THE EFFECTS OF MEMBRANE PHYSICAL PROPERTIES ON INSULIN RECEPTOR AUTOPHOSPHORYLATION AND SIGNALLING

By

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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 of diverse structure, which raise the lamellar to inverted hexagonal phase transition temperature (T_H) in model membranes, including carbobenzoxy-dipeptides, apolipoprotein A-I, acyl carnitines, and lysophosphatidylcholine, inhibited insulin-stimulated tyrosine phosphorylation of isolated receptors, as well as of receptors in cells over-expressing human insulin receptor. For compounds of similar structure, the inhibition of insulin receptor tyrosine phosphorylation correlates well with their bilayer stabilizing potency. All of the compounds tested exert their effects independently of changes in insulin binding to the receptor or changes in the basal tyrosine kinase activity of the receptor. We suggest that the membrane additives tested affect insulin receptor activity through alterations of the bulk physical properties of the membrane. This is further supported by the inability of these compounds to influence the soluble insulin receptor kinase domain activity, measured in the absence of phospholipids. In addition, stereoisomers of dipeptide analogues had identical effects on insulin receptor phosphorylation, suggesting that their mode of action did not involve specific interactions with the receptor.

Most of the compounds which inhibit tyrosine phosphorylation of the insulin receptor also inhibited glucose uptake in the same cells. Insulin-stimulated fluid phase pinocytosis was also inhibited by peptides which raise T_H. This suggests that alteration of membrane physical properties affects the divergent pathways involved in insulin receptor signal transduction.

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 phosphorylation or signalling to glucose uptake in intact cells. The effects of cationic amphiphiles were not readily predictable from their membrane modulating activity.

This thesis provides evidence that the mechanism of modulation of insulin signalling by these additives lies in the ability of such compounds 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.

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TABLE OF CONTENTS

		Page
1.0	**INTRODUCTION**	1
1.1	Overview	1
1.2	Structure of Biological Membranes	3
1.3	Lipid Polymorphism	4
	1.3.1 Phase Transitions	8
	1.3.2 Measurement of Phase Transitions	9
	1.3.3 Phase Transition Temperature as a Measure of Membrane Physical Properties	10
	1.3.4 Effects of Biophysical Properties on Membrane Activity	11
1.4	Insulin	12
1.5	The Insulin Receptor	13
	1.5.1 Synthesis and Processing of the Insulin Receptor	13
	1.5.2 Structure of the Insulin Receptor	14
	1.5.3 Insulin Receptor Function	15
1.6	Insulin Signal Transduction	15
1.7	Insulin Receptor Down-Regulation	20
1.8	Insulin Binding	20
	1.8.1 Analysis of Ligand-Receptor Interactions	21
	1.8.2 Lipid Effects on Insulin Binding	23
1.9	Autophosphorylation as a Measure of Insulin Receptor Activity	23
	1.9.1 Detergent Solubilized Assays of Insulin Receptor Autophosphorylation	24
	1.9.2 Vesicle Assay of Insulin Receptor Autophosphorylation	24
	1.9.3 Intact Cell Assay of Insulin Receptor Autophosphorylation	25

1.10 Lipid Effects on Insulin Receptor Autophosphorylation 26

1.11 Glucose Uptake 27

1.11.1 Lipid Effects on Glucose Transport 29

2.0 MATERIALS AND METHODS 30

2.1 Materials and Sources 30

2.1.1 Lipids 30
2.1.2 Chemicals and Solvents 31
2.1.3 Membrane Additives 31
2.1.4 Tissue Culture Reagents 32
2.1.5 Other Materials 32
2.1.6 Materials from Non-Commercial Sources 32
2.1.7 Commonly Used Buffers and Solutions 33

2.2 Isolation of Insulin Receptor From Human Placenta 35

2.2.1 Preparation of Placental Membranes 35
2.2.2 Solubilization and Affinity Chromatography 36
2.2.3 Insulin Binding Assay 36
2.2.4 Concentration and Storage of Insulin Receptor 37
2.2.5 Protein Assay 37
2.2.6 Competitive Insulin Binding Assay 38

2.3 Differential Scanning Calorimetry 38

2.4 Detergent Solubilized Assays of Insulin Receptor Tyrosine Kinase Activity 39

2.4.1 n-Octyl-β,D-glucopyranoside Assay 39
2.4.2 Triton Micellar Assay 39

2.5 Soluble Insulin Receptor Kinase Domain Activity 40

2.6 Culturing of NIH 3T3 HIR 3.5 Cells 41

2.6.1 Differentiation of NIH 3T3 HIR 3.5 Cells to Adipocytes 41

2.7 Intact Cell Insulin Receptor Phosphorylation 41

2.7.1 Phosphoamino Acid Analysis 42

2.8 Cell Viability Assay 43

2.9 Competitive Binding Studies 43

2.10 Glucose Uptake into NIH 3T3 HIR 3.5 Cells 44

2.11 Fluid Phase Endocytosis 45

2.12 Statistical Analysis 45

3.0 RESULTS AND DISCUSSION 46

3.1 Isolation of Insulin Receptor from Human Placenta 46

3.2 Triton Micellar Assay of Insulin Receptor Autophosphorylation 50

3.2.1 Lipid Sensitivity of Insulin Receptor Activity 50
3.2.2 Determination of Optimal Assay Conditions 55

3.3 Compounds Tested for Effects on Autophosphorylation 59

3.3.1 Effects of Peptide Derivatives on Insulin Receptor Activity in Mixed Micelles 63
3.3.2 Peptide Stereoisomer Effects 67
3.3.3 Effects of Other Bilayer Stabilizers on Insulin Receptor Activity in Mixed Micelles 71
3.3.4 Effects of Hexagonal Phase Promoters on Insulin Receptor Activity in Mixed Micelles 77
3.3.5 Membrane Physical Properties and Insulin Receptor Activity in Mixed Micelles 81

3.4 Soluble Insulin Receptor Kinase Domain Activity 85

3.4.1 Effects of Bilayer Stabilizers on SIRK Activity 86
3.4.2 Effects of Hexagonal Phase Promoters on SIRK Activity 90

3.5 Insulin Receptor Activity in NIH 3T3 HIR 3.5 Cells 92

3.5.1 Effects of CBZ-dipeptides on Insulin Receptor Activity in Intact Cells 95
3.5.2 Effects of Other Bilayer Stabilizers on Insulin Receptor Activity in Intact Cells 98
3.5.3 Effects of Hexagonal Phase Promoters on Insulin Receptor Activity in Intact Cells 107
3.5.4 Membrane Physical Properties and Insulin Receptor Activity in Intact Cells 110

3.6 Insulin Binding 116

3.6.1 Effects of Membrane Additives on Insulin-Receptor Interactions 116

3.7 Cell Viability 120

3.7.1 Effects of Membrane Additives on Cell Viability 121

3.8 Role of Phosphatase Activity in Insulin Receptor Phosphorylation 124

3.9 Correlation Between Insulin Receptor Phosphorylation in Micelles and in Intact Cells 126

3.9.1 Effects of CBZ-dipeptides on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells 129
3.9.2 Effects of Other Bilayer Stabilizers on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells 129
3.9.3 Effects of Hexagonal Phase Promoters on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells 131
3.9.4 Membrane Physical Properties and Insulin Receptor Autophosphorylation 132

3.10 Glucose Uptake in NIH 3T3 HIR 3.5 Cells 133

3.10.1 Effects of CBZ-dipeptides on Glucose Uptake by NIH 3T3 HIR 3.5 Cells 137

3.10.2 Effects of Other Bilayer Stabilizers on Glucose Uptake by NIH 3T3 HIR 3.5 Cells 140
3.10.3 Effects of Hexagonal Phase Promoters on Glucose Uptake by NIH 3T3 HIR 3.5 Cells 146
3.10.4 Membrane Physical Properties and Glucose Uptake by NIH 3T3 HIR 3.5 Cells 149
3.11 Effects of membrane Additives on Insulin Stimulation of Fluid Phase Pinocytosis 150

4.0 SUMMARY AND PERSPECTIVES 153

4.1 Insulin Receptor Phosphorylation and Glucose Uptake 153

4.2 Membrane Physical Properties, Insulin Receptor Activity and Glucose Uptake 156

5.0 BIBLIOGRAPHY 163

LIST OF EQUATIONS

		Page
1.3.1	Shape parameter	7
1.3.2.1	Entropy of phase transition	9
1.3.2.2	Estimation of van't Hoff enthalpy	10
1.8.1.1	Hormone-Receptor interactions	22
1.8.1.2	Mass Action equation	22
1.8.1.3	Scatchard equation	22
3.1.1	Beer's Law	46
3.3.1.1	Normalization of Autophosphorylation data	63

LIST OF FIGURES

		Page
1.3.1	Lipid Phases	6
1.6.1	Insulin Signal Transduction	19
3.1.1	Affi-10-insulin elution profile	48
3.1.2	Scatchard Plot of insulin-receptor binding	49
3.2.1.1	Effect of PE on IRTK activity in micelles	52
3.2.1.2	Effect of PS on IRTK activity in micelles	53
3.2.1.3	Effect of PC on IRTK activity in micelles	54
3.2.2.1	Dependence of insulin receptor autophosphorylation on receptor concentration and incubation time	57
3.2.2.2	Insulin receptor activity in Triton X-100	58
3.3.1	Structures of membrane additives	62
3.3.1.1	Effect of CBZ-Gly-L-Phe on IRTK activity in micelles	65
3.3.1.2	Effect of CBZ-L-Phe-Gly and L-Phe-Gly on IRTK activity in micelles	66
3.3.2.1	Effect of CBZ-dipeptide stereoisomers on IRTK activity in micelles	69
3.3.2.2	Dependence of T_H of DEPE on CBZ-Gly-D-Phe	70
3.3.3.1	Effect of Apo A-I on IRTK activity in micelles	73
3.3.3.2	Effect of Lyso PC on IRTK activity in micelles	74
3.3.3.3	Effects of Acyl carnitines on IRTK activity in micelles	75
3.3.3.4	Effects of cationic amphiphiles on IRTK activity in micelles	76

		Page
3.3.4.1	Effect of sphingosine on IRTK activity in micelles	79
3.3.4.2	Effect of dioctanoylglycerol on IRTK activity in micelles	80
3.3.5.1	Effects of branched DSPC species on IRTK activity in micelles	84
3.4.1.1	Effects of bilayer stabilizers on SIRK activity	88
3.4.1.2	Effects of cationic amphiphiles on SIRK activity	89
3.4.2.1	Effects of hexagonal phase promoters on SIRK activity	91
3.5.1	Electrophoresis and phosphoamino acid analysis of insulin receptor	94
3.5.1.1	Effect of CBZ-L-Phe-Gly on insulin receptor activity in intact cells	96
3.5.1.2	Effect of CBZ-Gly-L-Phe on insulin receptor activity in intact cells	97
3.5.2.1	Effect of Apo A-I on insulin receptor activity in intact cells	99
3.5.2.2	Effect of Lyso PC on insulin receptor activity in intact cells	100
3.5.2.3	Effect of palmitoyl carnitine on insulin receptor activity in intact cells	101
3.5.2.4	Effect of lauroyl carnitine on insulin receptor activity in intact cells	102
3.5.2.5	Effect of CATAM1 on insulin receptor activity in intact cells	105
3.5.2.6	Effect of CATAM2 on insulin receptor activity in intact cells	106
3.5.3.1	Effect of sphingosine on insulin receptor activity in intact cells	108
3.5.3.2	Effect of dioctanoylglycerol on insulin receptor activity in intact cells	109
3.6.1.1	Competitive binding studies	118
3.7.1.1	Effects of CBZ-Gly-L-Phe and decanol on cell viability	122
3.8.1	Dephosphorylation of insulin receptor over time	125

		Page
3.10.1	Time course of Glucose Uptake into NIH 3T3 HIR 3.5 cells	136
3.10.1.1	Effect of CBZ-Gly-L-Phe on Glucose Uptake	138
3.10.1.2	Effect of CBZ-L-Phe-Gly on Glucose Uptake	139
3.10.2.1	Effect of Lyso PC on Glucose Uptake	142
3.10.2.2	Effect of acyl carnitines on Glucose Uptake	143
3.10.2.3	Effect of Apo A-I on Glucose Uptake	144
3.10.2.4	Effect of cationic amphiphiles on Glucose Uptake	145
3.10.3.1	Effect of sphingosine on Glucose Uptake	147
3.10.3.2	Effect of dioctanoylglycerol on Glucose Uptake	148
3.11.1	Effect of CBZ-Gly-L-Phe on Fluid Phase Pinocytosis	151
3.11.2	Effect of CBZ-L-Phe-Gly on Fluid Phase Pinocytosis	152
4.2.1	Potency Correlations	158

LIST OF TABLES

		Page
3.5.4.1	Effects of various compounds on insulin receptor phosphorylation in NIH 3T3 HIR 3.5 cells	115
3.6.1.1	Effects of various compounds on insulin binding to insulin receptor	119
3.7.1.1	Effects of various compounds on cell viability	123
3.9.1	Effects of various compounds on insulin receptor phosphorylation in intact cells and micelles	128
4.1.1	Effects of various compounds on insulin receptor phosphorylation and glucose uptake	155
4.2.1	Summary of effects of various compounds on insulin signalling	159

LIST OF ABBREVIATIONS

Apo A-I	apolipoprotein A-I
ATP	adenosine triphosphate
BSA	bovine serum albumin
CATAM1	1-dimethylammonio-DL-2-propyl-cholesteryl-3-β-oxysuccinate
CATAM2	2-{[2-(trimethylammonio)ethyl]-methylamino}-ethyl-cholesteryl-3-β-oxysuccinate
C terminal	carboxy terminal
CBZ	carbobenzoxy
cDNA	complementary deoxyribonucleic acid
CPM	counts per minute
DAG	diacylglycerol
DEPE	L-α-dielaidoylphosphatidylethanolamine
DiC8	1,2-dioctanoylglycerol
DSC	differential scanning calorimetry
DSPC	distearoylphosphatidylcholine
ε	molar extinction coefficient
EC_{50}	effective concentration to attain 50% of response
EDTA	ethylenediaminetetraacetate
EthD-1	ethidium homodimer-1

FBS	fetal bovine serum
G-protein	guanosine-triphosphate-binding protein
GluT	glucose transporter
Gly	glycine
GRB2	growth factor receptor binding protein 2
H_I	hexagonal phase
H_{II}	inverted hexagonal phase
HEPES	4-(2-hydroxyethyl)-1-piperazine ethanesulfonic acid
IC_{50}	inhibitory concentration to attain 50%
IRS-1	insulin receptor substrate I
IRTK	insulin receptor tyrosine kinase
KRBB	Krebs- Ringer bicarbonate buffer
L	lamellar phase
L_α	lamellar liquid crystalline phase
L_β	lamellar gel phase
Lyso PC	lysophosphatidylcholine
MEM	minimum essential media
N terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PC	phosphatidylcholine

PE	phosphatidylethanolamine
Phe	phenylalanine
PI	phosphatidylinositol
PI(3)P	phosphatidylinositol-3-phosphate
$PI(3,4)P_2$	phosphatidylinositol-3,4-bisphosphate
$PI(3,4,5)P_3$	phosphatidylinositol-3,4,5-trisphosphate
PI(4)P	phosphatidylinositol-4-phosphate
$PI(4,5)P_2$	phosphatidylinositol-4,5-bisphosphate
PI3K	phosphatidylinositol-3'-kinase
PIPES	piperazine-N-N'-bis[2-ethane-sulphonic acid]
PLC	phospholipase C
PMSF	phenylmethylsulphonylfluoride
PS	phosphatidylserine
PTPase	protein tyrosine phosphatase
SDS	sodium dodecylsulphate
S.E.M.	standard error of the mean
SH2	src-homology-2
SHPTP2	src-homology-containing protein tyrosine phosphatase 2
SIRK	soluble insulin receptor kinase
TCA	trichloroacetic acid
TEMED	N,N,N'N'-tetramethylethylenediamine

T_H	bilayer to hexagonal phase transition temperature
TLC	thin layer chromatography
T_m	main phase transition temperature
TMA-DPH	trimethylamino-diphenylhexatriene
TRIS	Tris(hydroxymethyl)aminomethane

1.0 INTRODUCTION

1.1 Overview

Several membrane functions are modulated by the bulk biophysical properties of the membrane [Shinitzky, 1984]. The plasma membrane envelops the cell and separates it from its surroundings. In addition to forming a barrier around the cell, the plasma membrane moderates the movement of ions and molecules of various kinds into and out of the cell. The plasma membrane also transmits information across the barrier into the cell. For example the binding of hormones to receptors on the extracellular surface of the cell is transmitted through the membrane to intracellular signals to elicit the required response.

The peptide hormone insulin binds to receptors located in the plasma membrane of most mammalian cells, and in greatest amounts in insulin responsive tissues such as hepatocytes, adipocytes and muscle [Kahn *et al.*, 1981]. While much is known about the structure and basic properties of the insulin receptor, the mechanisms of insulin action remain largely unknown. The binding of insulin to its receptor is essential for insulin signalling within the cell. Much evidence suggests that the next step in the insulin response involves autophosphorylation of the receptor on tyrosine residues of the cytoplasmic portion of the β subunit [Olefsky, 1990, Kasuga *et al.*, 1982b]. The remainder of the insulin signal transduction pathway leading to the mitogenic and

metabolic effects of insulin is not well defined, but probably involves a phosphorylation cascade [Czech *et al.*, 1988].

The transmembrane topology of the insulin receptor renders it susceptible to changes in the membrane phospholipid bilayer. Upon insulin binding to the α subunit, there is a conformational change in the receptor [Pilch *et al.*, 1980; Baron *et al.*, 1990; 1992], presumed to be responsible for transmitting the signal to the intracellular tyrosine kinase domain of the β subunit. Modulation of the biophysical properties of the membrane may affect the relative populations of the active and inactive conformations of the receptor and thereby affect the efficiency of insulin signalling. The effectiveness of a series of peptide derivatives in inhibiting glucose uptake has been related to their effects on lipid polymorphism [Epand *et al.*, 1991a]. The mechanism of this inhibition of insulin signalling 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. We have studied the effects of these peptides, as well as other bilayer stabilizers of diverse structure, on insulin receptor autophosphorylation, which is the first step in insulin signalling [Olefsky, 1990; Kasuga *et al.*, 1982b].

It is important to understand the role of the various membrane constituents in plasma membrane activity. While the structure and function of membrane proteins have been greatly studied, one also must understand how properties of the membrane in which these proteins are located can affect their activity. The modulation of membrane physical

properties can be correlated to insulin receptor function.

1.2 Structure of Biological Membranes

Eukaryotic cells are organized around systems of membranes. The plasma membrane surrounds the cell and acts as a permeability barrier, and functions in the transduction of signals from the external environment to the interior of the cell. It is composed of proteins, lipids and carbohydrates. The lipid portion of the membrane contains phospholipids and glycolipids. Danielli and Davson [1935] first proposed that membranes are composed of a lipid bilayer, with the polar parts of the lipid molecules facing the surrounding aqueous phase and the hydrophobic portions facing the center of the bilayer. In 1972, Singer and Nicolson introduced a modified hypothesis for the structure of membranes. Their fluid mosaic model also proposed that phospholipid molecules are oriented with their polar ends facing the aqueous medium and their nonpolar hydrocarbon chains associated together in the membrane interior. This model adds, however, that the membrane lipids are in a mobile, fluid state, free to diffuse laterally through the bilayer, and that proteins are dispersed within the bilayer, rather than as continuous sheets, covering the membrane surfaces [Singer and Nicolson, 1972]. The fluid mosaic model also accounts for the asymmetry of membranes, by proposing that carbohydrate groups would be restricted to one side of the membrane. Revisions have been made to this model, including the idea that different proportions of the various phospholipids and other lipid molecules may be present in the two halves of a bilayer.

The latest summary of this model describes the membrane as consisting of three regions, the extracellular matrix, the lipid bilayer with integral membrane proteins, and the membrane cytoskeleton [Bloom *et al.*, 1991]. Even though the lipid bilayer represents only one portion of the plasma membrane, it is the core of the membrane, around which the other components are arranged. The three portions together determine the overall properties of the membrane.

This thesis is concerned with the physical properties of the lipid bilayer, and how they affect the activity of a particular protein within the plasma membrane.

1.3 Lipid Polymorphism

The self assembly of phospholipids in aqueous environments is driven by the hydrophobic effect [Cevc and Marsh, 1987]. This is an entropic effect arising from the hydrogen bonded structure of water. The strong hydrogen bonds between water molecules become distorted when a hydrocarbon molecule is dissolved in water. In opposition to this association is the entropy of mixing of each monomer with the solvent molecules, and the various electrostatic, steric, and hydration repulsive forces among the lipid molecules in the aggregate. Phospholipids can undergo gross morphological rearrangements to different forms. These include lamellar, micellar, cubic, and hexagonal phases. These phases are shown diagramatically in Figure 1.3.1. The lamellar (L) phase consists of bilayer sheets of lipid stacked in a one-dimensional lattice separated by layers of water. In spherical micelles the hydrocarbon chain interior is

isolated from water by a spherical shell formed from the lipid headgroups. Cubic phases contain two interpenetrating but unconnected water regions, separated by a single continuous lipid bilayer. The hexagonal (H_I) phase consists of long cylindrical micelles arranged on a hexagonal lattice, separated by water, while the inverted hexagonal (H_{II}) phase is composed of long cylindrical cores of water arranged on a hexagonal lattice with the polar headgroups coating the exterior of these cores and the hydrocarbon chains filling the interstitial spaces between the cores [Tate *et al.*, 1991]. The relative stability of the types of aggregates is determined by hydrophobic energy, surface free energy of the aggregate, electrostatic free energy and entropy of mixing [Bloom et al., 1991]. While biological membranes are lamellar, these membranes consist of large lipid regions, which in isolation do not readily form lamellar bilayers [Gruner, 1992]. Furthermore, even biological bilayers can exist in different lamellar states. These include the lamellar gel (L_β) phase, with the acyl chains of the phospholipids fully extended parallel to the bilayer normal in the all-*trans* conformation, and the lamellar liquid crystalline phase (L_α), where the phospholipids can undergo fast lateral and rotational diffusion [Schindler and Seelig, 1975], and the acyl chains contain *gauche* conformers. This allows the lipid headgroups to move farther apart as the effective hydrocarbon volume of the membrane increases. The bilayer thickness is also decreased relative to the L_β phase [Cevc and Marsh, 1987].

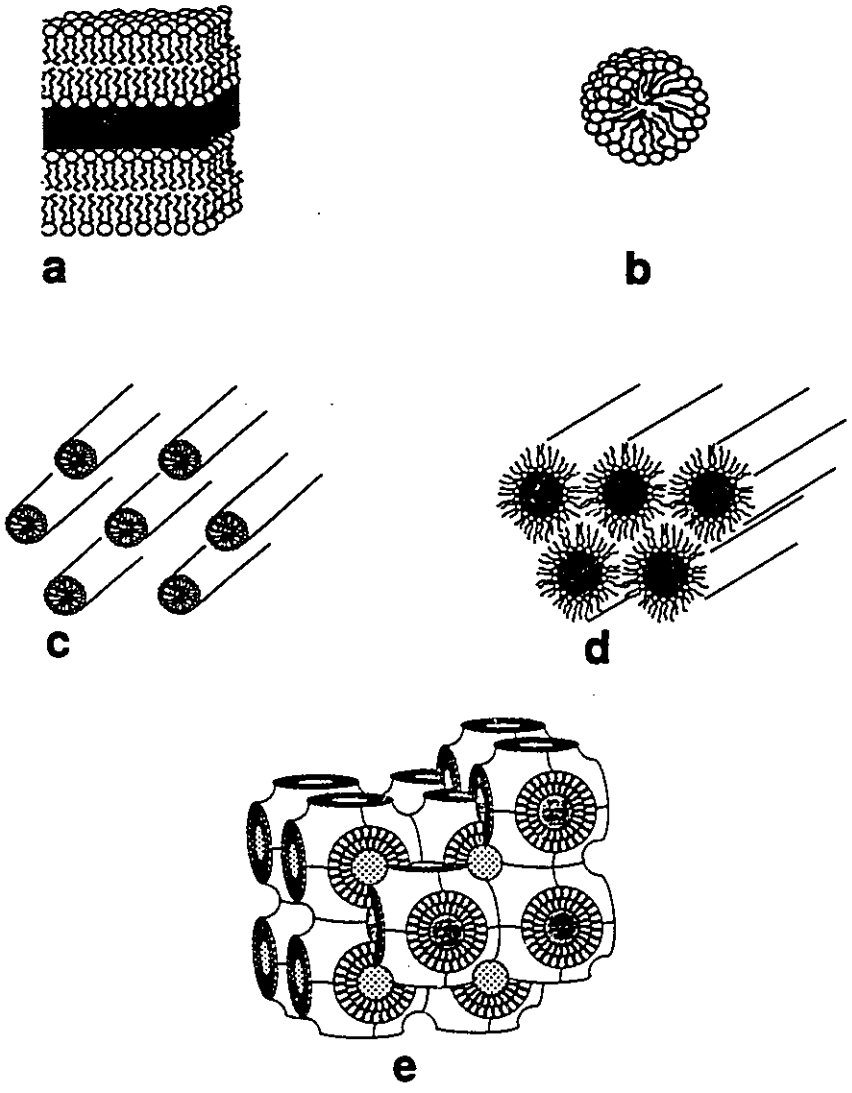


Figure 1.3.1

Diagramatic representations of various lipid phases [Tate *et al.*, 1991]. (a) Lamellar (L_α) phase, (b) Micellar phase, (c) Hexagonal (H_I) phase, (d) Inverted Hexagonal (H_{II}) phase, (e) Cubic phase. See section 1.3 for details.

The shape concept [Israelachvili *et al.*, 1980] suggests that lipid polymorphism is a result of the side-by-side packing of molecules whose cross-sectional areas vary systematically along the length of the molecules. Molecular shape can be expressed as

$$S = v / al \qquad (1.3.0.1)$$

where v is the volume per molecule, a is the area per headgroup and l is the approximate thickness of the lipid hydrocarbon layer. Values of v/al which are less than unity are associated with monolayers with a positive spontaneous curvature (such as in the H_I phase), while values of v/al which are greater than one lead to negative spontaneous curvatures (such as in the H_{II} phase). Finally, values of v/al approximately equal to one, having uniform cross-sectional areas along their length, are considered to have zero spontaneous curvature, and tend to form planar phases. In addition to spontaneous curvature, hydrocarbon packing constraints are a competing factor in determining the form adopted by the phospholipids. As the intrinsic radius of curvature increases, formation of the hexagonal phase is prevented by hydrocarbon packing constraints. These constraints arise as a result of the voids between the cylinders of the H_{II} phase, which must be filled by intercalation of the phospholipid acyl chains. X-ray diffraction studies have shown that H_{II} phase cylinders with a unit cell size smaller than 7.5 nm are essentially circular, while those with a larger unit cell size tend to be non-circular [Turner and Gruner, 1992]. Addition of alkanes to the larger H_{II} cylinders restored the circularity, by filling in the void regions and alleviating the hydrocarbon packing constraints [Turner *et al.*, 1992].

By studying the structural polymorphism of isolated lipids, one gains insight into the intermolecular forces which exist in, and affect the activity of, biomembranes. Transitions from one phase to another are observed among isolated phospholipids, in response to environmental changes, and are used as indicators of the physical properties of the membrane constituents.

1.3.1 Phase Transitions

Phospholipid phase transitions are induced by changes in temperature, hydration, or pressure, or by changes in the ionic stength or pH of the aqueous phase [Lewis and McElhaney, 1992]. Conversion from the gel (L_β) to liquid crystalline (L_α) state may be thermally induced. The temperature at which such a change occurs is characteristic for a particular phospholipid, and is termed the gel-to-liquid crystalline lipid phase transition temperature, or the main phase transition temperature T_m. The nature of the headgroup, the length of the fatty acyl chain, and the number, position and type of double bonds in the acyl chain of a phospholipid determine its main phase transition temperature. With further increase in temperature, phospholipids may convert from the L_α to the H_{II} phase. This occurs when there is a balance between the entropic reduction in free energy and the decrease in bilayer cohesive energy. Chain isomerisation accounts for the entropic term, while the decrease in bilayer cohesive energy arises from the lateral expansion of the hydrocarbon chains and from the energy cost of creating *gauche* conformers in the chains [Cevc and Marsh, 1987]. The stability

of a given phase is determined by the Gibbs free energy at a given temperature and water content. Contributions to the relative free energy of the non-bilayer phases include hydrocarbon packing constraints and intrinsic radius of curvature.

1.3.2 Measurement of Phase Transitions

The temperature and enthalpy of phase transitions are measured by differential scanning calorimetry (DSC). The sample and an inert reference (usually the buffer system used) are simultaneously heated at identical predetermined rates. The temperature difference between the sample and reference is maintained at zero ± 0.0001 °C. As the sample undergoes an endothermic event, the control system senses the resulting difference in temperature between the sample and reference cell and supplies more heat to the sample cell to maintain the temperature difference at zero. The excess specific heat (C_p) is recorded as a function of temperature. Differential scanning calorimetry can provide measures of the phase transition temperature (T_m), the enthalpy of the transition (ΔH_{cal}), the entropy change (ΔS), and the cooperativity of the phase transition. T_m is the temperature at which the excess specific heat reaches a maximum. The enthalpy of the transition is obtained from the area under the DSC peak of C_p versus temperature. Since the change in free energy (ΔG) of the system is zero at the phase transition midpoint temperature, the entropy associated with the transition can be calculated as shown in equation 1.3.2.1.

$$\Delta S = \Delta H_{cal} / T_m. \qquad (1.3.2.1)$$

The cooperativity of the transition is indicated in the sharpness of the DSC peak. This sharpness can be expressed as the temperature width at half-height, $\Delta T_{1/2}$. The smaller the value of $\Delta T_{1/2}$, the greater the cooperativity of the phase transition. The values of T_m and $\Delta T_{1/2}$ can then be used to calculate the van't Hoff enthalpy, ΔH_{vH} from the equation

$$\Delta H_{vH} = 4RT_m^2 / \Delta T_{1/2}, \qquad (1.3.2.2)$$

where R is the gas constant. Finally, the ratio of $\Delta H_{vH} / \Delta H_{cal}$ gives a measure of the degree of intermolecular cooperation between phospholipid molecules in a bilayer, referred to as the cooperative unit size. Both the phase transition temperature and the cooperativity of the bilayer-to-hexagonal phase transition can be affected by the presence of various additives to the phospholipid bilayer [Epand, 1985].

1.3.3 Phase Transition Temperature as a Measure of Membrane Physical Properties

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). Addition of compounds to the membrane can affect the monolayer curvature strain. It has been shown that increased negative monolayer curvature (v /al > 1) can lead to an instability in membrane bilayers that can be detected calorimetrically [Epand and Epand, 1994]. The effects of membrane additives on the temperature at which phospholipids undergo a change from bilayer to hexagonal phase (T_H) is an

indicator of their effect on curvature strain. Measurement of T_H of pure lipid systems in response to membrane additives is used as an indication of the effects of such additives on membrane physical properties. 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.

1.3.4 Effects of Biophysical Properties on Membrane Activity

Studies of membrane protein activity have been focused mainly on the aqueous environment of these proteins. However, the activity of an intrinsic membrane enzyme must be considered within the context of the lipids comprising the membrane [Carruthers and Melchoir, 1986]. Many integral membrane proteins are involved in several specific and nonspecific molecular interactions, both with their lipid and aqueous environments. Nonspecific interactions relate to colligative physical properties such as bilayer thickness, surface charge, dielectric constant profile, permeability. Specific interactions include binding of membrane components to specific sites on proteins. It has been suggested that the formation of non-bilayer phases may have a functional role in biological phenomena such as membrane fusion and transbilayer transport processes [Cullis and de Kruijff, 1979]. Several membrane functions are modulated by the bulk biophysical properties of the membrane [Shinitzky, 1984]. Some membrane functions have been shown to be correlated with the propensity of the membrane to undergo

rearrangement to the hexagonal phase [Yeagle, 1989; Epand, 1990]. Viral fusion, protein kinase C activity and insulin signalling in adipocytes have been shown to be sensitive to the presence of substances in the membrane which alter its propensity for forming the hexagonal phase [Epand, 1991]. Membrane functions may be facilitated in the presence of nonbilayer-forming lipids, since these lipids increase the energy of the membrane. This energy may be released by coupling with another membrane process [Epand and Epand, 1994].

1.4. Insulin

The peptide hormone insulin is secreted from pancreatic β cells when blood glucose levels are high. It signals the fed state and stimulates the storage of fuels and the synthesis of proteins. Insulin is synthesized as preproinsulin, which is 108 amino acid residues in length. The amino terminal signal sequence of preproinsulin is removed in the lumen of the endoplasmic reticulum. The remaining 84 amino acids constitute proinsulin, a molecule in which all of the correct disulfide bridges have been formed. While the hormone is being packaged for secretion, 33 residues are removed (the C chain), yielding insulin, consisting of an A chain of 21 amino acids and a B chain of 30 residues, held together by two disulfide bridges. Insulin binds to specific receptors on the plasma membranes of mammalian cells. The main insulin responsive tissues are hepatocytes, adipocytes, and muscle. The biological effects of insulin include stimulation of anabolic processes such as glucose uptake, glycolysis, and glycogen, fatty acid and

protein synthesis [Brogden and Heel, 1987], as well as regulation of gene expression [O'Brien and Granner, 1991]. Furthermore, insulin inhibits gluconeogenesis and catabolic processes, including lipolysis and glycogenolysis.

1.5 The Insulin Receptor

1.5.1 Synthesis and Processing of the Insulin Receptor

The insulin receptor gene is located on the short arm of chromosome 19, and contains 22 exons [Seino *et al.*, 1989]. Two isoforms of the human insulin receptor have been identified, as a result of alternative splicing of exon 11 of the insulin receptor gene [Mosthaf *et al.*, 1990]. Hematopoietic tissues express only the Ex11- transcript, lacking 12 amino acids at the carboxy-terminus of the receptor, while the liver expresses predominantly the Ex11+ transcript, and tissues such as placenta or muscle express both mRNAs [Moller *et al.*, 1989, Mosthaf *et al.*, 1990, Goldstein and Dudley, 1990]. The insulin receptor is synthesised on membrane-bound ribosomes as a single-chain precursor polypeptide [Kasuga *et al.*, 1982c]. Glycosylation [Hedo *et al.*, 1981], acylation [Hedo *et al.*, 1987], and formation of interchain disulfide bonds occur co-translationally. The proreceptor contains N- and O-linked sugar moieties and covalently bound fatty acids [Hedo *et al.*, 1981; 1987]. The proreceptor is cleaved in the Golgi to produce pre α and β subunits [Hedo *et al.*, 1987] joined by disulphide bonds. The mature receptor is transferred from the Golgi to the plasma membrane, where it is inserted. Two $\alpha\beta$ monomers then associate and the formation of disulfide bonds between the extracellular

α subunits yields the complete heterodimer.

1.5.2 Structure of the Insulin Receptor

The receptor to which insulin binds is a heterotetrameric glycoprotein with an apparent molecular mass of 350 kDa [Kahn *et al.*, 1981]. It is composed of two α subunits of 135 kDa, and two 95 kDa β subunits [Ullrich *et al.*, 1985; Ebina *et al.*, 1985]. The extracellular α subunits are disulfide bonded to each other and each to a membrane-spanning β subunit. The model for the insulin receptor structure is based upon biochemical and immunological studies, and has been confirmed by amino acid analysis [Ullrich *et al.*, 1985; Ebina *et al.*, 1985]. The quaternary structure of the detergent-solubilized receptor determined by electron microscopy suggests that the insulin receptor is shaped like the letter "T" [Christiansen *et al.*, 1991; Schaefer *et al.*, 1992]. Each receptor is approximately 24 nm in height and 18 nm in width with a thickness of the stem and the crossbar of 3-4 nm [Christiansen *et al.*, 1991]. Reconstituted insulin receptors project up to 12 nm above the membrane surface [Tranum-Jensen *et al.*, 1994]. The α subunits contain the cysteine-rich insulin binding domain [Yip *et al.*, 1978; Waugh *et al.*, 1989; Gustafson and Rutter, 1990], while the juxtamembrane region of the intracellular portion of the β subunits possess the ATP-binding and tyrosine kinase activities [Backer *et al.*, 1991].

1.5.3 Insulin Receptor Function

The insulin receptor acts to transduce the extracellular signal of the fed state across the plasma membrane, to the interior of the cell. Upon binding of insulin to its receptor, there is a conformational change in the receptor [Pilch *et al.*, 1980; Baron *et al.*, 1990, 1992]. Insulin binding causes a decrease in the Stokes radius and an increase in the sedimentation coefficient of the receptor, both of which are reversed upon dissociation of the receptor-hormone complex [Florke *et al.*, 1990]. These structural changes occur in the α subunit [Pilch *et al.*, 1980] and in the C-terminal portion of the β subunit [Baron *et al.*, 1990, 1992]. The changes in the receptor cytoplasmic domain could influence interactions between the receptor and cellular structures and, as such, play a key role in signal transduction. The conformational change is suggested to be required for activation of the tyrosine kinase activity of the insulin receptor [Baron *et al.*, 1990, 1992]. This leads to phosphorylation cascades which may account for some of the metabolic and mitogenic actions of insulin.

1.6 Insulin Signal Transduction

The first step in insulin signalling, following binding of insulin to the receptor, is activation of the autophosphorylation activity of the receptor [Kasuga *et al.*, 1982a]. The insulin receptor phosphorylates itself on several tyrosine residues. This is primarily by an intramolecular β subunit *trans*-phosphorylation reaction within the insulin holoreceptor [Shoelson *et al.*, 1991; Frattali *et al.*, 1992]. The major β subunit

autophosphorylation sites have been localized to tyrosine residues 1158, 1162, 1163, 1328, and 1334 [Tavare *et al.*, 1988; White *et al.*, 1988] using the nomenclature of Ebina [Ebina *et al.*, 1985]. Autophosphorylation of residues 1162 and 1163 play a critical role in the activation of the receptor kinase, while tyrosine residue 1158 may be involved in regulating the rate of such phosphorylation [Zhang *et al.*, 1991]. These three tyrosines represent the most rapidly autophosphorylated residues following insulin binding, and their phosphorylation is required for activation of wild-type insulin receptors [Dickens and Tavare, 1992]. The simultaneous mutation of these three tyrosine residues results in a receptor unable to mediate insulin signalling [Murakami and Rosen, 1991]. The requirement of autophosphorylation in insulin signalling is unclear, as there have been some reports of insulin signalling without tyrosine kinase activity. For example, insulin receptor tyrosine kinase (IRTK) activity does not seem to play an obligatory role in the insulin-signalling pathway that stimulates pyruvate dehydrogenase activity [Gottschalk, 1991]. Furthermore, autophosphorylation-defective insulin receptors mutated in the three major autophosphorylation sites have been reported to signal normal or enhanced levels of amino acid transport and mitogenesis [Rafaeloff *et al.*, 1991]. Since an increase in serine phosphorylation of several intracellular proteins is observed in response to insulin binding to its receptor [Czech *et al.*, 1988], it is hypothesized that the insulin receptor tyrosine kinase phosphorylates tyrosine residues on one or more serine kinases, leading to stimulation of the serine kinase(s), and ultimately in a serine phosphorylation cascade [Taylor, 1991]. Such a cascade can lead to activation and

inactivation of various proteins involved in metabolic pathways. The insulin receptor has been found to interact with, and activate, a 40 kDa guanosine-triphosphate-binding protein (G-protein) [Rothenberg and Kahn, 1988; Luttrell *et al.*, 1990; Kellerer *et al.*, 1991; Jo *et al.*, 1993a; Jo *et al.*, 1993b]. This G-protein may activate a specific phospholipase C [Fox *et al.*, 1987], which cleaves phosphatidylinositol glycan to yield the second messengers diacylglycerol and glycosyl phosphatidylinositol. Evidence for this activity includes the observation that insulin stimulates the generation of glycosyl phosphatidylinositol from hepatic plasma membranes [Saltiel and Cuatrecasas, 1986; Low and Saltiel, 1988], and that inositol glycans have insulin-mimetic actions [reviewed in Saltiel and Cuatrecasas, 1988]. Diacylglycerol has been indirectly demonstrated to stimulate glucose transporter efficiency [Stralfors, 1988]. Diacylglycerol is a known activator of protein kinase C. It has been suggested that insulin stimulation of glucose transport into cells involves protein kinase C, at the level of glucose transporter recruitment from intracellular stores [Ishizuka *et al.*, 1990]. The major substrate for the IRTK is a 185 kDa protein termed insulin receptor substrate-1 (IRS-1) [White *et al.*, 1985, 1987]. The tyrosine residues of IRS-1 which are phosphorylated by the IRTK are located within YMXM motifs, which are recognized by src-homology-2 (SH2) domains [Matsuda *et al.*, 1991]. SH2 regions are noncatalytic domains that are conserved among a series of cytoplasmic signalling proteins regulated by receptor protein-tyrosine kinases. They mediate the formation of heteromeric protein complexes at or near the plasma membrane. The formation of such complexes may control the activation of signal

transduction pathways by tyrosine kinases [Koch *et al.*, 1991]. Phosphorylated IRS-1 has been shown to interact with the SH2 domains of various enzymes involved in signal transduction [Sun *et al.*, 1991, 1993; Lowenstein *et al.*, 1992]. This is atypical, since other receptor protein tyrosine kinases interact directly with SH2 domains of signalling proteins [Schlessinger, 1994]. In this way IRS-1 can be thought of as a docking protein. Proteins which are activated upon association with IRS-1 in this manner include phosphatidylinositol-3'-kinase (PI3K), a protein tyrosine phosphatase (SHPTP2), and growth factor receptor-binding protein-2 (GRB2) [Sun *et al.*, 1991, 1993; Lowenstein *et al.*, 1992]. PI3K phosphorylates the inositol ring of PI, PI(4)P and PI(4,5)P_2 to produce the second messengers PI(3)P, PI(3,4)P_2 and PI(3,4,5)P_3 [Whitman, 1988; Stephens *et al.*, 1991]. PI3K is also activated by binding directly to the COOH terminus of the insulin receptor [Levy-Toledano *et al.*]. GRB2 interacts via its SH3 domain with mSOS (Son of Sevenless). This results in activation of Ras, which may function as an intermediate in insulin signalling to promote glucose transport [Kozma *et al.*, 1993; Manchester *et al.*, 1994]. The insulin receptor also phosphorylates Shc (Src homology 2/α-collagen-related protein), which then competes with IRS-1 for binding of GRB2 and activation of the Ras signalling pathway, leading to activation of MAP kinase and c-fos transcription [Yamauchi and Pessin, 1994]. The Ras signalling pathway has been shown to mimic insulin action on glucose transporter translocation [Kozma *et al.*, 1993]. These various pathways are summarized in Figure 1.6.1, and may account for the pleiotropic actions of insulin via regulation of protein activity and gene expression.

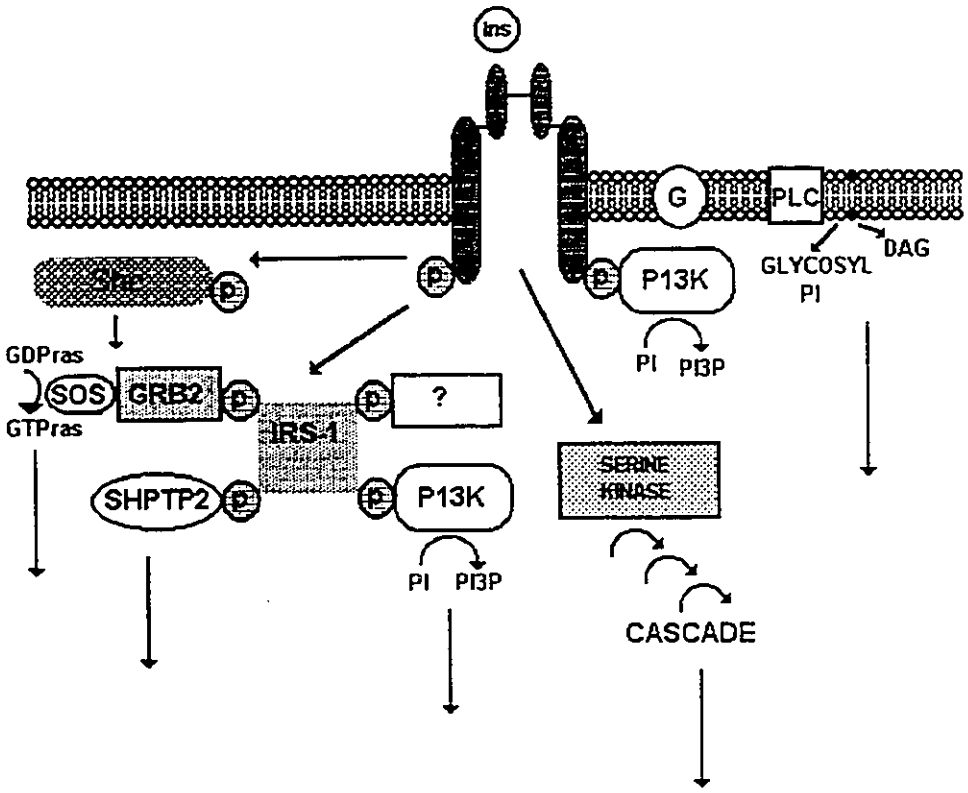


Figure 1.6.1

Insulin signal transduction. See section 1.6 for details.

1.7 Insulin Receptor Down Regulation

When cells are exposed to insulin, the half-life of the receptor is reduced [Kasuga *et al.*, 1981]. This down-regulation enables cells to regulate their sensitivity to insulin. Occupied receptors are internalized by coated pits as well as coated pit-independent processes. Once internalized, insulin dissociates from the receptor in endosomes. The receptors recycle to the cell surface [Sonne, 1988], and most of the insulin is degraded [Wang *et al.*, 1983]. It has been suggested that internalized receptor and insulin may be responsible for some aspects of insulin action [King and Johnson, 1985; Miller and Sykes, 1991].

1.8 Insulin Binding

Insulin binding to its receptor exhibits complex kinetics [Kahn, 1976]. Insulin binds to the α subunit of the insulin receptor [Yip *et al.*, 1978]. The cysteine-rich region of the α subunit is important for insulin binding [Waugh *et al.*, 1989; Gustafson and Rutter, 1990]. It is still unclear whether one or two insulin molecules bind to the apparently symmetrical receptor dimer [White and Kahn, 1994]. Linear or negatively co-operative binding isotherms have been obtained for insulin binding to insulin receptor, depending on the tissue source [Kohanski and Lane, 1983; Pang and Shafer, 1984] and prior exposure of receptor to hormone [Stevens *et al.*, 1983]. It is therefore unclear whether there are several populations of insulin receptors with distinct affinities for insulin in different tissues, or a single group of receptors coupled in different ways to

various metabolic activities. Hepatocyte plasma membranes contain a single class of homogeneous, noninteracting insulin receptors with two affinity states which may result from the temperature-dependent interaction of the receptor with an affinity regulator of insulin binding [Helmerhorst and Yip, 1993]. In general, however, the curvilinear Scatchard plots obtained for insulin binding [De Meyts *et al.*, 1973; Kahn, 1976] are interpreted as negative co-operative behaviour, with binding of one molecule of insulin inhibiting the affinity of the receptor for binding a second molecule of insulin [White and Kahn, 1994].

1.8.1 Analysis of Ligand-Receptor Interactions

Insulin binds to its receptor specifically, and may also form non-specific interactions with the receptor, and with other cellular structures. Therefore, in analyzing insulin-receptor interactions, as with any ligand-receptor interactions, specific binding must be distinguished from non-specific binding. This can be achieved by isotopic dilution. The amount of radioactive insulin bound to an insulin receptor preparation is measured in the presence and absence of an excess of unlabeled compound. It is presumed that the radioactively labeled compound competes with equal affinity at the receptor site as for the unlabeled compound. In the presence of sufficient excess of unlabeled ligand, the amount of labeled ligand will be significantly reduced. However, there will remain an amount of radioactivity adsorbed to the receptor preparation for which the unlabeled insulin cannot compete, even at very high concentrations. This non-

removable ligand is considered to be bound to the insulin receptor preparation in a nonspecific manner. The amount of nonspecific binding can then be subtracted from the total binding to determine the amount of specific binding of insulin to its receptor.

The interaction of a hormone (H) with its receptor (R) can be shown as:

$$R + H \rightleftharpoons RH \qquad (1.8.1.1)$$

where RH represents the receptor-hormone complex. The law of mass action gives:

$$K_a = [RH] / [R][H] \qquad (1.8.1.2)$$

where K_a is the association constant at equilibrium, [RH] is the concentration of receptor-hormone complex, [R] is the concentration of free receptors, and [H] is the concentration of hormone. The total number of receptors (B_{max}) is the sum of the bound and free receptors so that $B_{max} = [R] + [RH]$.

Therefore $K_a = [RH] / (B_{max} - [RH])\,[H]$.

Substituting B for the concentration of bound hormone ([RH]), and F for the concentration of free hormone ([H]), $K_a = B / (B_{max} - B)\,F$.

This is rearranged to the Scatchard equation:

$$B / F = K_a (B_{max} - B). \qquad (1.8.1.3)$$

Using this formulation, plotting bound/free hormone against bound hormone will generate a straight line with a slope of $-K_a$ and the intercept on the bound axis as B_{max}, for a simple bimolecular interaction with the ligand binding to only one type of site with constant affinity [Scatchard, 1949]. Cooperative interactions (negative or positive), or the presence of two or more classes of receptor sites results in non-linear Scatchard plots.

The effects of membrane additives on insulin binding to its receptor can be assessed by competition assays, where the displacement of radiolabeled insulin by increasing concentrations of unlabeled insulin is measured in the presence and absence of the additives. A plot of percent specific binding versus unlabeled insulin concentration will shift to the right if the additive lowers the affinity of the insulin receptor for insulin. That is, the EC_{50} (the concentration of unlabeled insulin required to displace 50% of the specifically bound radiolabeled insulin) will be increased.

1.8.2 Lipid Effects on Insulin Binding

The phospholipid environment has been shown to alter the binding of insulin to purified receptors [Lewis *et al.*, 1987; Ginsberg *et al.*, 1981]. Dietary fat can affect adipocyte phospholipid composition. Such modulation results in alterations of insulin binding to adipocyte insulin receptors [Field *et al.*, 1989, 1990; Cheema *et al.*, 1992]. Specifically, an increase in the polyunsaturated fat: saturated fat ratio of phospholipids was found to increase total insulin binding in rats.

1.9 Autophosphorylation as a Measure of Insulin Receptor Activity

Insulin receptor autophosphorylation on tyrosine residues is the first step in insulin signalling [Kasuga *et al.*, 1982b; Olefsky, 1990]. Autophosphorylation can therefore be used as a measure of insulin receptor activity in response to insulin binding. An inhibition of this activity, distinct from insulin binding, suggests an inhibition of the

conformational change required for signal transduction.

1.9.1 Detergent Solubilized Assays of Insulin Receptor Autophosphorylation

Micellar assays represent a simple, defined method for measuring enzymatic function. Different detergents and phospholipids can be used in mixed micellar assays. Insulin receptor activity has been previously measured in micelles containing phosphatidylinositol and n-octyl-β,D-glucopyranoside [Sweet *et al.*, 1987]. This detergent has recently been shown to decrease insulin binding and kinase activation of the insulin receptor, while Triton X-100 allowed an increase in these activities, even above the critical micelle concentration [Leray *et al.*, 1992]. Triton X-100 is a well characterized detergent, and therefore a good candidate for assaying insulin receptor autophosphorylation. Lipid sensitivity of the insulin receptor can be assesed by varying the nature and concentration of phospholipids, relative to Triton X-100. Furthermore this assay is useful in measuring the effects of membrane additives on insulin receptor activity in a specific lipid environment. To this end, hydrophobic compounds may be included in preparation of a lipid film, allowing for a uniform distribution of the compounds upon hydration in the presence of detergent.

1.9.2 Vesicle Assay of Insulin Receptor Autophosphorylation

Measuring insulin receptor activity in vesicles poses a problem. Since the insulin receptor spans the membrane, access to both sides of the membrane is required

to measure insulin receptor autophosphorylation. Insulin must be able to bind the α subunit while ATP must be able to bind the β subunit. This requires detergent solubilization of the vesicles, such as with Triton X-100 [Leray *et al.*, 1993], since vesicles are impermeable to insulin, ATP and magnesium [Tranum-Jensen *et al.*, 1994]. While ATP could be entrapped in the vesicles and insulin added to the outside, this is rather messy, and costly, given that the excess ATP, which is labelled with ^{32}P must be separated from the vesicles after they are formed. Furthermore, vesicles represent highly curved membranes, relative to cellular membranes, and thus curvature may become an important factor in determining insulin receptor activity in the presence or absence of membrane additives.

1.9.3 Intact Cell Assay of Insulin Receptor Autophosphorylation

Insulin receptor autophosphorylation can be measured in intact cells [Ellis *et al.*, 1986]. It is advantageous to measure the activity of the insulin receptor in its natural milieu, for comparison of natural and isolated receptor activities. Cell lines which over-express insulin receptor are useful. High level expression of human insulin receptors in eukaryotic cells has been demonstrated by transfection of mouse NIH 3T3 cells with a bovine papilloma virus vector containing full-length human kidney insulin receptor cDNA [Whittaker *et al.*, 1987]. Using intact cells introduces several variables into activity assays, however. The membrane environment becomes much more complicated, with varying lipid and protein compositions. The concentration of intracellular components

is difficult to control in cells. Furthermore, for measuring autophosphoryation, the presence and activity of other cellular kinases becomes important in distinguishing the insulin receptor activity.

1.10 Lipid Effects on Insulin Receptor Autophosphorylation

The insulin receptor is sensitive to its lipid environment. 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 *et al.*, 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 *et al.*, 1993]. The importance of this property to the functioning of membranes is suggested by the fact that the membranes of micro-organisms adjust their lipid composition to have considerable negative monolayer curvature strain and are close to the transition temperature of the inverted hexagonal (H_{II}) phase [Rilfors *et al.*, 1994]. 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.*, 1991a].

1.11 Glucose Uptake

All mammalian cells transport glucose. Glucose transporters are required for the facilitated diffusion of glucose into cells. Glucose transporters are a multifamily of gene products. GluT1 is expressed in high levels in erythrocytes, and in the blood-brain barrier, as well as in smaller amounts in many other tissues [Mueckler, 1990; Bell *et al.*, 1990; Thorens *et al.*, 1990]. GluT2 is expressed in organs involved in release of glucose, such as the intestine, liver and kidney, and pancreatic β cells, which release insulin [Mueckler, 1990; Bell *et al.*, 1990; Thorens *et al.*, 1990]. GluT3 is present at highest levels in neuronal cells, while GluT4 is the major transporter in muscle and adipose cells [Mueckler, 1990; Bell *et al.*, 1990; Thorens *et al.*, 1990]. The expression and function of GluT4 are modulated by physiological factors, and its regulation may be a major determinant of insulin responsiveness. A fifth glucose transporter, GluT5, is expressed mainly in the small intestine and kidney [Bell *et al.*, 1990]. These glucose transporters consist of 12 transmembrane domains, with N and C terminal ends on the cytoplasmic side [Meuckler *et al.*, 1985]. The glucose transporter is envisaged as a conformational oscillator that shifts the binding pocket for glucose between opposite sides of the plasma membrane [Lienhard *et al.*, 1992]. Glucose first binds to the outward facing glucose binding site of the transporter. The complex of transporter and glucose undergoes a conformational change, allowing glucose to occupy the binding site which

faces into the cell. After glucose is released from the transporter into the cell, the transporter changes back to the original comformation, enabling it to transport another molecule of glucose.

Glucose transporter function is regulated by localized expression of different transporter isoforms within a tissue, and by the subcellular distribution of different transporter isoforms [Kahn, 1992]. In insulin responsive tissues such as muscle and adipose, insulin binding to its receptor initiates a signal resulting in translocation of glucose transporters from an intracellular pool associated with membrane vesicles to the plasma membrane [Suzuki and Kono, 1980; Cushman and Wardzala, 1980]. Recent studies suggest that there are actually more than just these two pools of glucose transporters, and that subcellular trafficking of transporters can only be adequately described by a minimum of four intermediate pools, with two localized to the plasma membrane and two localized inside the cell [Holman *et al.*, 1994]. Both GluT1 and GluT4 undergo recruitment from intracellular pools to the plasma membrane in adipose cells exposed to insulin, but the magnitude of the GluT4 response is much greater [Khan *et al.*, 1989: Piper *et al.*, 1991]. GluT1 seems to play a role mostly in basal glucose transport [Assimacopoulos-Jeannet *et al.*, 1991]. The activity of glucose transporters does not always correlate simply with their expression [Simpson and Cushman, 1989]. The translocated transporters also require activation, which may result from conformational changes affecting the accesibility of the transporters to glucose [Kahn, 1992].

1.11.1 Lipid Effects on Glucose Transport

Glucose uptake by cells has been shown to be sensitive to the lipid environment. Diet-induced increases in polyunsaturated fatty acid composition facilitates glucose transport in adipocytes [Lee and Dupont, 1991]. However, a high-fat diet had no effect on the levels of GluT2 and GluT4 in liver and muscle cells [Okamoto et al., 1992]. Furthermore, insulin stimulation of glucose uptake has been shown to be inhibited by sphingosine [Robertson et al., 1989] and stimulated by diacylglycerol [Stralfors, 1988]. The membrane properties can affect glucose uptake at several stages including insulin receptor activity, glucose transporter recruitment and recycling, and glucose transporter activation.

2.0 MATERIALS AND METHODS

2.1 Materials and Sources

The materials and the sources used are listed in the tables below. All commercially obtained materials were reagent grade or better. Where required, materials were used according to the manufacturers' specifications.

2.1.1 Lipids

All lipids used were of the highest quality available.

Lipid	Supplier
DEPE	Avanti Polar Lipids
1,2-Dioctanoylglycerol	Sigma Chemical Company
Egg PC	Avanti Polar Lipids
Egg PE	Avanti Polar Lipids
Lauroyl Carnitine	Sigma Chemical Company
Lyso PC	Sigma Chemical Company
Palmitoyl Carnitine	Sigma Chemical Company
PI, plant, sodium salt	Avanti Polar Lipids
PS, bovine brain	Avanti Polar Lipids
Sphingomyelin, bovine brain	Sigma Chemical Company

2.1.2 Chemicals and Solvents

Chemical	Supplier
Acrylamide	Gibco-BRL
Ammonium persulfate	Gibco-BRL
Bacitracin	Sigma Chemical Company
Bovine serum albumin (Fraction V)	Sigma Chemical Company
Bromophenol blue	BioRad
Chloroform	Fisher
Coomassie Brilliant Blue	BioRad
EDTA	Fisher
HEPES	Sigma Chemical Company
Leupeptin	Sigma Chemical Company
N,N'-Methylenebisacrylamide	Gibco-BRL
n-Octyl-β,D-glucopyranoside	Sigma Chemical Company
Pepstatin	Sigma Chemical Company
PMSF	Sigma Chemical Company
Sodium dodecylsulfate	Gibco-BRL
TEMED	Gibco-BRL
TRIS	Sigma Chemical Company
Triton X-100	BioRad

All other chemicals and solvents were of the highest grade available.

2.1.3 Membrane Additives

Compound	Supplier
CBZ-Gly-D-Phe	Bachem
CBZ-Gly-L-Phe	Research Plus
CBZ-L-Phe-Gly	Research Plus
CBZ-D-Phe-L-Phe-Gly	Bachem, Peptide Institute, Armand-Frappier
1,2-Dioctanoylglycerol	Sigma Chemical Company
threo-Dihydrosphingosine	Sigma Chemical Company
Lauroyl Carnitine	Sigma Chemical Company
L-Phe-Gly	Research Plus
Lyso PC	Sigma Chemical Company
Palmitoyl Carnitine	Sigma Chemical Company

2.1.4 Tissue Culture Reagents

Minimum Essential Media	GIBCO-BRL
Fetal Bovine Serum	GIBCO-BRL
Hanks' Balanced Salts Solution	GIBCO-BRL
Trypsin	GIBCO-BRL
Genestein (G418)	GIBCO-BRL
Dexamethasone	Sigma Chemical Company
Isobutylmethylxanthine	Sigma Chemical Company

2.1.5 Other Materials

Material	**Supplier**
Affigel-10	BioRad
Porcine Insulin	Sigma Chemical Company
Gamma-Globulins	Sigma Chemical Company
GF/B Filters	Whatmann
YM-10 Filters	Amicon
BCA Protein Assay Kit	Pierce
Live/Dead Cell Viability Kit	Molecular Probes
Protein A-Sepharose	Pharmacia
Silica TLC Plates	Analtech
Trypsin	Sigma Chemical Company
[1,2-^{3}H]-2-Deoxy-D-Glucose	ICN
[^{125}I]-Tyr^{A14}insulin	ICN
[γ-^{32}P]-ATP	ICN
[^{32}P]-Orthophosphoric acid	Du Pont-NEN

2.1.6 Materials from Non-commercial Sources

Apolipoprotein A-I was a gift from Drs. G.M. Anantharamaiah and J.P. Segrest, University of Alabama, Birmingham, AL. The monoclonal antibody Ab83-14 was a gift from Dr. K. Siddle, Cambridge. Dr. J. Whittaker provided the NIH 3T3 HIR 3.5 cells,

and the SIRK was obtained from Dr. Melanie H. Cobb, University of Texas Southwestern Medical Center. The cationic amphiphiles CATAM1 and CATAM2 were synthesized by Mr. Remo Bottega [Bottega and Epand, 1992].

2.1.7 Commonly Used Buffers and Solutions

KRBB/BSA	107 mM NaCl 10 mM glucose 7 mM $NaHCO_3$ 5 mM KCl 3 mM $CaCl_2$ 1 mM $MgSO_4$ 1 mM Na_3VO_4 0.1% BSA pH 7.4
Phosphate Buffered Saline (PBS)	37 mM NaCl 2.6 mM KCl 1.47 mM KH_2PO_4 8 mM Na_2HPO_4 pH 7.4
Tris/NaCl	20 mM Tris 150 mM NaCl pH 7.4
Solubilization Buffer (for intact cell autophosphorylation assay)	50 mM Hepes 10 mM EDTA 10 mM NaF 30 mM $Na_4P_2O_7$ 1 mM Na_3VO_4 1 mM PMSF 100 μM ATP 1 mg/mL bacitracin 1% Triton X-100 pH 7.6

In vitro Autophosphorylation Buffer	50 mM Hepes 10 mM $MgCl_2$ 10 mM $MnCl_2$ pH 7.4
Laemmli Sample Buffer	3.2 g glycerol 0.5 mL β-mercaptoethanol 1.25 mL 1M sodium phosphate buffer, pH 7.0 2 mL 0.1 % bromophenol blue 0.6 g urea in 10 mL
Electrophoresis Resolving Gel	375 mM Tris 2.0 mM EDTA 0.1 mM SDS 10 % glycerol (v/v) 7.5 % acrylamide (w/v) 0.2 % N,N'-methylenebisacrylamide (w/v) 0.067 % TEMED (v/v) 0.1 % ammonium persulfate (w/v) pH 8.9
Electrophoresis Stacking Gel	50 mM Tris 2.0 μM EDTA 0.1 mM SDS 4.0 % acrylamide (w/v) 0.1 % N,N'-methylenebisacrylamide (w/v) 0.067 % TEMED (v/v) 0.1 % ammonium persulfate (w/v) pH 6.7
Gel Running Buffer	0.05 M Tris 0.38 M Glycine 2.5 mM EDTA 3.5 mM SDS

Gel Stain	0.25 % Coomassie Brilliant Blue 50 % TCA
Gel Destain	20 % methanol 7 % acetic acid

2.2 Isolation of Insulin Receptor From Human Placenta

Insulin receptor was isolated from human placenta by the method of Fujita-Yamaguchi *et al.* [1983], with minor modifications, as detailed below.

2.2.1 Preparation of placental membranes

One human placenta was obtained within 30 minutes 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, then placed in a Waring blender for 3 minutes, in 50 mM Tris, pH 7.4, with 0.25 M sucrose and 0.1 mM PMSF. The slurry was then homogenized 3 times for 1 minute each, with a Polytron homogenizer, until a uniform consistency was obtained. The homogenate was centrifuged at 7000 X g for 45 minutes at 4°C. The resulting supernatant was then centrifuged at 100 000 X g for 30 minutes at 4°C. The pellets were resuspended in 10 volumes of 50 mM Tris, pH 7.4, with 0.1 mM PMSF, homogenized using a teflon/glass homogenizer, and the placental membranes were pelleted by centrifugation at 100 000 X g for 30 minutes at 4°C.

2.2.2 Solubilization and Affinity Chromatography

The placental membrane pellet was stirred in 50 mL 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 minutes at room temperature. The solubilized membranes were centrifuged at 100 000 X g, for 45 minutes 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 BioRad Affigel-10, according to the 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 mL fractions were collected into 1 mL 0.5 M Tris-HCl, pH 7.4.

2.2.3 Insulin Binding Assay

The Affi-10-insulin fractions were assayed for insulin binding activity, as described by Lewis and Czech [1987]. Fractions were incubated with [^{125}I]-insulin (2200 Ci/mmole) in a total volume of 0.5 mL of 50 mM Tris, 1% BSA, pH 7.6, at 22°C. Non-specific binding was determined by the addition of 100 nM unlabelled porcine insulin to identical tubes. Precipitation of the labelled ligand-receptor complex was facilitated by rapid addition of 0.2 mL ice-cold gamma-globulin (2.5 mg/mL) in 50 mM Tris, pH 7.6, and 0.5 mL of 25% (v/v) ice-cold polyethylene glycol in 50 mM Tris, pH 7.6. The mixture was vortexed and placed on ice for 10 minutes. Free insulin was

separated from that bound to the insulin receptor by filtration through GF/C filters which had been presoaked in 0.3% polyethyleneimine for 30 minutes. Each filter was washed twice with 8% polyethylene glycol, 0.025% Triton X-100 in 50 mM Tris, pH 7.6. Radioactivity bound to the filters was determined by scintillation counting.

2.2.4 Concentration and Storage of Insulin Receptor

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, according to the manufacturer's protocol. The concentrated receptors were aliquoted and frozen at -70°C, with 20% glycerol.

2.2.5 Protein Assay

Protein content was measured using the BCA protein assay kit, according to the manufacturer's protocol. 0.1 mL SDS was added to 0.1 mL of BSA standards or 0.1 mL of sample to be assayed. Fifty parts of Reagent A (sodium carbonate, sodium bicarbonate, BCA detection reagent, sodium tartrate, in 0.1 N NaOH) were mixed with 1 part of Reagent B (40 % $CuSO_4$), and 2 mL was added to each sample. Following 30 minutes at 37°C, the absorbance of each sample was measured at a wavelength of 562 nm. The BSA samples were used to construct a standard curve of protein concentration versus absorbance. The protein contents of the samples were determined by

extrapolation from the standard curve.

2.2.6 Competitive Insulin Binding Assay

The competitive binding assay was performed as described by Fujita-Yamaguchi *et al.* [1983]. The insulin receptor preparation was incubated at 4°C for 16 hours with [^{125}I]-Tyr^{A14}insulin (30 pmol) in a final volume of 0.4 mL of 50 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100, 0.1% BSA, and various amounts of unlabelled insulin, giving final concentrations of 1 to 200 ng/mL. The receptor-[^{125}I]-insulin complex was separated from free [^{125}I]-insulin by addition of 0.1 mL of 0.4% gamma-globulin and 0.5 mL of 20% polyethylene glycol, followed by centrifugation for 2 minutes at 14 000 r.p.m.. The amount of ^{125}I-insulin bound to receptors was quantitated by scintillation counting of the pellets. The data was analyzed by the methods of Scatchard [1949] and Hill [1910].

2.3 Differential Scanning Calorimetry

DEPE and the compound of interest were codissolved 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 hour. The lipid films were suspended in 20 mM PIPES, 1 mM EDTA, 150 mM NaCl, 0.002% NaN_3 at pH 7.4 by vortexing at 45°C for 30 seconds. 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.

2.4 Detergent Solubilized Assays of Insulin Receptor Tyrosine Kinase Activity

2.4.1 n-Octyl-β,D-glucopyranoside Assay

Insulin receptor was incubated with 0.2 μM insulin and 50 μM PI, in *In vitro* Autophosphorylation Buffer containing 0.65% n-octyl-β,D-glucopyranoside, in a final volume of 40 μL, for 1 hour at room temperature, as described by Sweet *et al.* [1987]. The reaction was initiated by the addition of 10 μM [γ-^{32}P]ATP (10 μCi), and terminated after 10 minutes by addition of 20 μL Laemmli sample buffer with 5% β-mercaptoethanol. The samples were boiled for 3 minutes and subjected to SDS-PAGE. The gels were stained with 0.25 % Coomassie Brilliant Blue in 50% TCA for 5 minutes, and destained in 7% acetic acid, 20% methanol. The gels were then dried and autoradiographed. The autoradiographs were scanned with a densitometer, or the sections of the dried gel corresponding to the β subunit of the insulin receptor were excised, and ^{32}P incorporation was quantified by scintillation counting.

2.4.2 Triton-Micellar 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 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 hour to remove residual solvent. 3% Triton X-100 in autophosphorylation buffer 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, 2 μg isolated receptor, with or without water-soluble compounds to be tested, for 1 hour at 25°C. 10 μM [γ-^{32}P]ATP (10 μCi) was added and the mixture was incubated for 15 minutes at 25°C. The reaction was terminated by addition of 50 μL of Laemmli sample buffer plus 5% β-mercaptoethanol and boiled for 3 minutes. 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.

2.5 Soluble Insulin Receptor Kinase Domain Activity

SIRK activity was assayed by the method of Cobb *et al.* [1989]. 20 pmol of the soluble cytoplasmic tyrosine kinase domain of the insulin receptor was incubated with 10 μM [γ-^{32}P]ATP (5 μCi) in 10 mM Hepes, pH 8.0, 3 mM $MgCl_2$, 100 μg/mL bovine serum albumin, for 15 minutes at 27°C. The reaction was terminated with the addition of 20 μL of Laemmli sample buffer, and boiled for 3 minutes. 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.

2.6 Culturing of NIH 3T3 HIR 3.5 Cells

NIH 3T3 HIR 3.5 cells were grown in α-MEM medium containing 8% fetal bovine serum (FBS) in 100 mm Petri dishes. Every 2 days the cells were removed from the plates with 1 mL of 0.25% trypsin in PBS. Medium was added and the cells were pelleted by centrifugation. The cell pellet was resuspended in fresh medium and divided among 4 new Petri dishes. For insulin receptor autophosphorylation or glucose uptake assays, the cells were grown to confluency on 24-well plates.

2.6.1 Differentiation of NIH 3T3 HIR 3.5 Cells to Adipocytes

The cells were grown in 24-well plates and fed every 2 days until confluent. Two days post-confluency (day 0), differentiation was induced with 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 1 μg/mL insulin in α-MEM containing 10% FBS, as described by Frost and Lane [1985]. On day 2 the isobutylmethylxanthine and dexamethasone were removed. Insulin was maintained for a further 2 days, and removed on day 4. α-MEM containing 10% FBS was then replaced every 2 days. Over 90% of the cells expressed the adipocyte phenotype after day 8.

2.7 Intact Cell Insulin Receptor Phosphorylation

Phosphorylation of the insulin receptor in 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). The cells were then incubated at 37°C for 4 hours in KRBB/BSA with 0.2 mCi [^{32}P]orthophosphate. Insulin and/or compounds of interest were added to a final volume of 1 mL. After 5 minutes at 37°C the solution was removed from the cells. The cells were washed twice with ice-cold Tris/NaCl and solubilized on ice with 250 µL of solubilization buffer. The solubilized cells were transferred to microfuge tubes and centrifuged for 2 minutes. 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 minutes at 4°C. Protein A-Sepharose was added (3 mg/tube), and the mixture incubated a further 30 minutes. The samples were then centrifuged. The pellets were washed three times with 1 mL 50 mM Hepes, pH 7.6, containing 1 M NaCl, 0.1 % SDS, 0.5% Triton X-100, 0.1% BSA, and once with 1 mL 100 mM Tris, pH 6.8. The pellets were suspended in 50 µL of Laemmli sample buffer and boiled for 3 minutes prior to SDS-PAGE. The 95 kDa bands containing the insulin receptor β subunit were visualized by autoradiography.

2.7.1 Phosphoamino Acid Analysis

The 95 kDa polypeptide was hydrolysed to amino acids as described by Lewis *et al.* [1990]. The 95 kDa bands were excised from the polyacrylamide gel and washed

alternately in acetone and water, three times each, for 15 minutes each, then minced and incubated with 40 μg/mL trypsin in 25 mM N-ethylmorpholine, pH 8.2, at 37°C for 16 hours. The tryptic eluate was obtained by centrifugation and lyophilized. The phosphopeptides were hydrolysed in 6 N HCl at 110°C for 60 minutes. The amino acids were then lyophilized and reconstituted in 5 μL of phosphoamino acid standards (1 mg/mL each of phosphotyrosine, phosphoserine, and phosphothreonine), and 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.

2.8 Cell Viability Assay

Cell viability was assessed using the *Live/Dead* kit from Molecular Probes, Inc. The cells were incubated with or without various concentrations of each compound to be tested, as for the insulin receptor phosphorylation assay, then incubated with calcein AM and EthD-1 (ethidium homodimer), according to the supplier's protocol. The live and dead cells were visualized under a fluorescent microscope with 530 and 590 nm filters, respectively, and counted. The percentage of dead cells was calculated.

2.9 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 35 pM [^{125}I-Tyr^{A14}]insulin (0.0155 μCi), and varying amounts of unlabelled insulin, with or without the addition of the compound of interest, in binding buffer for 16 hours at 4°C. The cells were then washed 3 times with 1 mL of ice-cold phosphate buffered saline, then solubilized at 37°C with 0.5 mL 0.1 M NaOH for 1 hour. The solubilized cells were transferred to scintillation vials for gamma counting. The percent of specifically bound insulin was calculated, and expressed as a percent of that in the absence of any compound.

2.10 Glucose Uptake into NIH 3T3 HIR 3.5 Cells

Glucose uptake was measured as described by Frost and Lane [1985], with modifications. Confluent NIH 3T3 HIR 3.5 cells were serum-starved for 2 hours, then washed with 2 mL 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 pre-incubated for 15 minutes 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 minutes, followed by two washes with 1 mL ice-cold PBS, to remove extracellular glucose. The cells were solubilized with 300 μL 0.1 % SDS. The [^{3}H]-2-deoxyglucose that had been internalized by the cells was quantitated by scintillation counting.

2.11 Fluid Phase Endocytosis

Internalization of the lipophilic probe trimethylamino-diphenylhexatriene (TMA-DPH) was used as a measure of fluid phase pinocytosis, as described by Illinger *et al.* [1990] with modifications. NIH 3T3 HIR 3.5 cells were grown to confluence (1 - 5 x 10^6 cells/ dish) in 60 x 15 mm Petri dishes in α-MEM containing 10 % fetal bovine serum. Cells were incubated with insulin and/or compound of interest plus 4 μM TMA-DPH in the same medium for various times. The medium was then aspirated, and the cells were washed with 3 mL PBS, followed by two washes of 2.5 mL of PBS containing 3% fatty acid-free BSA, and finally with another 3 mL of PBS, to extract uninternalized TMA-DPH from the plasma membrane. The cells were removed from the petri dish with a rubber stopper into 3 mL of PBS. The fluorescence emission was measured in a luminescence spectrometer, with an excitation wavelength of 360 nm and an emission wavelength of 435 nm. The amount of internalized probe was calculated as a percent of total associated probe, by dividing the fluorescence quantum yield of the washed cells by that of unwashed cells.

2.12 Statistical Analysis

T-tests of the data were performed using the Minitab (Version 7.2) statistical software. Experiments were carried out in triplicate. Differences between data sets were calculated to be statistically significant at the 95% confidence level.

3.0 RESULTS AND DISCUSSION

3.1 Isolation of Insulin Receptor from Human Placenta

Insulin receptors were isolated from human placental membranes. Purification of the receptor by affinity chromatography required the preparation of an insulin column. For this purpose, insulin was coupled to Affi-gel-10, according to the manufacturer's protocol, as described in Materials and Methods section 2.2.2. The efficiency of coupling was assessed by quantitation of soluble insulin before and after incubation with the column material, by absorbance readings at 280 nm. Beers law was used to relate the absorbance readings to concentration of insulin. The molar extinction coefficient (ε) for insulin was first determined by measuring the absorbance at 280 nm (A_{280}) at a given concentration (c) of insulin and calculating:

$$\varepsilon = A_{280} / bc, \qquad (3.1.1)$$

where b is the path length of light through the sample, in centimeters. For example, for 2.2×10^{-4} M insulin and a path length of 1 cm, the absorbance reading was 0.272, yielding $\varepsilon = 1236\ M^{-1}cm^{-1}$. This value of ε was then used to determine the concentration of insulin in the buffer following incubation with the column material. Here an absorbance of 0.15 corresponds to 1.2×10^{-4} M unreacted insulin. This is 55% of the initial 250 mg of insulin. Therefore 45% of the insulin, corresponding to 112 mg, bound to 25 mL of the Affi-10 slurry.

Solubilized placental membranes were applied to the Affi-10-insulin column.

The column was washed, and 130 fractions of 2 mL each were collected. Subsequently, the column was eluted with sodium acetate buffer, and 2 mL fractions were collected. The ratio of specific to non-specific insulin binding was measured for each fraction, as described in Materials and Methods section 2.2.3. A peak of specific insulin binding was obtained between fractions 140 and 170. A typical Affi-10-insulin elution profile is shown in Figure 3.1.1. On average this peak contained 2 mg of protein, as determined by the BCA protein assay kit. Few other proteins were visible by Coomassie or silver staining of polyacrylamide gels. Scatchard analysis yielded a curvilinear relationship for insulin binding, as shown in Figure 3.1.2. This is in agreement with the findings of Kahn [1976]. Therefore the protein isolated from human placenta in this manner exhibited binding characteristics typical of insulin receptors. The curvilinearity of the Scatchard plot is discussed in section 1.8 of the Introduction.

Isolated insulin receptors were stored in 50 % glycerol, at -70°C, for up to one year, with no effect on insulin receptor binding and autophosphorylation activity.

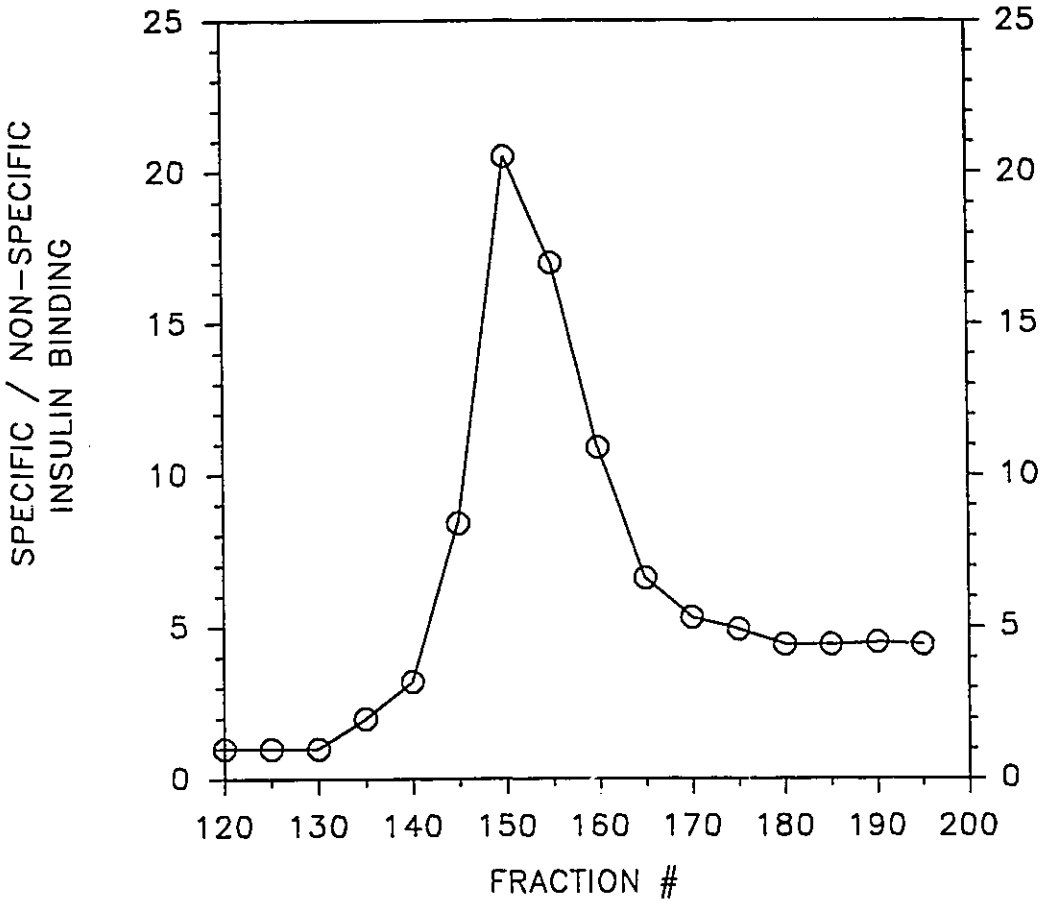


Figure 3.1.1

Affi-10-insulin elution profile. Specific insulin binding activity of affi-10-insulin fractions was measured as described in Materials and Methods, section 2.2.3. Elution with 50 mM sodium acetate buffer, pH 5.0 was initiated at fraction # 130.

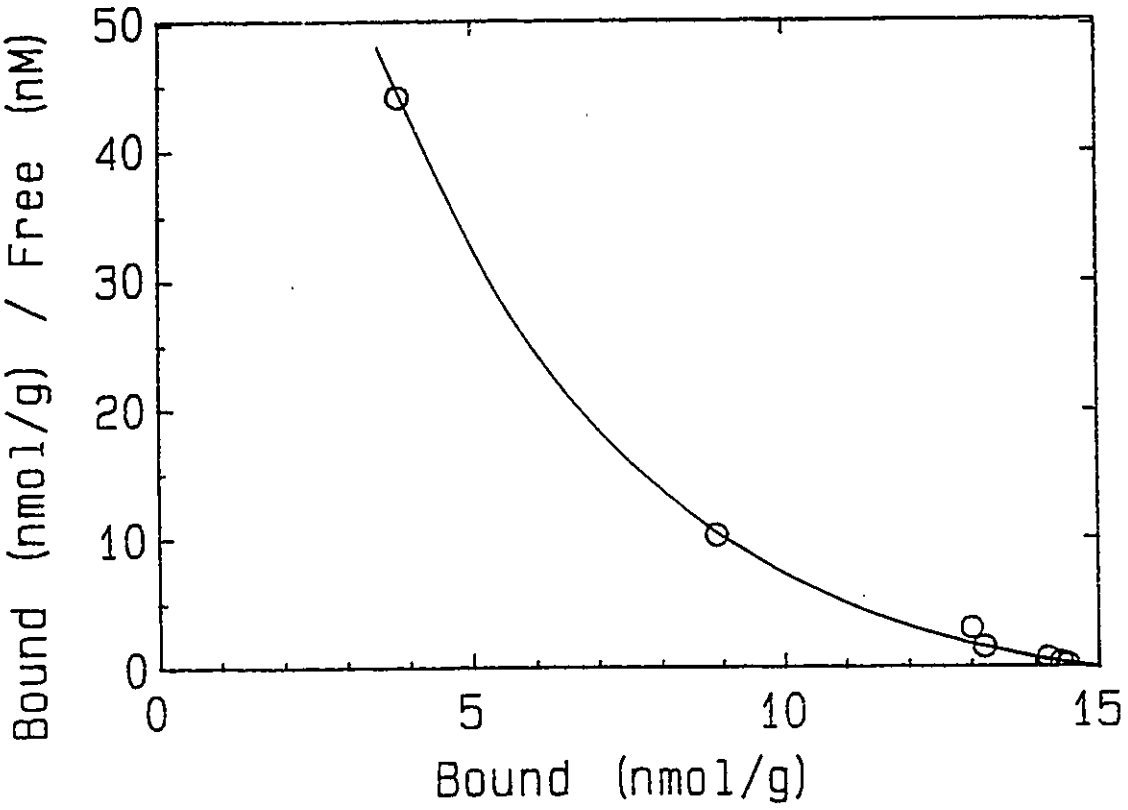


Figure 3.1.2

Scatchard analysis of insulin binding by isolated insulin receptor. The fraction of ^{125}I-insulin bound to unbound, or free insulin is plotted versus the concentration of bound insulin.

3.2 Triton Micellar Assay of Insulin Receptor Autophosphorylation

Insulin receptor activity has been previously measured in micelles containing the detergent n-octyl-β,D-glucopyranoside [Sweet *et al.*, 1987]. Dry phospholipids were suspended in buffer containing the detergent. However, this detergent has recently been shown to decrease insulin binding and kinase activation of the insulin receptor [Leray *et al.*, 1992]. Triton X-100 is a well characterized detergent, and therefore a good candidate for assaying insulin receptor autophosphorylation. Buffer containing Triton X-100 can be added to hydrate lipid films. Hydrophobic additives can be included in the preparation of the lipid films. Hydration in this manner then allows for a more uniform distribution of additives among the lipid and detergent, than in the n-octyl-β,D-glucopyranoside assay. The details of the assay are outlined in section 2.4.2 of Materials and Methods. 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. Firstly, protein kinase C activity has been successfully assayed in a similar system [Hannun *et al.*, 1985]. Secondly, the effects of membrane additives on insulin receptor activity in the micellar assay are similar to those found in intact cells, as discussed below.

3.2.1 Lipid Sensitivity of Insulin Receptor Activity

Lipid sensitivity of the insulin receptor was assessed by varying the nature and concentration of phospholipids, relative to Triton X-100.

Initially, 8 mole % of lipid relative to Triton X-100 was used. The negatively

charged phospholipid phosphatidylinositol (PI) was shown to support insulin receptor autophosphorylation in the presence of n-octyl-β,D-glucopyranoside [Sweet *et al.*, 1987]. No difference in insulin receptor autophosphorylation activity was observed between 8 mole percent phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) or PI, nor between 3:1 ratios of PC:PE, PC:PI, and PC:PS. However, at higher mole percent lipid, relative to Triton X-100, differences in lipid sensitivity were found. Figure 3.2.1.1 illustrates the effect of increasing the amount of PE in the assay. This resulted in a slight decrease in insulin-stimulated autophosphorylation, measured as counts per minute (CPM) incorporated into the β subunit of the insulin receptor. Phosphatidylserine was shown to elicit a biphasic effect, increasing the amount of autophosphorylation initially, then inhibiting at values above 25 mole %, as shown in Figure 3.2.1.2. The negatively charged head group of phosphatidylserine may be responsible for the loss of insulin receptor activity at higher concentrations, due to interactions between the receptor protein and PS. Increasing the concentration of PC led to enhancement of insulin-stimulated autophosphorylation, with little effect on the basal activity, as observed in Figure 3.2.1.3. It was found that up to 97.5 mole % of this zwitterionic phospholipid could be added to the assay without adverse effects. Since this comprises a fairly simple system, it was used to test the effects of various membrane additives on insulin receptor activity.

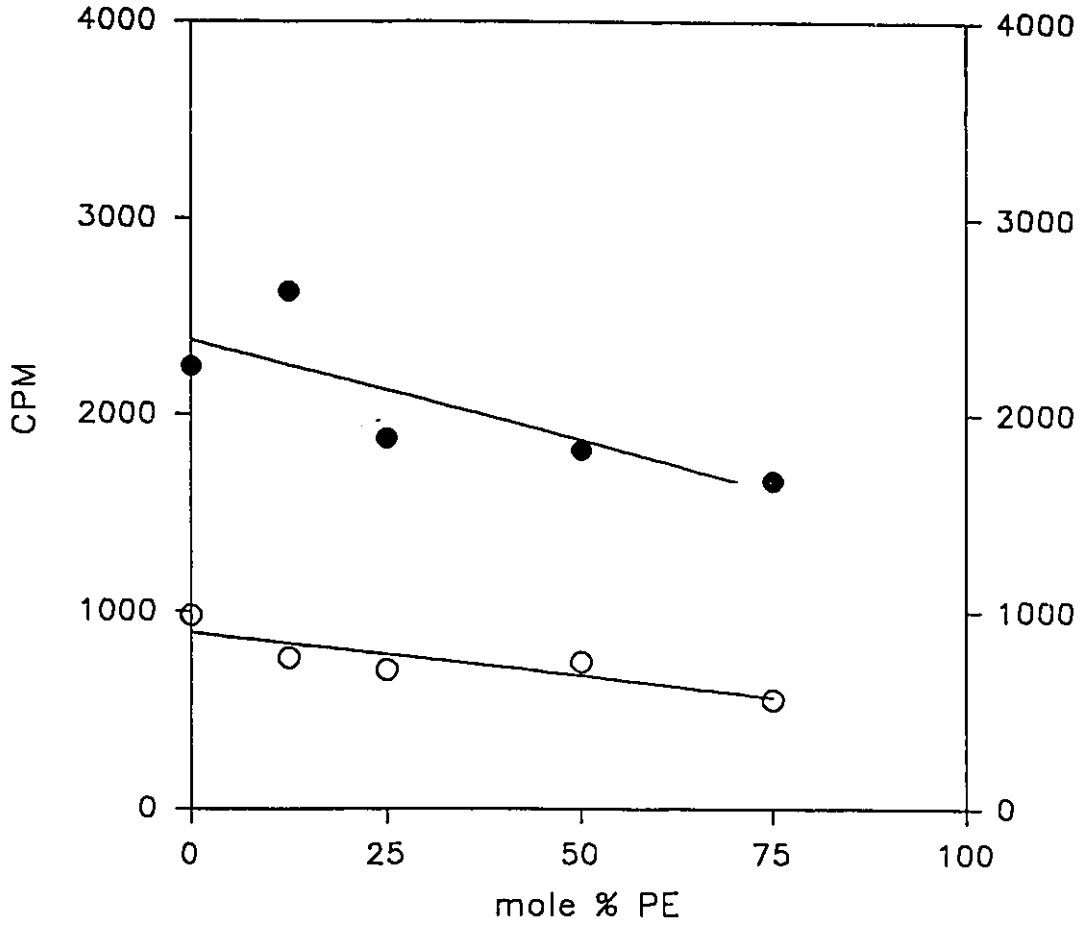


Figure 3.2.1.1

Phosphatidylethanolamine (PE) slightly inhibits insulin stimulation of insulin receptor autophosphorylation. The counts per minute (CPM) of ^{32}P incorporated into the insulin receptor in the absence (open symbols) and presence (filled symbols) of insulin is plotted versus the mole % of PE, relative to the amount of Triton X-100 in the assay.

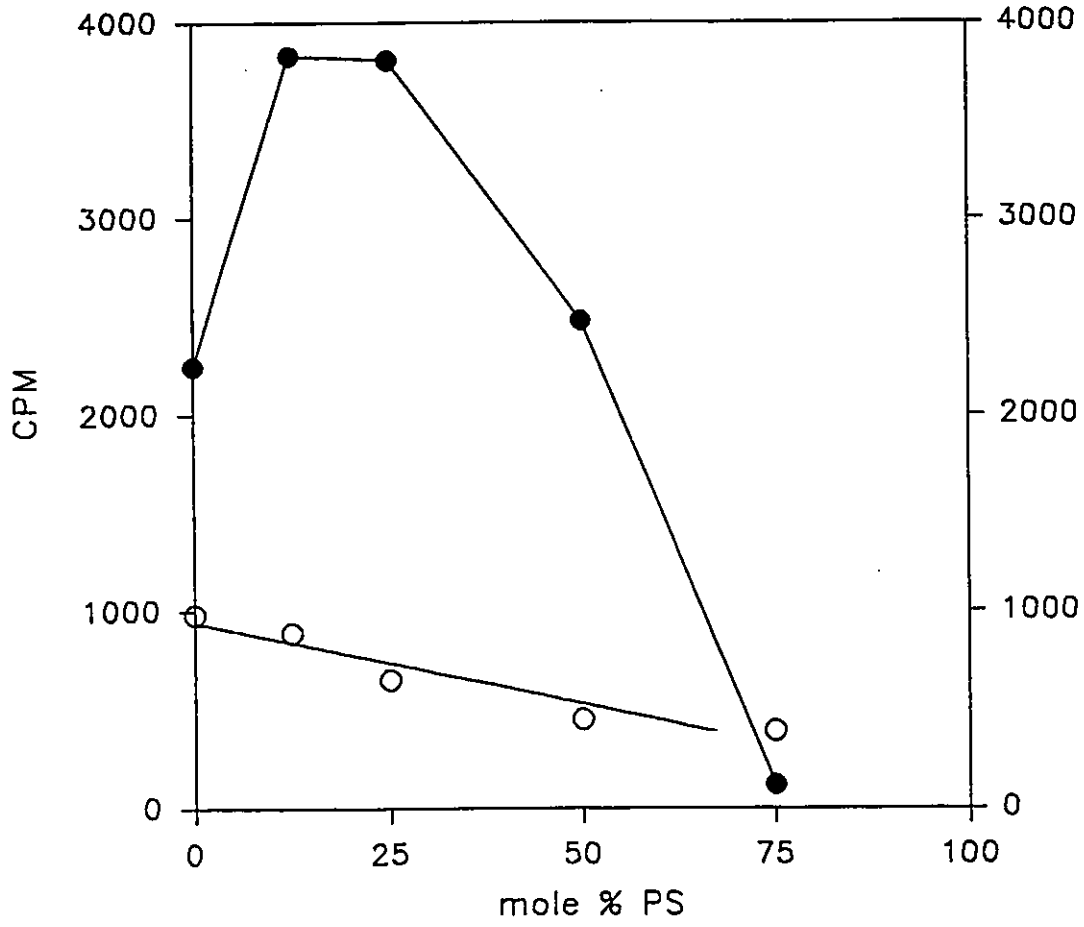


Figure 3.2.1.2

Phosphatidylserine (PS) has a biphasic effect on insulin stimulation of insulin receptor autophosphorylation. The counts per minute (CPM) of ^{32}P incorporated into the insulin receptor in the absence (open symbols) and presence (filled symbols) of insulin is plotted versus the mole % of PS, relative to the amount of Triton X-100 in the assay.

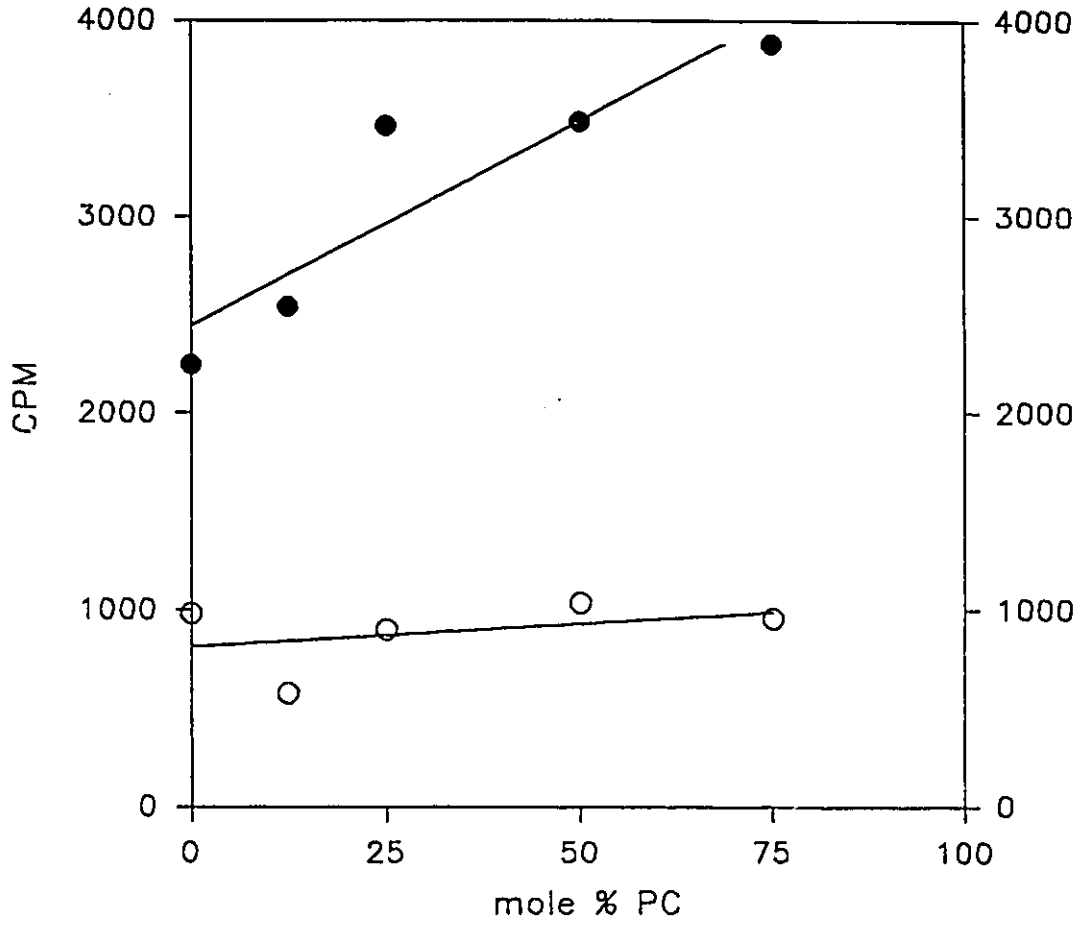


Figure 3.2.1.3

Phosphatidylcholine (PC) enhances insulin stimulation of insulin receptor autophosphorylation. The counts per minute (CPM) of ^{32}P incorporated into the insulin receptor in the absence (open symbols) and presence (filled symbols) of insulin is plotted versus the mole % of PC, relative to the amount of Triton X-100 in the assay.

3.2.2 Determination of Optimal Assay Conditions

The zwitterionic phospholipid, phosphatidylcholine (PC), supported a large stimulation of autophosphorylation in response to insulin at high mole percent lipid, with no effect on basal insulin receptor phosphorylation, as discussed in section 3.2.1. The effects of receptor concentration and incubation time were tested, with 75 mole % PC, relative to Triton X-100. As shown in Figure 3.2.2.1, it was determined that 2 μg of insulin receptor and an incubation time of 15 minutes were within the linear range of activity. These conditions were therefore used to assess the effects of various membrane additives on insulin receptor autophosphorylation. The effects of Triton X-100 concentration on insulin receptor activity were also assessed. It was found that increasing concentrations of Triton sustained receptor autophosphorylation in response to insulin, and in the absence of phospholipid (Figure 3.2.2.2). This is in agreement with the findings of Leray et al. [1992], who suggest that Triton X-100 favors the active $\alpha_2\beta_2$ oligomeric form of the insulin receptor. The fact that detergent can sustain the kinase activity is interesting. However, the relevance of this activity to physiological systems is questionable, since the insulin receptor is located in the phospholipid bilayer in the plasma membrane of cells. We therefore minimized the concentration of detergent in the assay.

Variations in insulin sensitivity were observed between insulin receptors isolated at different times, from different placentas. The amount of isolated protein required for a strong autoradiographic signal in a reasonable exposure time was determined for each batch of insulin receptors. In each assay, the basal activity was

compared to the insulin-stimulated activity.

The order of addition of assay components was not found to influence the results. Receptors were always pre-incubated with insulin in the presence of compounds of interest. However, the same effects were observed upon addition of the membrane additives after insulin binding. This suggests that the membrane additives did not affect insulin-receptor interactions. This was confirmed as discussed in section 3.6.1, Effects of Membrane Additives on Insulin-Receptor Interactions.

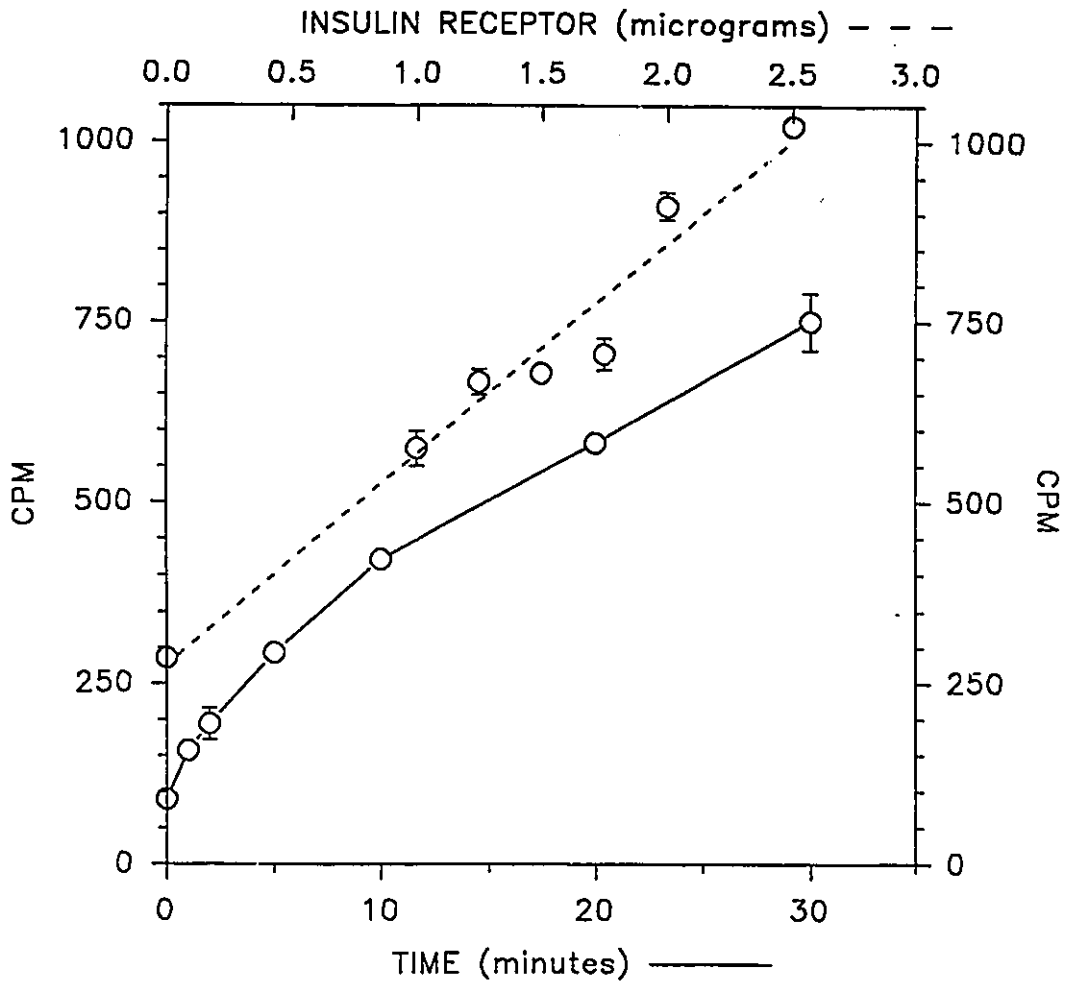


Figure 3.2.2.1

Dependence of insulin receptor phosphorylation on incubation time (solid curve) and receptor concentration (dashed curve), in mixed micelles containing 75 mole % phosphatidylcholine. Phosphorylation was measured by scintillation counting of ^{32}P incorporation into the insulin receptor β subunit, where CPM = counts per minute. Values are mean ± S.E.M. for triplicate samples.

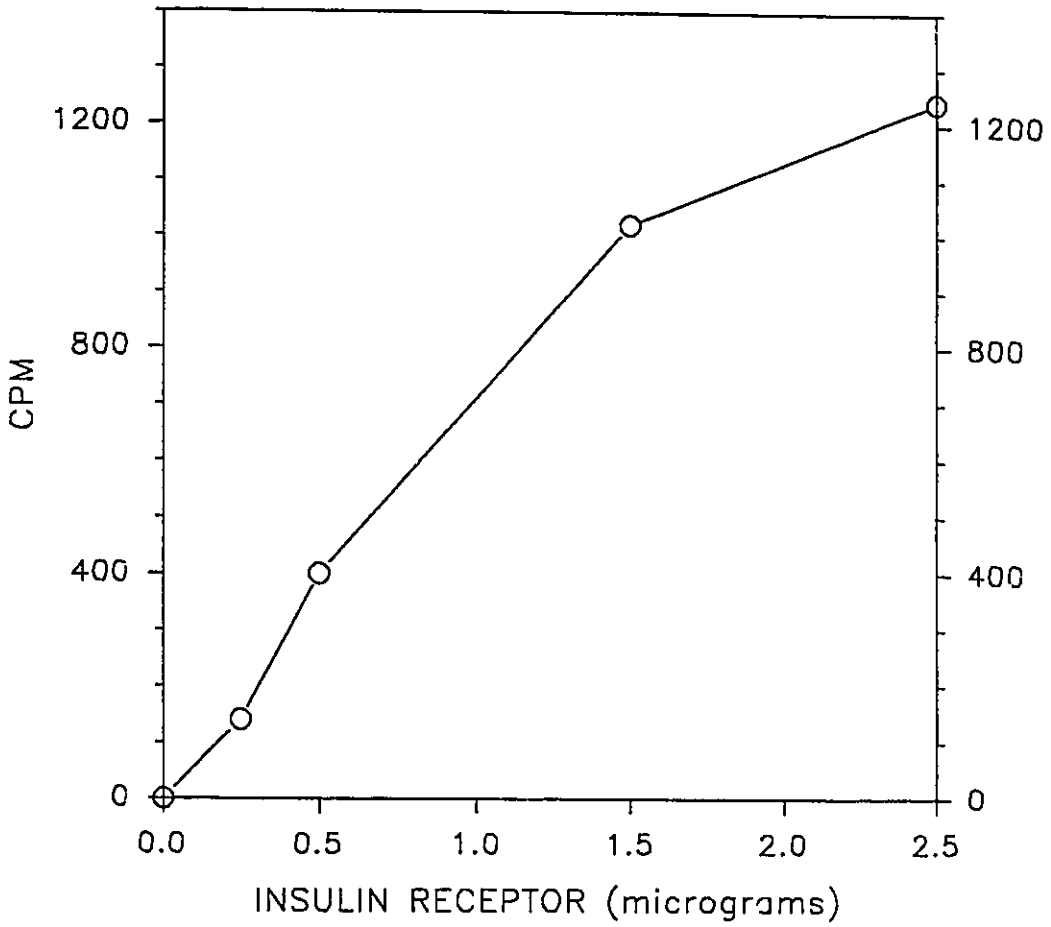


Figure 3.2.2.2

Dependence of insulin receptor activity on insulin receptor concentration in 0.1 % Triton X-100, in the absence of phospholipid. Phosphorylation was measured by scintillation counting of ^{32}P incorporation into the insulin receptor β subunit, where CPM = counts per minute.

3.3 Compounds tested for effects on autophosphorylation

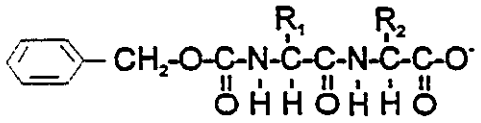
The compounds which were tested for their effects on insulin receptor activity are shown in Figure 3.3.1. It was previously shown that insulin-dependent functions of rat adipocytes are altered by membrane additives [Epand *et al.*, 1991]. The effects of a series of small peptides on insulin signalling correlated with their effects on the hexagonal phase forming tendency in model membranes. These peptides were therefore investigated for their ability to modulate insulin stimulation of the first step in insulin signalling, which is receptor autophosphorylation. These included the carbobenzoxy (CBZ)-dipeptides, CBZ-glycyl-L-phenylalanine (CBZ-Gly-L-Phe) and CBZ-L-phenylalanylglycine (CBZ-L-Phe-Gly). Both of these compounds raise the hexagonal phase transition temperature of DEPE. CBZ-Gly-L-Phe raises T_H by 15 ± 3 °C/mole fraction of additive, while CBZ-L-Phe-Gly raises the transition temperature by 34 ± 3 °C/mole fraction [Epand *et al.*, 1991]. The overall hydrophobicity of the carbobenzoxy-dipeptides is sufficient for partitioning into the membrane phase, where they would likely be situated near the surface of the membrane so that the charged terminal carboxyl group is exposed, and the amide bonds can be hydrogen bonded to water. The peptides remain near the surface of the membrane, and expand this region to a greater extent than the hydrocarbon center of the membrane, thus causing the bilayer (L_α) phase to be favored over the hexagonal (H_{II}) phase [Epand and Epand, 1991]. The dipeptide L-Phe-Gly, lacking the carbobenzoxy group, is not hydrophobic enough to spontaneously partition from the aqueous to the membrane phase, and has no effect on T_H. Bilayer-stabilizing compounds of different

structures were also tested for their effects on insulin receptor activity. Human serum apolipoprotein A-I (apo A-I), the major protein component of high density lipoprotein particles, contains amphipathic helical segments [Segrest *et al.*, 1974], and is an extremely strong bilayer stabilizer [Tytler *et al.*, 1993]. It was therefore also tested for its effects on insulin stimulation of insulin receptor autophosphorylation. Lysophosphatidylcholine (Lyso PC) was included in the study, since this detergent-like phospholipid stabilizes the bilayer relative to the hexagonal phase [Epand *et al.*, 1985]. Acyl carnitines show an increasing capacity to raise the bilayer to hexagonal phase transition temperature of membranes, with increasing acyl chain lengths [Epand *et al.*, 1989]. Lauroyl carnitine, with a chain length of 12 carbon atoms, raises T_H of phosphatidylethanolamine by approximately 60 °C/mole fraction, while the 16 carbon-chained palmitoyl carnitine raises T_H by approximately 280 °C/mole fraction [Epand *et al.*, 1989]. Sphingosine is a cationic amphiphile with the opposite effect on membrane physical properties. That is, sphingosine lowers the bilayer to hexagonal phase transition temperature of model membranes [Epand *et al.*, 1991]. We therefore tested to see if this compound could stimulate insulin receptor phosphorylation. To further assess the role of positive charge in the action of membrane additives, two bilayer-stabilizing cationic amphiphiles were also investigated for their ability to modulate insulin receptor activity. These included 1-dimethylammonio-DL-2-propyl-cholesteryl-3β-oxysuccinate, (CATAM1) and 2-{[2-(trimethylammonio)ethyl]-methylamino}-ethyl-cholesteryl-3β-oxysuccinate, (CATAM2) [Bottega and Epand, 1992]. The uncharged, potent hexagonal phase promoter

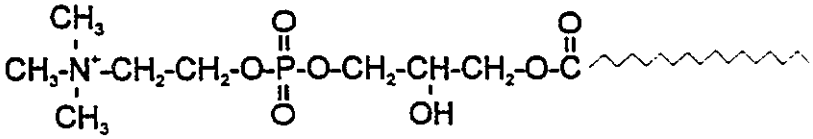
dioctanoylglycerol (DiC8) [Epand *et al.*, 1985] was also included in this study.

Figure 3.3.1 Structures of Various Compounds

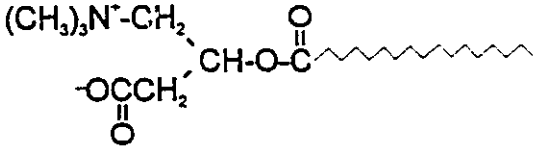
CBZ-dipeptide



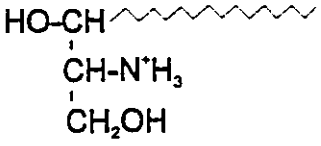
Lyso PC



Acyl Carnitine

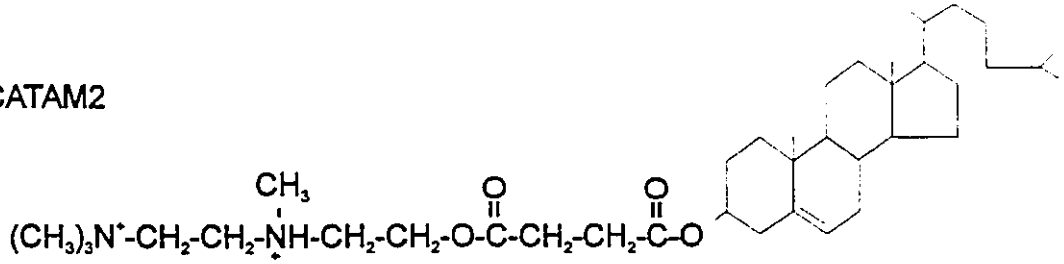


Sphingosine

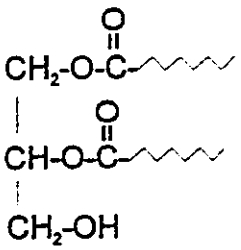


CATAM1

CATAM2



Dioctanolylglycerol



3.3.1 Effects of Peptide Derivatives on Insulin Receptor Activity in Mixed Micelles

The presence of carbobenzoxy-dipeptides in the mixed micellar assay affected insulin stimulation of insulin receptor autophosphorylation. Neither of the peptide derivatives 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, as follows:

$$\% \text{ Phosphorylation} = (CPM_{ins+} - CPM_{basal+}) / (CPM_{ins-} - CPM_{basal-}) \times 100\% \qquad 3.3.1.1$$

where CPM is the counts per minute, as determined by scintillation counting, ins is in the presence of insulin, and basal is in the absence of insulin. The subscripts + and - denote the presence and absence, respectively, of added compound to be tested.

Increasing concentrations of CBZ-Gly-L-Phe led to a decrease in the amount of insulin receptor autophosphorylation, as shown in Figure 3.3.1.1. At the highest concentration of peptide tested, 10 μM, insulin receptor activity was reduced to 47 ± 6 %, relative to that in the absence of the peptide. Similarly CBZ-L-Phe-Gly inhibited insulin stimulation of insulin receptor autophosphorylation. As shown in Figure 3.3.1.2, this peptide analogue inhibited insulin receptor activity to 31 ± 15 % at 10μM. The relative concentrations and potencies of the additives will be discussed in detail in section 3.9. CBZ-L-Phe-Gly is a slightly stronger bilayer stabilizer than CBZ-Gly-L-Phe. The increased hydrophobicity and bulkiness near the carbobenzoxy group in CBZ-L-Phe-Gly

allows it to expand the membrane near the surface to a greater extent than the hydrocarbon center of the membrane. This favors the bilayer phase over the hexagonal phase. Model membranes containing these peptides must therefore be heated to higher temperatures to undergo transition to the hexagonal phase. These CBZ-dipeptides might therefore inhibit insulin receptor activity as a result of their effects on membrane physical properties.

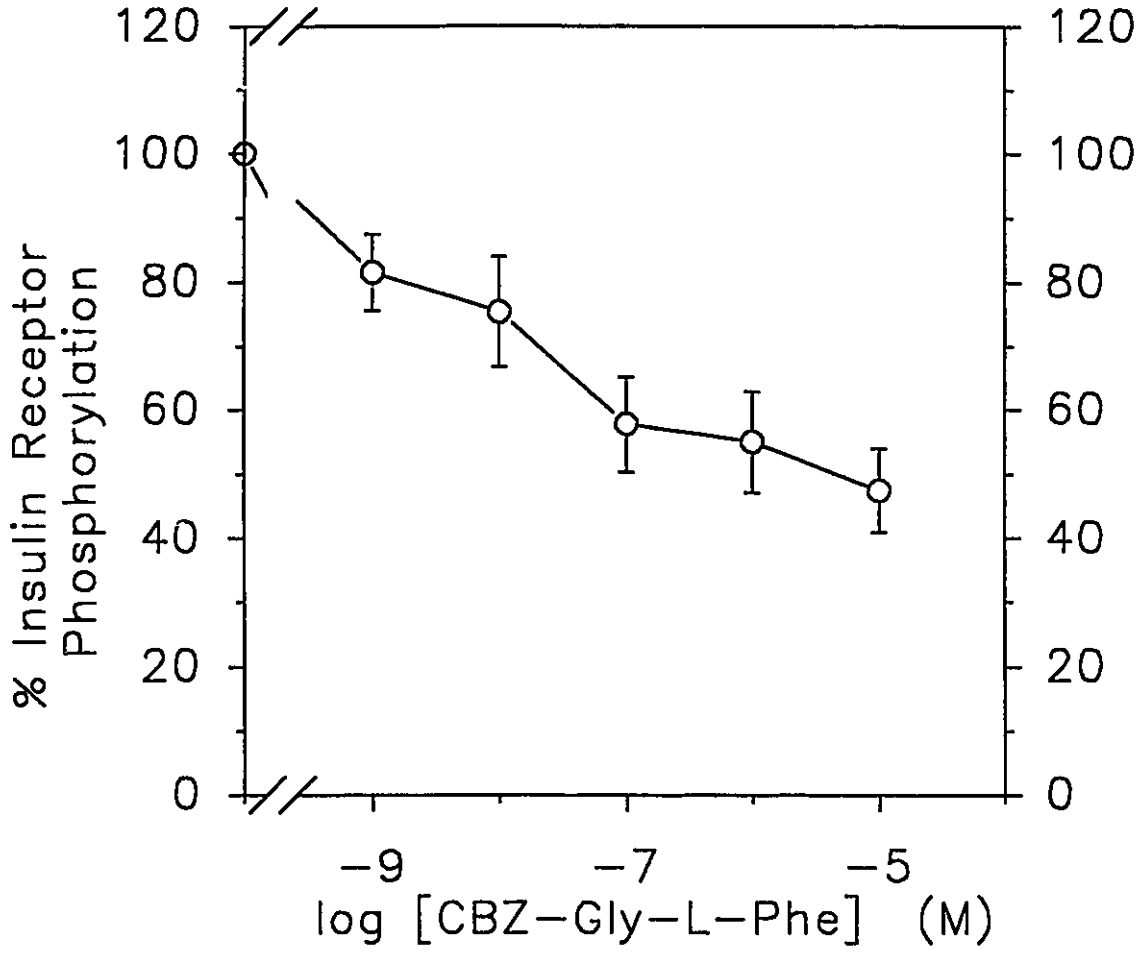


Figure 3.3.1.1

CBZ-Gly-L-Phe inhibits insulin receptor autophosphorylation in mixed micelles. A in the subsequent figures, 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 ± S.E.M. for triplicate experiments.

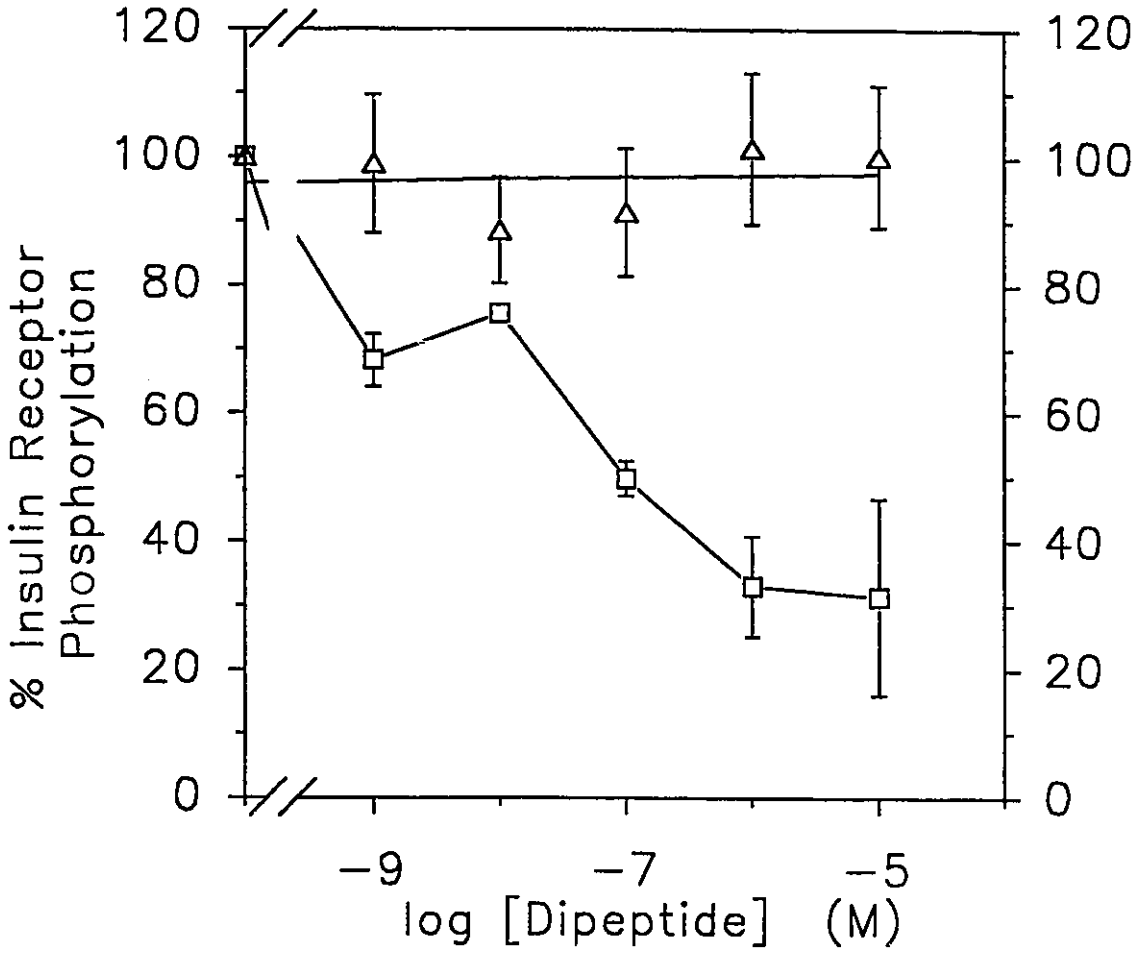


Figure 3.3.1.2

Effects of CBZ-L-Phe-Gly (□) and L-Phe-Gly (Δ) on insulin receptor autophosphorylation in mixed micelles. Values are mean ± S.E.M. for triplicate experiments.

3.3.2 Peptide Stereoisomer Effects

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. The stereoisomer CBZ-Gly-D-Phe was therefore tested for its effect on insulin stimulation of insulin receptor autophosphorylation. As shown in Figure 3.3.2.1, it had essentially the same inhibitory profile as the L isomer. To determine whether the L and D isomers had the same effect on membrane physical properties, we measured the transition temperature of DEPE in the presence of increasing concentrations of CBZ-Gly-D-Phe. A plot of transition temperature versus mole fraction of the dipeptide is shown in Figure 3.3.2.2. The slope of this plot was 20 ± 3 °C/mole fraction, indistinguishable from 15 ± 3 °C/mole fraction for that of CBZ-Gly-L-Phe [Epand *et al.*, 1991], suggesting that these stereoisomers have the same effect on the bulk biophysical properties of the membrane. This supports the hypothesis that these peptides inhibit insulin receptor activity by modulating the bulk properties of the membrane. This was further supported by the inability of L-Phe-Gly to affect insulin receptor activity, as shown in Figure 3.3.1.2. L-Phe-Gly lacks the hydrophobic carbobenzoxy group, and therefore does not partition into the membrane.

CBZ-D-Phe-L-Phe-Gly had previously been shown to inhibit insulin signalling in rat adipocytes (Epand *et al.*, 1991). However, inhibition of receptor autophosphorylation by this bilayer stabilizer from several commercial sources was

irreproducible in both micelles and intact cells. Further tests were therefore not completed with this tripeptide.

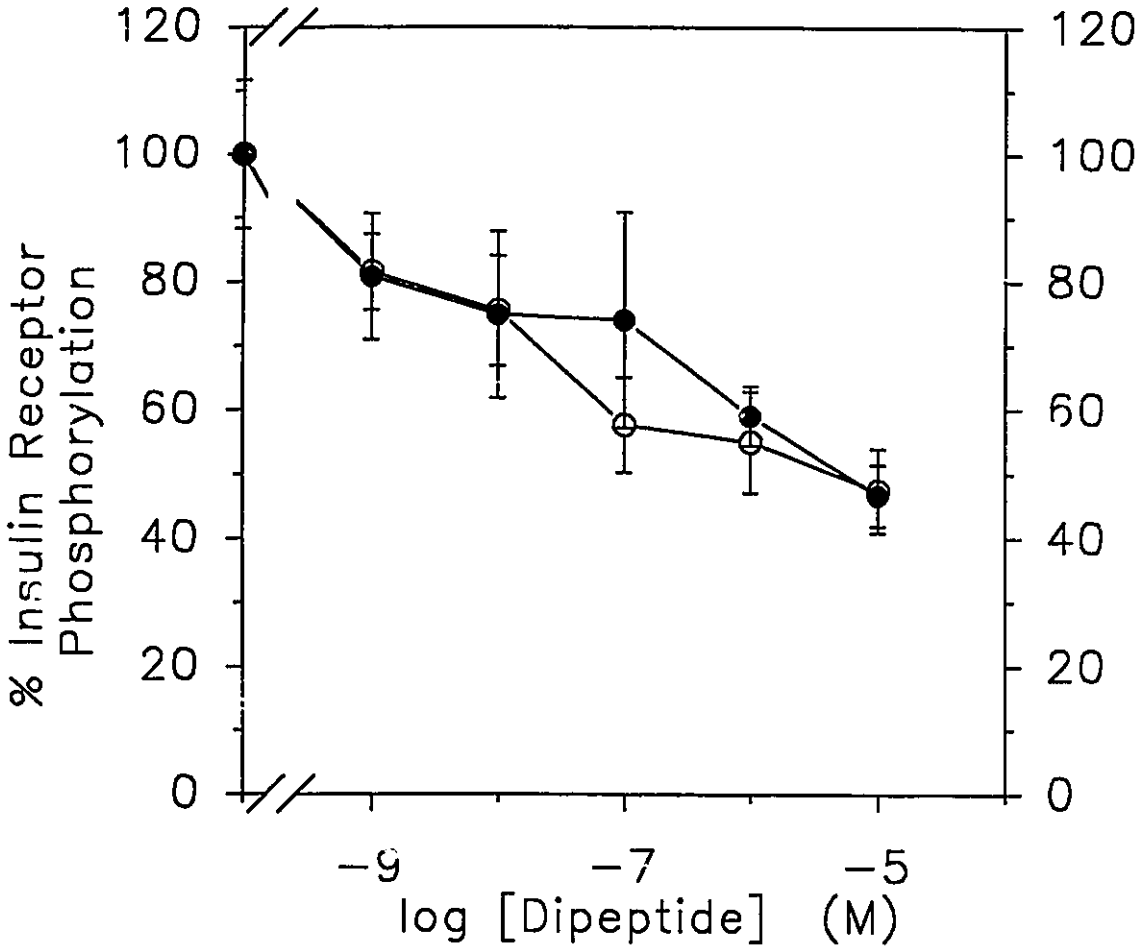


Figure 3.3.2.1

Stereo isomers of CBZ-Gly-Phe had identical effects on insulin receptor autophosphorylation in mixed micelles. ○, CBZ-Gly-L-Phe; ●, CBZ-Gly-D-Phe. Values are mean ± S.E.M. for triplicate experiments.

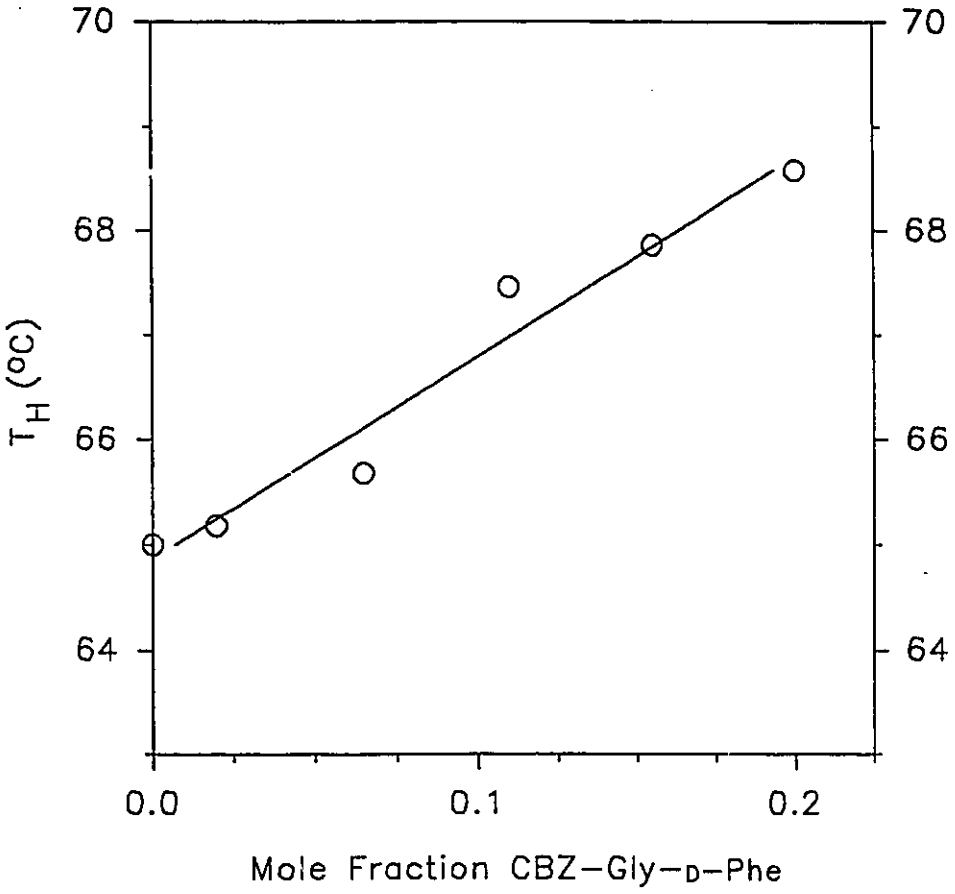


Figure 3.3.2.2

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 T_H .

3.3.3 Effects of Other Bilayer Stabilizers on Insulin Receptor Activity in Mixed Micelles

Bilayer stabilizers of diverse structure were investigated for their ability to alter insulin receptor autophosphorylation activity. The strongest bilayer stabilizer tested was apolipoprotein A-I. It was found to inhibit receptor activity, with the dose dependence shown in Figure 3.3.3.1. While apo A-I is more than 1000-fold more potent than the CBZ-dipeptides in stabilizing the bilayer relative to the hexagonal phase, it is not much more potent in inhibiting insulin receptor tyrosine kinase activity. Apo A-1 inhibited insulin receptor phosphorylation to 23 ± 2 % of that in the absence of apolipoprotein. The non-peptide bilayer stabilizer Lyso PC also was able to reduce the stimulation of receptor phosphorylation in response to insulin. Figure 3.3.3.2 illustrates the marked inhibition of insulin receptor tyrosine kinase activity in the presence of increasing concentrations of this detergent. Acyl carnitines, which are single-chain amphiphiles with large polar head groups, were also investigated for their effects on insulin receptor activity. The results are shown in Figure 3.3.3.3. Lauroyl carnitine, containing a 12 carbon fatty acid chain, was not able to inhibit insulin receptor activity at the concentrations tested. Instead, lauroyl carnitine stimulated this activity to approximately 130 %. While the short fatty acyl chain of lauroyl carnitine may render it less able to relieve curvature strain in the micelles consisting of longer acyl chains, it is unclear how this could lead to stimulation of insulin receptor activity. Increasing concentrations of the 16-carbon containing palmitoyl carnitine led to inhibition of insulin receptor tyrosine

phosphorylation, to 74 ± 2 % of control values. The increased inhibitory potency of the longer acyl carnitine correlates with the greater bilayer stabilizing potency of palmitoyl carnitine, as described in section 3.3. This provides further evidence for the mechanism of action of these various bilayer stabilizers on insulin receptor phosphorylation lying in their ability to alter the bulk biophysical properties of the membrane. Positively charged bilayer stabilizers were also included in this study. Two cationic amphiphiles which raise the bilayer to hexagonal phase transition temperature of model membranes had been shown to inhibit protein kinase C activity in a mixed micellar assay [Bottega and Epand, 1992]. CATAM1 and CATAM2 were found to augment insulin stimulation of receptor phosphorylation at low concentrations. At 10 μM however, CATAM1 inhibited insulin receptor autophosphorylation while CATAM2 had no effect. These results are presented in Figure 3.3.3.4. The biphasic action of CATAM1 and CATAM2 differed from the inhibition observed with most of the other bilayer stabilizers. While stimulation of insulin receptor kinase activity by low concentrations of these cationic additives is the same as has been observed with cationic polyamino acids [Morrison *et al.*, 1989; Kohanski, 1989; Biener and Zick, 1990], the latter did not lead to inhibition of kinase activity at higher concentrations. It appears that the effects of cationic amphiphiles on kinase activity are complex and not predictable from their ability to modulate membrane physical properties.

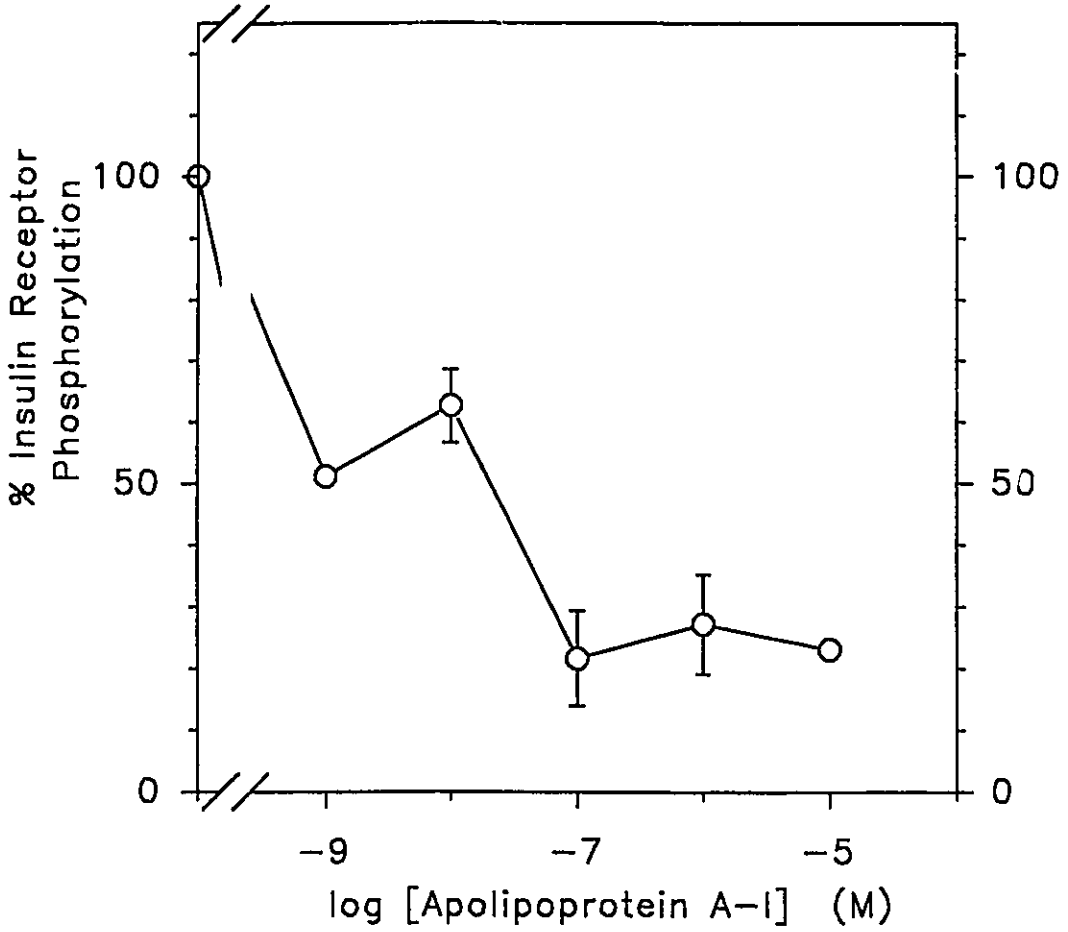


Figure 3.3.3.1

Inhibition of insulin receptor autophosphorylation by apolipoprotein A-I in mixed micelles. Values are mean ± S.E.M. for triplicate experiments.

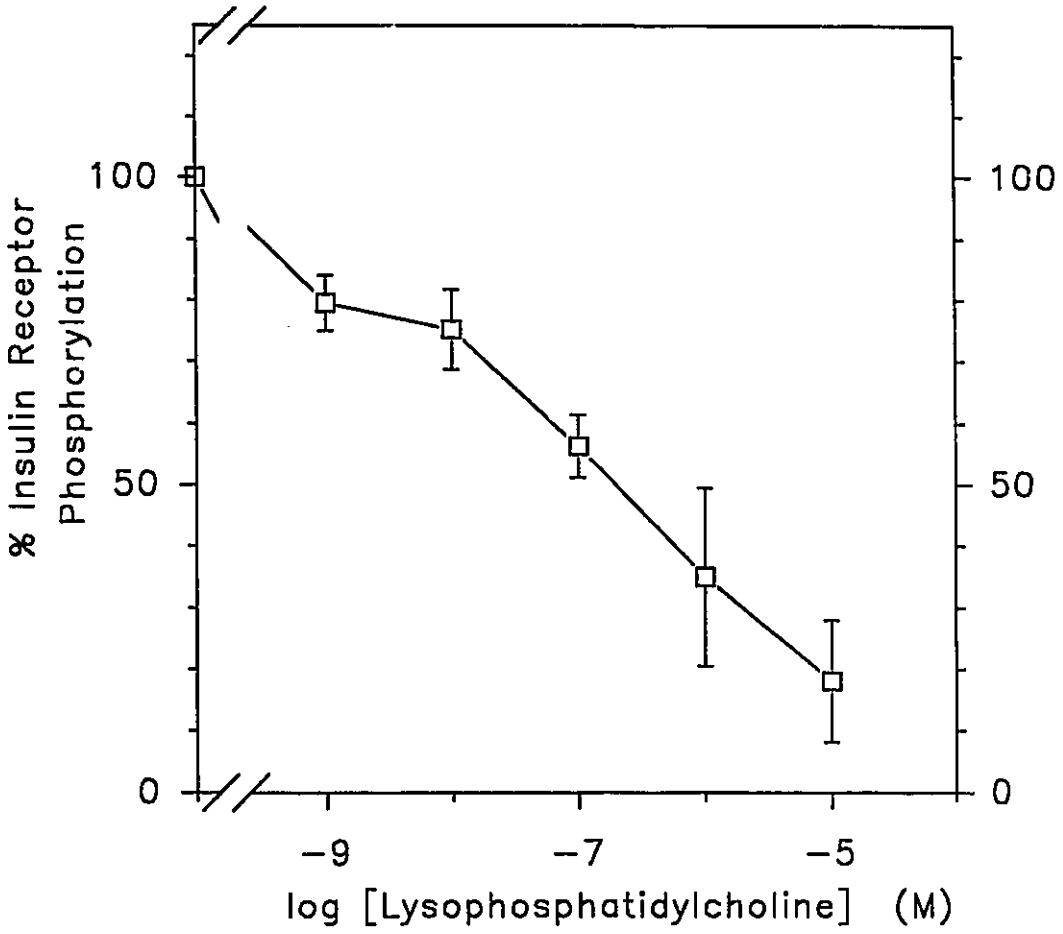


Figure 3.3.3.2

Inhibition of insulin receptor autophosphorylation by lysophosphatidylcholine in mixed micelles. Values are mean ± S.E.M. for triplicate experiments.

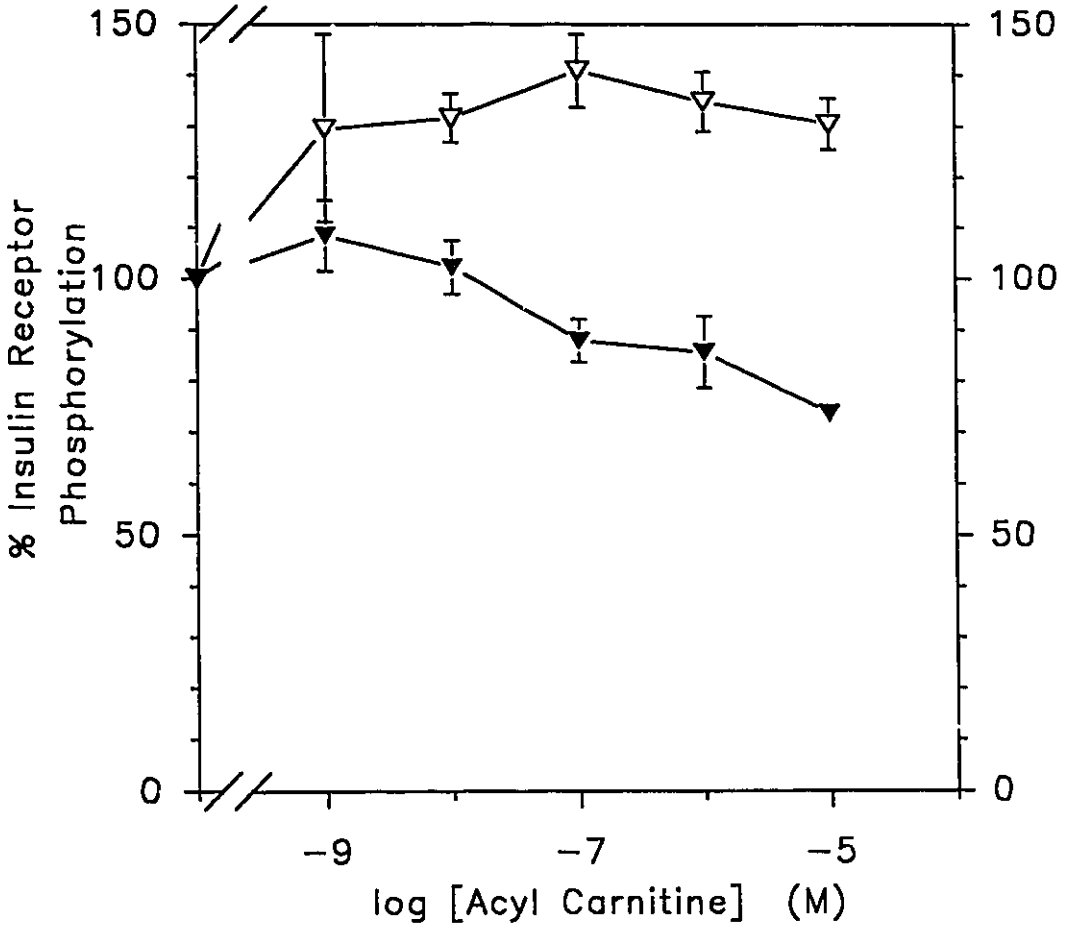


Figure 3.3.3.3

Effects of acyl carnitines on insulin receptor autophosphorylation in mixed micelles. ∇, Lauroyl carnitine; ▼, Palmitoyl carnitine. Values are mean ± S.E.M. for triplicate experiments.

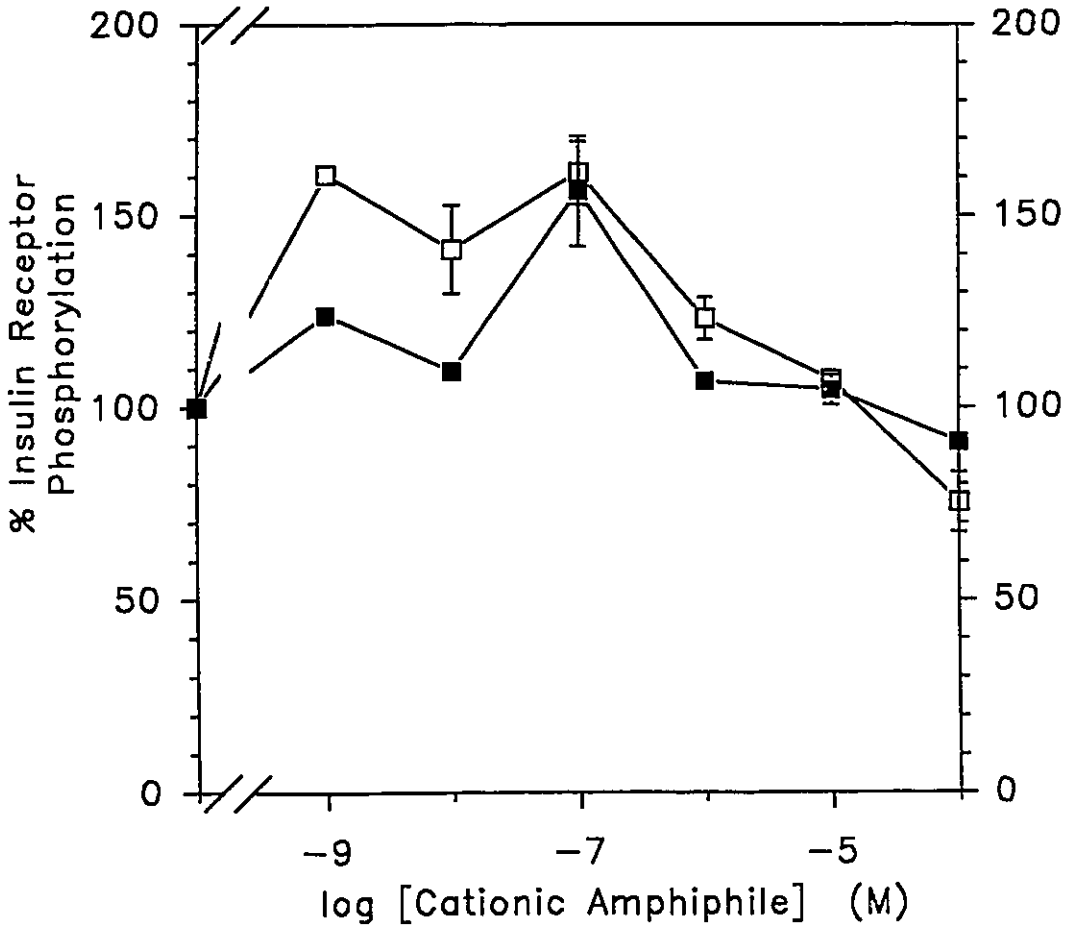


Figure 3.3.3.4

Effects of cationic amphiphiles on insulin receptor autophosphorylation in mixed micelles. □, CATAM1; ■, CATAM2. Values are mean ± S.E.M. for triplicate experiments.

3.3.4 Effects of Hexagonal Phase Promoters on Insulin Receptor Activity in Mixed Micelles

To further investigate the effects of positively charged amphiphiles on insulin receptor activity, a cationic hexagonal phase promoter was included in the tyrosine kinase assay. Sphingosine lowers the hexagonal phase transition temperature of model membranes [Epand *et al.*, 1991]. Since bilayer stabilizers were found to inhibit insulin receptor autophosphorylation, it was proposed that hexagonal phase promoters, with the opposite effects on membrane physical properties, would stimulate this activity. Up to concentrations of 1 nM, sphingosine further simulated phosphorylation in response to insulin, with maximal stimulation of 181 ± 10 % at 1 nM. Higher concentrations of sphingosine had the opposite effect, inhibiting insulin receptor autophosphorylation to approximately 17 % of control values, at 10 μM sphingosine, as shown in Figure 3.3.4.1. The inhibition at higher sphingosine concentrations agrees with the findings of Arnold and Newton [1991], who used much higher concentrations of sphingosine. Taken with the results of CATAM1 and CATAM2, discussed in section 3.3.3, it appears that cationic amphiphiles belong to a separate class of compounds whose effects on insulin receptor tyrosine kinase activity are not readily predictable from their ability to modulate membrane physical properties. An uncharged hexagonal phase promoter was therefore investigated for its ability to modulate insulin stimulation of insulin receptor autophosphorylation. Dioctanoylglycerol (DiC8), which lowers T_H, was found to have the opposite effect on tyrosine kinase activity than the bilayer stabilizing compounds. DiC8

stimulated the insulin effect, with increasing concentrations, to 230 ± 16 % of control phosphorylation, at 10 μM, as shown in Figure 3.3.4.2. The stimulation observed in the presence of DiC8 agrees with its effect in rat adipocytes [Terry *et al.*, 1991] and human mononuclear cells [Grunberger and Levy, 1990]. It is interesting to note that DiC8 was not able to stimulate receptor phosphorylation in intact cells, as described in section 3.5.3. 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.

Other hexagonal phase promoting additives could not be tested for their ability to affect insulin receptor activity, since such compounds tend to be cytotoxic, and therefore the results of the mixed micellar assays could not be correlated with effects in intact cells.

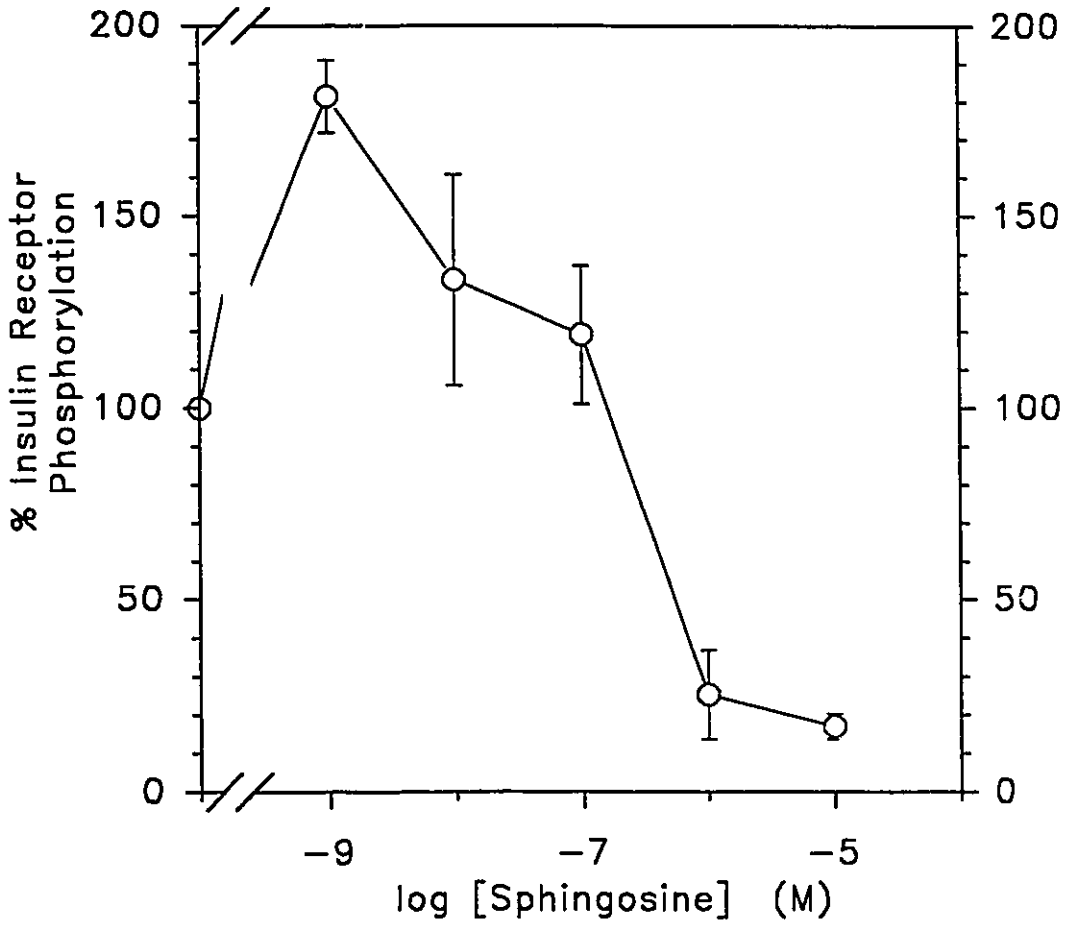


Figure 3.3.4.1

Effect of sphingosine on insulin receptor autophosphorylation in mixed micelles. Values are mean ± S.E.M. for triplicate experiments.

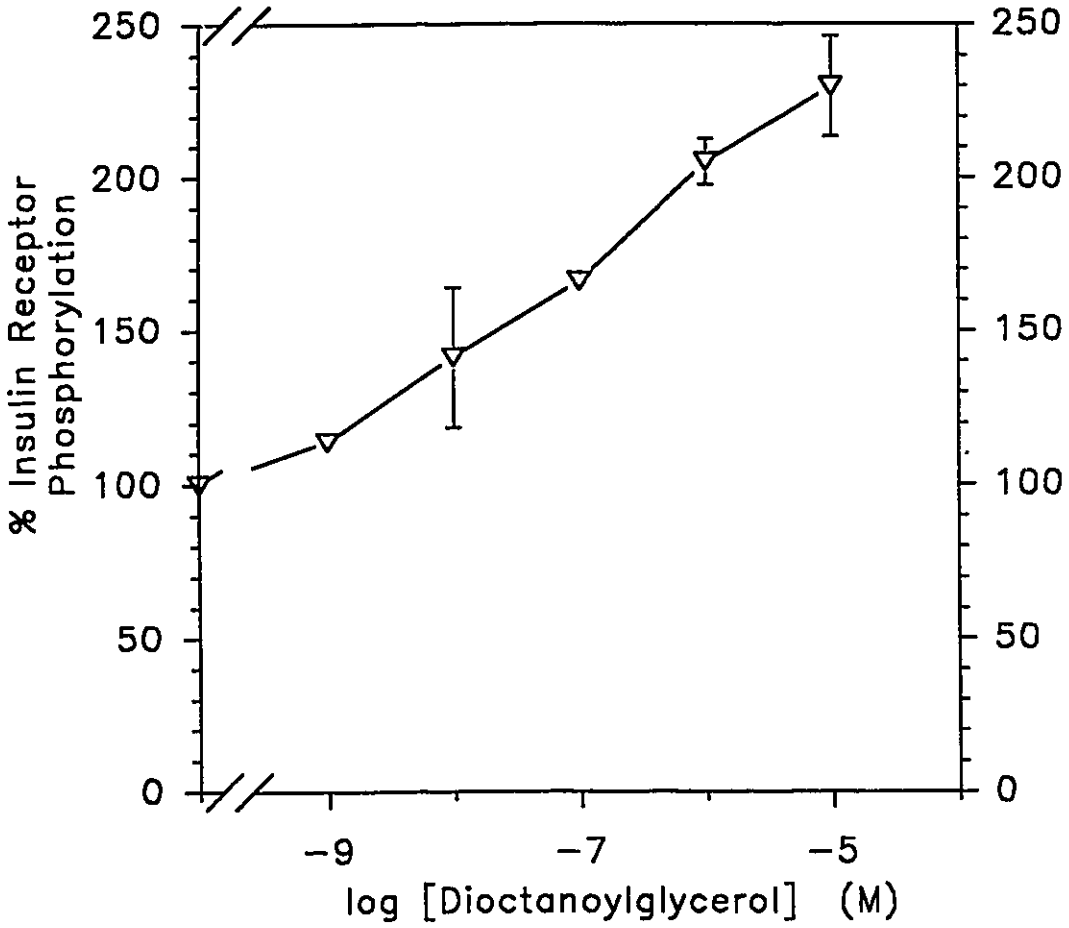


Figure 3.3.4.2

Dioctanoylglycerol enhances insulin stimulation of insulin receptor autophosphorylation in mixed micelles. Values are mean ± S.E.M. for triplicate experiments.

3.3.5 Membrane Physical Properties and Insuiin Receptor Activity in Mixed Micelles

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.

The membrane stabilizing potencies of compounds can be compared by their ability to raise the bilayer to hexagonal phase transition temperature per mole fraction of additive. The strongest bilayer stabilizers have the largest positive slopes for the plot of transition temperature versus mole fraction of additive. The bilayer stabilizing potency does not correlate quantitatively with the extent of insulin receptor tyrosine kinase inhibition, when comparing compounds of widely differing structure. For example the rank order of potency for bilayer stabilization is: Apo A-I > palmitoyl carnitine > CATAM1 > Lyso PC > CATAM2 > lauroyl carnitine > CBZ-L-Phe-Gly > CBZ-Gly-D-Phe = CBZ-Gly-L-Phe > sphingosine > DiC8. The rank order for kinase inhibitory potency in micelles is: sphingosine ≅ lyso PC > apo A-I > CBZ-L-Phe-Gly > CBZ-Gly-D-Phe = CBZ-Gly-L-Phe > palmitoyl carnitine > CATAM1 = CATAM2 = lauroyl carnitine > DiC8, when compared at 10 μM of compound. The lack of correlation may be due to the fact that the concentrations of added compound, rather than the mole fraction of compound which has partitioned into the membrane, are compared. The less hydrophobic peptides may not partition totally into the membrane. This may also 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. For compounds of similar structure, however, such as the CBZ-dipeptides or the acyl carnitines, the bilayer stabilizing potency correlates with insulin receptor inhibitory potency. This supports the idea that modulation of the bulk biophysical properties of the membrane by hydrophobic compounds alters the activity of the insulin receptor.

To further support the hypothesis that the compounds tested affect insulin receptor phosphorylation via modulation of membrane physical properties, a homologous series of compounds including a bilayer stabilizer and a hexagonal phase promoter have been included in the mixed micellar assay of insulin receptor tyrosine kinase activity. Distearoylphosphatidylcholine (DSPC) raises the bilayer to hexagonal phase transition temperature of DEPE. Addition of the branched side chain in the middle of the acyl chain of DSPC disrupts hydrocarbon packing and increases hydrocarbon volume. Therefore di(8-methylstearoyl)phosphatidylcholine (8-methyl-DSPC) is less effective than DSPC in stabilizing the bilayer phase [Epand *et al.*, 1991b]. Furthermore di(8-*n*-butylstearoyl)phosphatidylcholine (8-*n*-butyl-DSPC), with the bulky butyl substituent, lowers the bilayer to hexagonal phase transition temperature [Epand *et al.*, 1991b]. It was expected that DSPC and 8-methyl-DSPC would inhibit insulin stimulation of tyrosine kinase activity, and that 8-*n*-butyl-DSPC would augment the insulin response. It was

found that DSPC and 8-methyl-DSPC inhibited insulin receptor phosphorylation, as shown in Figure 3.3.5.1. The bilayer stabilizing potencies of these two amphiphiles did not correlate with their kinase inhibitory potency. Rather than stimulating insulin receptor activity, the hexagonal phase promoter 8-butyl-DSPC also inhibited insulin stimulation of insulin receptor autophosphorylation, as shown in Figure 3.3.5.1. It should be noted, however, that the samples of branched phospholipids used were quite old, and changed the consistency of the assay media, making it very viscous, suggesting a change in the micellar morphology. This may account for the inhibitory action of the hexagonal phase promoter, and the stronger inhibition of 8-methyl-DSPC, even though it is a weaker bilayer stabilizer than unbranched DSPC. These effects may represent an artifact of the micellar assay, and are not necessarily representative of what would be observed in a bilayer. Additional samples of this material (not commercially available) could not be obtained from the original source. Therefore this has not been investigated further, and although the results of the DSPC series of compounds contradict the proposal that compounds can affect insulin receptor activity by modulation of membrane physical properties, these results may not be reliable for the above-stated reasons.

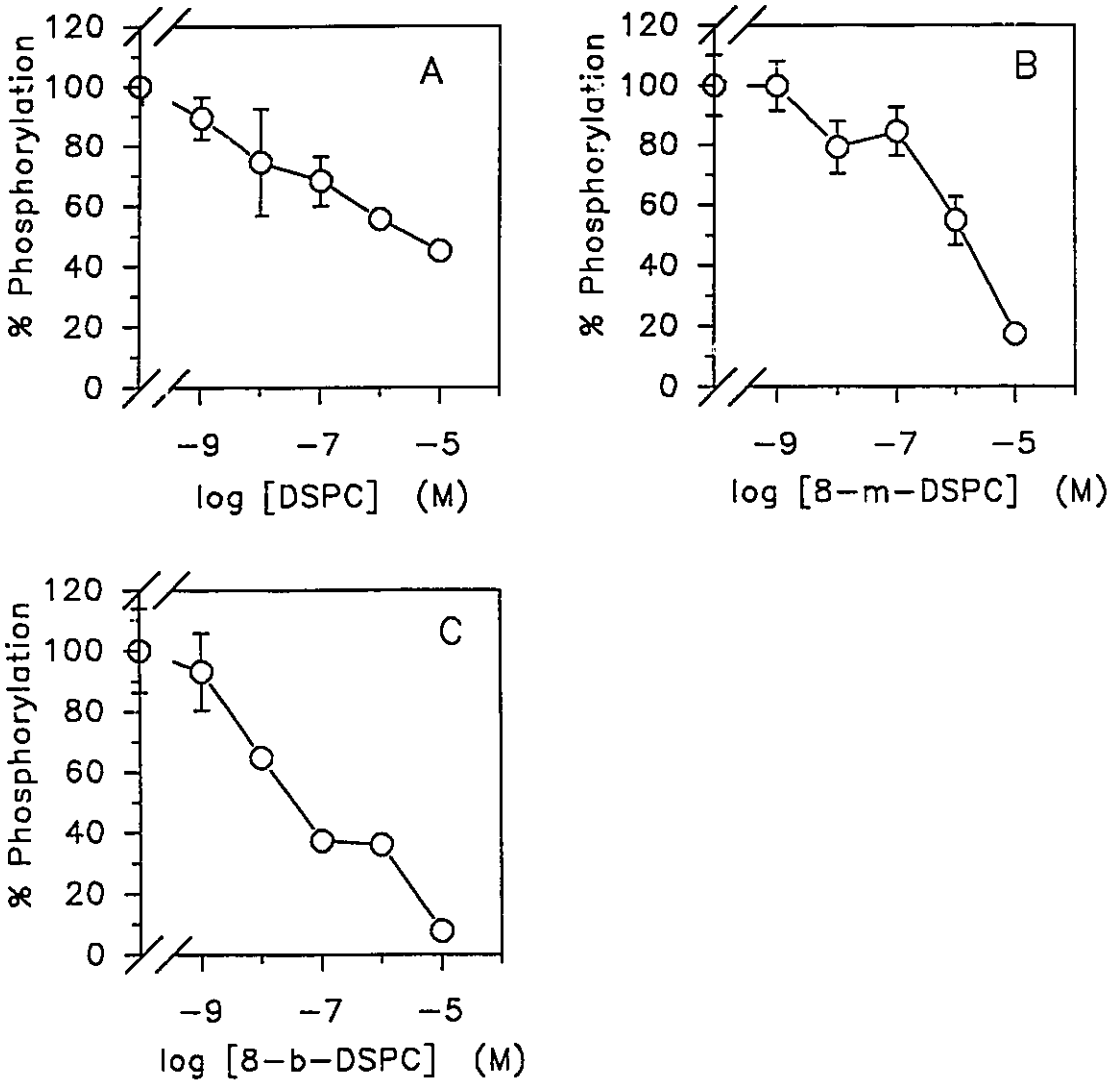


Figure 3.3.5.1

Effects of unbranched and branched phosphatidylcholines on insulin receptor activity in mixed micelles. A, distearoylphosphatidylcholine; B, di(8-methylstearoyl)-phosphatidylcholine; C, di(8-*n*-butylstearoyl)phosphatidylcholine. Values are mean ± S.E.M. for triplicate experiments.

3.4 Soluble Insulin Receptor Kinase Domain Activity

To determine whether any of the compounds tested 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. This is comprised of residues 959-1355 of the human insulin receptor, and is synthesized in Sf9 cells by the use of a *Baculovirus* expression vector [Ellis *et al.*, 1988]. This 48 kDa protein is constitutively active, and phosphorylates itself on tyrosine residues in an intermolecular reaction [Cobb *et al.*, 1989]. Autophosphorylation results in an increase in enzymatic activity, as in the native insulin receptor [Cobb *et al.*, 1989]. Since SIRK lacks the transmembrane domain, activity is measured in the absence of phospholipids. Thus, if the compounds tested here exert their effects by modulation of membrane properties, they should have no effect on the kinase activity of the soluble insulin receptor kinase domain. The dependence of SIRK activity on bilayer stabilizers and hexagonal phase promoters was measured as described in Materials and Methods, section 2.5. The percent of SIRK phosphorylation was calculated as the percent of ^{32}P incorporation into SIRK in the presence of compound relative to that in the absence of compound to be tested. Since SIRK is constitutively active, the kinase activity measured by this assay is independent of insulin binding. The effects of the membrane additives on insulin binding will be discussed in section 3.6.1.

3.4.1 Effects of Bilayer Stabilizers on SIRK Activity

The bilayer-stabilizing compounds CBZ-Gly-L-Phe, CBZ-L-Phe-Gly, apo A-I, lauroyl carnitine and palmitoyl carnitine were unable to affect the constitutively active SIRK, as shown in Figure 3.4.1.1. This suggests that these compounds inhibit receptor tyrosine kinase activity by interacting non-specifically with the membrane, or by interacting with the extracellular or transmembrane domain of the native insulin receptor. However, it is unlikely that compounds of varying structure could all inhibit receptor kinase activity by interacting with a specific site on the receptor. Lyso PC, however, inhibited the activity of this kinase, to approximately 70% of the activity in the absence of Lyso PC (Figure 3.4.1.1). It is possible that a portion of the inhibition of insulin receptor activity in the presence of Lyso PC is due to interaction with the tyrosine kinase domain of the insulin receptor, but inhibition of the intact receptor activity is much stronger, as was observed in Figure 3.3.3.2. The positively charged bilayer stabilizers CATAM1 and CATAM2 had different effects on SIRK activity. Figure 3.4.1.2 illustrates the slight inhibition of SIRK autophosphorylation by increasing concentrations of CATAM1 and CATAM2. The inhibition of SIRK activity by cationic amphiphiles suggests that the effects of high concentrations of positively charged compounds on the intact receptor activity may not be due to membrane modulation.

The lack of effect of most of the bilayer-stabilizing additives on SIRK activity supports the hypothesis that such additives exert their inhibitory effects on the native insulin receptor by modulating the bulk biophysical properties of the membrane, rather

than by direct interaction with the kinase domain of the receptor itself.

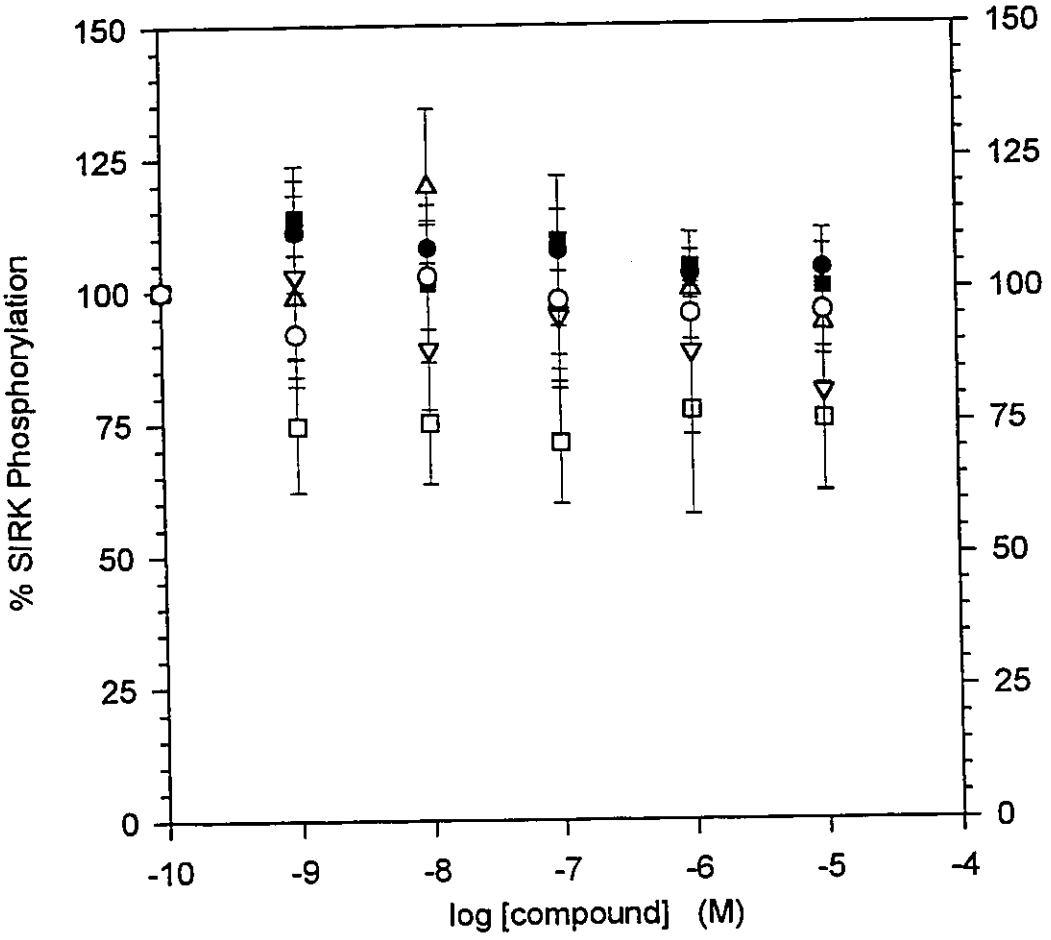


Figure 3.4.1.1

Effects of bilayer-stabilizing compounds on soluble insulin receptor kinase (SIRK) domain activity. Incorporation of ^{32}P into the SIRK domain was expressed as a percent of the activity in the absence of added compound. Values are mean ± S.E.M. for triplicate experiments. ●, CBZ-Gly-L-Phe; ■, CBZ-L-Phe-Gly; □, Lyso PC; O, Apo A-I; Δ, Palmitoyl carnitine; ∇, Lauroyl carnitine.

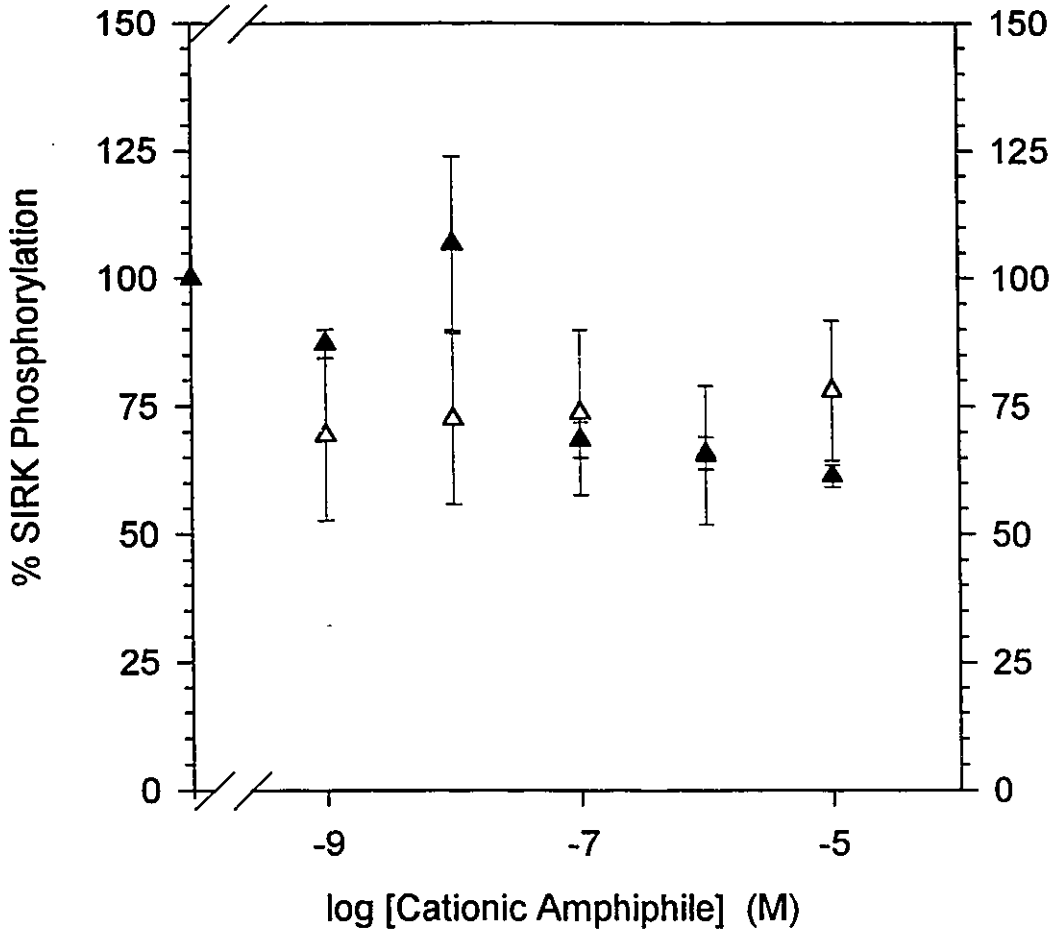


Figure 3.4.1.2

Effects of cationic amphiphiles on soluble insulin receptor kinase (SIRK) domain activity. Incorporation of ^{32}P into the SIRK domain was expressed as a percent of the activity in the absence of added compound. Values are mean ± S.E.M. for triplicate experiments. ▲, CATAM1; △, CATAM2.

3.4.2 Effects of Hexagonal Phase Promoters on SIRK Activity

Sphingosine reduced SIRK activity, with a 60 percent reduction in kinase activity, at 10 μM of this hexagonal phase promoter (Figure 3.4.2.1). As discussed in section 3.4.1, cationic amphiphiles may alter insulin receptor activity in a membrane-independent manner. In the absence of a hydrophobic environment, positively charged amphiphiles may interact with acidic residues of the soluble insulin receptor tyrosine kinase domain, with the hydrophobic portion at the liquid air interface. Residues 1274-1286 are particularly rich in glutamic acid. In the presence of phospholipids however, these hydrophobic compounds would be more likely to interact with the phospholipids, than with the intracellular tyrosine kinase domain. Thus it is unlikely that the mechanism of inhibition of cationic amphiphiles includes direct interaction with the insulin receptor kinase domain. The hexagonal phase promoter DiC8 inhibited SIRK activity, as shown in Figure 3.4.2.1. This is in contrast to its stimulatory effect on the native receptor (section 3.3.4). 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, due to the hydrophobic effect.

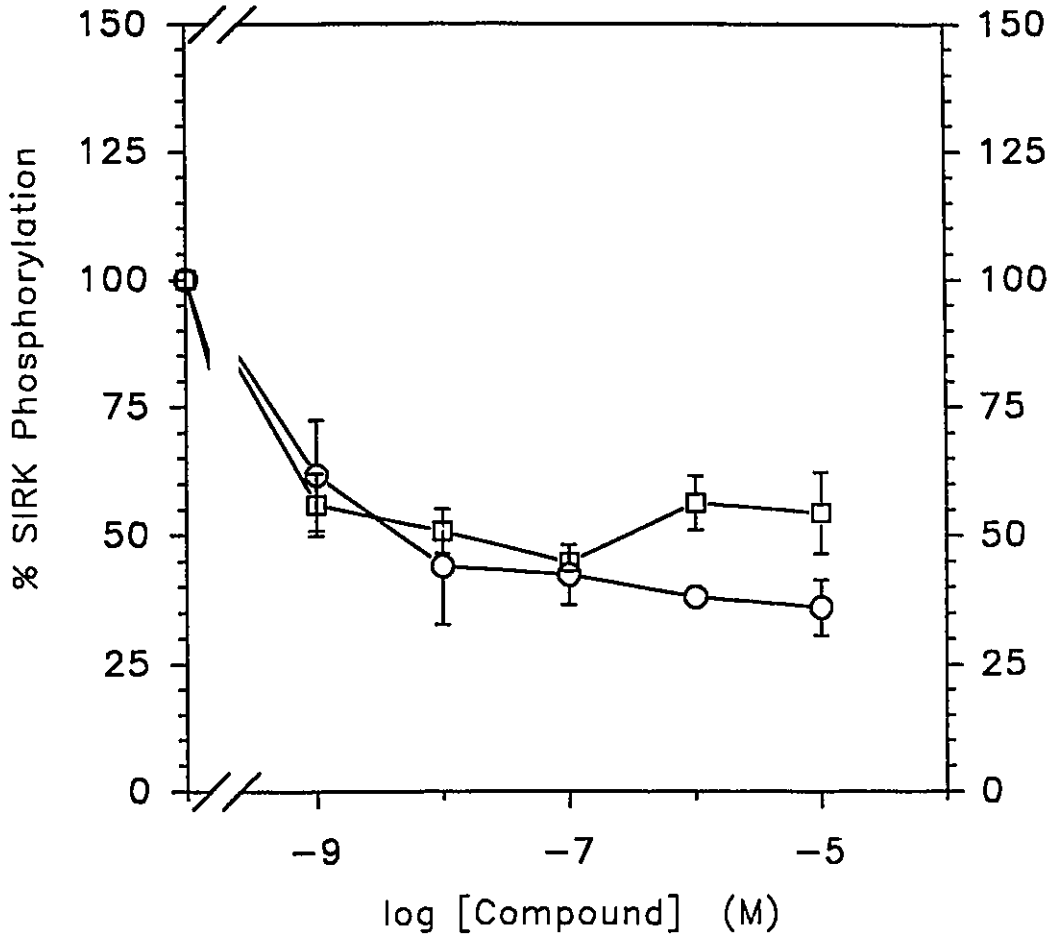


Figure 3.4.2.1

Effects of hexagonal phase promoters on soluble insulin receptor kinase (SIRK) domain activity. Incorporation of ^{32}P into the SIRK domain was expressed as a percent of the activity in the absence of added compound. Values are mean ± S.E.M. for triplicate experiments. O, Sphingosine; □, Dioctanoylglycerol.

3.5 Insulin Receptor Activity in NIH 3T3 HIR 3.5 Cells

NIH 3T3 HIR 3.5 cells over-express human insulin receptors, resulting in an average of 6 x 10^6 receptors per cell surface [Whittaker *et al.*, 1987]. These cells are therefore useful to study the phosphorylation of insulin receptors in response to insulin binding, since the large number of receptors leads to a large signal, and a reasonable amount of protein for phosphoamino acid analysis. The latter was required since the insulin receptor is a substrate for serine/threonine kinases in the cell, in addition to its own autophosphorylation activity [Czech *et al.*, 1988; Pillay and Siddle, 1991]. In order to determine the effects of the membrane additives on the autophosphorylation activity, it was necessary to discriminate between tyrosine phosphorylation and serine/threonine phosphorylation. The details of the intact cell insulin receptor phosphorylation assay are outlined in the Materials and Methods, section 2.7. Following incorporation of ^{32}P by the cells, insulin receptor activity was measured by isolation of receptors by immunoprecipitation and gel electrophoresis. A typical autoradiograph of such a gel is shown in Figure 3.5.1. The main band showing ^{32}P incorporation was 95 kDa, which corresponds to the β subunit of the insulin receptor. Phosphorylated insulin receptor was hydrolyzed to individual amino acids, and the phosphoamino acids were separated by TLC, and visualized by autoradiography. The corresponding autoradiograph of the TLC plate is also shown in Figure 3.5.1. The amount of phosphate incorporated into tyrosine and serine residues of the receptor was quantitated by scintillation counting of the scraped TLC spots. While threonine phosphorylation of the insulin receptor has been reported

[Kasuga *et al.*, 1982c; Jacobs and 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.

NIH 3T3 HIR 3.5 fibroblasts were used for these studies. While these cells can be differentiated to adipocytes [Frost and 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.

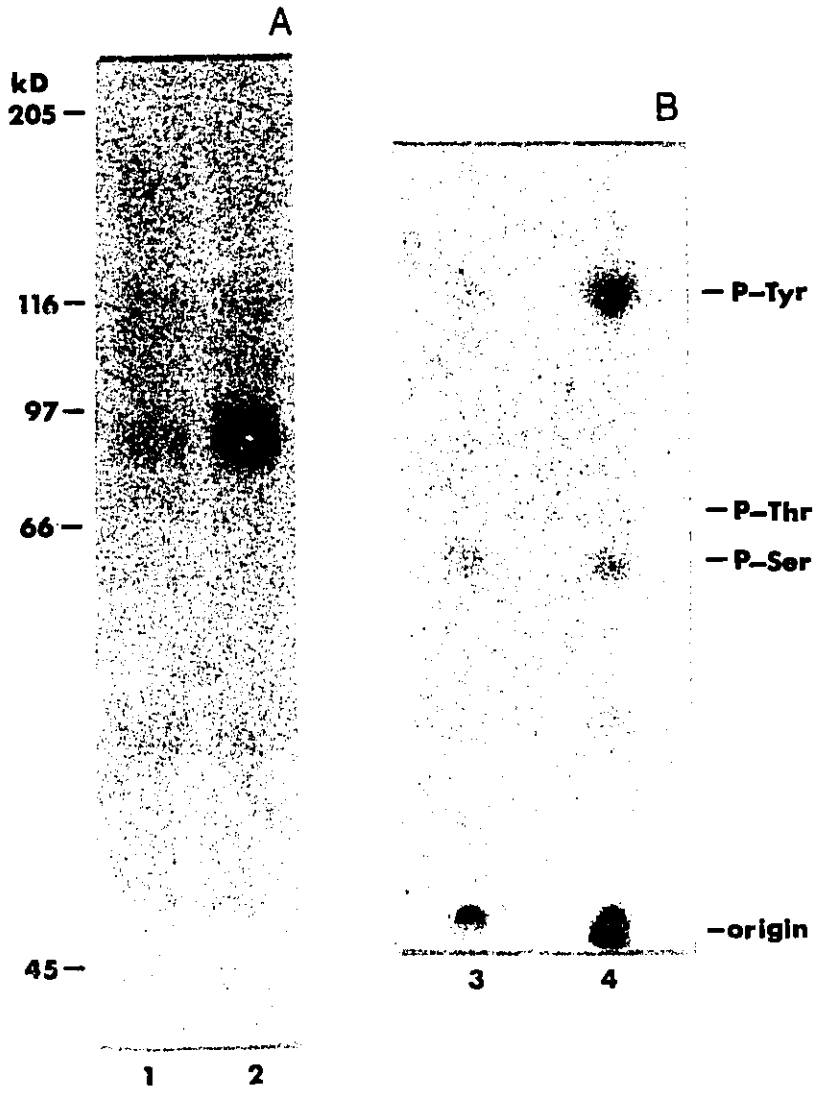


Figure 3.5.1

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. 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 thin layer chromatography.

3.5.1 Effects of CBZ-dipeptides on Insulin Receptor Activity in Intact Cells

Increasing concentrations of the bilayer stabilizing peptide derivative carbobenzoxy-L-Phe-Gly reduced the level of insulin-stimulated insulin receptor tyrosine phosphorylation in intact cells, almost to that in the absence of insulin, with no effect on serine phosphorylation. These results are presented in Figure 3.5.1.1. The weaker bilayer stabilizer CBZ-Gly-L-Phe was less inhibitory than CBZ-L-Phe-Gly with respect to insulin receptor phosphorylation activity, as in mixed micelles. Figure 3.5.1.2 shows the dose dependence of phosphorylation on CBZ-Gly-L-Phe. Neither of the peptide derivatives affected serine phosphorylation or basal tyrosine phosphorylation of the receptor. The fact that the stronger bilayer stabilizer inhibits insulin receptor phosphorylation to a greater extent provides further evidence for the mechanism of action of these additives lying in their ability to modulate membrane bulk biophysical properties.

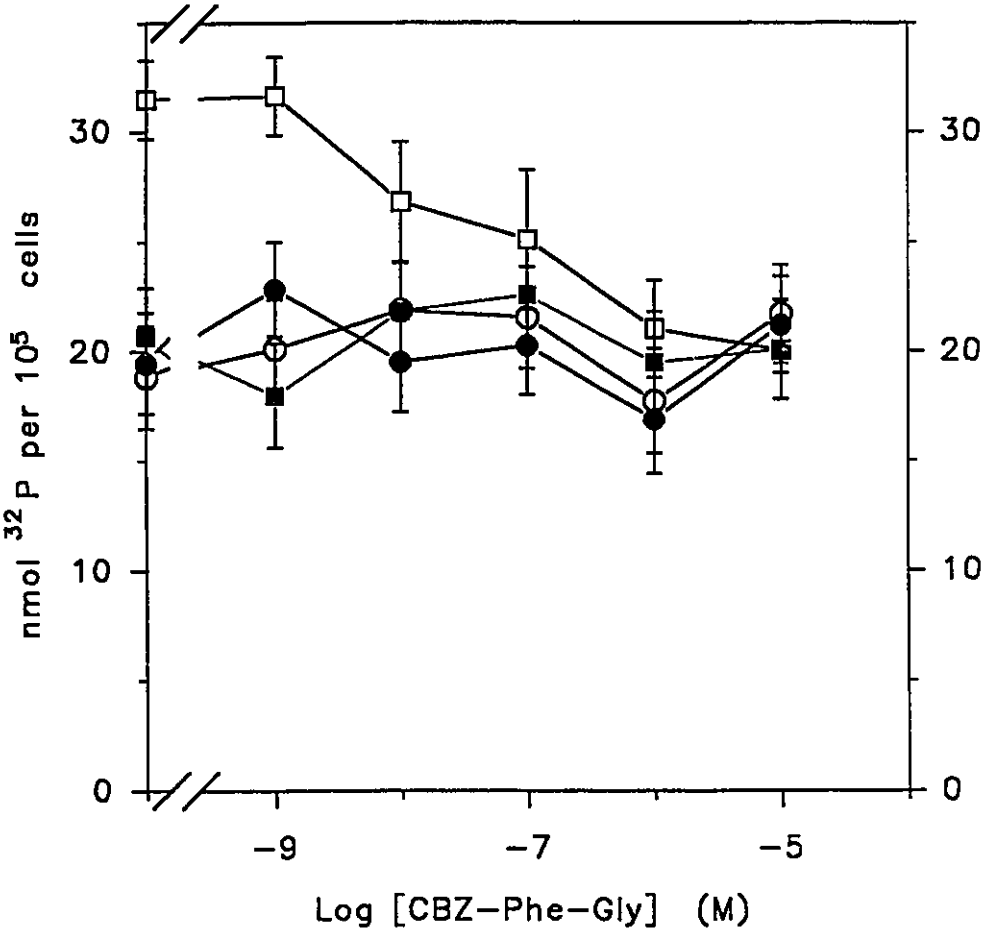


Figure 3.5.1.1

Effect of CBZ-L-Phe-Gly on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

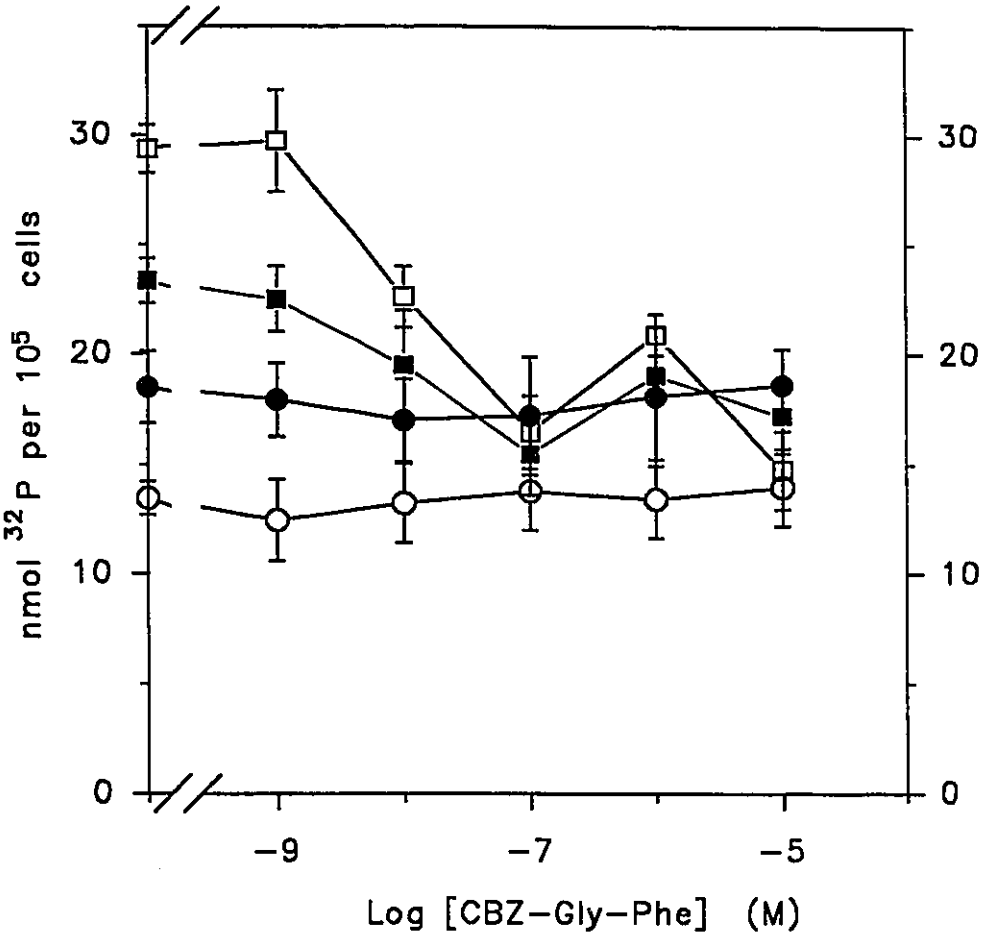


Figure 3.5.1.2

Effect of CBZ-Gly-L-Phe on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

3.5.2 Effects of Other Bilayer Stabilizers on Insulin Receptor Activity in Intact Cells

The strongest bilayer stabilizer tested, apolipoprotein A-I, inhibited tyrosine phosphorylation of the receptor to the greatest extent (Figure 3.5.2.1). Apo A-I was the only compound which also inhibited the insulin stimulation of serine residues of the receptor. However, this was the only case where serine phosphorylation was significantly stimulated in response to insulin. This suggests that apo A-I affects not only insulin receptor tyrosine kinase, but a serine kinase as well. A membrane-associated serine kinase should also be sensitive to membrane physical properties. Such is the case for protein kinase C [Epand and Lester, 1990; Epand *et al.*, 1991]. Figure 3.5.2.2 shows that lysophosphatidylcholine was less potent in inhibiting tyrosine phosphorylation of the receptor, as it did not inhibit to basal levels at the concentrations tested. Palmitoyl carnitine, which raises T_H more per mole fraction than does the shorter-chained lauroyl carnitine [Epand *et al.*, 1989] was more effective at inhibiting insulin stimulation of insulin receptor phosphorylation, as found in comparing Figures 3.5.2.3 and 3.5.2.4. None of the bilayer stabilizing additives affected basal levels of phosphotyrosine or phosphoserine in the β subunit of the insulin receptor.

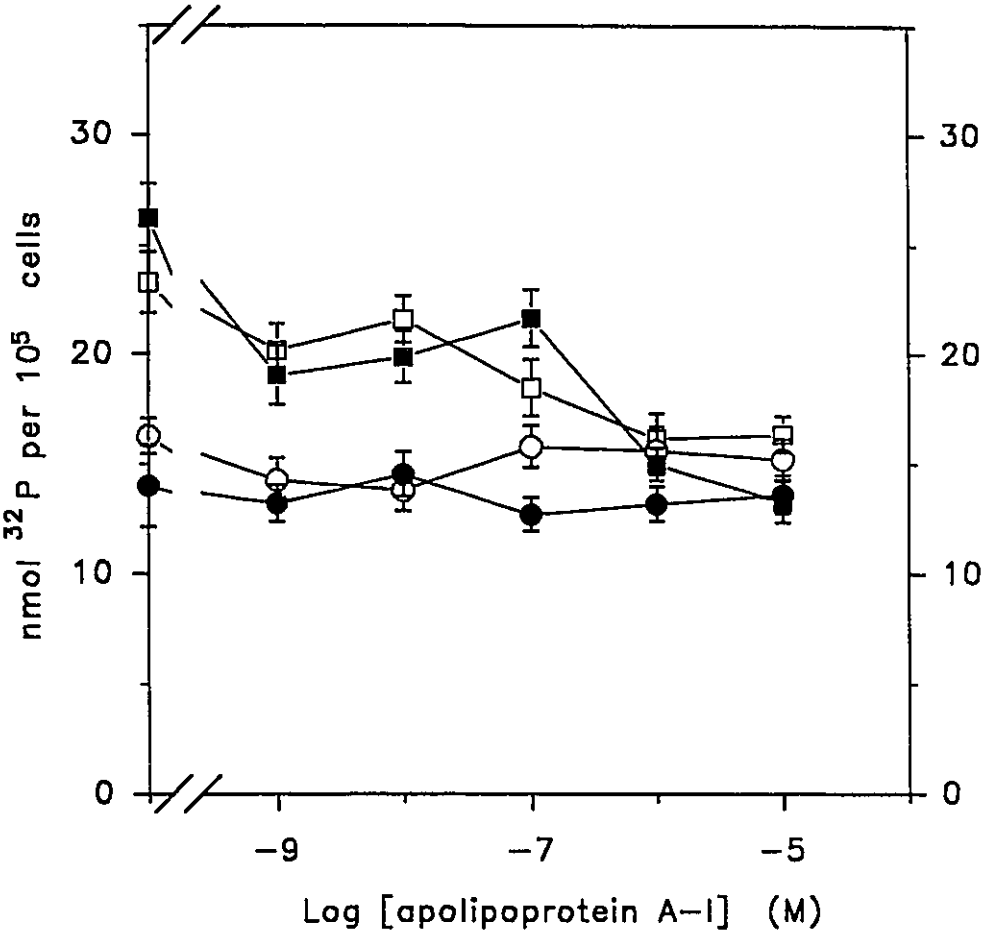


Figure 3.5.2.1

Effect of apolipoprotein A-I on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

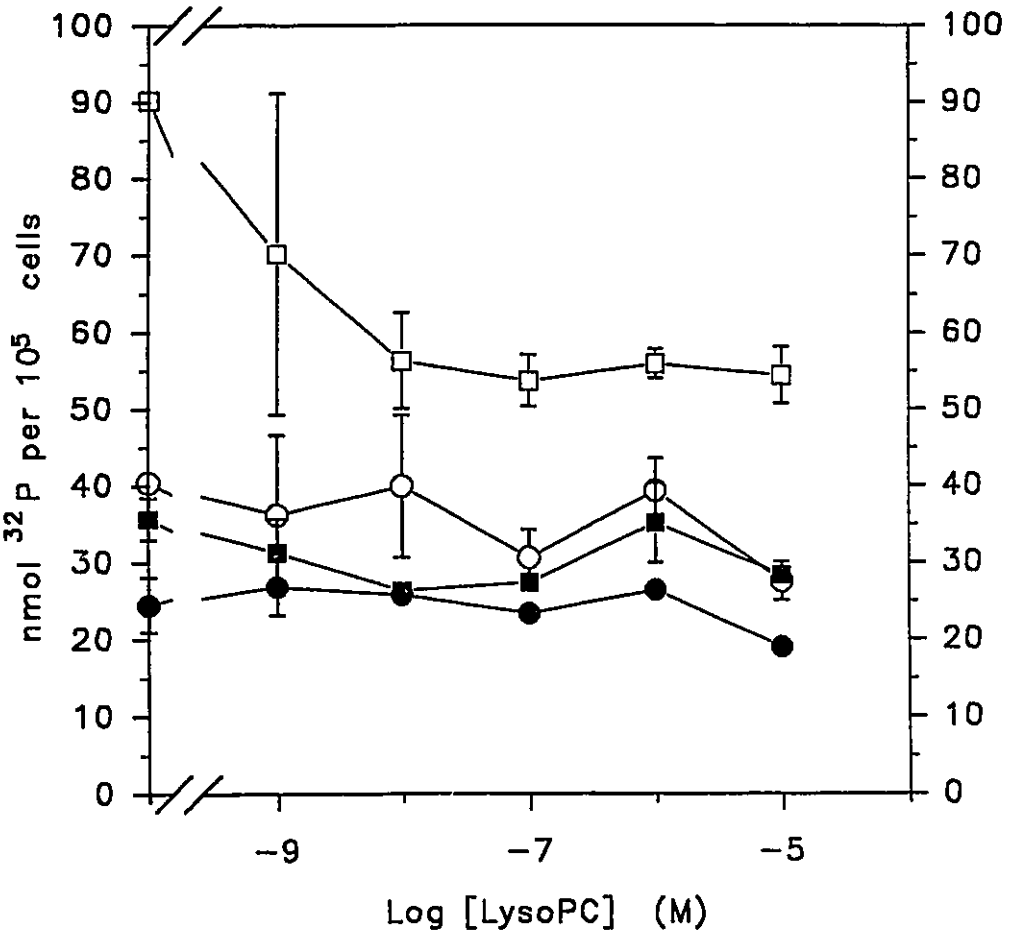


Figure 3.5.2.2

Effect of lysophosphatidylcholine on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

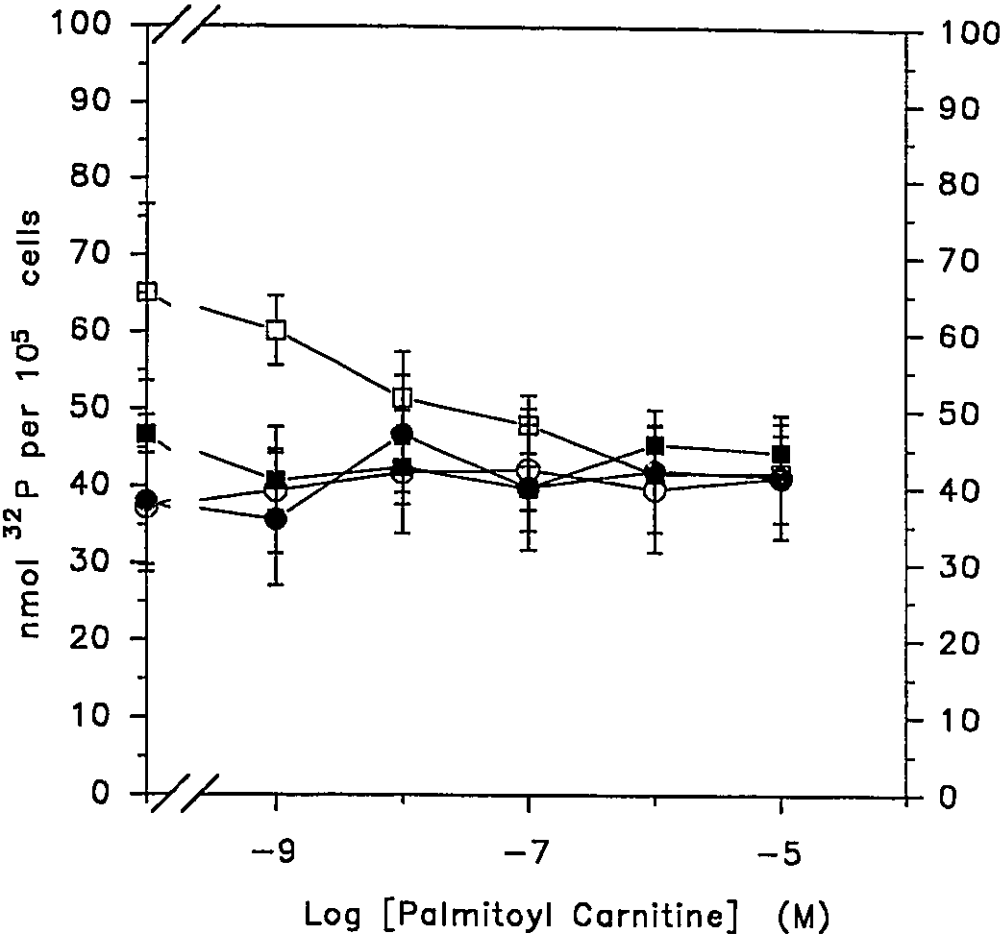


Figure 3.5.2.3

Effect of palmitoyl carnitine on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

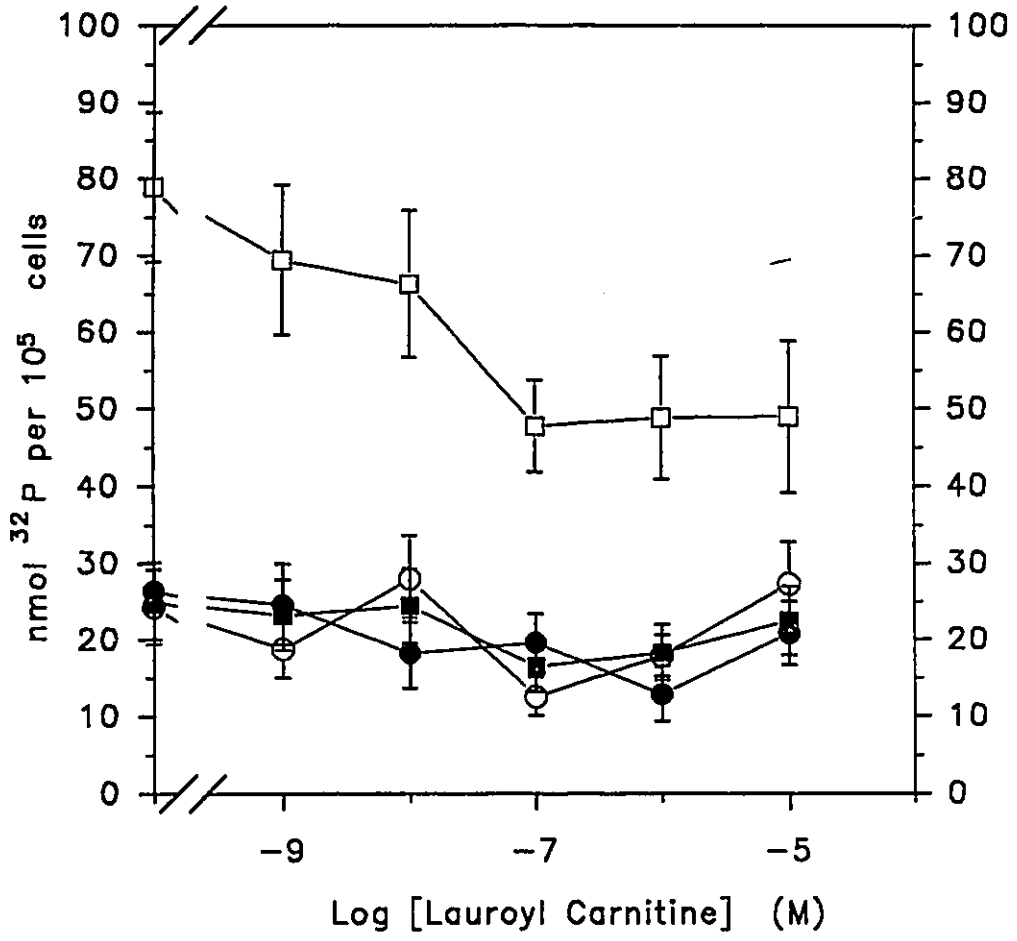


Figure 3.5.2.4

Effect of lauroyl carnitine on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

The two cationic amphiphiles which had been shown to inhibit protein kinase C activity [Bottega and Epand, 1992] were also investigated in this system. These amphiphiles were shown to have no effect on basal or insulin-stimulated serine phosphorylation of the insulin receptor. CATAM1 inhibited tyrosine phosphorylation to basal levels (Figure 3.5.2.5). Despite the similarity in structure and effect on protein kinase C activity [Bottega and Epand, 1992], CATAM2 stimulated insulin receptor tyrosine phosphorylation to 227 ± 56 % ($p < 0.05$) at submicromolar concentrations, with no effect at higher concentrations, as shown in Figure 3.5.2.6. The lack of effect of CATAM1 and CATAM2 on serine phosphorylation of the receptor was interesting, given that these cationic amphiphiles inhibit protein kinase C [Bottega and 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 over-express 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. It is noteworthy that in some cases the level of tyrosine phosphorylation in the absence of added compound is greater than in others. For example compare Figures 3.5.2.1 and 3.5.2.2. These differences likely result from differences in the cells, despite precautions to serum starve cells prior to the experiment so that they would all be very insulin-responsive. The results are reproducible, so that the activity in the presence of the compounds can be compared to that in the absence of compound, within each experiment,

without worrying about day-to-day variation in initial insulin sensitivity.

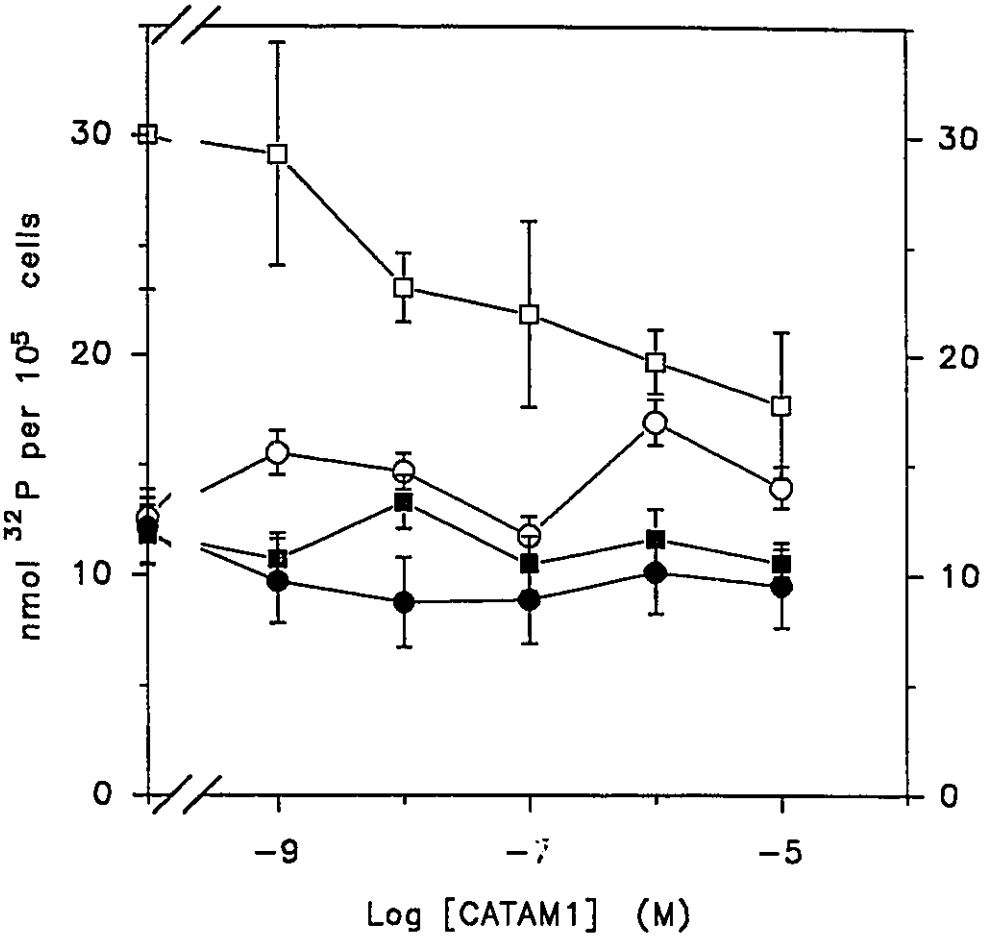


Figure 3.5.2.5

Effect of CATAM1 on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

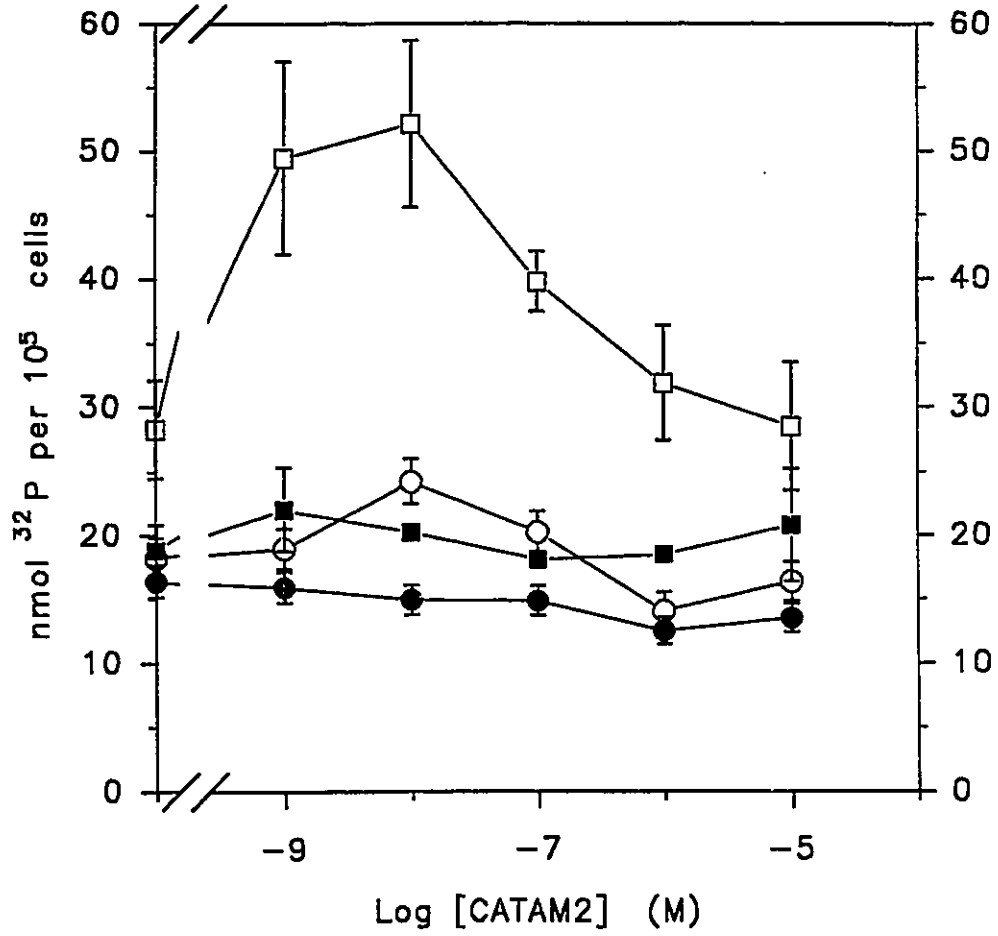


Figure 3.5.2.6

Effect of CATAM2 on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

3.5.3 Effects of Hexagonal Phase Promoters on Insulin Receptor Activity in Intact Cells

Sphingosine inhibited insulin-stimulated insulin receptor tyrosine phosphorylation to near-basal levels, as shown in Figure 3.5.3.1, in agreement with its effects in mixed micelles (Figure 3.3.4.1), and the findings of Arnold and Newton [1991]. The apparent stimulation at 1 nM was not significant ($p > 0.05$). This is in contrast to the stimulation of isolated receptor autophosphorylation in micelles (Figure 3.3.4.1). The uncharged hexagonal phase promoter DiC8 had no significant effect on insulin receptor phosphorylation in these cells, as depicted in Figure 3.5.3.2. Although we did not observe any effect of DiC8 on receptor phosphorylation, studies in rat adipocytes [Terry *et al.*, 1991] and mixed micelles (section 3.3.4) showed a stimulation of insulin receptor tyrosine phosphorylation in response to this additive. A series of hexagonal phase promoters could not be tested as these additives are generally cytotoxic.

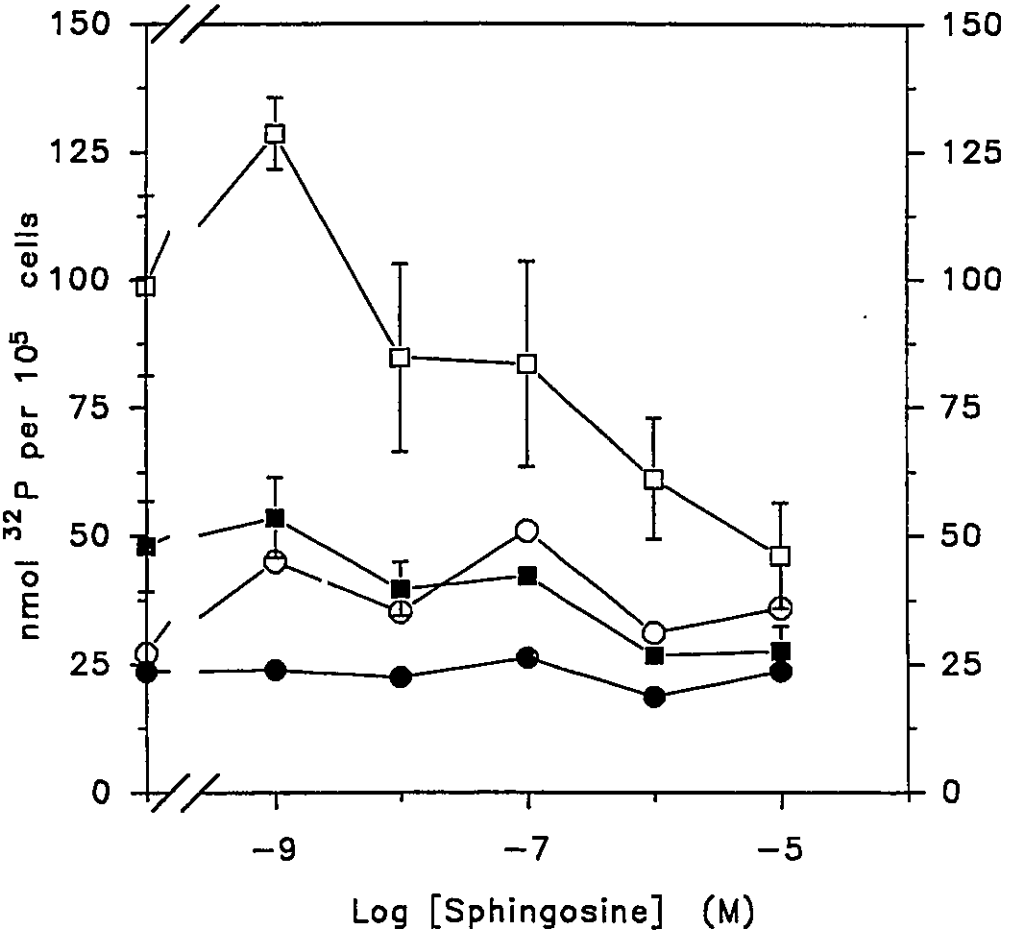


Figure 3.5.3.1

Effect of sphingosine on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

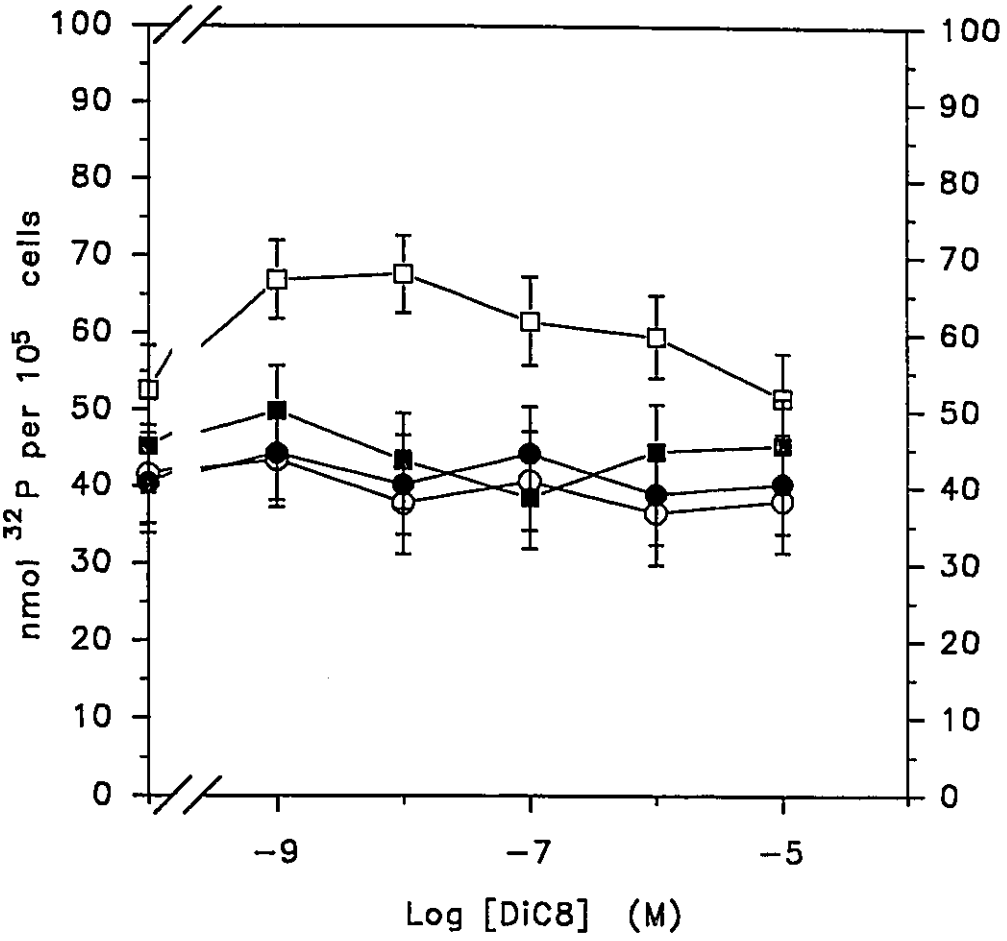


Figure 3.5.3.2

Effect of dioctanoylglycerol on insulin receptor tyrosine (open symbols) and serine (filled symbols) phosphorylation in NIH 3T3 HIR 3.5 cells, in the absence (circles) and presence (squares) of 1 μM insulin. Values are mean ± S.E.M. for triplicate experiments.

3.5.4 Membrane Physical Properties and Insulin Receptor Activity in Intact Cells

It had 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 signalling is related to the ability of the peptide derivatives 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 signalling 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 signalling by inhibiting autophosphorylation of the receptor in response to insulin. All of the compounds which inhibited insulin receptor autophosphorylation have similar effects on lipid polymorphism. Thus it is possible that the compounds exert their effects on insulin signalling non-specifically, 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 as readily. 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 and Brown, 1993] and protein kinase C [Senisterra and Epand, 1993].

The relative insulin receptor tyrosine kinase activity was compared by subtracting basal tyrosine 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 3.5.4.1, for the highest concentrations of compounds tested. In general, apart from cationic amphiphiles, the effects of compounds on insulin receptor tyrosine phosphorylation correlates with their effects on lipid polymorphism. The rank order of bilayer stabilizing potency is: Apo A-I > palmitoyl carnitine > CATAM1 > Lyso PC > CATAM2 > lauroyl carnitine > CBZ-L-Phe-Gly > CBZ-Gly-L-Phe > sphingosine > DiC8. For compounds of similar structure, the rank order of potency of bilayer stabilization correlated directly with that of inhibitory potency. For example the rank order for insulin receptor kinase inhibitory potency is: Apo A-I > CBZ-L-Phe-Gly ≅ palmitoyl carnitine > CBZ-Gly-L-Phe > CATAM1 > lyso PC ≥ sphingosine ≥ lauroyl carnitine > CATAM2 ≅ DiC8. Thus for compounds of similar structure, stronger bilayer stabilizers inhibited insulin receptor tyrosine kinase activity to a greater extent. For example, the inhibitory potencies of apo A-I, and the peptides CBZ-L-Phe-Gly and CBZ-Gly-L-Phe correlate with their ability to raise T_H. Apo A-I is the strongest bilayer stabilizer, and it was found to inhibit insulin receptor activity to a greater extent than CBZ-L-Phe-Gly, which in turn was more potent than the weaker bilayer stabilizer CBZ-Gly-L-Phe. Similarly, palmitoyl carnitine had a greater effect than

lauroyl carnitine on both bilayer stabilization and kinase activity. Furthermore, lyso PC is a stronger bilayer stabilizer than lauroyl carnitine, but not as strong as palmitoyl carnitine. Accordingly lyso PC was intermediate in its effects on insulin receptor autophosphorylation. There were even consistencies among the cationic amphiphiles, in that CATAM1 inhibited insulin receptor activity and raised the bilayer to hexagonal phase transition temperature of model membranes more than did CATAM2, at a concentration of 10 μM. 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. This may be a consequence of comparing the effects of the compounds at one particular concentration, rather than correlating EC_{50} values for each compound. Furthermore, the amount of each compound that actually partitioned into the membrane may vary. The concentration of compounds on the abscissae of the dose-response curves refer to the bulk concentration in the buffer overlaying the monolayer of cells. At 10 μM, this would correspond to approximately 6 mole percent relative to the phospholipid of the cell plasma membrane, and a compound to insulin receptor ratio of 62:1, if all of the compound partitioned into the membrane. The fraction of the less hydrophobic CBZ-dipeptides and apo A-I in the membrane would be less than this estimate. 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 cationic amphiphiles sphingosine, CATAM1 and CATAM2 appear to belong to a separate class of compounds whose effects on insulin signalling are not readily predictable from their membrane modulating activity. Even certain cationic polypeptides and proteins affect the tyrosine kinase activity of the insulin receptor [Morrison *et al.*, 1989]. The differential effects of CATAM1 and CATAM2, despite their similar structures, suggests that their mechanism of action involves more than membrane modulation.

Compounds which lower T_H tend to be cytotoxic. Therefore the only non-cationic hexagonal phase promoter included in these studies was DiC8. While bilayer stabilizers inhibit insulin receptor activity, we were unable to show whether hexagonal phase promoters could stimulate this activity. Since NIH 3T3 HIR 3.5 cells express insulin receptor at such high levels, the stimulation observed in response to insulin may already be near maximal, such that further stimulation is unobservable.

None of the additives exerted their effects by altering insulin binding to the receptor, as discussed in section 3.6. In addition, inhibition of kinase activity in the presence of bilayer stabilizing compounds was not a result of a decreased cell viability (see section 3.7), or increased phosphatase activity (see section 3.8). Furthermore, it was shown that direct interaction of compounds with the kinase domain of the receptor was not responsible for modulation of insulin receptor activity, since the compounds were

unable to affect SIRK activity similarly, as described in section 3.4. This emphasizes the importance of the membrane in the action of these additives.

COMPOUND	% TYROSINE PHOSPHORYLATION
CBZ-L-Phe-Gly	0.3 ± 4.5
CBZ-Gly-L-Phe	4.9 ± 0.6
Apolipoprotein A-I	0.01 ± 1.8
Lysophosphatidylcholine	30 ± 6
Lauroyl Carnitine	40 ± 6
Palmitoyl Carnitine	2.0 ± 0.5
Sphingosine	35 ± 8
CATAM1	20 ± 2
CATAM2	no effect[a]
Dioctanoylglycerol	no effect[b]

a CATAM2 had no significant effect at 10 μM, as determined by independent t-test, but stimulated to 227.95 ± 56.5 % at 0.1 nM.

[b] Dioctanoylglycerol had no significant effect at 10 μM, as determined by independent t-test.

Table 3.5.4.1

Effects of various compounds on tyrosine phosphorylation of the insulin receptor β subunit in NIH 3T3 HIR 3.5 cells. The % tyrosine phosphorylation was 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 ± S.E.M. for 10 μM compound.

3.6 Insulin Binding

Inhibition of insulin receptor phosphorylation, as observed in the presence of various bilayer-stabilizing membrane additives, could result from alterations in the affinity of the insulin receptor for insulin. To determine whether the compounds could inhibit insulin receptor phosphorylation at the level of insulin binding to the receptor, the additives were assayed for their effects on insulin binding in competitive binding studies, as described in Materials and Methods, section 2.9.

3.6.1 Effects of Membrane Additives on Insulin-Receptor Interactions

The inhibition of tyrosine kinase activity by various bilayer stabilizing compounds was not a result of inhibition of insulin binding to its receptor, as determined by competitive binding assays. Most of the compounds had little or no effect on the concentration of unlabelled insulin required to displace 50 % of the ^{125}I-insulin (EC_{50}), nor on the specific binding, as a percentage of total binding. As shown in Figure 3.6.1.1, CBZ-L-Phe-Gly did not significantly affect the amount of insulin required to displace 50% of the labeled insulin. The EC_{50} values in the absence and presence of 10 μM CBZ-L-Phe-Gly were 0.91 ± 0.14 nM and 0.94 ± 0.17 nM, respectively. Most of the other membrane additives were similarly unable to alter either the EC_{50}, or specific binding, as summarized in Table 3.6.1.1. Apo A-I, sphingosine and DiC8, however, increased the EC_{50} slightly, suggesting a decreased affinity of the insulin receptor for insulin in the presence of these compounds. Even higher concentrations of these

compounds, however, did not affect the total specific binding of insulin to its receptor. This suggests that these compounds do not act as competitive inhibitors.

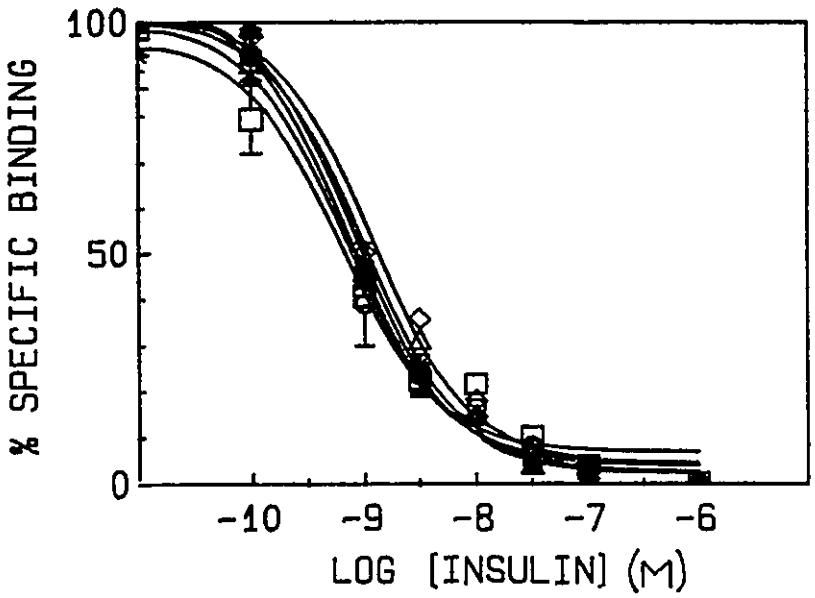


Figure 3.6.1.1

Bilayer-stabilizing compounds do not affect insulin-receptor interactions. Competetive binding assays were performed as described in Materials and Methods, section 2.9. Compounds were tested at 10 μM. O, no additive; Δ, CBZ-L-Phe-Gly; □, CBZ-Gly-L-Phe; *, Apo A-I; ●, Lauroyl carnitine; ◊, Palmitoyl carnitine.

COMPOUND	EC_{50} (nM)	% SPECIFIC BINDING
No Additive	0.91 ± 0.14	98 ± 1.2
CBZ-L-Phe-Gly	0.94 ± 0.17	99 ± 6.4
CBZ-Gly-L-Phe	0.64 ± 0.36	98 ± 5.3
Apolipoprotein A-I	2.9 ± 0.36	98 ± 1.9
Lysophosphatidylcholine	1.0 ± 0.15	98 ± 1.4
Lauroyl Carnitine	0.86 ± 0.10	99 ± 1.1
Palmitoyl Carnitine	1.2 ± 0.27	98 ± 1.7
Sphingosine	1.8 ± 0.27	98 ± 4.9
CATAM1	1.1 ± 0.30	98 ± 1.3
CATAM2	1.0 ± 0.21	98 ± 5.1
Dioctanoylglycerol	1.7 ± 0.21	98 ± 5.3

Table 3.6.1.1

Effects of various compounds on insulin binding to insulin receptor in NIH 3T3 HIR 3.5 cells. Insulin binding was measured as described in Materials and Methods section 2.9. Specific Binding = Total Binding - Non-specific binding. Values given are mean ± S.E.M. for 10 μM compound.

3.7 Cell Viability

Measurement of insulin receptor activity in an intact cell assay required that the viability of the cells under various assay conditions be assessed. This was particularly important for the cases where the membrane additives appeared to inhibit insulin receptor tyrosine phosphorylation. An increase in cell death with increasing concentrations of compounds could have been misinterpreted as a decrease in insulin receptor activity. Cell viability was therefore measured in the presence of increasing concentrations of membrane additives, to determine whether the observed decreases in receptor activity could be a result of cell death. Live and dead cells were identified microscopically by the use of specific fluorescent compounds, as described in Materials and Methods section 2.8. The *Live/Dead* viability kit is more sensitive than trypan blue exclusion. With trypan blue, the timing of exposure of cells to the dye is critical, as cells continue to take up stain over time [Patterson, 1979]. The live dead kit includes probes specific for each live and dead cells. Ethidium homodimer-I is a high affinity red fluorescent DNA stain which is only able to pass through the compromised membranes of dead cells. Therefore, dead cells emit red fluorescence at 590 nm. A second probe, calcein AM is a fluorogenic substrate that is cleaved only in viable cells, since cleavage relies on esterase activity. Cleavage of calcein AM produces a green fluorescent membrane impermeant product, so that live cells produce green fluorescence at 530 nm. The amount of dead cells as a percent of the total number of cells viewed was calculated.

3.7.1 Effects of Membrane Additives on Cell Viability

An example of a cell viability curve is shown in Figure 3.7.1.1. CBZ-Gly-L-Phe was found to have no effect on cell viability at the concentrations used in the intact cell phosphorylation assay. Similarly, none of the other compounds whose effect on insulin receptor phosphorylation is reported, affected the percentage of dead cells. The hexagonal phase promoter decanol had originally been tested for its effects on insulin receptor autophosphorylation in intact cells. A decrease in the level of tyrosine phosphorylation of the receptor was observed with increasing concentrations of decanol. This apparent decrease in activity was actually due to a decrease in cell viability caused by the decanol, as observed in Figure 3.7.1.1. Decanol was therefore not included in any of the studies of insulin receptor activity. Digitonin was used as a control for total cell death. The effects of the highest concentrations of compounds tested, 10 μM, on the percentage of cell death, are summarized in Table 3.7.1.1.

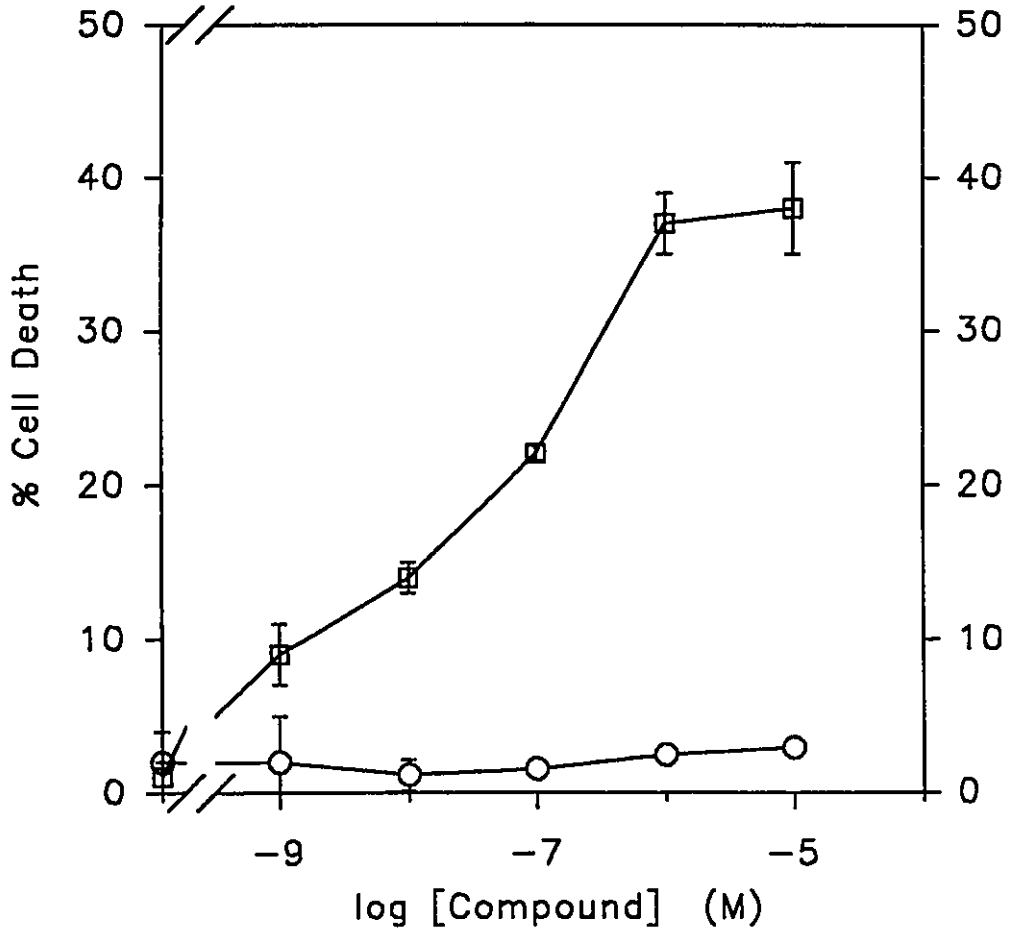


Figure 3.7.1.1

Dependence of cell death on concentration of membrane additives. Cell viability was assessed by the *Live/Dead* fluorescence assay. Values are mean ± S.E.M. for triplicate wells of cells incubated in the presence of various compounds under the conditions used for the phosphorylation assay. O, CBZ-Gly-L-Phe; □, Decanol.

COMPOUND	% CELL DEATH
No Additive	3.6 ± 0.9
CBZ-L-Phe-Gly	4.5 ± 0.1
CBZ-Gly-L-Phe	2.9 ± 0.5
Apolipoprotein A-I	1.3 ± 0.3
Lysophosphatidylcholine	6.0 ± 1.0
Lauroyl Carnitine	3.4 ± 1.0
Palmitoyl Carnitine	5.0 ± 1.7
Sphingosine	3.9 ± 1.3
CATAM1	4.0 ± 1.1
CATAM2	4.3 ± 0.3
Dioctanoylglycerol	1.3 ± 0.1
0.5% Digitonin	100 ± 0

Table 3.7.1.1

Effects of various compounds on NIH 3T3 HIR 3.5 cell viability. Cell viability was assessed by the *Live/Dead* fluorescence assay. Values are mean ± S.E.M. for triplicate wells of cells incubated in the presence of 10 μM compound.

3.8 Role of Phosphatase Activity in Insulin Receptor Phosphorylation

Protein tyrosine phosphatases (PTPases) cleave phosphate groups from tyrosine residues of proteins. Stimulation of a PTPase could result in decreased levels of insulin receptor phosphotyrosine. This could be misinterpreted as a decrease in insulin receptor autophosphorylation activity. The insulin receptor has been shown to be a substrate for leukocyte common antigen-related phosphatase [Hashimoto *et al.*, 1992a], PTPase1B, PTP-α, T cell protein tyrosine phosphatase, and CD45 [Ramachandran *et al.*, 1992; Hashimoto *et al.*, 1992b]. The number and identity of phosphatases in NIH 3T3 HIR 3.5 cells which act on the insulin receptor is unknown. A non-specific phosphatase inhibitor, orthovanadate, was added to the incubation and solubilization buffers for the intact cell insulin receptor autophosphorylation assay. Orthovanadate effectively inhibits all tyrosine phosphatases so far tested [Pot and Dixon, 1992]. The dephosphorylation of insulin receptor over time was measured in the presence of orthovanadate, and found to be negligible over the incubation time of this assay. The results of the dephosphorylation assay are shown in Figure 3.8.1. This suggests that any change in phosphorylation state of the insulin receptor in response to membrane additives was not a result of alterations in phosphatase activity.

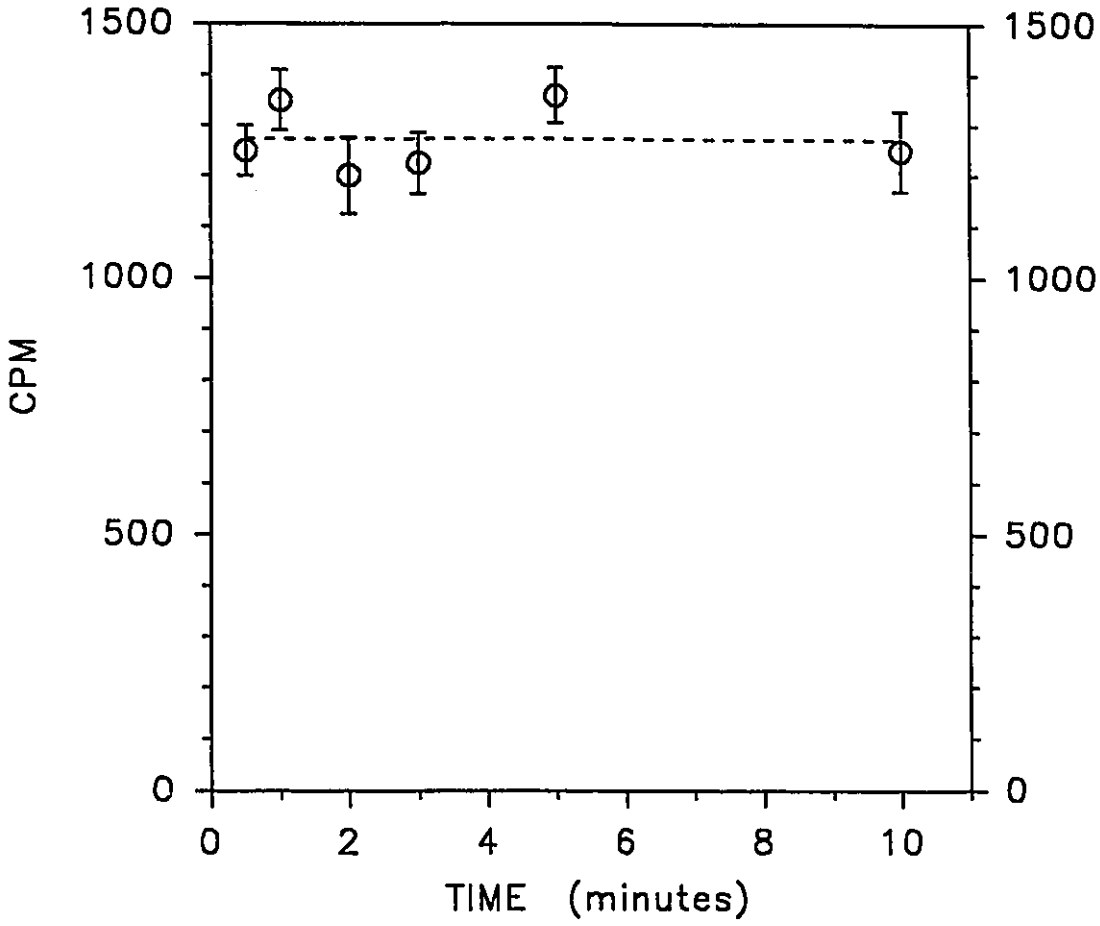


Figure 3.8.1

Phosphatase activity in NIH 3T3 HIR 3.5 cells. Loss of ^{32}P from insulin receptor β subunit was measured over time, in the presence of 1 mM sodium orthovanadate. Counts per minute (CPM) were measured by scintillation counting. Values are mean ± S.E.M. for triplicate experiments.

3.9 Correlation Between Insulin Receptor Phosphorylation in Micelles and in Intact Cells

There are several differences between an intact cell assay and a mixed micellar assay. In cells, the phospholipid composition, presence of phosphatases and other kinases within the cell, and the intracellular concentrations of small molecules such as ATP, as well as ions such as calcium, are all variable. In the micellar assay the phospholipid composition, calcium and ATP levels are all defined, and no phosphatases or other kinases are present. There are also differences in the molecular organization and physical properties of cell membranes and micelles. For example, in micelles protein kinase C is specific for PS, while in vesicles any anionic lipid will support protein kinase C activity [Mosior and Epand, 1993]. Despite the differences, the mixed micellar assay is still useful for measuring enzymatic function, particularly for the testing of membrane additives, as has been shown for studies of protein kinase C [Hannun et al., 1985]. Here also, it is shown that hydrophobic compounds have similar effects on insulin receptor autophosphorylation activity in Triton X-100 mixed micelles and in NIH 3T3 HIR 3.5 cells. There is good agreement between the inhibitory action of the bilayer stabilizing additives in the two assays (with the exception of lauroyl carnitine, as discussed below). High concentrations of the cationic amphiphiles also had the same effects in both assay systems, while the H_{II} promoter DiC8 was only able to affect insulin receptor activity in mixed micelles. The effects of various compounds on the percent of tyrosine phosphorylation of the insulin receptor were compared at the highest

concentrations of additive tested, as shown in Table 3.9.1. The concentration of compound refers to its concentration in the media overlaying the cells in the intact cell assay, and the concentration in the total assay volume, for the mixed micellar assay. At 10 μM compound, if all of the additive partitions into the membrane, this corresponds to 5.72 mole % of compound relative to phospholipid in the intact cell assay, and only 0.27 mole % of compound relative to phosphatidylcholine in the mixed micellar assay. PC constitutes the major fraction of the micelle material. Furthermore, the compound to insulin receptor ratio in the fibroblasts was 62:1, compared to 23:1 in the micelles. Therefore there was more compound relative to phospholipid and relative to insulin receptor in the cells. This may explain the greater susceptibility of the cells to bilayer stabilizers, but doesn't account for the cells being less susceptible to hexagonal phase promoters, as discussed below. This may result from the differences in curvature between micelles and biological membranes, and by the ability of cells to metabolize DiC8, as discussed in section 3.9.3.

COMPOUND	% PHOSPHORYLATION IN INTACT CELLS	% PHOSPHORYLATION IN MICELLES
CBZ-L-Phe-Gly	0.3 ± 4.5	31 ± 15
CBZ-Gly-L-Phe	4.9 ± 0.6	47 ± 6
Apolipoprotein A-I	0.01 ± 1.8	23 ± 2
Lysophosphatidylcholine	30 ± 6	18 ± 9
Lauroyl Carnitine	40 ± 6	130 ± 5
Palmitoyl Carnitine	2.0 ± 0.5	74 ± 2
Sphingosine	35 ± 8	17 ± 3
CATAM1	20 ± 2	75 ± 8
CATAM2	no effect[a]	no effect[a]
Dioctanoylglycerol	no effect[b]	230 ± 16

[a] CATAM2 had no significant effect at 10 μM, as determined by independent t-test, but stimulated to 227.95 ± 56.5 % at 0.1 nM in intact cells and stimulated to 156 ± 14 % at 0.1 μM in micelles.

[b] No significant effect at 10 μM, as determined by independent t-test

Table 3.9.1

Effects of membrane additives on insulin receptor tyrosine phosphorylation in NIH 3T3 HIR 3.5 cells and in Triton X-100 mixed micelles. % Phosphorylation was calculated from I_a-B_a / I_c-B_c x 100 %, where I is insulin-stimulated, B is basal, a is in the presence of 10 μM compound, c is control, with no added compound. Values are mean ± S.E.M. for triplicate experiments.

3.9.1 Effects of CBZ-dipeptides on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells

In both the intact cell and the micellar environment, CBZ-L-Phe-Gly inhibited IRTK more than did CBZ-Gly-L-Phe. When comparing the percent of phosphorylation, normalized with respect to basal activity, it is obvious that the additives had a greater effect on the insulin receptor in intact cells than in the isolated system. As mentioned above this may be a result of the relative concentrations of peptides, in terms of mole fraction of phospholipid.

3.9.2 Effects of Other Bilayer Stabilizers on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells

Apo A-I inhibited autophosphorylation activity of isolated insulin receptors, and of insulin receptors in NIH 3T3 HIR 3.5 cells. As with the CBZ-dipeptides, the inhibition was greater in the cells, than in the micellar assay. Lauroyl carnitine had no effect in the mixed micellar assay, in contrast to its inhibitory action in the cells. The differential effects of lauroyl carnitine in the two assay systems 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 containing PC, compared with intact cells, which contain a variety of phospholipids of differing intrinsic curvatures. The longer-chained palmitoyl carnitine had a much stronger inhibitory effect in the cellular assay, and weak inhibition in the mixed micellar assay. Palmitoyl carnitine had a greater effect than

lauroyl carnitine, for both assays, correlating with the stronger ability of palmitoyl carnitine to raise T_H in model membranes. Lysophosphatidylcholine exhibited similar inhibition in the two assays. It was the only bilayer stabilizer which didn't exhibit a greater effect on the cells than the isolated receptors. It is possible that lyso PC is metabolized to PC in the NIH 3T3 HIR 3.5 cells, lowering the effective concentration of lyso PC in the cell membrane and thereby rendering it less potent than in the micelle assay.

In summary, all of the bilayer stabilizers, with the exception of lyso PC, inhibited insulin receptor autophosphorylation in intact cells more than that of isolated receptors in mixed micelles. As mentioned for the CBZ-dipeptides, this may be a consequence of the relative concentrations of these compounds, in terms of mole fraction of phospholipid. Furthermore, the phospholipid composition of biological membranes may be such that the membranes are close to the border of the H_{II} transition. Evidence of this comes from the membranes of microorganisms, which adjust their lipid composition to have considerable negative monolayer curvature strain [Rilfors *et al.*, 1994]. The negative curvature strain of such membranes would be relieved by bilayer stabilizers, while micelles, which already have positive curvature, may not be greatly affected by compounds which stabilize the bilayer relative to the hexagonal phase.

3.9.3 Effects of Hexagonal Phase Promoters on Insulin Receptor Autophosphorylation in Micelles versus in Intact Cells

Sphingosine elicited similar inhibitory effects on insulin receptor autophosphorylation in cells and in mixed micelles at 10 μM. However, sphingosine was unable to stimulate receptor kinase at low concentrations in intact cells as it did in mixed micelles. Dioctanoylglycerol was only able to stimulate insulin receptor activity in mixed micelles, not in intact cells. So, hexagonal phase promoters could not stimulate insulin receptor tyrosine kinase activity in cells, despite the fact that a greater ratio of compound to phospholipid was achieved in the cells, as described in section 3.9. In intact cells diacylglycerol may be phosphorylated to form phosphatidic acid. This would decrease the concentration of DiC8 in the membrane, which would account for the lack of effect of this hexagonal phase promoter on insulin receptor activity. Also, at high concentrations of phosphatidic acid, the electrostatic repulsion among the headgroups and PS stabilize the bilayer relative to the hexagonal phase, raising T_H. Phosphatidic acid in the presence of diacylglycerol and elevated calcium concentrations reduces the activity of protein kinase C [Epand and Stafford, 1990]. Hexagonal phase promoters may be less able to increase negative curvature strain in biological membranes, compared to in micelles. Micelles, which have positive curvature, will be drastically altered by compounds that have negative cuvature. Since cell membranes may be close to the border of the H_{II} transition, as mentioned above, the negative curvature cannot be increased much without killing the cell by destruction of the plasma membrane.

3.9.4 Membrane Physical Properties and Insulin Receptor Autophosphorylation

A variety of hydrophobic compounds of diverse chemical structure affect the insulin-promoted phosphorylation of tyrosine residues on the insulin receptor. These effects occur independently of changes in insulin binding to the receptor or changes in the non-stimulated 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 and Czech, 1987; Leray *et al.*, 1993]. 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 affect 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 was 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 autophosphorylation. For compounds of similar structure, the inhibition of autophosphorylation correlates with the ability of the compounds to raise the bilayer to hexagonal phase transition temperature in model membranes.

3.10 Glucose Uptake in NIH 3T3 HIR 3.5 Cells

Insulin binding to its receptor results in an increased internalization of glucose in insulin responsive cells. Translocation of glucose transporters from an intracellular pool associated with membrane vesicles to the plasma membrane, as well as activation of the glucose transporters occur in response to insulin binding [Suzuki and Kono, 1980; Cushman and Wardzala, 1980]. However, the details of the signalling between insulin receptor activation and glucose transport are not yet defined. While removal of key autophosphorylation sites from the insulin receptor were found to abolish insulin stimulation of 2-deoxyglucose uptake [Murakami and Rosen, 1991], other mutational studies have implied that receptor kinase activity is not required for insulin stimulation of glucose transport [Moller *et al.*, 1991; Debant *et al.*, 1988, 1989]. The recent finding that ras proteins can act as intermediates in signalling to glucose uptake [Kozma *et al.*, 1993; Manchester *et al.*, 1994] suggests that a phosphorylation cascade is required for the stimulation of sugar transport. It has also been suggested that protein kinase C may be involved in recruitment of glucose transporters to the plasma membrane [Ishizuka *et al.*, 1990]. Formation of the protein kinase C activator diacylglycerol as a result of phospholipase C stimulation [Fox *et al.*, 1987] and the ability of diacylglycerol to

stimulate glucose transporter efficiency [Stralfors *et al.*, 1988] suggest a role for protein kinase C in glucose transport.

It had previously been shown that CBZ-dipeptides inhibit [^{3}H]-2-deoxyglucose uptake in rat adipocytes, and that the inhibition correlated with bilayer stabilizing potency [Epand *et al.*, 1991a]. The mechanism of this inhibition of insulin signalling is unknown but is presumed to be related to the ability of the peptide derivatives to alter the biophysical properties of the plasma membrane. The CBZ-dipeptides may inhibit glucose uptake at the level of insulin receptor phosphorylation, since these peptide derivatives inhibited insulin receptor tyrosine kinase activity in NIH 3T3 HIR 3.5 cells (section 3.5.1). It was necessary to determine whether these CBZ-dipeptides could also inhibit insulin stimulated glucose uptake in the same cells. Bilayer stabilizing compounds of diverse structure, as well as hexagonal phase promoters, were also investigated for their ability to alter insulin-stimulated uptake of glucose by NIH 3T3 HIR 3.5 cells. The effects of these compounds on glucose uptake have been correlated with their effects on insulin receptor autophosphorylation in the same cells, and with their effects on membrane biophysical properties.

The uptake of [^{3}H]-2-deoxyglucose by cells was measured as described in Materials and Methods section 2.10. A time-course of glucose uptake into NIH 3T3 HIR 3.5 fibroblasts is shown in Figure 3.10.1. The effects of membrane additives on the percent of glucose taken up by the cells in 30 minutes were compared. None of the additives affected basal levels of glucose uptake, therefore the data was normalized by

subtracting the amount of glucose taken up in the absence of insulin, from that in the presence of 1 μM insulin. This value was then expressed as a percentage of that in the absence of added compound of interest.

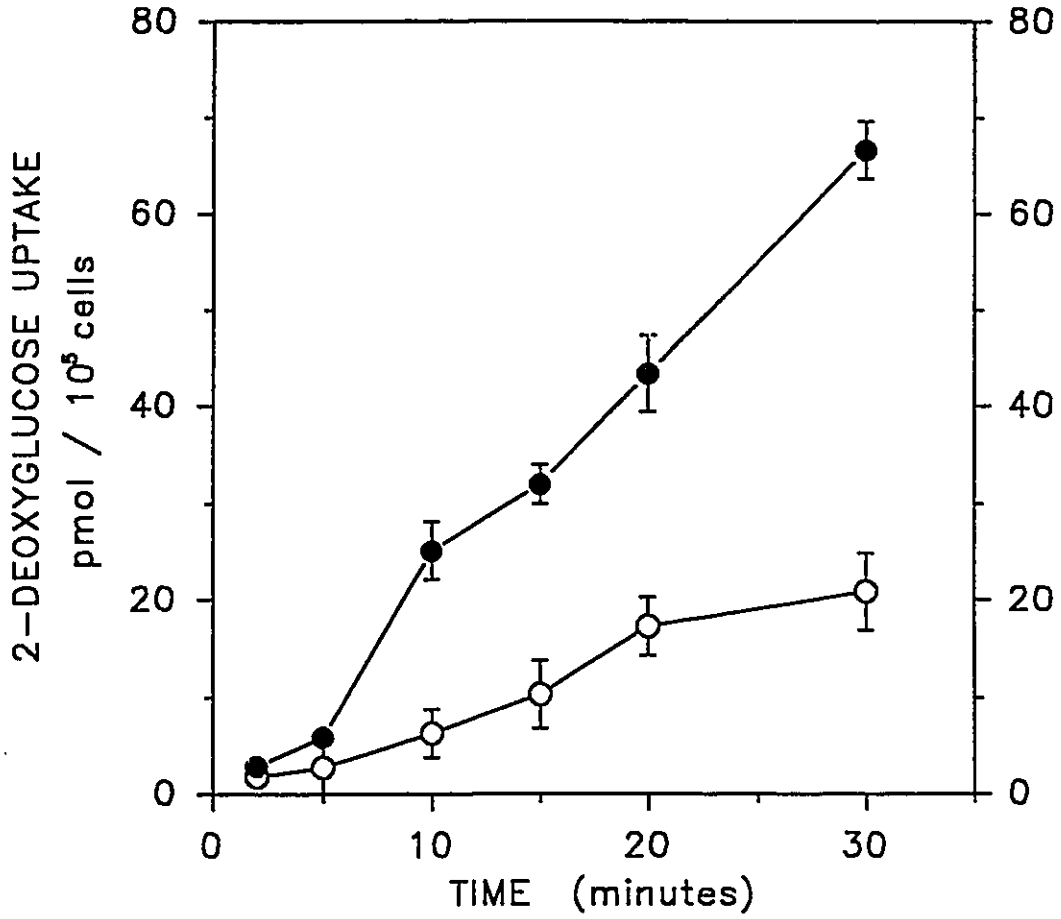


Figure 3.10.1

Time dependence of [^{3}H]-2-deoxyglucose uptake by NIH 3T3 HIR 3.5 cells in the absence (O) and presence (●) of insulin. Values are mean ± S.E.M. for triplicate wells of 10^5 cells.

3.10.1 Effects of CBZ-dipeptides on Glucose Uptake by NIH 3T3 HIR 3.5 Cells

The carbobenzoxy-dipeptides inhibited insulin stimulation of glucose uptake into fibroblasts. This was in agreement with their effects in rat adipocytes [Epand *et al.*, 1991]. CBZ-Gly-L-Phe inhibited glucose uptake to approximately 16% of the activity in the absence of this dipeptide. The dose response curve is shown in Figure 3.10.1.1. The estimated concentration of CBZ-Gly-L-Phe that inhibited glucose uptake by 50 % (IC_{50}) was 0.44 μM. The stronger bilayer-stabilizer CBZ-L-Phe-Gly inhibited glucose uptake to 11% as shown in Figure 3.10.1.2, with an estimated IC_{50} of 0.22 μM.

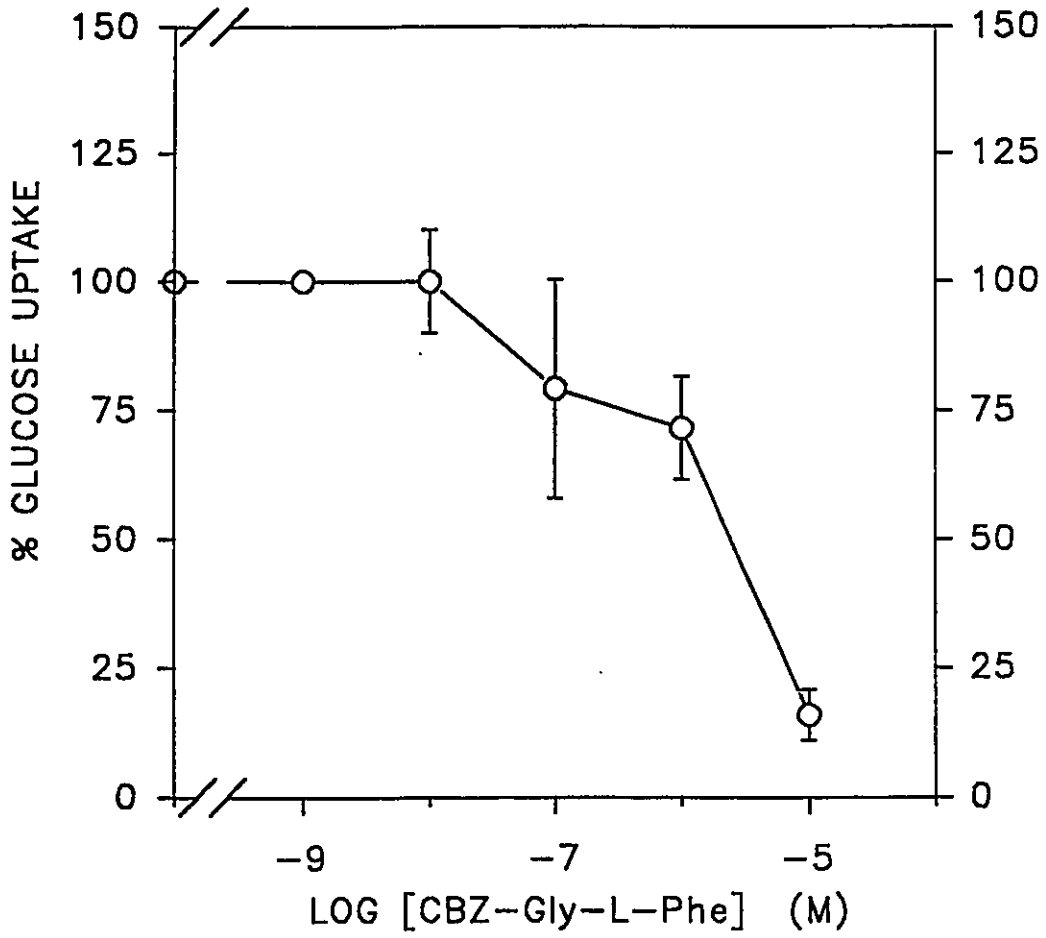


Figure 3.10.1.1

Effect of CBZ-Gly-L-Phe on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

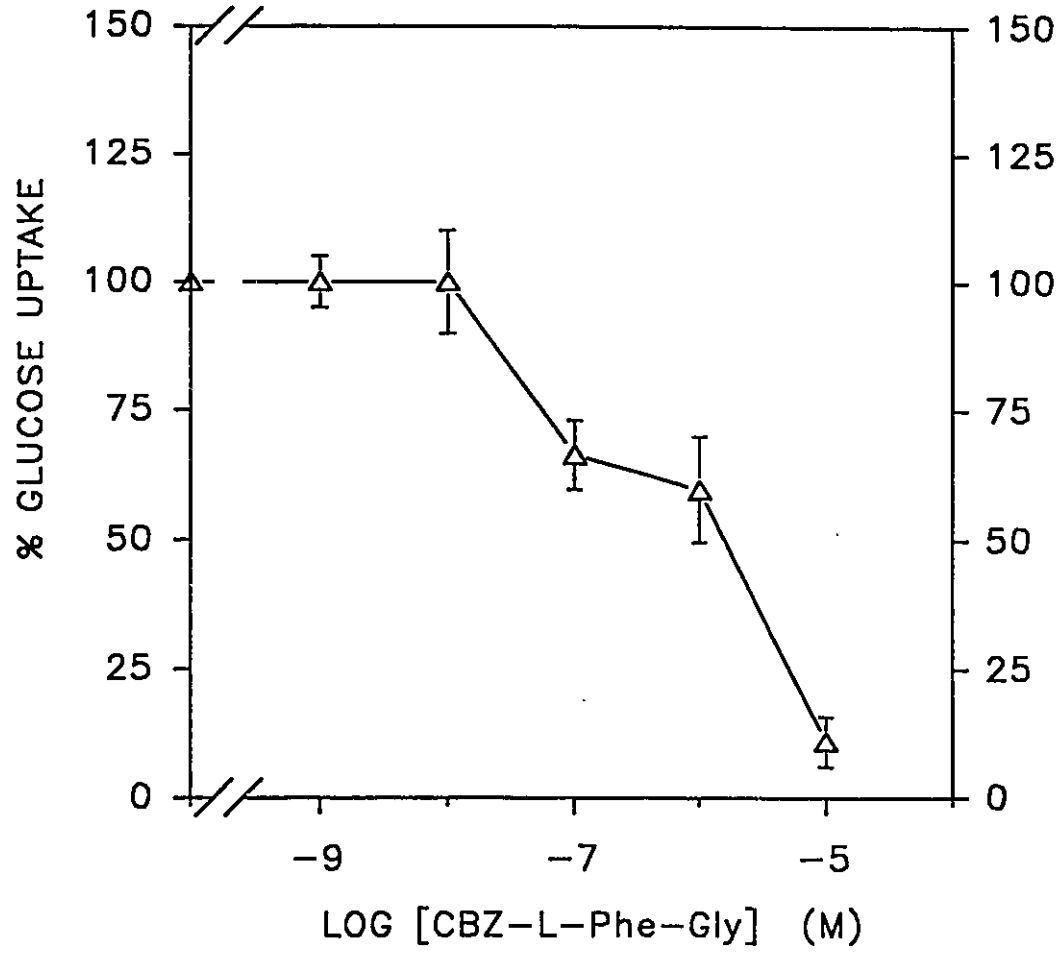


Figure 3.10.1.2

Effect of CBZ-L-Phe-Gly on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

3.10.2 Effects of Other Bilayer Stabilizers on Glucose Uptake

The mechanism of inhibition of glucose uptake by the CBZ-dipeptides is unkown, but is presumed to be related to the ability of these additives to raise the bilayer to hexagonal phase transition temperature. To examine the possibility that membrane modulation can affect uptake of glucose by insulin-responsive cells, bilayer stabilizing compounds of diverse structure were investigated for their effects on [^{3}H]-2-deoxyglucose uptake. Lyso PC, which raises T_H of model membranes, and inhibited insulin receptor autophosphorylation in micelles (section 3.4.1) and in NIH 3T3 HIR 3.5 cells (section 3.5.2), also inhibited glucose uptake in these cells. Figure 3.10.2.1 illustrates the inhibitory effect of Lyso PC, with an 80 % reduction in the level of insulin stimulated glucose uptake at the highest concentration tested. The concentration required to inhibit glucose uptake to 50 % was approximately 0.28 μM. The bilayer stabilizer lauroyl carnitine also inhibited the uptake of 2-deoxyglucose in response to insulin. 10 μM lauroyl carnitine inhibited glucose uptake to 27 ± 5 % of control values, with an approximate IC_{50} of 0.55 μM, as shown in Figure 3.10.2.2. The longer-chained palmitoyl carnitine, which raises T_H more per mole fraction than lauroyl carnitine [Epand *et al.*, 1989], had a greater inhibitory effect on glucose uptake over the concentration range from 1 nM to 1 μM. This agrees with the greater bilayer stabilizing ability and inhibition of insulin receptor tyrosine phosphorylation in response to palmitoyl carnitine relative to lauroyl carnitine (sections 3.3.3 and 3.5.2). At the highest concentration tested, however, lauroyl carnitine was more inhibitory than palmitoyl carnitine. Such

an effect was not observed for insulin receptor tyrosine phosphorylation. 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 3.10.2.3. This result was significant ($p < 0.05$) and reproducible. Apo A-I may extract lipids from the plasma membrane [Hara and Yokoyama, 1991], 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 was not sufficient to stimulate glucose transport in the absence of insulin.

The two cationic amphiphiles had different effects on glucose uptake activity. CATAM1, which inhibited insulin receptor tyrosine phosphorylation, also inhibited glucose uptake to 35 ± 4 %, with an apparent IC_{50} of 50 nM, while CATAM2, which stimulated receptor phosphorylation at low concentrations, had no effects on glucose transport. These results are shown in Figure 3.10.2.4. The different effects of the similar cationic amphiphiles supports the idea that these compounds fall into their own separate class, affecting membrane activities in a manner not predictable from their effects on the bilayer to hexagonal phase transition temperature.

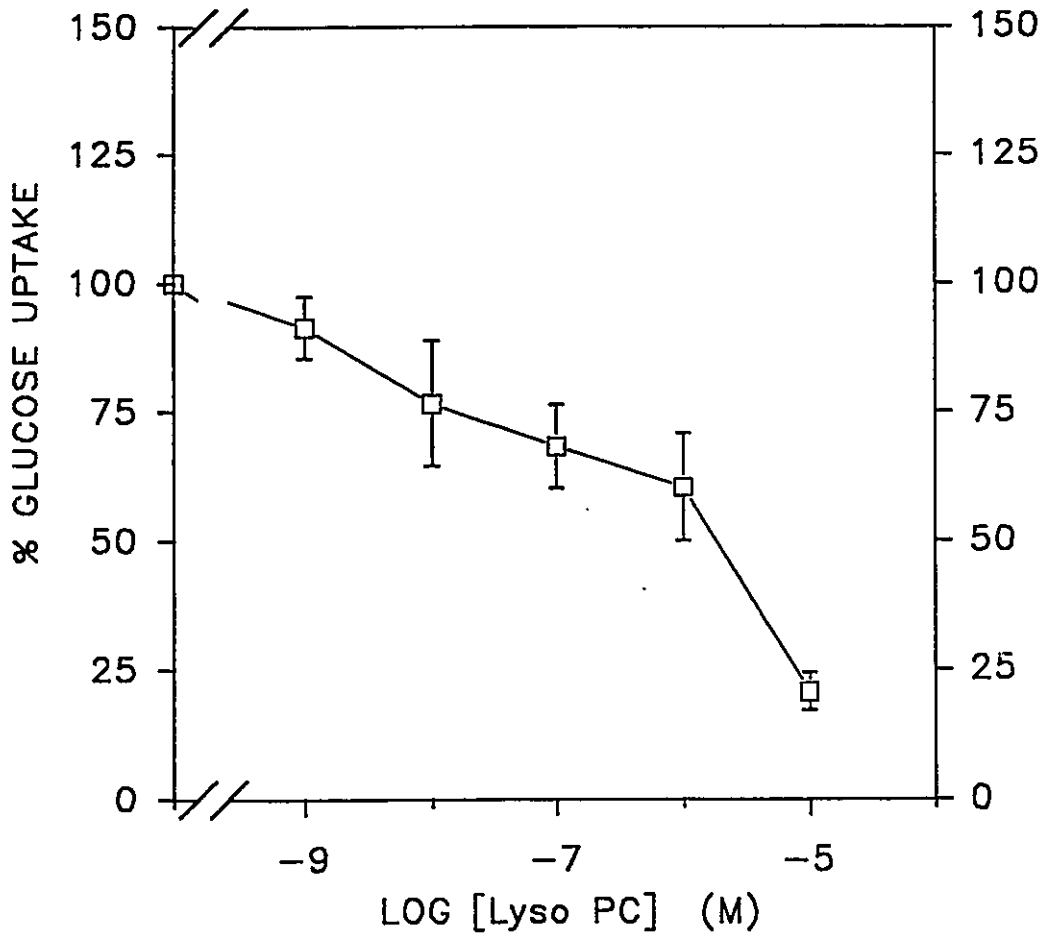


Figure 3.10.2.1

Effect of lysophosphatidylcholine on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

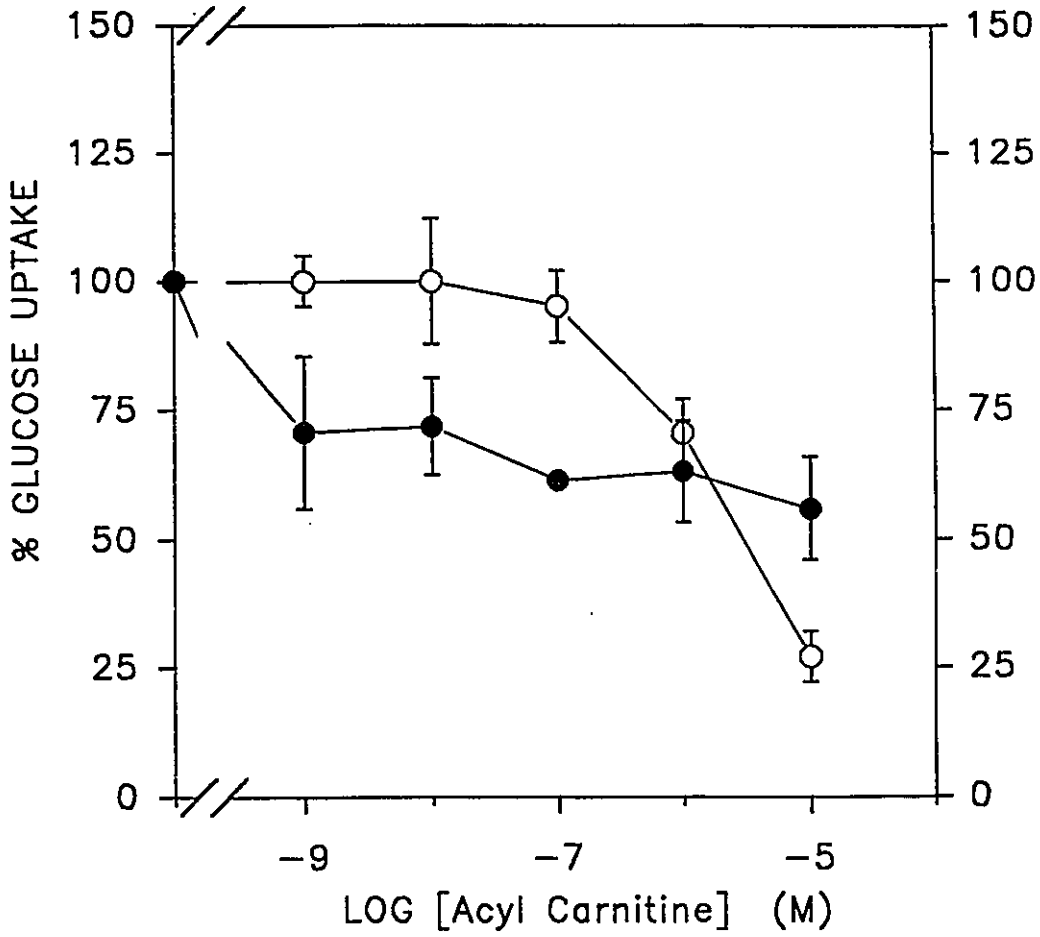


Figure 3.10.2.2

Effects of lauroyl carnitine (O) and palmitoyl carnitine (●) on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

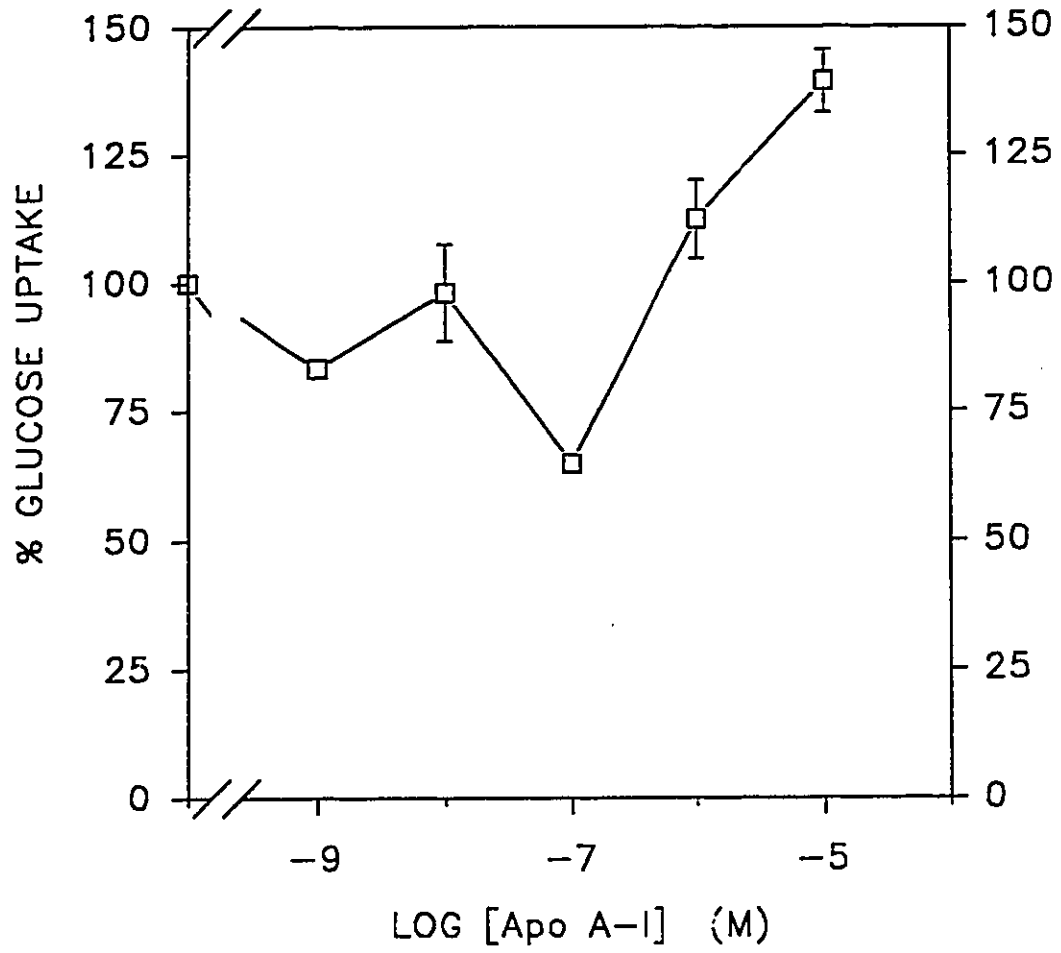


Figure 3.10.2.3

Effect of apolipoprotein A-I on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

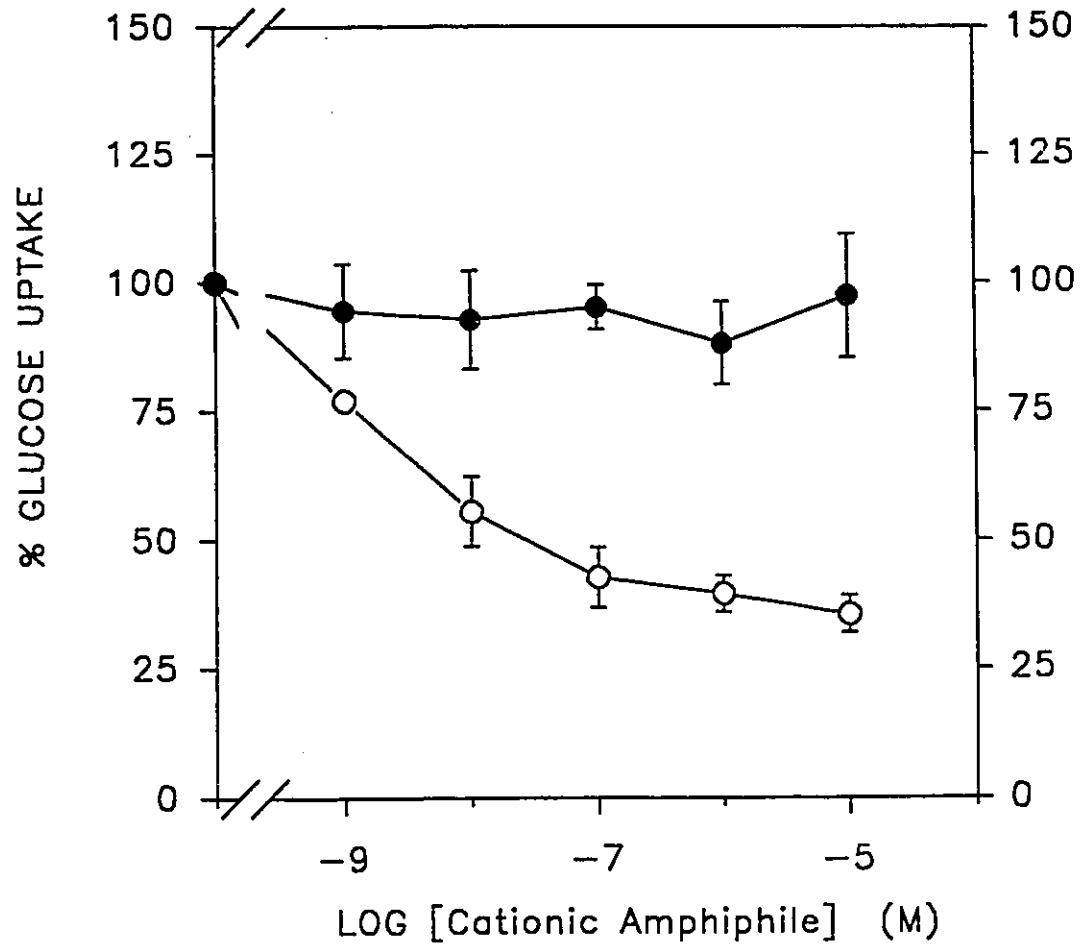


Figure 3.10.2.4

Effects of cationic amphiphiles on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expresssed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells. CATAM1, O; CATAM2, ●.

3.10.3 Effects of Hexagonal Phase Promoters on Glucose Uptake

The positively charged hexagonal phase promoter sphingosine had no significant effect on insulin stimulation of 2-deoxyglucose uptake into NIH 3T3 HIR 3.5 cells, as shown in Figure 3.10.3.1. This is in contrast to its stimulatory action on glucose transport in rat adipocytes [Epand *et al.*, 1991a], and its ability to inhibit insulin receptor tyrosine phosphorylation in NIH 3T3 HIR 3.5 cells. Furthermore, the lack of effect of sphingosine on basal glucose uptake does not agree with its effect in rat adipocytes, where it has a stimulatory insulin-like action on the basal rate of glucose transport [Robertson *et al.*, 1989; Epand *et al.*, 1991a]. DiC8 also had no effect on insulin stimulation of glucose uptake, as shown in Figure 3.10.3.2, consistent with its inability to alter tyrosine phosphorylation of the insulin receptor in these cells. The lack of stimulation may be explained by a dependence of glucose uptake on autophosphorylation. As mentioned above, DiC8 was unable to stimulate the insulin receptor tyrosine kinase. While these hexagonal phase promoters were unable to stimulate insulin signalling to glucose uptake in fibroblasts over-expressing insulin receptor, the hexagonal phase promoters hexane and sphingosine have been shown to activate the basal rate of glucose uptake in rat adipocytes [Epand *et al.*, 1991a], suggesting a role for membrane destabilization in facilitation of glucose transport.

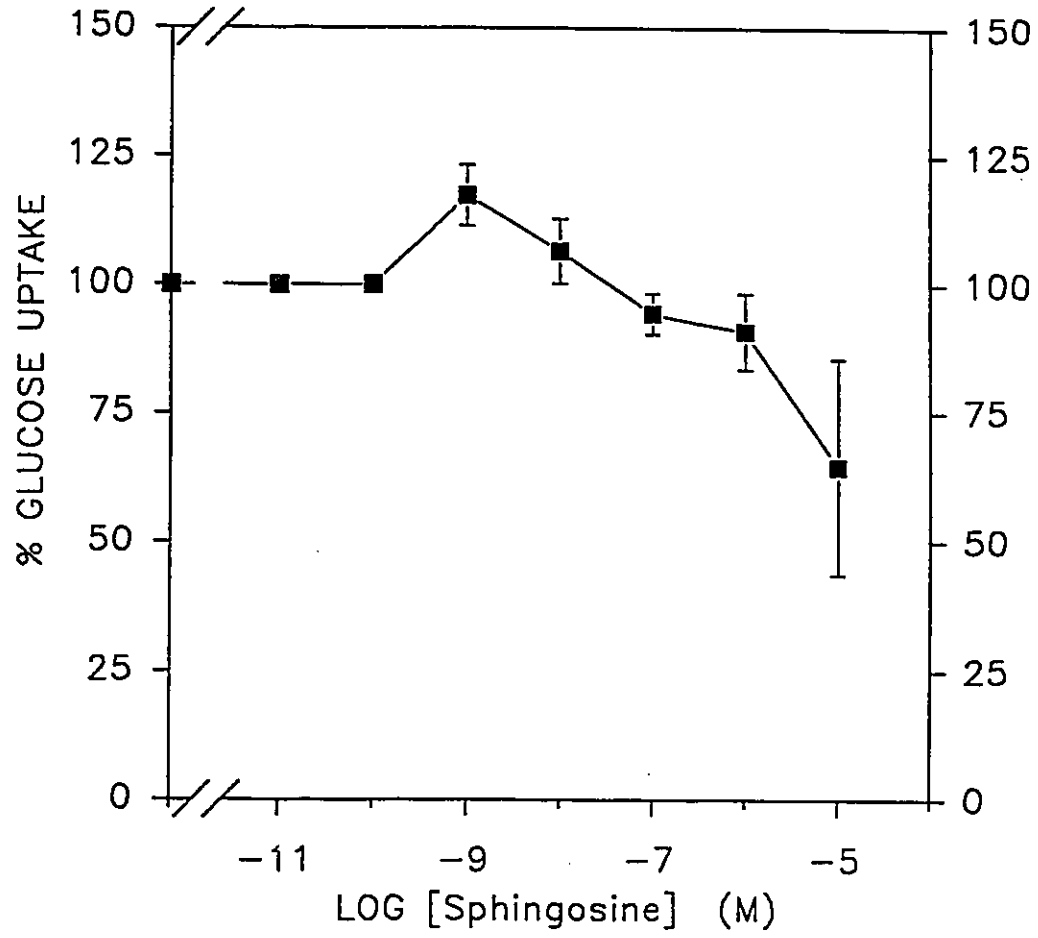


Figure 3.10.3.1

Effect of sphingosine on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expressed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

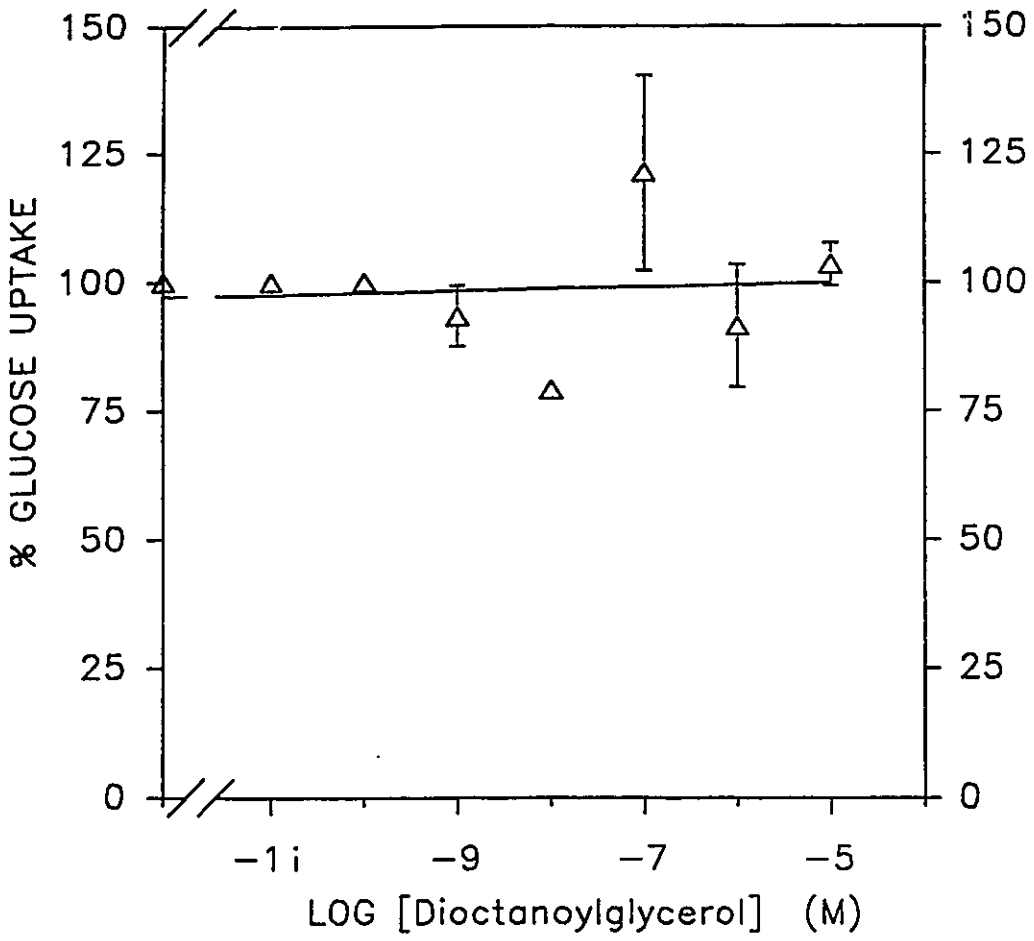


Figure 3.10.3.2

Effect of dioctanoylglycerol on [^{3}H]-2-deoxyglucose uptake by 10^5 NIH 3T3 HIR 3.5 cells in 30 minutes. For each concentration, the amount of glucose uptake in the absence of insulin was subtracted from that in the presence of 1 μM insulin, and expressed as a percent of glucose uptake in the absence of any additive. Values are mean ± S.E.M. for triplicate wells of cells.

3.10.4 Membrane Physical Properties and Glucose Uptake

Compounds which stabilize the bilayer relative to the hexagonal phase in model membranes were found to inhibit insulin stimulation of glucose uptake in cells over-expressing human insulin receptor. This inhibition may result from an inhibition of insulin receptor autophosphorylation by these membrane additives, if insulin receptor kinase activity is in fact required for insulin signalling to glucose transport. Since glucose transporters are transmembrane proteins, they would also be susceptible to changes in membrane physical properties. Compounds introduced into the plasma membrane which relieve negative curvature strain could inhibit the fusion of intracellular vesicles to the plasma membrane, which occurs in recruitment of glucose transporters from intracellular stores [Suzuki and Kono, 1980; Cushman and Wardzala, 1980]. The inhibition of glucose transport observed in the presence of various membrane additives may result from a combination of inhibition at the level of the insulin receptor and direct effects on the properties of the plasma membrane. The lack of stimulation of glucose uptake in the presence of hexagonal phase promoters agrees with the inability of these compounds to stimulate insulin receptor phosphorylation in these cells, which may support the idea that insulin stimulation of glucose uptake requires receptor tyrosine kinase activity. Additionally, glucose transporters may be less sensitive to increases in membrane monolayer negative curvature than to bilayer stabilization.

3.11 Effects of Membrane Additives on Insulin Stimulation of Fluid Phase Pinocytosis

To further relate the changes in insulin receptor phosphorylation to effects on insulin action, insulin stimulation of fluid phase pinocytosis was observed. Internalization of the lipophilic fluorescent probe trimethylamino-diphenylhexatriene (TMA-DPH) was used as a measure of fluid phase pinocytosis, as outlined in Materials and Methods, section 2.11. Internalization of this probe follows endocytosis of the plasma membrane, and results in increased fluorescence quantum yield as the probe goes from aqueous media to the phospholipid membrane [Illinger *et al.*, 1990]. CBZ-Gly-L-Phe inhibited insulin stimulated fluid phase pinocytosis in NIH 3T3 HIR 3.5 cells, as illustrated in Figure 3.11.1. Similar inhibition of pinocytosis was observed with CBZ-L-Phe-Gly (Figure 3.11.2). This inhibition of another insulin-stimulated event, in addition to glucose uptake, suggests that insulin receptor autophosphorylation, which was also inhibited by these dipeptide derivatives, is required for insulin signalling. Alternatively, bilayer stabilization may inhibit fluid phase pinocytosis irrespective of the effects on the activity of the insulin receptor. The inhibition of TMA-DPH internalization by both CBZ-dipeptides in the absence of insulin suggests that this is the case, especially since the CBZ-dipeptides had no effect on the basal level of insulin receptor phosphorylation.

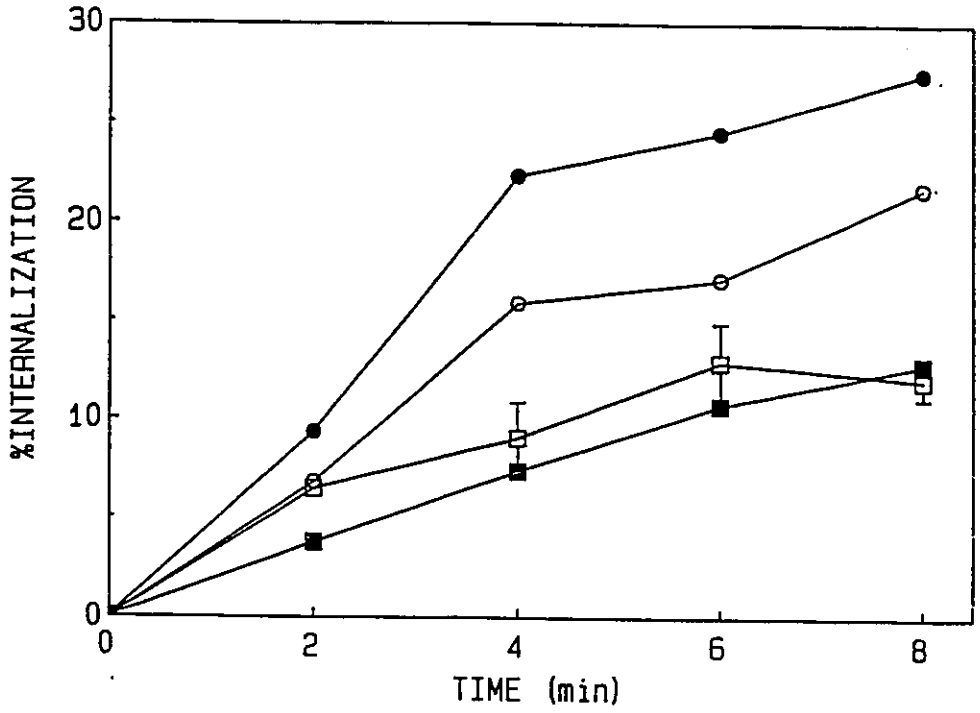


Figure 3.11.1

Effect of 10 μM CBZ-Gly-L-Phe on fluid phase pinocytosis in NIH 3T3 HIR 3.5 cells. Fluid phase pinocytosis was measured as the internalization of TMA-DPH, following incubation in the absence (open symbols) and presence (filled symbols) of insulin, with (squares) and without (circles) CBZ-Gly-L-Phe. Values are mean ± S.E.M. for triplicate experiments.

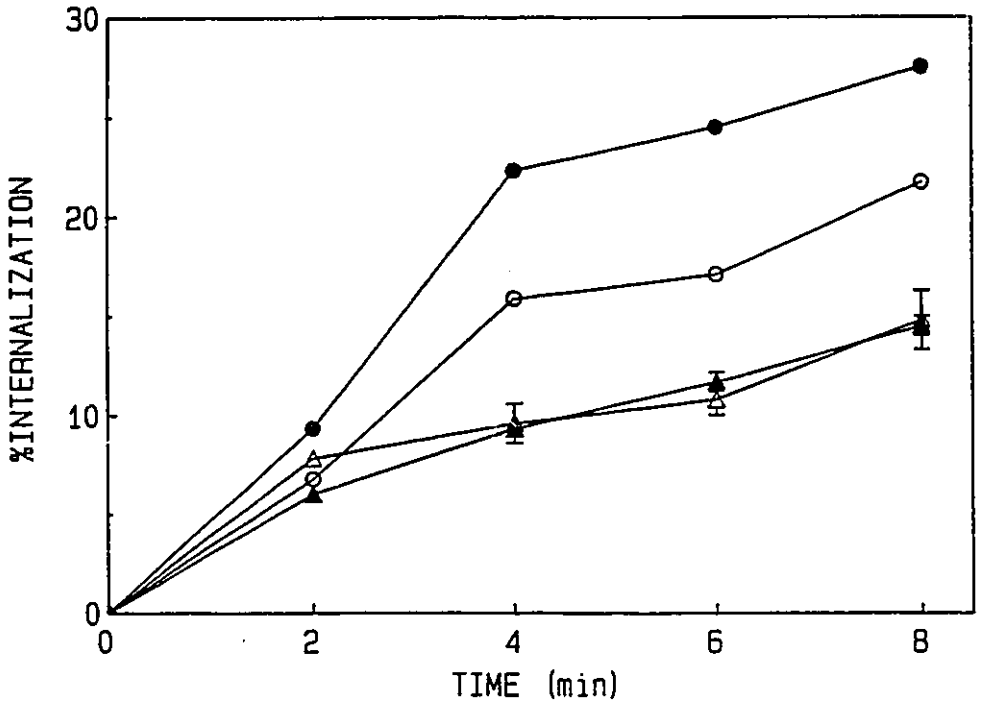


Figure 3.11.2

Effect of 10 μM CBZ-L-Phe-Gly on fluid phase pinocytosis in NIH 3T3 HIR 3.5 cells. Fluid phase pinocytosis was measured as the internalization of TMA-DPH, following incubation in the absence (open symbols) and presence (filled symbols) of insulin, with (triangles) and without (circles) CBZ-L-Phe-Gly. Values are mean ± S.E.M. for triplicate experiments.

4.0 SUMMARY AND PERSPECTIVES

4.1 Insulin Receptor Phosphorylation and Glucose Uptake

The requirement for insulin receptor autophosphorylation in glucose uptake is not completely established, as discussed in section 3.10. The 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 signalling to promote glucose transport. The effects of the various membrane additives on insulin receptor tyrosine phosphorylation and glucose uptake in NIH 3T3 HIR 3.5 cells is compared in Table 4.1.1, for the highest concentration of compounds tested. CBZ-Gly-L-Phe, CBZ-L-Phe-Gly, lyso PC, palmitoyl carnitine and lauroyl carnitine all inhibited both insulin receptor tyrosine phosphorylation and glucose uptake in the intact cells. In general, glucose uptake activity was less sensitive to the additives than was autophosphorylation, as higher concentrations were required to inhibit glucose uptake. This may be a result of the over-expression 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, capable of signalling to glucose uptake. 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 signalling, but by

directly affecting the activity of the transmembrane glucose transporter or its recruitment to the plasma membrane, which requires membrane fusion. Apo A-I had differential effects on insulin receptor tyrosine kinase and glucose uptake activities. As mentioned in section 3.10.2, this may result from the sensitivity of glucose transport to the extraction of phospholipids from the plasma membrane. The cationic amphiphiles CATAM1, CATAM2 and sphingosine, had similar effects on tyrosine phosphorylation of the insulin receptor and the rate of glucose uptake by the cells. All three additives inhibited both of these activities, despite the fact that sphingosine has the opposite effect on membrane monolayer curvature than CATAM1 and CATAM2. Therefore, cationic amphiphiles appear to affect insulin signalling in a complex manner, not predictable from their effects on membrane physical properties. The hexagonal phase promoter DiC8 was unable to stimulate either insulin receptor phosphorylation or glucose uptake. This further suggests a dependence of glucose transport activity on insulin receptor autophosphorylation.

As is evident from Table 4.1.1, with the exception of apo A-I, bilayer stabilizers which inhibit insulin receptor phosphorylation also inhibited glucose uptake in the same cells. Furthermore, for many of the compounds, the rank order of inhibitory potencies correlates for receptor autophosphorylation and glucose uptake. For example, CBZ-L-Phe-Gly inhibited both activities to a greater extent than did CBZ-Gly-L-Phe, which, in turn, was more inhibitory than lyso PC or lauroyl carnitine.

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
Lauroyl Carnitine	40 ± 6	27 ± 5
Palmitoyl Carnitine	2.0 ± 0.5	56 ± 10
CATAM1	20 ± 2	35 ± 4
CATAM2	no effect[a]	95 ± 5
Sphingosine	35 ± 8	76 ± 2
Dioctanoylglycerol	no effect[a]	103 ± 4

[a] No significant effect at 10 μM, as determined by independent t-test.

Table 4.1.1

Effects of various compounds on tyrosine phosphorylation of the insulin receptor β subunit and glucose uptake in NIH 3T3 HIR 3.5 cells. 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 ± S.E.M., for 10μM compound.

4.2 Membrane Physical Properties, Insulin Receptor Activity and Glucose Uptake

The bilayer stabilization potencies of the various membrane additives were compared as the increase in T_H per mole fraction of additive. The rank orders of potency of the compounds for glucose uptake, insulin receptor phosphorylation and bilayer stabilization are compared in Figure 4.2.1. These potency orders do not necessarily correlate with each other. That is, the strongest bilayer stabilizers were not necessarily the strongest inhibitors of receptor phosphorylation or glucose uptake. Possible reasons for the differences in potencies include the fact that the effects of the compounds on glucose uptake and insulin receptor activity are compared at one concentration, and that the actual concentration of compound in the membrane may vary, depending on the hydrophobicity. Furthermore, over-expression of insulin receptors in the cells may render them more susceptible to changes in insulin receptor activity, than to glucose uptake, in response to alterations in membrane physical properties. Insulin signalling in NIH 3T3 HIR 3.5 cells may not be relevant to that in insulin responsive tissue, due to the abnormal ratio of insulin receptors to pathway proteins in these cells, and possibly due to the isotype of glucose transporter expressed [Tavare and Siddle, 1993]. Finally, glucose transport may be affected directly by alterations in membrane physical properties, or indirectly via effects on insulin receptor activity. While the effects of the various compounds on insulin action may not correlate quantitatively with their ability to raise T_H , for bilayer stabilizers of similar structure, at least, the correlations hold. Table 4.2.1

summarizes the trends that each of the compounds tested had on insulin receptor phosphorylation, both in micelles and in intact cells, and on glucose uptake into cells. From this generalized table, it is obvious that the direction of the effects (inhibition or stimulation) correlate well between each of these activities.

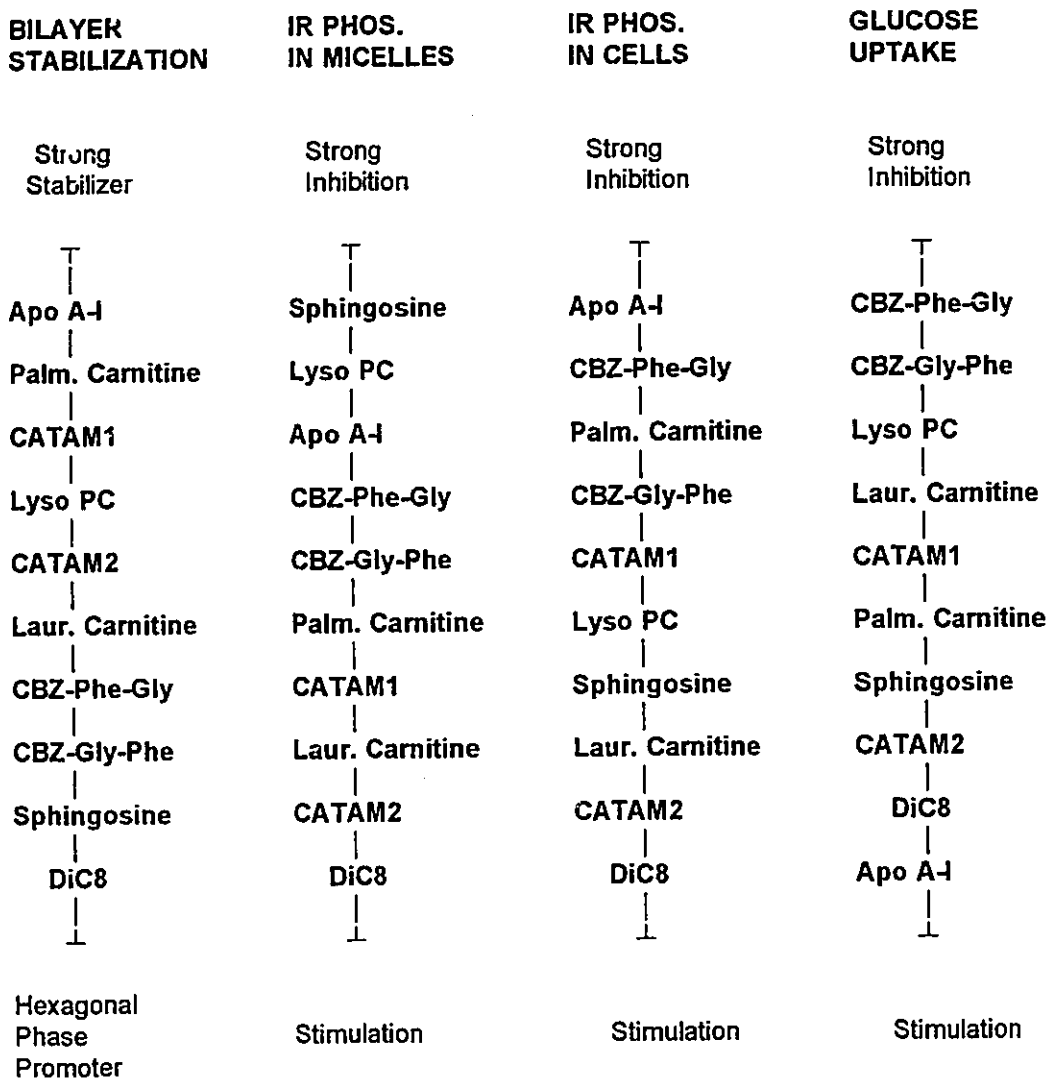


Figure 4.2.1

Potencies of various compounds for bilayer stabilization, insulin receptor phosphorylation (IR PHOS) in mixed micelles and intact cells, and glucose uptake. Compounds are listed as most potent bilayer stabilizer to most potent hexagonal phase promoter, or from most inhibitory to least inhibitory (or stimulatory), top to bottom. Compiled from effects of compounds at 10 μM.

COMPOUND	CELL IR PHOS	MICELLE IR PHOS	GLUCOSE UPTAKE
CBZ-L-Phe-Gly	↓	↓	↓
CBZ-Gly-L-Phe	↓	↓	↓
Apolipoprotein AI	↓	↓	↑
. . . Lysophosphatidylcholine	↓	↓	↓
Lauroyl Carnitine	↓	NO EFFECT	↓
Palmitoyl Carnitine	↓	↓	↓
CATAM 1	↓	↓	↓
CATAM 2	NO EFFECT	NO EFFECT	NO EFFECT
Sphingosine	↓	↓	↓
Dioctanoylglycerol	NO EFFECT	↑	NO EFFECT

Table 4.2.1

Summary of effects of various compounds on insulin signalling. ↓: Inhibition, ↑: Stimulation, IR: Insulin receptor, PHOS: Phosphorylation. Effects are compared at 10 μM compound.

It was not the aim of this work to determine the role of insulin receptor tyrosine kinase activity in insulin signalling to glucose transport, but rather to determine the dependence of insulin receptor activity, measured by the first step in insulin signalling, on membrane physical properties. Since originally the carbobenzoxy-dipeptides were found to inhibit insulin stimulation of glucose transport in rat adipocytes, we investigated the mechanism of this inhibition. While there are multiple factors that determine the rate of glucose uptake, we have identified one step in insulin signalling which is affected by these peptide derivatives. CBZ-dipeptides, and other bilayer stabilizers of diverse structure, inhibit insulin-stimulated tyrosine phosphorylation of the insulin receptor in fibroblasts transfected with the human insulin receptor gene, and in receptors isolated from human placenta. The lack of effect of these compounds on insulin binding and cell viability provides evidence for the mechanism of action of such additives lying in their effect on signal transduction through their ability to alter the bulk physical properties of the membrane. This was further supported by the similar action of peptide stereoisomers on insulin receptor phosphorylation and by the inability of these compounds to affect the activity of the constitutively active soluble insulin receptor kinase domain. The fact that the CBZ-dipeptides also inhibited fluid phase pinocytosis in fibroblasts over-expressing human insulin receptor suggests that insulin receptor autophosphorylation is also required for this insulin-stimulated event.

While previous studies have shown that the tyrosine kinase activity of reconstituted insulin receptors was modulated by phospholipids [Lewis and Czech, 1987;

Leray *et al.*, 1993], results presented here demonstrate that such modulation of insulin receptor function can also result from the incorporation of other substances into the membrane. Although the intact cell 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.

Future experiments with the insulin receptor may yield additional information on how certain bilayer stabilizers inhibit insulin signalling. It is proposed that bilayer stabilization via relief of membrane monolayer curvature strain inhibits receptor activity by inhibiting conformational changes necessary for signal transduction through the membrane. Conformational changes could be measured in the presence and absence of bilayer stabilizers to determine the validity of this proposal. Studies in insulin responsive cells may be useful to further investigate the relationship between membrane physical properties, insulin receptor autophosphorylation and glucose uptake.

The activity of the insulin receptor, like any intrinsic membrane protein, must be considered within the context of the membrane. The insulin receptor is not unique in its dependence on membrane physical properties. The activity of several membrane-bound enzymes has been shown to increase in the presence of hexagonal phase forming lipids. These include phospholipase A_2 [Dawson *et al.*, 1983], calcium ATPase [Navarro *et al.*, 1984], and protein kinase C [Epand *et al.*, 1991b]. The specific activity of

reconstituted mitochondrial adenine nucleotide translocator is also affected by the intrinsic radius of curvature of the bilayer [Streigher-Scott *et al.*, 1994]. Other membrane functions, such as membrane fusion are modulated in a predictable manner by agents which affect the bilayer-hexagonal phase equilibrium [Epand, 1986; Cheetham and Epand, 1987]. 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].

5.0 BIBLIOGRAPHY

Arnold, R.S. and Newton, A.C. (1991) Inhibition of the insulin receptor tyrosine kinase by sphingosine, Biochemistry 30, 7747-7754.

Assimacopoulos-Jeannet, F., Cusin, I., Greco-Perotto, R.M., Terrettaz, J., Rohner-Jeanrenaud, F., Zarjevski, N. and Jeanrenaud, B. (1991) Glucose transporters: structure, function, and regulation, Biochimie, 73, 67-70.

Backer, J.M., Schroeder, G.G., Cahill, D.A., Ullrich, A., Siddle, K. and White, M.F. (1991) Cytoplasmic juxtamembrane region of the insulin receptor: a critical role in ATP binding, endogenous substrate phosphorylation, and insulin-stimulated bioeffects in CHO cells, Biochemistry, 30, 6366-6372.

Baron, V., Gautier, N., Komoriya, A., Hainaut, P., Scimeca, J.C., Mervic, M., Lavielle, S., Dolais-Kitabgi, J. and Van Obberghen, E. (1989) Insulin binding to its receptor induces a conformational change in the receptor C-terminus, Biochemistry, 29, 4634-4641.

Baron, V., Kaliman, P., Gautier, N. and Van Obberghen, E. (1992) The insulin receptor activation process involves localized conformational changes, J. Biol. Chem., 267, 23290-23294.

Bell, G.I., Kayano, T., Buse, J.B., Burant, C.F., Takeda, J., Lin, D., Fukumoto, H. and Seino, S. (1990) Molecular biology of mammalian glucose transporters, Diabetes Care, 13, 198-208.

Biener, Y. and Zick, Y. (1990) Basic polycations activate the insulin receptor kinase and a tightly associated serine kinase, Eur. J. Biochem., 194, 243-250.

Bloom, M., Evans, E. and Mouritsen, O.G. (1991) Physical properties of the fluid lipid-bilayer component of cell membranes: a perspective, Q. Rev. Biophys., 24, 293-297.

Bottega, R. and Epand, R.M. (1992) Inhibition of Protein Kinase C by cationic amphiphiles, Biochemistry, 31, 9025-9030.

Brogden, R.N. and Heel, R.C. (1987) Human insulin. A review of its biological activity, pharmacokinetics and therapeutic use, Drugs, 34, 350-371.

Burgering, B. M. Th., Medema, R.H., Maassen, J.A., van de Wetering, M. L., van der Eb, A.J., McCormick, F. and Bos, J.L. (1991) Insulin stimulation of gene expression mediated by p21ras activation, EMBO J., 10, 1103-1109.

Carruthers, A. and Melchoir, D.L. (1986) How bilayer lipids affect membrane protein activity, Trends Biol. Sci., 11, 331-335.

Cevc, G. and Marsh, D. (1987) In *Phospholipid Bilayers: physical principles and models*, E.E. Bittar, ed., John Wiley & Sons, Inc., New York.

Cheema, S.K., Venkatraman, J. and Clandinin, M. T. (1992) Insulin binding to liver nuclei from lean and obese mice is altered by dietary fat, Biochim. Biophys. Acta., 1117, 37-41.

Cheetham, J.J. and Epand, R.M. (1987) Comparison of the interaction of the anti-viral chemotherapeutic agents amantadine and tromantadine with model phospholipid membranes, Biosci. Rep., 7, 225-230.

Christiansen, K., Tranum-Jensen, J., Carlsen, J. and Vinten, J. (1991) A model for the quaternary structure of human placental insulin receptor deduced from electron microscopy, Proc. Natl. Acad. Sci. USA, 88, 249-252.

Cobb, M.H., Sang, B.-C., Gonzales, R., Goldsmith, E. and Ellis, L. (1989) Autophosphorylation activates the soluble cytoplasmic domain of the insulin receptor in an intermolecular reaction, J. Biol. Chem., 264, 18701-18706.

Cullis, P.R. and deKruijff, B. (1979) Lipid polymorphism and the functional roles of lipids in biological membranes, Biochim. Biophys. Acta., 559, 399-420.

Cushman, S.W. and Wardzala, L.J. (1980) Potential mechanisms of insulin action on glucose transporter in isolated rat adipose cell: apparent translocation of intracellular transport systems to the plasma membrane, J. Biol. Chem., 256, 4758-4762.

Czech, M.P., Klarlund, J.K., Yagaloff, K.A., Bradford, A.P. and Lewis, R.E. (1988) Insulin receptor signaling. Activation of multiple serine kinases, J. Biol. Chem., 263, 11017-11020.

Danielli, J.F. and Davson, H. (1935) A contribution to the theory of permeability of thin films, J. Cell. Comp. Physiol., 5, 495-508.

Dawson, R.M.C., Hemington, N.L. and Irvine, R.F. (1983) Diacylglycerol potentiates phospholipase attack upon phospholipid bilayers: possible connection with cell stimulation, Biochem. Biophys. Res. Commun., 117, 196-201.

Debant, A., Clauser, E., Ponzio, G., Filloux, C., Auzan, C., Contreres, J.-O. and Rossi, B. (1988) Replacement of insulin receptor tyrosine residues 1162 and 1163 does not alter the mitogenic effect of the hormone, Proc. Natl. Acad. Sci. USA, 85, 8032-8036

Debant, A., Ponzio, G., Clauser, E., Contreres, J.-O. and Rossi, B. (1989) Receptor cross-linking restores an insulin metabolic effect altered by mutation on Tyrosine 1162 and Tyrosine 1163, Biochemistry, 28, 14-17.

DeMeyts, P., Roth, J., Neville, D.M., Jr., Gavin, J.R. and Lesniak, M.A. (1973) Insulin interactions with its receptors: Experimental evidence for negative cooperativity, Biochem. Biophys. Res. Commun., 55, 154.

Dickens, M. and Tavare, J.M. (1992) Analysis of the order of autophosphorylation of human insulin receptor tyrosines 1158, 1162 and 1163, Biochem. Biophys. Res. Comm., 186, 244-250.

Ebina, Y., Ellis, L., Jarnagin, K., Edery, M., Graf, L., Clauser, E., Ou, J.H., Masearz, R., Kan, Y.W., Goldfine, I.D., Roth, R.A. and Rutter, W.J. (1985) The human insulin receptor cDNA: the structural basis for hormone-activated transmembrane signaling, Cell, 40, 747-758.

Ellis, L., Clauser, E., Morgan, D.O., Ettery, M., Roth, R.A. and Rutter, W.J. (1986) Replacement of insulin receptor tyrosine residues 1162 and 1163 compromises insulin-stimulated kinase activity and uptake of 2-deoxyglucose, Cell, 45, 721-732.

Ellis, L., Levitan, A., Cobb, M.H. and Ramos, P. (1988) Efficient expression in insect cells of a soluble, active human insulin receptor protein-tyrosine kinase domain by use of a baculovirus vector, J. Virol., 62, 1634-1639.

Epand, R.M. (1985) Diacylglycerols, lysolecithin or hydrocarbons markedly alter the bilayer to hexagonal phase transition temperature of phosphatidylethanolamines, Biochemistry, 24, 7092-7095.

Epand, R.M. (1986) Virus replication inhibitory peptide inhibits the conversion of phospholipid bilayers to the hexagonal phase, Biosci. Rep., 6, 647-653.

Epand, R.M. (1990) Relationship of phospholipid hexagonal phases to biological phenomena, Biochem. Cell. Biol., 68, 17-23.

Epand, R.M. (1991) Biological consequences of alterations in the physical properties of membranes, in *Cell and Model Membrane Interactions*, S. Ohki, Ed., Plenum Press, New York, pp. 135-147.

Epand, R.M., Robinson, K.S., Andrews, M.E. and Epand, R.F. (1989) Dependence of the bilayer to hexagonal phase transition on amphiphile chain length, Biochemistry, 28, 9398-9402.

Epand, R.M. and Lester, D.S. (1990) The role of membrane biophysical properties in the regulation of protein kinase C activity, Trends Pharmacol. Sci., 11, 317-320.

Epand, R.M. and Stafford, A.R. (1990) Counter-regulatory effects of phosphatidic acid on protein kinase C activity in the presence of calcium and diolein, Biochem. Biophys. Res. Commun., 171, 487-490.

Epand, R.M. and Epand, R.F. (1991) Lipid-peptide interactions, in *The Structure of Biological Membranes*, P. Yeagle, Ed., CRC Press, Boca Raton, FL., pp. 573-601.

Epand, R.M., Stafford, A.R. and Debanne, M.T. (1991a) Action of insulin in rat adipocytes and membrane properties, Biochemistry, 30, 2092-2098.

Epand, R.M., Epand, R.F., Leon. B. T.-C. and Kuo, J.F. (1991b) Evidence for the regulation of the activity of protein kinase C through changes in membrane properties, Biosci. Rep., 11, 59-64.

Epand, R.M. and Epand, R.F. (1994) Calorimetric detection of curvature strain in phospholipid bilayers, Biophys. J., 66, 1450-1456.

Field, C.J., Toyomizu, M. and Clandinin, M.T. (1989) Relationship between dietary fat, adipocyte membrane compositin and insulin binding in the rat, J. Nutr., 119, 1489-1489.

Field, C.J., Ryan, E.A., Thomson, A.R. and Clandinin, M.T. (1990) Diet fat composition alters membrane phospholipid composition, insulin binding and glucose metabolism in adipocytes from control and diabetic animals, J. Biol. Chem., 265, 11143-11150.

Florke, R.-R., Klein, H.W. and Reinauer, H. (1990) Structural requirements for signal transduction of the insulin receptor, Eur. J. Biochem., 191, 473-482.

Fox, J.A., Soliz, N.M. and Saltiel, A.R. (1987) Purification of a phosphatidylinositol-glycan specific phospholipase C from liver plasma membranes: a possible target of insulin action, Proc. Natl. Acad. Sci. USA, 84, 2663-2667.

Frattali, A.L., Treadway, J.L. and Pessin, J.E. (1992) Transmembrane signaling by the human insulin receptor kinase. Relationship between intramolecular β subunit *trans*- and *cis*-autophosphorylation and substrate kinase activation, J. Biol. Chem., 267, 19521-19528.

Frost, S.C. and Lane, M.D. (1985) Evidence for the involvement of vicinal sulfhydryl groups in insulin-activated hexose transport in 3T3-L1 adipocytes, J. Biol. Chem., 260, 2646-2652.

Fujita-Yamaguchi, Y., Choi, S., Yoshikazu, S. and Itakura, K. (1983) Purification of insulin receptor with full binding activity, J. Biol. Chem., 258, 5045-5049.

Gibson, N.J. and Brown, M.F. (1993) Lipid headgroup and acyl chain composition modulate the MI-MII equilibrium of rhodopsin in recombinant membranes, Biochemistry, 32, 2438-2454.

Ginsberg, B.H., Brown, T.J., Simon, I. and Spector, A.A. (1981) Effect of the membrane lipid environment on the properties of insulin receptors, Diabetes, 30, 773-780.

Goldstein, B.J. and Dudley, A.L. (1990) The rat insulin receptor: primary structure and conservation of tissue-specific alternative messenger RNA splicing, Mol. Endocrinol., 4, 235-244.

Gottschalk, W.K. (1991) The pathway mediating insulin's effects on pyruvate dehydrogenase bypasses the insulin receptor tyrosine kinase, J. Biol. Chem., 266, 8814-8819.

Gruner, S.M. (1992) Nonlamellar lipid phases in *The Structure of Biological Membranes*, P.L. Yeagle, ed., CRC Press, Boca Raton, pp. 211-250.

Gustafson, T.A. and Rutter, W.J. (1990) The cysteine-rich domains of the insulin and insulin-like growth factor I receptors are primary determinants of hormone binding specificity. Evidence from receptor chimeras, J. Biol. Chem., 265, 18663-18667.

Hannun, Y. A., Loomis, C.R. and Bell, R.M. (1985) Activation of protein kinase C by Triton X-100 mixed micelles containing diacylglycerol and phosphatidylserine, J. Biol. Chem., 260, 10039-10043.

Hara, H. and Yokoyama, S. (1991) Interaction of free apolipoproteins with macrophages. Formation of high density lipoprotein-like lipoproteins and reduction of cellular cholesterol, J. Biol. Chem., 266, 3080-3086.

Hashimoto, N., Feener, E.P., Zhang, W.-R. and Goldstein, B.J. (1992a) Insulin receptor protein-tyrosine phosphatases. Leukocyte common antigen-related phosphatase rapidly deactivates the insulin receptor kinase by preferential dephosphorylation of the receptor regulatory domain, J. Biol. Chem., 267, 13811-13814.

Hashimoto, N., Zhang, W.-R. and Goldstein, B.J. (1992b) Insulin receptor and epidermal growth factor receptor phosphorylation by three major rat liver protein-tyrosine phosphatases expressed in a recombinant bacterial system, Biochem. J., 284, 569-576.

Hedo, J.A., Kasuga, M., Van Obberghen, E., Roth, J. and Kahn, C.R. (1981) Direct demonstration of glycosylation of insulin receptor subunits by biosynthetic and external labeling: Evidence for heterogeneity, Proc. Natl. Acad. Sci. USA, 78, 4791-4795

Hedo, J.A., Collier, E. and Watkinson, A. (1987) Myristyl and palmityl acylation of the insulin receptor, J. Biol. Chem., 262, 954-961.

Helmerhorst, E. and Yip, C. (1993) Insulin binding to rat liver plasma membranes predicts a homogeneous class of binding sites in different affinity states that may be related to a regulator of insulin binding, Biochemistry, 32, 2356-2362.

Hill, A.V. (1910) The possible effects of the aggregation of the molecules of haemoglobin on its dissociation curves, J. Physiol., 40, iv-vii.

Holman, G.D., Leggio, L.L. and Cushman, S.W. (1994) Insulin-stimulated GluT4 glucose transporter recycling. A problem in membrane protein subcellular trafficking through multiple pools, J. Biol. Chem., 269, 17516-17524.

Hubert, P., Bruneau-Wack, C., Cremel, G., Le Marchand-Brustel, Y. and Staedel, C. (1991) Lipid-induced insulin resistance in cultured hepatoma cells is associated with a decreased insulin receptor tyrosine kinase activity, Cell Regulation, 2, 56-[illegible].

Illinger, D., Poindron, P., Fonteneau, P., Modollel, M. and Kuhry, J.-G. (1990) Internalization of the lipophilic fluorescent probe trimethylamino-diphenylhexatriene follows the endocytosis and recycling of the plasma membrane in cells, Biochim. Biophys. Acta., 1030, 73-81.

Ishizuka, T., Cooper, D.R., Hernandez, H., Buckley, D., Standaert, M. and Farese, R.V. (1990) Effects of insulin on diacylglycerol-protein kinase C signalling in rat diaphragm and soleus muscles and relationship to glucose transport, Diabetes 39, 181-190.

Israelachvili, J.N., Marcelja, S. and Horn, R.G. (1980) Physical principles of membrane organization, Q. Rev. Biophys., 13, 121-200.

Jo, H., Byer, S. and McDonald, J.M. (1993a) Insulin stimulates association of a 41 kDa G-protein with the insulin receptor, Biochem. Biophys. Res. Comm., 196, 99-106.

Jo, H., Radding, W., Anantharamaiah, G.M. and McDonald, J.M. (1993b) An insulin receptor peptide (1135-1156) stimulates guanosine 5'-[γ-thio]triphosphate binding to the 67 kDa G-protein associated with the insulin receptor, Biochem. J., 294, 19-24.

Kahn, B.B., Charron, M.J., Lodish, H.F., Cushman, S.W. and Flier, J.S. (1989) Differential regulation of two glucose transporters in adipose cells from diabetic and insulin-treated diabetic rats, J. Clin. Invest., 84, 404-411.

Kahn, B.B. (1992) Facilitative glucose transporters: Regulatory mechanisms and dysregulation in diabetes, J. Clin. Invest., 89, 1367-1374.

Kahn, C.R. (1979) Membrane receptors for hormone and neurotransmitters, J. Cell Biol., 70, 161-186.

Kahn, C.R., Baird, K.L., Fier, J.S., Grunfeld, C., Harmon, J.T., Harrison, L.C., Karlsson, F.A., Kasuga, M., King, G.L., Lang, U.C., Podskalny, J.M. and Van Obberghen, E. (1981) Insulin receptors, receptor antibodies, and the mechanism of insulin action, Rec. Prog. Horm. Res., 37, 477.

Kasuga, M., Kahn, C.R., Hedo, J., Van Obberghen, E. and Yamada, K.M. (1981) Insulin-induced receptor loss in cultured human lymphocytes is due to accelerated receptor degradation, Proc. Natl. Acad. Sci. U.S.A., 78, 6917.

Kasuga, M., Karlsson, F.A. and Kahn, C.R. (1982a) Insulin stimulates the phosphorylation of the 95,000-Dalton subunit of its own receptor, Science, 215, 185-187.

Kasuga, M., Zick, Y., Blithe, D.L., Crettaz, M. and Kahn, C.R. (1982b) Insulin stimulates the tyrosine phosphorylation of the insulin receptor in a cell-free system, Nature, 298, 667-669.

Kasuga, M., Zick, Y., Blithe, D.L., Karlsson, F.A., Haring, H.U. and Kahn, C.R. (1982c) Insulin stimulation of phosphorylation of the β subunit of the insulin receptor. Formation of both phosphoserine and phosphotyrosine, J. Biol. Chem., 257, 9891-9894.

Kellerer, M., Obermaier-Kusser, B. and Profrock, A. (1991) Insulin activates GTP-binding to a 40 000 Dalton protein in fat cells, Biochem. J., 276, 103-108.

King, G.L. and Johnson, S.M. (1985) Receptor mediated transport of insulin across endothelial cells, Science, 227, 1583-1586.

Koch, C.A., Anderson, D., Moran, M.F., Ellis, C. and Pawson, T. (1991) SH2 and SH3 domains: elements that control interactions of cytoplasmic signaling proteins, Science, 252, 668-674.

Kohanski, R.A. (1989) Insulin receptor aggregation and autophosphorylation in the presence of cationic polyamino acids, J. Biol. Chem., 264, 20984-20991.

Kozma, L., Baltensperger, K., Klarlund, J., Porras, A., Santos, E. and Czech, M.P. (1993) The Ras signaling pathway mimics insulin action on glucose transporter translocation, Proc. Natl. Acad. Sci. USA, 90, 4460-4464.

Lee, H.-C. and Dupont, J. (1991) Effects of dietary fatty acids on the activity of glucose transport in adipocytes, J. Nutr. Biochem., 2, 38-43.

Leray, V., Hubert, P., Cremel, G. and Staedel, C. (1992) Detergents affect insulin binding, tyrosine kinase activity and oligomeric structure of partially purified insulin receptors, Arch. Biochem. Biophys., 294, 22-29.

Leray, V., Hubert, P., Burgun, C., Staedel, C. and Cremel, G. (1993) Reconstitution studies of lipid effects on insulin-receptor kinase activation, Eur. J. Biochem., 213, 277-284.

Levy-Toledano, R., Taouis, M., Blaettler, D.H., Gorden, P. and Taylor, S.I. (1994) Insulin-induced activation of phosphatidyl inositol 3-kinase, J. Biol. Chem., 269, 31178-31182.

Lewis, R.N.A.H. and McElhaney, R.N. (1992) The mesomorphic phase behavior of lipid bilayers, in *The Structure of Biological Membranes*, P.L. Yeagle, ed., CRC Press, Boca Raton, pp. 73-155.

Lewis, R.E. and Czech, M.P. (1987) Phospholipid environment alters hormone-sensitivity of the purified insulin receptor kinase, Biochem. J., 248, 829-836.

Lewis, R.E., Wu, P., MacDonald, R.G. and Czech, M.P. (1990) Insulin-sensitive phosphorylation of serine 1293-1294 on the human insulin receptor by a tightly associated serine kinase, J. Biol. Chem., 265, 947-954.

Lienhard, J.E., Slot, J.W., James, D.E. and Mueckler, M.M. (1992) How cells absorb glucose, Scientific American, 1992, 86-91.

Low, M.G. and Saltiel, A.R. (1988) Structural and functional roles of glycosyl-phosphatidylinositol in membranes, Science, 239, 268-275.

Lowenstein, E.J., Daly, R.J., Batzer, A.F., Li, W., Margolis, B., Lammers, R., Ullrich, A., Skolnik, E.Y., Bar-Sagi, D. and Schlessinger, J. (1992) The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling, Cell, 70, 431-442.

Luttrell, L., Kilgour, E., Larner, J. and Romero, G. (1990) A pertussis-toxin sensitive G-protein mediates some aspects of insulin action in BC_3H-1 murine myocytes, J. Biol. Chem., 265, 16873-16879.

Manchester, J., Kong, X., Lowry, O.H. and Lawrence, J.C., Jr. (1994) Ras signaling in the activation of glucose transport by insulin, J. Biol. Chem., 264, 4644-4648.

Matsuda, M., Mayer, B.J. and Hanafusa, H. (1991) Identification of domains of the v-*crk*
oncogene product sufficient for association with phosphotyrosine-containing proteins, Mol. Cell. Biol., 11, 1607-1613.

Meuckler, M., Caruso, C., Baldwin, S.A., Panico, M., Blench, I., Morris, H.R., Allard, W.J., Lienhard, G.E. and Lodish, H.F. (1985) Sequence and structure of a human glucose transporter, Science, 229, 941-945.

Miller, D.S. and Sykes, D.B. (1991) Stimulation of protein synthesis by internalized insulin, J. Cell. Physiol., 147, 487-494.

Moller, D.E., Yokota, A., Caro, J.F. and Flier, J.S. (1989) Tissue-specific expression of two alternatively spliced insulin receptor mRNA's in man, Mol. Endocrinol, 3, 1263-1269.

Moller, D.E., Benecke, H. and Flier, J.S. (1991) Biologic activities of naturally occuring human insulin receptor mutations. Evidence that metabolic effects of insulin can be mediated by a kinase-deficient insulin receptor, J. Biol. Chem., 266, 10995-11001.

Morrison, B.D., Feltz, S.M. and Pessin, J.E. (1989) Polylysine specifically activates the insulin-dependent insulin receptor protein kinase, J. Biol. Chem., 264, 9994-10001.

Mosoir, M. and Epand, R.M. (1993) Mechanism of activation of protein kinase C: Roles of diolein and phosphatidylserine, Biochemistry, 32, 66-75.

Mosthaf, L., Grako, K., Dull, T.J., Coussens, L., Ullrich, A. and McClain, D.A. (1990) Functionally distinct insulin receptors generated by tissue-specific alternative splicing, EMBO J., 9, 2409-2413.

Mueckler, M. (1990) Family of glucose-transporter genes. Implications for glucose homeostasis and diabetes, Diabetes, 39, 6-11.

Munoz,G. and Marshall, S.H. (1990) An alternative method for a fast separation of phosphotyrosine, Anal. Biochem., 190, 233-237.

Murakami, M.S. and Rosen, O.M. (1991) The role of insulin receptor autophosphorylation in signal transduction, J. Biol. Chem., 266, 22653-22660.

Navarro, J., Toivio-Kinnucan, M. and Racker, E. (1984) Effect of lipid composition on the calcium/adenosine 5'-triphosphate coupling ratio of the Ca^{2+}-ATPase of sarcoplasmic reticulum, Biochemistry, 23, 130-135.

O'Brien, R.M. and Granner, D.K. (1991) Regulation of gene expression by insulin, Biochem. J., 278, 609-619.

Okamoto, M., Kono, S., Inoue, G., Hayashi, T., Kosaki, A., Maeda, I., Kubota, M., Kuzuya, H. and Imura, H. (1992) Effects of a high-fat diet on insulin receptor kinase and the glucose transporter in rats, J. Nutr. Biochem., 3, 241-250.

Olefsky, J.M. (1990) The insulin receptor. A multifunctional protein, Diabetes, 39, 1009-1016.

Patterson, M.K.Jr. (1979) Measurements of growth and viability of cells in culture, Methods in Enzymol., LVIII, 141-152.

Pilch, P.F. and Czech, M.P. (1980) Hormone binding alters the conformation of the insulin receptor, Science, 210, 1152-1153.

Pillay, T.S. and Siddle, K. (1991) Insulin-stimulated serine/threonine phosphorylation of the insulin receptor: paucity of threonine 1348 phosphorylation in vitro indicates the involvement of more than one serine/threonine kinase in vivo, Biochem. Biophys. Res. Commun., 179, 962-971.

Piper, R.C., Hess, L.J. and James, D.E. (1991) Differential sorting of two glucose transporters expressed in insulin-sensitive cells, Am. J. Physiol., 260, C570-C580.

Pot, D.A. and Dixon, J.E. (1992) A thousand and two protein tyrosine phosphatases, Biochim. Biophys. Acta, 1136, 35-43.

Rafaeloff, R., Maddux, B.A., Brunetti, A., Sbraccia, P., Sung, C.K., Patel, R., Hawley, D.M. and Goldfine, I.D. (1991) Transmembrane signalling by insulin via an insulin receptor mutated at tyrosines 1158, 1162, and 1163, Biochem. Biophys. Res. Comm., 179, 912-918.

Ramachandran, C., Aebersold, R., Tonks, N.K. and Pot, D.A. (1992) Sequential dephosphorylation of a multiply phosphorylated insulin receptor peptide by protein tyrosine phosphatases, Biochemistry, 31, 4232-4238.

Rilfors, L., Hauksson, J.B. and Lindblom, G. (1994) Regulation and phase equilibria of membrane lipids from Bacillus megaterium and Acholeplasma laidlawii strain A containing methyl-branched acyl chains, Biochemistry, 33, 6110-6120.

Robertson, D.G., DiGirolamo, M., Merrill, A.H. Jr. and Lambeth, J. D. (1989) Insulin-stimulated hexose transport and glucose oxidation in rat adipocytes is inhibited by sphingosine at a step after insulin binding, J. Biol. Chem., 264, 6773-6779.

Rothenberg, P.L. and Kahn, C.R. (1988) Insulin inhibits pertussis toxin-catalyzed ADP-ribosylation of G-proteins. Evidence for a novel interaction between insulin receptors and G-proteins,J. Biol. Chem., 263, 15546-15552.

Saltiel, A.R. and Cuatrecasas, P. (1986) Insulin stimulates the generation from hepatic plasma membranes of modulators derived from an inositol glycolipid, Proc. Natl. Acad. Sci. USA, 83, 5793-5797.

Saltiel, A.R. and Cuatrecasas, P. (1988) In search of a second messenger for insulin, Am. J. Physiol., 255, C1-C11.

Scatchard, G. (1949) The attractions of proteins for small molecules and ions, Ann. N. Y. Acad. Sci., 51, 660-672.

Schaefer, E.M., Erickson, H.P., Federwisch, M., Wollmer, A. and Ellis, L. (1992) Structural organization of the human insulin receptor ectodomain, J. Biol. Chem., 267, 23393-23402.

Schlessinger, J. (1994) SH2/SH3 signaling proteins, Curr. Op. Gen. Dev., 4, 25-30.

Segrest, J.P., Jackson, R.L., Morrisett, J.D. and Gotto, A.M. Jr. (1974) A molecular theory of lipid-protein interactions in the plasma lipoproteins, FEBS Lett., 38, 247-253

Seino, S., Seino, M., Nishi, S. and Bell, G.I. (1989) Structure of human insulin receptor gene and characterization of its promotor, Proc. Natl. Acad. Sci. USA, 86, 114-118.

Senisterra, G. and Epand, R.M. (1993) Role of membrane defects in the regulation of the activity of protein kinase C, Arch. Biochem. Biophys., 300, 378-383.

Shindler, H. and Seelig, J. (1975) Deuterium order parameters in relation to thermodynamic properties of a phospholipid bilayer. A statistical mechanical interpretation, Biochemistry, 14, 2283-2287.

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

Singer, S.J. and Nicolson, G.L. (1972) The fluid mosaic model of the structure of cell membranes, Science, 175, 720-731.

Shoelson, S.E., Boni-Schnetzler, M., Pilch, P.F. and Kahn, C.R. (1991) Autophosphorylation within insulin receptor β-subunits can occur as an intramolecular process. Biochemistry, 30, 7740-7746.

Sonne, O. (1988) Receptor mediated endocytosis and degredation of insulin, Physiol. Rev., 68, 1129-1196.

Stephens, L.R., Hughes, K.T. and Irvine, R.F. (1991) Pathway of phosphatidylinositol (3,4,5)-triphosphate synthesis in activated neutrophils, Nature, 351, 33-39.

Stralfors, P. (1988) Insulin stimulation of glucose uptake can be mediated by diacylglycerol in adipocytes, Nature 335, 554-556.

Streicher-Scott, J., Lapidus, R. and Sokolove, P.M. (1994) The reconstituted mitochondrial adenine nucleotide translocator: effects of lipid polymorphism, Arch. Biochem. Biophys., 315, 548-554.

Sun, X.J., Crimmins, D.L., Myers, M.G., Miralpiex, M. and White, M.F. (1993) Pleiotropic insulin signals are engaged by multisite phoshorylation of IRS-1, Mol. Cell Biol., 13, 7418-7428.

Sun, X.J., Rothenberg, P., Kahn, C.R., Backer, J.M., Araki, E., Wilden, P.A., Cahill, D.A., Goldstein, B.J. and White, M.F. (1991) Structure of the insulin receptor substrate IRS-1 defines a unique signal transduction protein, Nature, 352, 73-77.

Suzuki, K. and Kono, T. (1980) Evidence that insulin causes translocation of glucose transport activity to the plasma membrane, Proc. Natl. Acad. Sci. USA, 77, 2542-2545.

Sweet, L.J., Dudley, D.T., Pessin, J.E. and Spector, A.A. (1987) Phospholipid activation of the insulin receptor kinase: regulation by phosphatidylinositol, Faseb J., 1, 55-59.

Tate, M.W., Eikenberry, E.F., Turner, D.C., Shyamsunder, E. and Gruner, S.M. (1991) Nonbilayer phases of membrane lipids, Chem. Phys. Lipids, 57, 147-164.

Tavare, J.M., O'Brien, R.M., Siddle, K. and Denton, R.M. (1988) Analysis of insulin receptor phosphorylation sites in intact cells by two-dimensional phosphopeptide mapping, Biochem. J., 253, 783-788.

Tavare, J.M. and Siddle, K. (1993) Mutational analysis of insulin receptor function: consensus and controversy, Biochim. Biophys. Acta., 1178, 21-39.

Taylor, R. (1991) Insulin Action 1991, Clin. Endocrinol., 34, 159-171.

Terry, M.L., Levy, J., and Grunberger, G. (1991) Diacylglycerols modulate insulin action in rat adipocytes, Horm. Metab. Res., 23, 266-270.

Thorens, B., Charron, M.J. and Lodish, H.F. (1990) Molecular physiology of glucose transporters, Diabetes Care, 13, 209-218.

Tranum-Jensen, J., Christiansen, K., Carlsen, J., Brenzel, G., Vinten, J. (1994) Membrane topology of insulin receptors reconstituted into lipid vesicles, J. Membrane Biol., 140, 215-223.

Turner, D.C. and Gruner, S.M. (1992) X-ray diffraction reconstruction of the inverted hexagonal (H_{II}) phase of lipid-water systems, Biochemistry 31, 1340-1355.

Turner, D.C., Gruner, S.M. and Huang, J.S. (1992) Distribution of decane within the unit cell of the inverted hexagonal (H_{II}) phase of lipid-water-decane systems determined by neutron diffraction, Biochemistry, 31, 1356-1363.

Tytler, E.M., Segrest, J.P., Epand, R.M., Nie, S.-Q., Epand, R.F., Mishra, V.K., Venkatachalapathi, Y.V. and Anantharamaiah, G.M. (1993) Reciprocal effects of apolipoprotein and lytic peptide analogs on membranes: cross-sectional molecular shapes of amphipathic α-helices control membrane stability, J. Biol. Chem., 268, 22112-22118.

Ullrich, A., Bell, J.R., Chen, E.Y., Herrera, R., Petruzzelli, L.M., Dull, T.J., Gray, A., Coussens, L., Liao, Y.-C., Tsubokawa, M., Mason, A., Seeburg, P.H., Grunfeld, C., Rosen, O.M. and Ramachandran, J. (1985) Human insulin receptor and its relationship to the tyrosine kinase family of oncogenes, Nature, 313, 756-761.

Wang, C.-C., Sonne, O., Hedo, J.A., Cushman, S.W. and Simpson, I.A. (1983) Insulin-induced internalization of the insulin receptor in the isolated rat adipose cell, J. Biol. Chem., 258, 5129-5134.

Waugh, S.M. and Pilch, P.F. (1989) Insulin binding changes the interface region between α subunits of the insulin receptor, Biochemistry, 28, 2722-2727.

White, M.F., Maron, R. and Kahn, C.R. (1985) Insulin rapidly stimulates tyrosine phosphorylation of a Mr 185,000 protein in intact cells, Nature, 318, 183-186.

White, M.F., Stegmann, E.W., Dull, T.J., Ullrich, A. and Kahn, C.R. (1987) Characterization of an endogenous substrate of the insulin receptor in cultured cells, J. Biol. Chem., 262, 9769-9777.

White, M.F., Shoelson, S.E., Keutmann, H. and Kahn, C.R. (1988) A cascade of tyrosine autophosphorylation in the β-subunit activates the phosphotransferase of the insulin receptor, J. Biol. Chem., 263, 2969-2980.

White, M.F. and Kahn, C.R. (1994) The insulin signaling system, J. Biol. Chem., 269, 1-4.

Whitman, M., Downes, C.P., Keller, M., Keller, T. and Cantley, L. (1988) Type I phosphatidylinositol kinase makes a novel inositol phospholipid, phosphatidylinositol-3-phosphate, Nature 332, 644-646.

Whittaker, J., Okamoto, A.K., Thys, R., Bell, G.I., Steiner, D.F. and Hofmann, C.A. (1987) High-level expression of human insulin receptor cDNA in mouse NIH 3T3 cells, Proc. Natl. Acad. Sci. U.S.A., 84, 5237-5241.

Yamauchi, K. and Pessin, J.E. (1994) Insulin receptor substrate-1 (IRS1) and Shc compete for a limited pool of Grb2 in mediating insulin downstream signaling, J. Biol. Chem., 269, 31107-31114.

Yeagle, P.L. (1989) Lipid regulation of cell membrane structure and function, FASEB J., 3, 1833-1842.

Yip, C.C., Yeung, C.W.T. and Moule, M.L. (1978) Photoaffinity labeling of insulin receptor of rat adipocyte plasma membrane, J. Biol. Chem., 253, 1743-1745.

Zhang, B., Tavare, J.M., Ellis, L. and Roth, R.A. (1991) The regulatory role of known tyrosine autophosphorylation sites of the insulin receptor kinase domain, J. Biol. Chem., 266, 990-996.