein into a phospholipid bilayer will allow us to perform topographical studies to better elucidate both structural and functional domains of the purified human placental insulin receptor.

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