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Insulin Receptor Autophosphorylation and Signaling Is Altered by Modulation of Membrane Physical Properties†

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ABSTRACT: Many membrane functions are modulated by the bulk biophysical properties of the membrane. Various compounds which alter membrane physical properties were investigated for their ability to modulate insulin receptor autophosphorylation and signaling. Compounds which raise the bilayer to hexagonal phase transition temperature in model membranes, including carbobenzoxydipeptides, apolipoprotein A-I, acyl carnitines, and lysophosphatidylcholine, inhibited insulin stimulation of insulin receptor tyrosine phosphorylation of isolated receptors as well as in cells overexpressing human insulin receptor. For compounds of similar structure, the inhibition of insulin receptor tyrosine phosphorylation correlates well with their bilayer-stabilizing potency. Most of the compounds which inhibit tyrosine phosphorylation of the insulin receptor also inhibited glucose uptake in the same cells. Compounds which lower the bilayer to hexagonal phase transition temperature in model membranes enhanced insulin stimulation of autophosphorylation in isolated receptors, with no effect on insulin receptor activity in NIH 3T3 HIR 3.5 cells. The effects of cationic amphiphiles were not readily predictable from their membrane modulating activity. All of the compounds tested exert their effects independent of changes in insulin binding to the receptor or changes in the basal tyrosine kinase activity of the receptor. This provides evidence that mechanism of modulation of insulin signaling by these additives lies in their ability to alter the bulk physical properties of the membrane. The results suggest that membrane monolayer curvature strain is a factor contributing to the efficiency of insulin signal transduction.

The insulin receptor is a transmembrane heterotetrameric glycoprotein consisting of two insulin-binding α subunits which are disulfide bonded to two β subunits possessing tyrosine kinase activity (Ullrich et al., 1985; Ebina et al., 1985). The first step in insulin signaling, following binding of the hormone to the extracellular α subunit, involves autophosphorylation of tyrosine residues on the cytoplasmic portion of the β subunit (Kasuga et al., 1982a; Olefsky, 1990). The remainder of the signal transduction pathway leading to the mitogenic and metabolic effects of insulin is not well understood but probably involves a phosphorylation cascade (Czech et al., 1988). A 185-kDa protein termed insulin receptor substrate-1 (IRS-1)[1] is phosphorylated by

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[1] Abbreviations: Apo A-I, apolipoprotein A-I; CATAM1, 1-(dimethylamino)-3-(trimethylammonio)-DL-2-propylcholesteryl-3β-oxysuccinate; CATAM2, 2-{[2-(trimethylammonio)ethyl]methylamino}-ethylcholesteryl-3β-oxysuccinate; CBZ-Gly-L-Phe, carbobenzoxyglycyl-L-phenylalanine; CBZ-L-Phe-Gly, carbobenzoxy-L-phenylalanylglycine; DiC8, dioctanoylglycerol; GRB2, growth factor receptor binding protein-2; H_{II}, inverted hexagonal phase; IRS-1, insulin receptor substrate-1; lyso-PC, lysophosphatidylcholine; PI3′-kinase, phosphatidylinositol 3′-kinase; PKC, protein kinase C; PMSF, phenylmethanesulfonyl fluoride; SDS−PAGE, sodium dodecylsulfate−polyacrylamide gel electrophoresis; SH2, src homology 2; SIRK, soluble insulin receptor kinase; T_H, bilayer to hexagonal phase transition temperature.

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the insulin receptor on at least eight tyrosine residues (White et al., 1985). Unlike other receptor tyrosine kinases, which interact directly with signaling molecules via SH2 domains, it is the tyrosine phosphorylation sites on IRS-1 that are recognized by SH2 domains, allowing binding of several SH2 proteins involved in signal transduction (White & Kahn, 1994). PI3′-kinase, a protein tyrosine phosphatase, and growth factor receptor-binding protein-2 (GRB-2) are stimulated by insulin in this manner (Sun et al., 1991, 1993; Lowenstein et al., 1992). GRB-2 has been implicated in activation of Ras, which may function as an intermediate in insulin signaling to promote glucose transport (Kozma et al., 1993; Manchester et al., 1994). Activation of a specific phospholipase C may occur in response to insulin binding to its receptor (Salteil, 1990). Cleavage of a glycosyl phosphatidyl inositol by this phospholipase liberates a glycosylinositol phosphate as well as a diacylglycerol, which may act as second messengers (Salteil, 1990; Taylor, 1991). These various signaling pathways may account for the pleiotropic actions of insulin (Salteil, 1990).

The transmembrane topology of the insulin receptor renders it susceptible to changes in the membrane phospholipid bilayer. The insulin receptor has been observed to undergo a conformational change upon insulin binding which is presumed to be required for the transmission of the signal to the β subunit (Pilch & Czech, 1980; Baron et al., 1989, 1992). Modulation of the membrane properties may affect the relative populations of the active and inactive conformations of the receptor and thereby affect the efficiency of insulin signaling. Evidence supporting this idea includes the observation that lipid-induced insulin resistance is associated with a decreased tyrosine kinase activity (Hubert et al., 1991). Furthermore, with purified receptor it has been shown that the phospholipid environment alters binding and kinase activity of the insulin receptor (Lewis & Czech, 1987; Sweet et al., 1987; Ginsberg et al., 1981; Leray et al., 1993).

The mechanism by which insulin receptor activity is coupled to membrane properties is not known. One possibility is that membrane monolayer curvature strain (Gruner, 1992) affects the conformational rearrangement of transmembrane proteins. The photoisomerization of rhodopsin has been shown to be sensitive to this parameter (Gibson & Brown, 1993). The importance of this property to the functioning of membranes is suggested by the fact that microorganisms adjust their lipid composition to have considerable negative monolayer curvature strain and are close to the transition temperature to the inverted hexagonal (H_{II}) phase (Rilfors et al., 1994). The effects of membrane additives on the temperature at which phospholipids undergo a change from bilayer to hexagonal phase is an indicator of their effect on curvature strain.

Addition of compounds to the membrane can affect the monolayer curvature strain. Phospholipid bilayers will rearrange to other forms, such as the H_{II} phase, when the curvature strain of the individual monolayers of the membrane becomes too large (Gruner, 1992). The hexagonal phase cannot form in the membranes of living cells, since this would lead to loss of membrane integrity. It is the propensity toward H_{II} phase formation, rather than the rearrangement to the hexagonal phase per se, that affects biological membrane function. For example, it has been shown that increased negative monolayer curvature can lead to an instability in membrane bilayers that can be detected calorimetrically (Epand & Epand, 1994).

Previously in this laboratory it was shown that small hydrophobic peptide derivatives which stabilize the bilayer relative to the hexagonal phase in model membranes inhibited insulin-stimulated glucose uptake and protein synthesis (Epand et al., 1991). The effectiveness of a series of peptide derivatives in inhibiting glucose uptake was related to their effects on lipid polymorphism. The mechanism of this inhibition of insulin signaling is unknown but is presumed to be related to the ability of the peptide derivatives to alter the biophysical properties of the plasma membrane. However, there are multiple factors determining the rate of glucose uptake or protein synthesis. While there have been a few reports suggesting that insulin receptor autophosphorylation is not an absolute requirement for insulin signaling (Moller et al., 1991; Debant et al., 1988, 1989), it is generally believed that insulin receptor tyrosine kinase activity may be required but is not sufficient for activation of glucose uptake (Wilden et al., 1992).

The relationship between membrane biophysical properties, insulin receptor autophosphorylation, and insulin action was investigated by assessing the ability of bilayer stabilizers and hexagonal phase promoters to modulate insulin stimulation of insulin receptor autophosphorylation and glucose uptake. The importance of the membrane in the action of such additives was demonstrated by the insensitivity of the soluble insulin receptor kinase domain to these additives and by the lack of effect of a less hydrophobic derivative of one of the inhibitory peptides.

EXPERIMENTAL PROCEDURES

Materials. The following chemicals were obtained from commercial sources: minimum essential media, fetal bovine serum, and Hanks' balanced salts solution, from Gibco, Grand Island, NY; [^{32}P]orthophosphoric acid from Du Pont-New England Nuclear, Quebec; [^{125}I]Tyr^{A14} insulin, [γ-^{32}P]-adenosine 5′-triphosphate, and [1,2-^{3}H]2-deoxy-D-glucose from ICN Biomedicals, Mississauga, ON; Affi-Gel-10, from Bio-Rad Laboratories Ltd., Mississauga, ON; YM10 filter from Amicon, Danvers, MA; phospholipids, from Avanti Polar Lipids, Alabaster, AL; bovine serum albumin, porcine insulin, *threo*-dihydrosphingosine, lysophosphatidylcholine, acyl carnitines, dioctanoylglycerol, and phosphoamino acids from Sigma Chemical Co., St. Louis, MO; carbobenzoxyglycyl-L-phenylalanine (CBZ-Gly-L-Phe) and carbobenzoxy-L-phenylalanylglycine (CBZ-L-Phe-Gly) from Research Plus Inc., Bayonne, NJ; carbobenzoxyglycyl-D-phenylalanine (CBZ-Gly-D-Phe), from Bachem Inc., Torrance, CA.; Protein A-Sepharose from Pharmacia, Quebec; Live/Dead cell viability kit from Molecular Probes, Eugene, OR; BCA protein assay kit from Pierce, Rockford, IL. Apolipoprotein A-I was a generous gift from Drs. G. M. Anantharamaiah and J. P. Segrest, University of Alabama, Birmingham, AL. All other chemicals were reagent grade.

Culturing of NIH 3T3 HIR 3.5 Cells. NIH 3T3 HIR 3.5 cells which have been transfected to express the human insulin receptor (Whittaker et al., 1987) were generously provided by Dr. J. Whittaker and were grown in α-MEM medium containing 10% fetal bovine serum at 37 °C with 5% CO_2. For phosphorylation and binding assays, cells were grown to confluence in 24-well plates.

Insulin Receptor Phosphorylation in Intact Cells. Phosphorylation of the insulin receptor in intact NIH 3T3 HIR

3.5 cells was carried out as described by Ellis et al. (1986) and Tavare et al. (1988). Confluent cells in 24-well plates were washed twice with 1 mL of Krebs-Ringer bicarbonate buffer/BSA (KRBB/BSA: 107 mM NaCl, 5 mM KCl, 3 mM $CaCl_2$, 1 mM $MgSO_4$, 7 mM $NaHCO_3$, 10 mM glucose, 1 mM Na_3VO_4, 0.1% bovine serum albumin, pH 7.4). The cells were then incubated at 37 °C for 4 h in KRBB/BSA with 0.2 mCi of [^{32}P]orthophosphate. Insulin and/or compounds of interest were added to a final volume of 1 mL. After 5 min at 37 °C the solution was removed from the cells. The cells were washed twice with ice-cold Tris/NaCl (20 mM Tris, 150 mM NaCl, pH 7.4) and solubilized on ice with 250 μL of solubilization buffer (50 mM Hepes, pH 7.6, 1% Triton X-100, 10 mM EDTA, 10 mM NaF, 30 mM $Na_4P_2O_7$, 1 mM Na_3VO_4, 1 mg/mL bacitracin, 1 mM phenylmethanesulfonyl fluoride, 100 μM ATP). The solubilized cells were transferred to microfuge tubes and centrifuged for 2 min. The supernatants were incubated with 7.5 μg of monoclonal antibody directed against the human insulin receptor (Ab 83-14, from Dr. K. Siddle, Cambridge) for 60 min at 4 °C. Protein A-Sepharose was added (3 mg/tube) and the mixture incubated a further 30 min. The samples were then centrifuged. The pellets were washed three times with 1 mL of 50 mM Hepes, pH 7.6, containing 1 M NaCl, 0.1% SDS, 0.5% Triton X-100, and 0.1% BSA and once with 1 mL of 100 mM Tris, pH 6.8. The pellets were suspended in 50 μL of Laemmli sample buffer and boiled for 3 min prior to SDS−PAGE. The 95-kDa bands containing the insulin receptor β subunit were visualized by autoradiography.

Phosphoamino Acid Analysis. The 95-kDa polypeptide was hydrolyzed to amino acids as described by Lewis et al. (1990). The excised 95-kDa bands were washed alternately in acetone and water, three times each, for 15 min each, and then minced and incubated with 40 μg/mL TPCK-treated trypsin in 25 mM *N*-ethylmorpholine, pH 8.2, at 37 °C for 16 h. The tryptic eluate was obtained by centrifugation and lyophilized. The phosphopeptides were hydrolyzed in 6 N HCl at 110 °C for 60 min. The amino acids were then lyophilized and reconstituted in 5 μL of phosphoamino acid standards (1 mg/mL each of phosphotyrosine, phosphoserine, and phosphothreonine), spotted on silica gel TLC plates (250 μm), and run three consecutive times in ethanol: ammonium hydroxide, 35:16 (v/v), as described by Munoz et al. (1990). The phosphoamino acid standards were visualized with ninhydrin, and the radiolabeled phosphoamino acids were visualized by autoradiography and quantitated by removal of the radioactive silica gel spots, followed by scintillation counting.

Competitive Binding Studies. Competitive binding studies were performed as described by Whittaker et al. (1987). Confluent cells on 24-well plates were washed twice with binding buffer (Hanks' balanced salts solution supplemented with 50 mM Hepes, pH 7.6, and 10 mg/mL BSA) and incubated with [^{125}I-Tyr-A14]insulin (30 pmol) and varying amounts of unlabeled insulin, with or without the addition of the compound of interest, in binding buffer for 16 h at 4 °C. The cells were then washed three times with 1 mL of ice-cold phosphate buffered saline and then solubilized at 37 °C with 500 μL of 0.1 M NaOH for 1 h. The solubilized cells were transferred to scintillation vials for γ counting. The percent of specifically bound insulin was calculated.

Cell Viability Assay. Cell viability in the presence of the additives was assessed using the Live/Dead kit from Molecular Probes, Inc. The cells were incubated with or without additives, as for the insulin receptor phosphorylation assay, then incubated with calcein AM and ethidium homodimer (EthD-1) according to supplier's protocol. The calcein AM is cleaved only in viable cells by intracellular esterases, yielding calcein, which produces green fluorescence in live cells. EthD-1 enters through the damaged membranes of dead cells and produces red fluorescence upon binding to nucleic acids. The live and dead cells were visualized under a fluorescent microscope with 530 and 590 nm filters, respectively. The numbers of live and dead cells were counted, and the percent of dead cells was calculated.

Glucose Uptake. Confluent NIH 3T3 HIR 3.5 cells were serum-starved for 2 h and then washed with 2 mL of uptake buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, 10 mM Na_2HPO_4, pH 7.4). The cells were preincubated for 15 min at 37 °C with 1 μM insulin and/or additives to be tested, in uptake buffer. Cells were then incubated with [^{3}H]2-deoxyglucose (0.5 μCi, 10 mM) for 30 min, followed by two washes with 1 mL of ice-cold PBS, to remove extracellular glucose. The cells were solubilized with 300 μL of 0.1% SDS. The [^{3}H]2-deoxyglucose that had been internalized by the cells was quantitated by scintillation counting.

Protein Determination. The amount of protein in one well of confluent cells (10^5 cells) was determined using the BCA protein assay kit.

Isolation of Insulin Receptor from Human Placenta. Insulin receptor was isolated from human placenta by the method of Fujita-Yamaguchi et al. (1983), with modifications. One human placenta was obtained within 30 min of delivery and placed in ice-cold 0.25 M sucrose. The placenta was trimmed of amnion and chorion, cut into small pieces, and rinsed twice with the sucrose solution, and then placed in a Waring blender for 3 min, in 50 mM Tris, pH 7.4, with 0.25 M sucrose and 0.1 mM phenylmethanesulfonyl fluoride (PMSF). The slurry was then homogenized three times for 1 min each, with a Polytron homogenizer, until a uniform consistency was obtained. The homogenate was centrifuged at 7000*g* for 45 min at 4 °C. The supernatant of this centrifugation was then centrifuged at 100000*g* for 30 min at 4 °C. The pellets were resuspended in 10 volumes of 50 mM Tris, pH 7.4, with 0.1 mM PMSF and homogenized using a teflon/glass homogenizer, and the suspension was recentrifuged at 100000*g* for 30 min at 4 °C. The resulting placental membrane pellet was stirred in 50 mL of solubilization buffer (50 mM Tris, pH 7.4, 2% Triton X-100, 0.1 mM PMSF, 1 mM bacitracin, 20 μg/mL pepstatin, 1.7 μg/mL leupeptin) for 45 min at room temperature. The solubilized membranes were centrifuged at 100000*g*, for 45 min at 4 °C. The supernatant containing the insulin receptors was added to 25 mL of Affi-10-insulin column material (prepared by coupling bovine insulin to Bio-Rad Affi-Gel-10, according to supplier's protocol), and rotated overnight at 4 °C. The column was washed with 50 mM Tris, pH 7.4, containing 0.1% Triton X-100 and 1 mM PMSF, and eluted with 50 mM sodium acetate buffer, pH 5.0. Two milliliter fractions were collected into 1 mL of 0.5 M Tris-HCl, pH 7.4. The fractions were assayed for insulin binding activity, as described by Lewis and Czech (1987). The Affi-10-insulin fractions showing specific insulin binding activity

were pooled and concentrated, using an Amicon YM-10 filter, under nitrogen. The protein content of the concentrated fractions was determined using the BCA protein assay kit.

Micellar Insulin Receptor Phosphorylation Assay. Lipid films were prepared by dissolving an appropriate amount of phospholipid, with or without hydrophobic compounds to be tested, in chloroform/methanol (2:1 v/v). The chloroform/methanol solutions were dispensed into assay tubes, and the solvent was evaporated with a stream of nitrogen. The lipid films were placed in a vacuum desiccator for 1 h to remove residual solvent. Three percent Triton X-100 in autophosphorylation buffer (50 mM Hepes, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$) was added to the lipid films with vigorous vortexing to yield mixed micelles. A 10 μL aliquot of this was incubated in a total volume of 50 μL containing 0.5 μM insulin and 10 μg of isolated receptor, with or without water-soluble compounds to be tested, for 1 h at 25 °C. [γ-^{32}P]ATP (10 μCi) was added, and the mixture was incubated for 15 min at 25 °C. The reaction was terminated by addition of 50 μL of Laemmli sample buffer and boiled for 3 min. The samples were subjected to SDS−PAGE, and the β subunit of the insulin receptor was visualized by autoradiography. Quantitation of phosphotyrosine was achieved via scintillation counting of the excised bands.

Soluble Insulin Receptor Kinase Domain Activity. Soluble insulin receptor kinase domain (SIRK) autophosphorylation was assayed by the method of Cobb et al. (1989). Twenty picomoles of the soluble cytoplasmic tyrosine kinase domain of the insulin receptor (a gift from Dr. Cobb) was incubated with 10 μM [γ -^{32}P]ATP in 10 mM Hepes, pH 8.0, 3 mM $MgCl_2$, and 100 μg/mL bovine serum albumin, for 15 min at 27 °C. The reaction was terminated with the addition of 20 μL of Laemmli sample buffer and boiled for 3 min. Following SDS−PAGE, the 48-kDa SIRK was visualized by autoradiography. Incorporation of ^{32}P into bands excised from gels was quantitated by scintillation counting.

Differential Scanning Calorimetry. Dielaidoylphosphatidylethanolamine (DEPE) and added compound were codissolved in a solution in chloroform/methanol (2:1, v/v). The solvent was evaporated with a stream of nitrogen, to deposit the solutes as a film on the wall of a glass test tube. Final traces of solvent were removed in a vacuum desiccator at 40 °C for 1 h. The lipid films were suspended in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, and 0.002% NaN_3 at pH 7.4 by vortexing at 45 °C for 30 s. The final lipid concentration was 5 mg/mL. The lipid suspensions were degassed under vacuum before loading into an MC-2 high sensitivity scanning calorimeter (Microcal Co., Amherst, MA). A scan rate of 39 K/h was employed. The temperature of the bilayer to hexagonal phase transition was fitted to a single van't Hoff component and the transition temperature reported as that for the fitted curve.

RESULTS

Phosphorylation of the insulin receptor β subunit in response to insulin was investigated in the presence of various compounds whose effects on the bilayer to hexagonal phase transition temperature (T_H) had been characterized. Cells transfected with the human insulin receptor gene were preincubated with [^{32}P]orthophosphoric acid and then with insulin and/or compounds of interest. The insulin receptor was immunoprecipitated from lysed cells and separated by

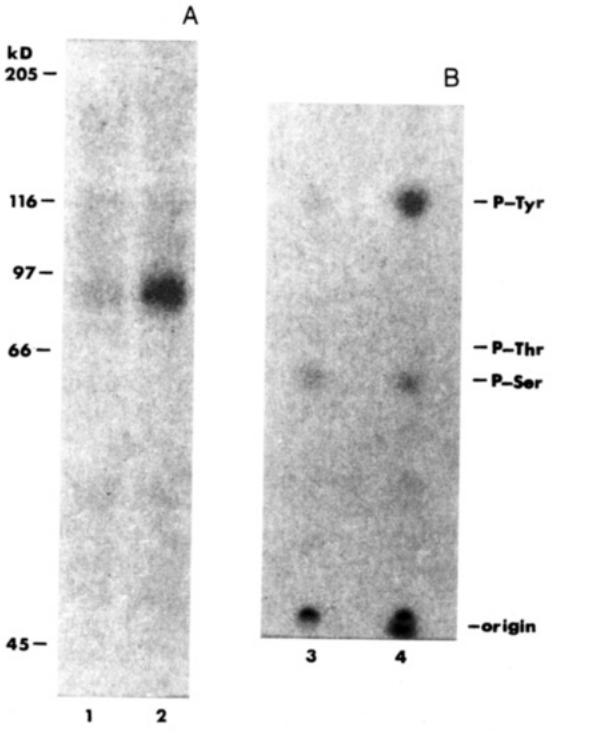

FIGURE 1: Insulin receptor phosphorylation in NIH 3T3 HIR 3.5 cells. (A) Gel electrophoresis of immunoprecipitated insulin receptor, following incubation of cells in the absence (lane 1) and presence (lane 2) of 1 μM insulin, as described under Experimental Procedures (B) Phosphoamino acid analysis of the β subunit of the insulin receptor. Following SDS−PAGE of insulin receptor immunoprecipitated from cells incubated in the absence (lane 3) and presence (lane 4) of insulin, the insulin receptor β subunit was removed from dried gel fragments and hydrolyzed. The phosphoamino acids were separated by TLC as described under Experimental Procedures.

SDS−PAGE. A typical autoradiograph of immunoprecipitated insulin receptor is shown in Figure 1A. The ^{32}P-labeled 95-kDa band corresponds to the insulin receptor β subunit. The phosphorylation of the insulin receptor results from both autophosphorylation, which occurs on tyrosine residues, and activation of cellular serine/threonine kinase(s). Phosphoamino acid analysis was therefore required to distinguish these activities. A typical autoradiograph of the TLC separation of phosphoamino acids from the hydrolysed-insulin receptor β subunit is shown in Figure 1B. The amount of phosphate incorporated into tyrosine and serine residues of the receptor was quantitated by scintillation counting of the scraped TLC spots. Figure 2 shows the effects of two of the additives on the levels of phosphotyrosine and phosphoserine, as nanomoles of ^{32}P transferred per 10^5 cells in 5 min. While threonine phosphorylation of the insulin receptor has been reported (Kasuga et al., 1982b; Jacobs & Cuatrecasas, 1986), the phosphothreonine was barely detectable and not quantifiable by this method. The relative tyrosine kinase activity (presumed to be insulin receptor autophosphorylation activity) was compared by subtracting basal phosphorylation from insulin-stimulated and expressing the result as a percent of insulin-promoted phosphate transferred in the absence of any compound. These values are compared in Table 1, for the highest concentrations of compounds tested. As shown in Figure 2A, increasing concentrations of the bilayer stabilizing peptide derivative carbobenzoxy-L-phenylalanylglycine (CBZ-L-Phe-Gly) reduced the level of insulin-stimulated insulin

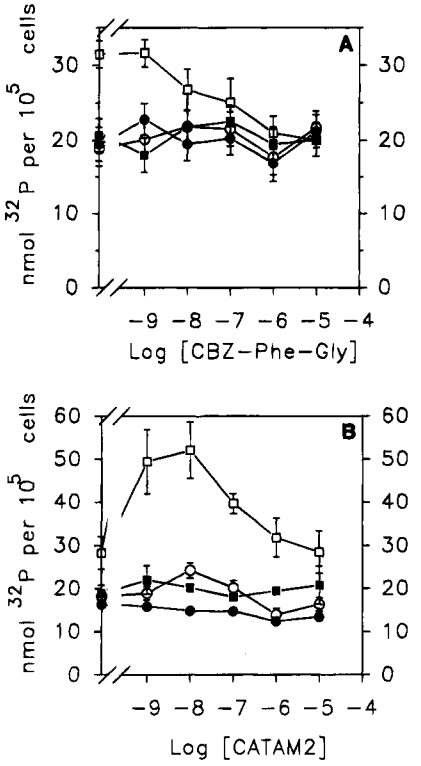

FIGURE 2: Effects of additives on insulin receptor tyrosine (open symbols) and serine (closed symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin, as described under Experimental Procedures. (A) CBZ-L-Phe-Gly; (B) CATAM2. Values are mean ± SEM for three experiments.

Table 1: Effect of Various Compounds on Tyrosine Phosphorylation of the Insulin Receptor β Subunit and Glucose Uptake in NIH 3T3 HIR 3.5 Cells[a]

compound	% tyrosine phosphorylation	% glucose uptake
CBZ-L-Phe-Gly	0.3 ± 4.5	11 ± 5
CBZ-Gly-L-Phe	4.9 ± 0.6	16 ± 5
apolipoprotein A-I	0.01 ± 1.8	139 ± 6
lysophosphatidylcholine	30 ± 6	21 ± 4
lauroylcarnitine	40 ± 6	27 ± 5
palmitoylcarnitine	2.0 ± 0.5	56 ± 10
sphingosine	35 ± 8	76 ± 23
CATAM1	20 ± 2	35 ± 4
CATAM2	no effect[b]	95 ± 5
dioctanoylglycerol	no effect[b]	103 ± 4

[a] The % tyrosine phosphorylation and % glucose uptake were calculated from $I_a - B_a/I_c - B_c \times 100\%$, where I is insulin-stimulated, B is basal, a is in the presence of 10 μM added compound, c is control. Values are MEAN ± SEM, for 10 μM compound. [b] No significant effect at 10 μM, as determined by independent t-test.

receptor tyrosine phosphorylation almost to that in the absence of insulin, with no effect on serine phosphorylation. Bilayer stabilizing strength relates to the ability of the compound to raise the bilayer to hexagonal phase transition temperature of model membranes, i.e., to stabilize the bilayer *relative to* the H_{II} phase. Strong bilayer stabilizers raise T_H at relatively low mole fractions, while hexagonal phase promoters lower this temperature (Epand, 1991). The peptide derivative carbobenzoxyglycylphenylalanine (CBZ-Gly-L-Phe) has less bilayer stabilizing strength than does CBZ-L-Phe-Gly (Epand et al., 1991). As shown in Table 1, CBZ-Gly-L-Phe was also less inhibitory than CBZ-L-Phe-Gly with respect to insulin receptor phosphorylation activity. The strongest bilayer stabilizer tested, apolipoprotein A-I (apo A-I), inhibited tyrosine phosphorylation of the receptor to the greatest extent. Lysophosphatidylcholine (lyso-PC) was less potent in inhibiting tyrosine phosphorylation of the receptor, as it did not inhibit to basal levels at the concentrations tested. Palmitoylcarnitine, which raises T_H more per mole fraction than does the shorter-chained lauroylcarnitine (Epand et al., 1989), was more effective at inhibiting insulin stimulation of insulin receptor phosphorylation. None of the bilayer stabilizing additives affected basal levels of phosphotyrosine or phosphoserine in the β subunit of the insulin receptor. Only apo A-I inhibited insulin stimulation of serine phosphorylation. However, this was the only case where serine phosphorylation was significantly stimulated in response to insulin (results not shown). Two cationic amphiphiles which had been shown to inhibit protein kinase C activity (Bottega & Epand, 1992) were also investigated in this system. These amphiphiles also raise the bilayer to hexagonal phase transition temperature of model membranes (Bottega & Epand, 1992). They were found to have no effect on basal or insulin-stimulated serine phosphorylation of the insulin receptor. 1-(Dimethylamino)-3-(trimethylammonio)-DL-2-propylcholesteryl-3β-oxysuccinate [CATAM1, referred to as amphiphile XI by Bottega and Epand (1992)] inhibited tyrosine phosphorylation to basal levels. Despite the similarity in structure and effect on protein kinase C (PKC) activity (Bottega & Epand, 1992), 2-{[2-(trimethylammonio)ethyl]-methylamino}ethylcholesteryl-3β-oxysuccinate [CATAM2, or amphiphile XIII in Bottega and Epand (1992)] had no effect on phosphorylation of the insulin receptor, as shown in Figure 2B. Sphingosine is a cationic amphiphile with the opposite effect on membrane physical properties. That is, sphingosine lowers the bilayer to hexagonal phase transition temperature (Epand et al., 1991). We tested to see if this compound could stimulate insulin receptor phosphorylation. We found that sphingosine inhibited insulin-stimulated insulin receptor tyrosine phosphorylation to near-basal levels, in agreement with the findings of Arnold and Newton (1991), who used much higher concentrations of sphingosine. The uncharged hexagonal phase promoter dioctanoylglycerol (DiC8) was also investigated in this system. DiC8 was found to have no significant effect (Table 1). However, a series of hexagonal phase promoters could not be tested as these additives are generally cytotoxic. The concentrations of compounds shown in Figure 2 refer to the bulk concentration in the buffer overlaying the monolayer of cells. At 10^{-5} M, this would correspond to approximately 6 mol % relative to the phospholipid of the cell plasma membrane, if all of the compound partitioned into the membrane. The fraction of the less hydrophobic CBZ-peptides and apo A-I in the membrane would be less than this estimate.

Bilayer stabilizing potency does not strictly correlate with the extent of kinase inhibition, when comparing compounds of widely differing structure. For example, the rank order of stabilizing potency is Apo A-I > palmitoylcarnitine > CATAM1 > lyso-PC > CATAM2 > lauroylcarnitine > CBZ-Phe-Gly > CBZ-Gly-Phe > sphingosine > DiC8, while the rank order for kinase inhibitory potency is Apo A-I ≃ CBZ-Phe-Gly ≃ palmitoylcarnitine > CBZ-Gly-Phe > CATAM1 > lyso-PC ≥ sphingosine ≥ lauroylcarnitine > CATAM2 ≃ DiC8. This order of potency refers to that at 10^{-5} M compound, rather than IC_{50} values, as the latter were difficult to accurately determine from the mostly nonsig-

moidal dose−response curves. The data suggest that, except for cationic amphiphiles, compounds which raise the bilayer to hexagonal phase transition temperature in model membranes inhibit insulin-stimulated insulin receptor tyrosine phosphorylation. In general, peptides are more effective inhibitors than are the amphiphiles, but within these classes the inhibitory potency follows the same rank order as their bilayer stabilizing ability.

The inhibition of kinase activity was not a result of inhibition of insulin binding to its receptor. The compounds had little or no effect on the concentration of unlabeled insulin required to displace 50% of the [^{125}I]insulin (EC_{50}) (results not shown).

Furthermore, the effects of the inhibitors of autophosphorylation on cell viability were assessed, to confirm that cell death was not responsible for the observed inhibition of insulin receptor phosphorylation. None of the compounds led to significant cell death at the concentrations used in the phosphorylation assay (results not shown).

Stimulation of a tyrosine phosphatase would also lead to decreased insulin receptor tyrosine phosphorylation. Therefore, a nonspecific tyrosine phosphatase inhibitor, sodium orthovanadate, was included at a concentration of 1 mM during incubations and solubilization of the cells. Even after a 10 min incubation with insulin there was very little phosphate loss from the β subunit of the insulin receptor (results not shown). Therefore protein tyrosine phosphatase stimulation is not involved in the mechanism of action of these compounds.

To correlate membrane biophysical properties to insulin receptor activity and to insulin signal transduction, we examined the effects of the same compounds on [^{3}H]2-deoxyglucose uptake into NIH 3T3 HIR 3.5 cells. It had previously been shown that CBZ-dipeptides inhibit glucose uptake in rat adipocytes and that the inhibition correlated with bilayer stabilizing potency (Epand et al., 1991). CBZ-Gly-L-Phe and CBZ-L-Phe-Gly also inhibited glucose uptake in these cells, to approximately 16% and 11% of that in the absence of these peptides. Representative dose−response curves are shown in Figure 3. None of the compounds affected basal glucose uptake (results not shown); therefore, the data were normalized by subtracting basal glucose uptake from that in the presence of insulin and expressed as a percentage of that in the absence of any added compound. Table 1 illustrates the effects of the additives on glucose uptake activity in these cells. The bilayer stabilizer lauroylcarnitine inhibited glucose uptake to 27 ± 5%, at 10 μM. The longer-chained palmitoylcarnitine inhibited insulin-stimulated glucose uptake with increasing concentrations, but only to approximately 56% of control. This is in contrast to the greater bilayer stabilizing ability and inhibition of insulin receptor tyrosine phosphorylation of palmitoylcarnitine relative to lauroylcarnitine. The strongest bilayer stabilizer tested, apo A-I, gave an uncharacteristic result, inhibiting glucose uptake at 0.1 μM and stimulating it to approximately 140% at 10 μM, as shown in Figure 3A. These were significant effects, as determined by unpaired *t*-tests. Furthermore, sphingosine, which inhibited receptor phosphorylation, had no effect on transport of glucose into these cells, while it had previously been shown to stimulate glucose uptake by rat adipocytes (Epand et al., 1991). Furthermore, the lack of effect of sphingosine on basal glucose uptake is in contrast to its effect in rat adipocytes, where it has a

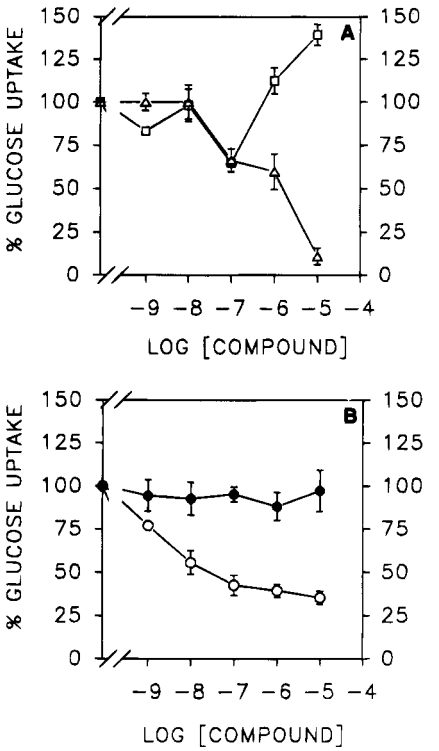

FIGURE 3: Effects of additives on insulin stimulated glucose uptake in NIH 3T3 HIR 3.5 cells. Internalization of [^{3}H]2-deoxyglucose by cells was measured as described under Experimental Procedures. Basal [^{3}H]2-deoxyglucose uptake was subtracted from that in the presence of 1 μM insulin and expressed as a percentage of that in the absence of added compound. Values are mean ± SEM for triplicate samples. (A) CBZ-L-Phe-Gly (△), apo A-I (□); (B) CATAM1 (○), CATAM2 (●).

stimulatory insulin-like action on the basal rate of glucose transport (Robertson et al., 1989; Epand et al., 1991). The two cationic amphiphiles had different effects on glucose uptake activity, as shown in Figure 3B. CATAM1, which inhibited insulin receptor tyrosine phosphorylation, also inhibited glucose uptake to 35 ± 4%, while CATAM2, which stimulated receptor phosphorylation at low concentrations, had no effect on glucose transport. DiC8 also had no effect on insulin stimulation of glucose uptake, consistent with its inability to alter tyrosine phosphorylation of the insulin receptor. These data suggest that, in general, bilayer stabilizers that inhibit insulin receptor tyrosine phosphorylation also inhibit glucose uptake, while hexagonal phase promoters have no effect. In addition, cationic amphiphiles affect insulin signaling in a manner not readily predictable from their effects on T_H.

Given the complexity of the intact cell system, we also assessed the ability of the compounds to affect insulin receptor phosphorylation in a simple, defined assay. Insulin stimulation of the autophosphorylation of purified placental insulin receptor was therefore measured in Triton mixed micelles. Various phospholipids were first tested for their effects on tyrosine phosphorylation of the insulin receptor in response to insulin. The zwitterionic phospholipid phosphatidylcholine (PC) supported a large stimulation of autophosphorylation in response to insulin at high mol % lipid, with no effect on basal phosphorylation. It was determined that 2 μg of insulin receptor and an incubation time of 15 min were within the linear range of activity, for micelles containing 75 mol % PC, relative to Triton X-100 (results not shown). These conditions were therefore used to confirm

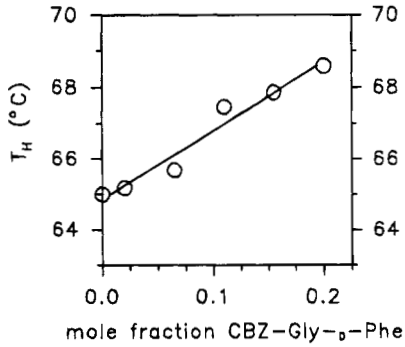

FIGURE 4: Shift in the bilayer to hexagonal phase transition temperature of DEPE as a function of different concentrations of CBZ-Gly-D-Phe. The lipid (5 mg/mL) and peptide were hydrated with Pipes buffer, pH 7.4, and subjected to differential scanning calorimetry for determination of the bilayer to hexagonal phase transition temperature (T_H), as described under Experimental Procedures. Duplicate samples were run to ensure reproducibility.

the effects of various membrane additives on insulin receptor autophosphorylation observed in intact cells.

None of the compounds tested affected basal phosphorylation levels; therefore, the amount of ^{32}P incorporation into the insulin receptor β subunit in the absence of insulin was subtracted from that in the presence of insulin, and this value was expressed as a percentage of the control, with no added compound. The ^{32}P incorporation into the β subunit of the receptor occurs on tyrosine residues, as determined by phosphoamino acid analysis (results not shown). The enantiomer of CBZ-Gly-L-Phe had not been previously characterized with respect to its ability to modulate membrane physical properties. Differential scanning calorimetry was therefore performed, to determine the ability of CBZ-Gly-D-Phe to raise the bilayer to hexagonal phase transition temperature (T_H) of DEPE. Increasing mole fractions of this peptide raised the transition temperature of DEPE. The slope of the plot of mole fraction of CBZ-Gly-D-Phe versus hexagonal phase transition temperature (Figure 4) was 20 ± 3 °C/mol, indistinguishable from 15 ± 3 °C/mol for that of CBZ-Gly-L-Phe (Epand et al., 1991), suggesting that these optical isomers have the same effect on the bulk biophysical properties of the membrane. As shown in Figure 5A and Table 2, CBZ-Gly-L-Phe and its enantiomer CBZ-Gly-D-Phe inhibited insulin receptor phosphorylation to similar extents over the range of concentrations tested. The slightly stronger bilayer stabilizer, CBZ-Phe-Gly, inhibited autophosphorylation to a somewhat greater extent, as in intact cells, while the water-soluble dipeptide L-Phe-Gly (lacking the hydrophobic CBZ group) was unable to affect insulin receptor kinase activity (Figure 5A). Apolipoprotein A-I (apo A-I) and lysophosphatidylcholine (lyso-PC) had the strongest effects on kinase activity (Table 2). Lauroylcarnitine had no effect in this assay, in contrast to its inhibitory action in NIH 3T3 HIR 3.5 cells, while palmitoylcarnitine only gave weak inhibition to 74 ± 2% ($p < 0.05$) at 10 μM, with no effect at lower concentrations. High concentrations of the hexagonal phase promoter sphingosine caused an inhibition of insulin receptor autophosphorylation (Figure 5B). This was is agreement with its effects in intact cells. The other two cationic amphiphiles, CATAM1 and CATAM2, augmented insulin stimulation of receptor phosphorylation at low concentrations. At 10 μM, however, CATAM1 inhibited this activity, while CATAM2 had no effect (Table 2). The effects at 10 μM agree with those in NIH 3T3 HIR 3.5 cells. DiC8

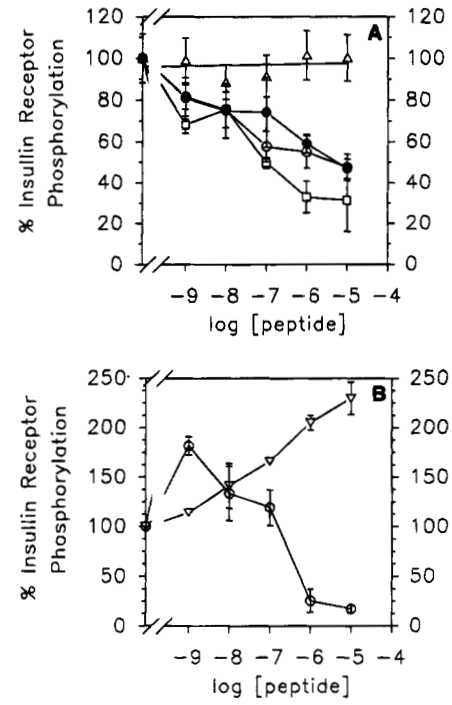

FIGURE 5: Effects of various compounds on insulin receptor phosphorylation in mixed micelles. Insulin receptor phosphorylation was measured as described under Experimental Procedures. The incorporation of ^{32}P into the insulin receptor β subunit in the absence of insulin was subtracted from that in the presence of insulin, and this was expressed as a percent of that in the absence of added compound. Values are mean ± SEM for triplicate experiments. (A) CBZ-Gly-D-Phe (●), CBZ-Gly-L-Phe (○), CBZ-L-Phe-Gly (□), L-Phe-Gly (△). (B) Sphingosine (○), DiC8 (▽).

stimulated the insulin effect, with increasing concentrations, to approximately 230% of control phosphorylation at 10 μM, as shown in Figure 5B. Such stimulation was not observed in the cell assay.

In summary, there is agreement between the inhibitory action of the bilayer stabilizing additives in the two assays (with the exception of lauroylcarnitine). At high concentrations the cationic amphiphiles also have the same effects in both systems, while the hexagonal promoter DiC8 was only able to affect insulin receptor activity in micelles.

Finally, to determine whether any of these compounds could exert their effects by directly affecting the enzymatic activity of the receptor, we tested their ability to modulate the tyrosine kinase activity of the soluble insulin receptor kinase (SIRK) domain. The bilayer-stabilizing compounds were unable to affect the constitutively active SIRK. DiC8, sphingosine, and CATAMI, however, inhibited SIRK activity (results not shown).

DISCUSSION

Several membrane functions are modulated by the bulk biophysical properties of the membrane (Shinitzky, 1984). It has previously been demonstrated that membrane additives can modify insulin-dependent functions of rat adipocytes in a manner predictable from the properties of the additives in model membranes (Epand et al., 1991). Shifts in the bilayer to hexagonal phase transition temperature in model membranes upon incorporation of peptide derivatives was correlated with effects on glucose uptake. While these results suggested that the mechanism of inhibition of insulin signaling is related to the ability of the peptide derivatives

Table 2: Effect of Various Compounds on Insulin Receptor Tyrosine Phosphorylation in Mixed Micelles[a]

compound	% tyrosine phosphorylation	compound	% tyrosine phosphorylation
CBZ-L-Phe-Gly	31 ± 15	lauroylcarnitine	no effect[b]
L-Phe-Gly	100 ± 10	palmitoylcarnitine	74 ± 2
CBZ-Gly-L-Phe	47 ± 6	sphingosine	17 ± 3
CBZ-Gly-D-Phe	47 ± 5	CATAM1	75 ± 8[c]
apolipoprotein A-I	23 ± 2	CATAM2	no effect[d]
lyophosphatidylcholine	18 ± 9	dioctanoylglycerol	230 ± 16

[a] The % tyrosine phosphorylation was calculated as in Table 1. Values are mean ± SEM, for 10 μM compound. [b] No significant effect, as determined by independent *t*-test. [c] CATAM1 stimulated insulin receptor phosphorylation at lower concentrations, with a maximum of 161 ± 8% at 0.1 μM. [d] CATAM2 had no significant effect at 10 μM, as determined by independent t-test, but maximally stimulated insulin receptor phosphorylation to 156 ± 14% at 0.1 μM.

to alter receptor function, there are also many other factors that can determine the rate of glucose uptake into cells. We have now identified one step in insulin signaling which is inhibited by these peptides, as well as by other bilayer stabilizing agents. Compounds which raise the bilayer to hexagonal phase transition temperature of model membranes inhibit insulin signaling by inhibiting autophosphorylation of the receptor in response to insulin. These compounds do not affect the affinity of insulin for its receptor. This is not surprising given the variability in the structures of these additives. It would be difficult to imagine how each of these compounds could interact with the insulin-binding domain of the α subunit of the insulin receptor. It is similarly unlikely that each of these compounds, of differing structures, could elicit their effects by interacting directly with other regions of the receptor. The inhibition of SIRK activity by cationic amphiphiles, including sphingosine, suggests that the effects of high concentrations of positively charged compounds on the intact receptor activity may not be due to membrane modulation. However, the inhibition of SIRK activity by DiC8 does not suggest that this hexagonal phase promoter interacts with the kinase domain of the insulin receptor. Such an interaction would not explain DiC8 stimulation of intact receptor activity, as the interaction of DiC8 with the receptor kinase domain would not likely occur in the presence of phospholipids. All of the compounds which inhibited insulin receptor autophosphorylation in intact cells have similar effects on lipid polymorphism. Thus it is possible that the compounds exert their effects on insulin signaling nonspecifically, via their effects on the bulk biophysical properties of the membrane. It is possible that agents which relieve negative curvature strain in membranes make the bilayer arrangement more stable so that protein conformational rearrangements, required for enzyme catalysis and/or signal transduction, cannot occur. The functioning of a number of other proteins are inhibited by agents which raise the bilayer to hexagonal phase transition temperature. These include rhodopsin (Gibson & Brown, 1993) and protein kinase C (Senisterra & Epand, 1993).

NIH 3T3 HIR 3.5 cells which were transfected to express the human insulin receptor were used for these studies. While these cells can be differentiated to adipocytes (Frost & Lane, 1985), we found that the fibroblasts themselves exhibited a sizeable stimulation of insulin receptor autophosphorylation in response to insulin. Furthermore, the lengthy differentiation period and fragility of the adipocytes were avoided by utilizing the fibroblasts for these studies. Given the lack of understanding of insulin signal transduction, and the complexity of signal transduction in general, a simplified, defined assay of insulin receptor phosphorylation was sought to confirm the effects of membrane-modulating compounds on insulin receptor activity in intact cells. A micellar assay, using purified human placental insulin receptor, was chosen over a vesicle assay. It is difficult to measure insulin receptor activity in lipid vesicles, since access to both sides of the membrane is required, to allow both for interaction of insulin with the α subunit as well as ATP with the β subunit on the opposite side of the membrane. Insulin receptor activity has been previously measured in a detergent solubilized assay containing *N*-acetyl-β,D-glucopyranoside and phosphatidylinositol (Sweet et al., 1987). In the present study, hydrophobic compounds are included in the lipid film preparation, allowing for a uniform distribution of the compounds with the phospholipid and detergent. The detergent included in this assay was Triton X-100 rather than *N*-acetyl-β,D-glucopyranoside, as the latter has been shown to decrease insulin binding and kinase activation (Leray et al., 1992). While mixed micelles differ from natural membranes, there is reason to believe that membrane additives have similar effects on kinase activity in micelles as in phospholipid bilayers. First, protein kinase C activity has been successfully assayed in a similar system (Hannun et al., 1985). Secondly, the effects of these compounds on insulin receptor activity in the micellar assay were found to be similar to those found in intact cells.

In general, apart from cationic amphiphiles, the effects of compounds on insulin receptor tyrosine phosphorylation correlates with their effects on lipid polymorphism. Thus the mechanism of action of these compounds may reside in their ability to modulate the physical properties of the membrane. This is supported by the results obtained for the optical isomers of CBZ-Gly-Phe. If CBZ-Gly-L-Phe inhibited insulin receptor kinase activity by direct interaction with the insulin receptor, its enantiomer CBZ-Gly-D-Phe should not be able to interact with the receptor in the same manner and therefore should not exert the same effect on kinase activity. In fact, the two isomers were found to have the same effect on insulin receptor phosphorylation, supporting the hypothesis that these compounds act via membrane modulation. This was further supported by the inability of the dipeptide L-Phe-Gly to affect insulin receptor activity. L-Phe-Gly lacks the hydrophobic carbobenzoxy group and would therefore not be likely to partition into the membrane. For compounds of similar structure, the rank order of potency of bilayer stabilization correlated directly with that of inhibitory potency. However, the bilayer stabilization strength (the slope of the plot of hexagonal phase transition temperature versus mole fraction of compound) did not quantitatively correlate with autophosphorylation inhibitory strength for either the micellar or the intact cell assay. This may be a

consequence of comparing the effects of the compounds at one particular concentration, rather than correlating EC_{50} values. The shapes of the dose–response curves for the membrane additives did not permit accurate determination of EC_{50} values for each compound. Furthermore, the amount of each compound that actually partitioned into the membrane may vary. In addition, there are other complexities in this system including effects of the additives on other bulk membrane physical properties besides curvature strain, the miscibility of the additives with other membrane components, i.e., different extents of partitioning into different domains of the membrane, and access of the additive to the membrane region surrounding the receptor, or, in certain cases, direct interaction with the receptor protein. The differential effect of lauroylcarnitine in the two assays may be explained by the short size of the hydrophobic fatty acyl chain of this compound. It may be less able to affect curvature strain in micelles, compared with intact cells.

The cationic amphiphiles sphingosine, CATAM1, and CATAM2 appear to belong to a separate class of compounds whose effects on insulin signaling are not readily predictable from their membrane modulating activity. Even certain cationic and hydrophobic polypeptides and proteins affect the tyrosine kinase activity of the insulin receptor (Morrison et al., 1989). The lack of effect of CATAM1 and 2 on insulin receptor serine phosphorylation was interesting, given that CATAM1 and 2 inhibit protein kinase C activity (Bottega & Epand, 1992). This suggests that the insulin receptor is not a substrate for PKC in these cells. This may be due to the imbalance in the relative amounts of these two proteins, since the NIH 3T3 HIR 3.5 cells overexpress the insulin receptor but not any of the PKC isoforms. Furthermore, the differential effects of the two cationic amphiphiles, despite their similar structures, suggests that their mechanism of action involves more than membrane modulation.

The requirement for insulin receptor autophosphorylation in glucose uptake is not completely established (see the introduction). Our findings that compounds which inhibit tyrosine phosphorylation of the insulin receptor also inhibit glucose uptake support the generally held belief that tyrosine kinase activity is in fact required for insulin signaling to promote glucose transport. The strong bilayer stabilizer and inhibitor of insulin receptor tyrosine phosphorylation, apo A-I, was an exception, augmenting insulin stimulation of glucose uptake. Apo A-I may extract lipids from the plasma membrane, and this may have more of an effect on glucose uptake than on insulin receptor activity. While the bilayer stabilization by apo A-I would be expected to inhibit insulin receptor activity, the lipid extraction may stimulate membrane cycling and recruitment of glucose transporters. However, the action of apo A-I is not sufficient to stimulate glucose transport in the absence of insulin, as shown by the lack of effect of apo A-I on basal glucose uptake. In general, glucose uptake was less sensitive to the additives than was autophosphorylation activity, as higher concentrations were required to inhibit glucose uptake (for example, compare Figures 2A and 3A). This may be a result of the overexpression of insulin receptor in these cells. A 70 or 80% inhibition of autophosphorylation activity still leaves a lot of autophosphorylated insulin receptors in these cells. Only at high concentrations of the additives could glucose transport also be inhibited. At such high concentrations, the modulation of membrane physical properties may account for the inhibition of glucose uptake, not only through inhibition of insulin signaling, but also by directly affecting the activity of the transmembrane glucose transporter or its recruitment to the plasma membrane. The cationic amphiphiles had differential effects on glucose uptake and insulin receptor phosphorylation, supporting the idea that these compounds, including sphingosine, fall into their own separate class.

Compounds which lower the bilayer to hexagonal phase transition temperature of model membranes tend to be cytotoxic. Therefore the only noncationic hexagonal phase promoter included in these studies was DiC8. Although we did not observe an effect of DiC8 on receptor phosphorylation in NIH 3T3 HIR 3.5 cells, studies in rat adipocytes showed a stimulation of insulin receptor tyrosine phosphorylation in response to this additive (Terry et al., 1991). Studies in cultured human monocytes and lymphoblastoid cells also showed increased tyrosine kinase activity both in intact cells and with the isolated insulin receptor (Grunberger & Levy, 1990). We have observed DiC8 stimulation of receptor activity in Triton mixed micelles. DiC8 increases the negative monolayer curvature strain and promotes hexagonal phase formation. Micelles may be more susceptible to additives which promote negative curvature strain than are the plasma membranes of cells, since micelles have positive spontaneous curvature.

We have demonstrated that a variety of hydrophobic compounds of diverse chemical structure affect the insulin-promoted phosphorylation of tyrosine residues on the insulin receptor. These effects occur independent of changes in insulin binding to the receptor or changes in the nonstimulated tyrosine kinase activity of the receptor. It thus appears that these agents are affecting the efficiency of signal transduction between the extracellular insulin binding site and the cytoplasmic tyrosine kinase domain of the receptor. Previous studies have shown that the tyrosine kinase activity of reconstituted insulin receptors was modulated by phospholipids (Lewis & Czech, 1987; Leray et al., 1993). Our work demonstrates that such modulation of insulin receptor function can also result from the incorporation of other substances into the membrane, suggesting a role for the modulation of membrane physical properties in determining receptor function. None of the compounds tested had any effect on basal insulin receptor tyrosine phosphorylation. This suggests that the mechanism of action of these compounds lies in their ability to affect the conformational change of the insulin receptor that occurs upon insulin binding to the α subunit. This is further supported by the inability of these compounds to affect the activity of the constitutively active soluble insulin receptor kinase domain. Although the system is complex and several factors contribute to determining receptor function, one of the factors that can be identified as being important for this change in receptor function is membrane monolayer curvature. In particular, uncharged or anionic substances that partition into the membrane and reduce negative curvature strain are inhibitors of insulin receptor signal transduction. Alterations in the membrane environment of the insulin receptor may be important in changing the insulin receptor tyrosine kinase activity in non-insulin-dependent diabetes (Grunberger et al., 1990) or in aging (Carrascosa et al., 1989; Nadiv et al., 1994).

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REFERENCES

Arnold, A. S., & Newton, A. C. (1991) *Biochemistry 30*, 7747−7754.

Baron, V., Gautier, N., Balloti, R. K., Rossi, B., Saint-Pierre, S., Van Obberghen, E., & Dolais-Kitabi, J. (1989) *Biochem. J. 260*, 749−756.

Baron, V., Kaliman, P., Gautier, N., & Van Obberghen, E. (1992) *J. Biol. Chem. 267*, 23290−23294.

Bottega, R., & Epand, R. M. (1992) *Biochemistry 31*, 9025−9030.

Carrascosa, J. M., Ruiz, P., Martinez, C., Pulido, J. A., Satrustegui, J., & Andres, A. (1989) *Biochem. Biophys. Res. Commun. 160*, 303−309.

Cobb, M. H., Sang, B.-C., Gonzales, R., Goldsmith, E., & Ellis, L. (1989) *J. Biol. Chem. 264*, 18701−18706.

Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P., & Lewis, R. E. (1988) *J. Biol. Chem. 263*, 11017−11020.

Debant, A., Clauser, E., Ponzio, G., Filloux, C., Auzan, C., Contreres, J.-O., & Rossi, B. (1988) *Proc. Natl. Acad. Sci. U.S.A. 85*, 8032−8036.

Debant, A., Ponzio, G., Clauser, E., Contreres, J.-O., & Rossi, B. (1989) *Biochemistry 28*, 14−17.

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 40*, 747−758.

Ellis, L., Clauser, E., Morgan, D. O., Ettery, M., Roth, R. A., & Rutter, W. J. (1986) *Cell 45*, 721−732.

Epand, R. M. (1991) in *Cell and Model Membrane Interactions* (Ohki, S., Ed.) pp 135−147, Plenum Press, New York.

Epand, R. M., & Epand, R. F. (1994) *Biophys. J. 66*, 1450−1456.

Epand, R. M., Robinson, K. S., Andrews, M. E., & Epand, R. F. (1989) *Biochemistry 28*, 9398−9402.

Epand, R. M., Stafford, A. R., & Debanne, M. T. (1991) *Biochemistry 30*, 2092−2098.

Frost, S. C., & Lane, D. (1985) *J. Biol. Chem. 260*, 2646−2652.

Fujita-Yamaguchi, Y., Choi, S., Sakamoto, Y., & Itakura, K. (1983) *J. Biol. Chem. 258*, 5045−5049.

Gibson, N. J., & Brown, M. F. (1993) *Biochemistry 32*, 2438−2454.

Ginsberg, B. H., Brown, T. J., Simon, I., & Spector, A. A. (1981) *Diabetes 30*, 773−780.

Grunberger, G., & Levy, J. (1990) *Eur. J. Biochem. 187*, 191−198.

Gruner, S. M. (1992) in *The Structure of Biological Membranes* (Yeagle, P. L., Ed.) pp 211−250, CRC Press, Boca Raton, FL.

Hannun, Y. A., Loomis, C. R., & Bell, R. M. (1985) *J. Biol. Chem. 260*, 10039−10043.

Hubert, P., Brunear-Wack, C., Cremel, G., Le Marchand-Brustel, Y., & Staedel, C. (1991) *Cell Regul. 2*, 56−72.

Jacobs, S., & Cuatrecasas, P. (1986) *J. Biol. Chem. 261*, 934−939.

Kasuga, M., Zick, Y., Blithe, D. L., Crettaz, M., & Kahn, C. R. (1982a) *Nature 298*, 667−669.

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

Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E., & Czech, M. P. (1993) *Proc. Natl. Acad. Sci. U.S.A. 90*, 4460−4464.

Leray, V., Hubert, P., Burgun, C., Staedel, C., & Cremel, G. (1993) *Eur. J. Biochem. 213*, 277−284.

Lewis, R. E., & Czech, M. P. (1987) *Biochem J. 248*, 829−836.

Lewis, R. E., MacDonald, R. G., & Czech, M. P. (1990) *J. Biol. Chem. 265*, 947−954.

Lowenstein, E. J., Daly, R. J., Batzer, A. G., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E. Y., Bar-Sagi, D., & Schlessinger, J. (1992) *Cell 70*, 431−442.

Manchester, J., Kong, X., Lowry, O. H., & Lawrence, J. C., Jr. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 4644−4648.

Moller, D. E., Benecke, H., & Flier, J. S. (1991) *J. Biol. Chem. 266*, 10995−11001.

Morrison, B. D., Feltz, S. M., & Pessin, J. E. (1989) *J. Biol. Chem. 264*, 9994−10001.

Munoz, G., & Marshall, S. H. (1990) *Anal. Biochem. 190*, 233−237.

Nadiv, O., Shinitzky, M., Manu, H., Hecht, D., Roberts, C. T., Jr., LeRoith, D., & Zick, Y. (1994) *Biochem. J. 298*, 443−450.

Olefsky, J. M. (1990) *Diabetes 39*, 1009−1016.

Pilch, P. F., & Czech, M. P. (1980) *Science 210*, 1152−1153.

Rilfors, L., Hauksson, J. B., & Lindblom, G. (1994) *Biochemistry 33*, 6110−6120.

Robertson, D. G., DiGirolamo, M., Merrill, A. H., Jr., & Lambeth, J. D. (1989) *J. Biol. Chem. 264*, 6773−6779.

Salteil, A. R. (1990) *J. Nutr. Biochem. 1*, 180−188.

Senisterra, G., & Epand, R. M. (1993) *Arch. Biochem. Biophys. 300*, 378−383.

Shinitzky, M. (1984) *Physiology of Membrane Fluidity*, Vols. I and II, CRC Press, Boca Raton, FL.

Sun, X. J., Rothenberg, P., Kahn, C. R., Backer, J. M., Araki, E., Wilden, P. A., Cahill, D. A., Goldstein, B. J., & White, M. F. (1991) *Nature 352*, 73−77.

Sun, X. J., Crimmins, D. L., Myers, M. G., Miralpiex, M., & White, M. F. (1993) *Mol. Cell. Biol. 13*, 7418−7428.

Sweet, L. J., Dudley, D. T., Pessin, J. E., & Spector, A. A. (1987) *Faseb J. 1*, 55−59.

Tavare, J. M., O'Brien, R. M., Siddle, K., & Denton, R. M. (1988) *Biochem. J. 253*, 783−788.

Taylor, R. (1991) *Clin. Endocrinol. 34*, 159−171.

Terry, M. L., Levy, J., & Grunberger, G. (1991) *Horm. Metab. Res. 23*, 266−270.

Ullrich, A., Bell, J. R., Chen, E. Y., Herrera, R., Petruzelli, 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 313*, 756−761.

White, M. F., & Kahn, C. R. (1994) *J. Biol. Chem. 269*, 1−4.

White, M. F., Maron, R., & Kahn, C. R. (1985) *Nature 318*, 183−186.

Whittaker, J., Okamoto, A. K., Thys, R., Bell, G. I., Steiner, D. F., & Hofmann, C. A. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 5237−5241.

Wilden, P. A., Kahn, R. C., Siddle, K., & White, M. F. (1992) *J. Biol. Chem. 267*, 16660−16668.

BI941856H