Ballas, S. K. (1987) *Comp. Biochem. Physiol. 4*, 837-842.
Benga, G., Popescu, O., Borza, V., & Pop, V. I. (1983) *Cell Biol. Int. Rep. 7*, 807-818.
Benga, G., Popescu, O., Borza, V., Pop, V. I., Muresan, A., Mocsy, I., Brain, A., & Wiggleworth, J. M. (1986) *Eur. J. Cell Biol. 41*, 252-262.
Clark, G. F., Krivan, H. C., Wilkins, T. D., & Smith, D. F. (1987) *Arch. Biochem. Biophys. 257*, 217-229.
Dix, J. A., Ausiello, D. A., Jung, C. Y., & Verkman, A. S. (1985) *Biochim. Biophys. Acta 821*, 243-252.
Durocher, J. R., Payne, R. C., & Conrad, M. E. (1975) *Blood 45*, 11-20.
Fettiplace, R., & Haydon, D. A. (1980) *Physiol. Rev. 60*, 510-550.
Fischbarg, J., Kuang, K., Vera, J. C., Arant, S., Silverstein, S. C., Loike, J., & Rosen, M. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 3244-3247.
Frohlich, O., Gunn, R. B., Gargus, J. J., & Rizzolo, L. J. (1988) *Biophys. J. 53*, 531a (Abstract).
Fukuda, K., Honma, K., Manabe, H., Utsumi, H., & Hamada, A. (1987) *Biochim. Biophys. Acta 926*, 132-138.
Gould, G. W., & Leinhard, G. E. (1989) *Biochemistry 28*, 9447-9452.
Handler, J. S. (1988) *Am. J. Physiol. 255*, F375-F382.
Jennings, M. L., Douglas, S. M., & McAndrew, P. E. (1986) *Am. J. Physiol. 251*, C32-C40.
Kuwahara, M., Berry, C. A., & Verkman, A. S. (1988) *Biophys. J. 54*, 595-602.
Lawrence, W. D., Schoenl, M., & Davis, P. J. (1989) *J. Biol. Chem. 264*, 4766-4768.
Macey, R. I. (1984) *Am. J. Physiol. 246*, C195-C203.
Mlekoday, H. J., Moore, R., & Levitt, D. G. (1983) *J. Gen. Physiol. 81*, 213-220.
Poole, R. C., & Halestrap, A. P. (1988) *Biochem. J. 254*, 385-390.
Rich, G. T., Sch'afi, R. I., Barton, T. C., & Solomon, A. K. (1967) *J. Gen. Physiol. 50*, 2391-2405.
Rosenberg, P. A., & Finkelstein, A. (1978) *J. Gen. Physiol. 72*, 341-350.
Sambrook, J., Fritsch, E. F., & Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd ed., p 7.19, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Shi, L.-B., & Verkman, A. S. (1989) *J. Gen. Physiol. 94*, 1101-1115.
Solomon, A. K., Chasan, B., Dix, J. A., Lukacovic, M. F., Toon, M. R., & Verkman, A. S. (1984) *Ann. N.Y. Acad. Sci. 414*, 79-124.
Steck, T. L., & Kant, J. A. (1974) *Methods Enzymol. 31*, 172-180.
Verkman, A. S. (1989) *Am. J. Physiol. 257*, C837-C850.
Verkman, A. S., Lencer, W., Brown, D., & Ausiello, D. A. (1988) *Nature 333*, 268-269.
Verkman, A. S., Weyer, P., Ausiello, D. A., & Brown, D. (1989) *J. Biol. Chem. 264*, 20608-20613.
Zhang, R., & Verkman, A. S. (1991) *Am. J. Physiol. 260*, 26-34.
Zhang, R., Logee, K., & Verkman, A. S. (1990) *J. Biol. Chem. 265*, 15375-15378.

Action of Insulin in Rat Adipocytes and Membrane Properties†

Richard M. Epand,* Alan R. Stafford, and Maria Teresa Debanne
Department of Biochemistry, McMaster University Health Sciences Centre, 1200 Main Street West, Hamilton, Ontario, Canada L8N 3Z5

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ABSTRACT: Several small peptides inhibit insulin-promoted glucose uptake in rat adipocytes. At 10 μM peptide concentration, the extent of their inhibition of the insulin effect is related to the ability of these peptides to raise the bilayer- to hexagonal-phase transition temperature in model membranes. Hexane and DL-*threo*-dihydrosphingosine lower this phase transition temperature in model membranes, and they promote glucose uptake in adipocytes. There is thus an empirical relationship between the action of membrane additives on glucose uptake in adipocytes and their effect on the hexagonal-phase-forming tendency in model membranes. The most potent of the bilayer-stabilizing peptides tested in this work is carbobenzoxy-D-Phe-L-Phe-Gly. This peptide also inhibits insulin-stimulated protein synthesis in adipocytes. In contrast, DL-*threo*-dihydrosphingosine stimulates protein synthesis. The uptake of [^{125}I]iodoinsulin by adipocytes is inhibited by carbobenzoxy-D-Phe-L-Phe-Gly. The mechanism of action of the bilayer-stabilizing peptides includes inhibition of insulin-dependent protein phosphorylation in adipocytes. The peptides are not specific inhibitors of a single function but are suggested to cause their effects by altering the physical properties of the membrane in a nonspecific manner. These results demonstrate that insulin-dependent functions of rat adipocytes can be modified by membrane additives in a manner predictable from the properties of these additives in model membranes.

Several membrane functions are modulated by the bulk biophysical properties of the membrane (Shinitzky, 1984). There are many such properties that may affect function. Much attention has been given to the modulation of membrane function by changes in the microviscosity or "fluidity" of the membrane. However, other physical properties that may be related to function are the molecular arrangement, stability, and polarity of the membrane–water interface. Subtle changes in these properties, which may have profound effects on function, are difficult to measure directly. However, the bilayer- to hexagonal-phase transition temperature in model

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* To whom correspondence should be addressed.

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membranes is particularly sensitive to the addition of substances that alter the hydrophobic–hydrophilic balance of the membrane (Epand, 1985). Shifts in the bilayer- to hexagonal-phase transition temperature in model membranes upon the incorporation of certain peptides can be compared with the changes these peptides induce in certain functional properties of biological membranes. Thus, although the hexagonal phase is not formed in biological membranes, changes in the relative stability of the bilayer and hexagonal phases upon the addition of additives can be used to predict how these additives will alter certain functions of biological membranes. Several membrane functions have been shown to be correlated with the propensity of the membrane to undergo rearrangement to the hexagonal phase (Yeagle, 1989; Hui & Sen, 1989; Epand, 1990).

A number of Z-dipeptide amides (Z is carbobenzoxy) inhibit insulin-activated glucose uptake in adipocytes (Aiello et al., 1986). There is a good correlation between the inhibitory effect of these peptides on glucose uptake and their ability to stabilize bilayers by raising the bilayer- to hexagonal-phase transition temperature of synthetic phosphatidylethanolamines (Epand et al., 1987). In the present work, we test the generality of the correlation between bilayer stabilization and the inhibition of insulin effects in adipocytes, and we identify insulin-dependent protein phosphorylation as a site of action of these inhibitors. A preliminary report on some of this work has recently appeared (Epand et al., 1990).

Experimental Procedures

Materials. The following chemicals were obtained from commercial sources: 2-deoxy-D-[G-^{3}H]glucose, ICN; Hepes, phloretin, cycloheximide, DL-*threo*-dihydrosphingosine, Z-Gly-Phe-NH_2, and BSA fraction V, Sigma; Z-L-Phe-Gly, Z-Gly-L-Phe, and L-Phe-Gly, Research Plus; L-Phe-L-Met-L-Arg-L-Phe-NH_2, Cambridge Research Biochemicals; Z-D-Phe-L-Phe-Gly, Peptide Institute; dinonyl phthalate, Fluka; silicone oil (high density), Aldrich; collagenase, Boehringer-Mannheim; porcine insulin, Eli Lilly Co.; L-[3,4,5-^{3}H]leucine, (3-[^{125}I]iodotyrosyl^{A14})insulin (porcine), sodium [^{32}P]phosphate, and [γ-^{32}P]ATP, Du Pont–New England Nuclear; Dulbecco's modified Eagle's medium, Gibco.

Isolation of Rat Adipocytes. Adipocytes were isolated according to the method of Rodbell (1964) from the epididymal fat pads of three to five male Sprague-Dawley rats weighing 180–230 g, fed ad libitum. For the ^{32}P-labeling experiments, cells from 24 rats were used. The buffer used was Krebs–Ringer–Hensleit (KRH) buffer, pH 7.4, gassed with 95% O_2:5% CO_2 supplemented with 1% BSA. Glucose (0.36 g/L) was added to the buffer during cell digestion with collagenase but was removed during the washing of the cells for the 2-deoxyglucose uptake experiments.

2-Deoxyglucose Uptake Experiments. This method was adapted from the method of Douen and Jones (1989). Cells were allowed to float to the top of a Falcon 2059 tube, and the volume was adjusted to give a 60% v/v solution in the KRH–BSA buffer. The cells were then gently shaken by hand to evenly disperse them in solution, and 1-mL aliquots were added to plastic centrifuge tubes. The cells were incubated either with or without 1 μM insulin in the presence or absence of various membrane-active agents. The cells were first preincubated for 30 min at 37 °C in a shaking water bath. After this time, 80 μL of the cell suspension (about 160 μg of protein) was transferred to Eppendorf tubes and placed in a water bath for 2 to 3 min to equilibrate. The reaction was started by adding 20 μL of a 0.5 mM 2-deoxyglucose solution containing 25 μCi/mL 2-deoxy[^{3}H]glucose. The final concentration of 2-deoxyglucose was 0.1 mM. The cells were continuously shaken throughout the experiment. At various time points, glucose transport was terminated by adding 0.75 mL of ice-cold phloretin solution (0.15 mM phloretin, 10 mM Hepes, 0.25 M sucrose, pH 7.4). The cells were then floated through 500 μL of a dinonyl phthalate–silicone oil solution (2.5:1 v/v) by microfuging for 30 s. The cells were transferred to scintillation vials by using 1-in. strips of pipe cleaners to scoop the cells from the top of the oil. ACS scintillation fluid was added, and the cells were counted. Nonmediated uptake into adipocytes was monitored by using [U-^{14}C]sucrose. The basal and insulin-stimulated rates of uptake were negligible compared with 2-deoxyglucose uptake. Sucrose has been used as a marker for fluid-phase endocytosis in rat adipocytes (Gibbs & Lienhard, 1984).

Protein Synthesis. The method followed was that of Marshall (1989). The only difference was that we separated the adipocytes after incubation as indicated above for the glucose transport.

Internalization of the Insulin Receptor. The procedure used was that of Marshall (1985) again with the modification of our oil flotation and cell transfer as mentioned above.

^{32}P Loading of Adipocytes. Adipocytes from 24 rats were resuspended in 30 mL and incubated at 37 °C for 80 min in Krebs–Ringer bicarbonate Hepes buffer, pH 7.4, containing sodium [^{32}P]phosphate (0.1 mCi/mL, 9000 Ci/mmol) as previously described (Joost et al., 1987).

Insulin-Dependent Protein Phosphorylation. After ^{32}P loading of the adipocytes, Z-D-Phe-L-Phe-Gly (10^{-4} M) was added to some cell aliquots and the samples were incubated 15 min. Insulin (10^{-5} M) was then added to some aliquots of the adipocyte suspension, and the cells were incubated an additional 25 min at 37 °C with gentle agitation. The cells were then separated by centrifugation and washed at 18 °C with a pH 7.4 buffer containing 20 mM Hepes, 255 mM sucrose, 1 mM EDTA, and 0.1 mM sodium orthovanadate. The cells isolated from the epididymal fat pads of 20 male, 200-g, Sprague-Dawley rats were suspended in 20 mL of this buffer and cooled to 4 °C. The cells were homogenized with 10 strokes of a Thomas grinder spinning at 2600 rpm. The suspension was centrifuged in an SW28 rotor at 28 000 rpm for 3 h at 4 °C. The supernate was retained for analysis of the phosphorylation of cytosolic proteins. The pellet was solubilized in 3 mL of a pH 7.4 buffer containing 50 mM Tris-HCl, 1% Triton X-100, 2 mM sodium orthovanadate, 2 mM EDTA, and 1 mM phenylmethanesulfonyl fluoride (PMSF). Small amounts of insoluble material were removed by centrifugation in a microfuge at 4 °C. This solubilized preparation was further fractionated as described below.

Affinity Chromatography of Insulin Receptor. The insulin receptor was partially purified by passage through a column of Affi-Gel 10–insulin according to a procedure kindly provided to us by Dr. C. C. Yip, University of Toronto. The affinity resin was prepared by washing with 25 mL of ice-cold 2-propanol, followed by washing three times with 25 mL of cold water. The gel was then equilibrated with 100 mL of 0.1 M sodium phosphate buffer containing 6 M urea. The column material was then coupled to insulin by adding 25 mL of the phosphate–urea buffer containing 250 mg of bovine insulin and incubating the mixture overnight at 4 °C with agitation. The modified resin was then poured into a column and washed with 800 mL of the phosphate–urea buffer, 500 mL of 0.1 M sodium acetate, pH 4.5, containing 1 M NaCl and 100 mL of 50 mM Tris-HCl, pH 7.4, containing 1 M NaCl, 0.1% Triton X-100, and 1 mM PMSF. This affinity column was

successfully used by us to purify the insulin receptor from human placenta (T. McCallum and R. M. Epand, unpublished results).

A suspension containing 200 μL of the affinity chromatography material was packed in a small column. The column was washed with 5 mL of the phosphate–urea buffer and then equilibrated with 5 mL of 50 mM Tris-HCl, pH 7.4, containing 0.1% Triton X-100 and 1 mM PMSF. One milliliter of the solubilized pellet, described in the preceding section, was applied to this column. The column eluent was collected and reapplied to the column two more times in an attempt to increase the yield of the insulin receptor. The column was finally washed with 5 mL of 50 mM Tris-HCl, pH 7.4, containing 1 M NaCl, 0.1% Triton X-100, and 1 mM PMSF. The column material was then removed, centrifuged quickly to remove any remaining buffer, suspended in 100 μL of electrophoresis sample buffer (described below), boiled for 3 min, and analyzed by SDS–PAGE.

Immunoprecipitation of Proteins Containing Phosphorylated Tyrosine. To 500 μL of the solubilized pellet (see above) was added 10 μL of a phosphotyrosine antibody solution (Oncogene Science). This antibody preparation contains 100 μg of mouse monoclonal IgG_1 in 1 mL of 0.05 M sodium phosphate buffer, pH 7.4, containing 0.1% sodium azide and 0.1% bovine serum albumin. As a control, a separate 500 μL of the solubilized pellet was mixed with 10 μL of normal mouse serum that had been diluted 100-fold. These mixtures were incubated overnight at 4 °C with shaking. Rabbit anti-mouse serum (11 μL of a 10-fold dilution) was then added to the tubes which were incubated for 2 h at room temperature. The antibodies were then bound to protein A–Sepharose by adding 200 μL of a suspension containing 100 mg of resin and incubating an additional 1 h at room temperature with shaking. The Sepharose was then pelleted by centrifugation and the pellet washed four times with phosphate-buffered saline containing 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, 0.2% sodium azide, and 0.004% sodium fluoride at pH 7.25 as described by Oncogene Science. The pellet was analyzed by SDS–PAGE.

Permeabilized Adipocytes. Adipocyte preparations were permeabilized by incubation at 37 °C with 20 μg/mL digitonin for 15 min similar to the method previously described (Mooney & Anderson, 1989). Insulin (10^{-7} M), Z-D-Phe-L-Phe-Gly (10^{-4} M), or a mixture of both was then added to some aliquots of cell suspensions, and the incubation was continued for an additional 15 min. The permeabilization medium was then removed and replaced by medium with or without insulin (10^{-7} M) and/or Z-D-Phe-L-Phe-Gly (10^{-4} M). Labeling of proteins with ^{32}P was then initiated with the addition of 75 μM [γ-^{32}P]ATP (8 μCi/nmol). After 1 min of incubation at 37 °C, the phosphorylation assays were stopped by floating the adipocytes by centrifugation through oil for 30 s as described above. The cell pellet was quickly frozen in a methanol–dry ice bath. The frozen cell pellet was removed from the top of the microfuge tubes, transferred to new containers, and then disrupted in ice-cold 30 mM Tris-HCl (pH 7.4), 1% Triton X-100, 50 mM NaF, 30 mM sodium phosphate, 10 mM ATP, 1 mg/mL benzamidine, 100 Kallikrein units/mL aprotinin, 1 mg/mL bacitracin, 2 mM vanadate, 1 mM PMSF, 10 mM pyrophosphate, 1 mM *p*-nitrophenyl phosphate, and 10 mM β-glycerophosphate. The insulin receptor was then purified by affinity chromatography and analyzed by SDS–PAGE as described above.

SDS–PAGE. Protein separation by SDS–PAGE was done by the method of Laemmli (1970). Samples of phosphorylated proteins were dissolved in 50–100 μL of 50 mM sodium phosphate, pH 7.0, containing 10% glycerol, 2% 2-mercaptoethanol, 2% SDS, 0.01% bromophenol blue, and 2.4% urea and boiled for 3 min. These samples were then applied to 7.5% or 11.7% acrylamide gels. Gels were dried, and autoradiography was performed at −70 °C with Kodak X-Omat AR film and a Cronex Lightning Plus enhancing screen.

Table I: Bilayer Stabilization and Glucose Uptake

compound	slope[a]	% basal[b]	% insulin[c]
Z-D-Phe-L-Phe-Gly	84 ± 5	112 ± 15	12 ± 15
Z-Gly-L-Phe-NH_2	52 ± 7	78 ± 10	42 ± 15
Z-L-Phe-Gly	34 ± 3	107 ± 12	49 ± 9
Z-Gly-L-Phe	15 ± 3	98 ± 10	70 ± 16
L-Phe-L-Met-L-Arg-L-Phe-NH_2	12 ± 3	141 ± 21	70 ± 19
L-Phe-Gly	1 ± 2	108 ± 14	119 ± 24

[a] Slope of plot of L_α–H_{II}-phase transition temperature of dielaidoylphosphatidylethanolamine vs mole fraction of peptide additive. Units are degrees per mole fraction. Except for L-Phe-L-Met-L-Arg-L-Phe-NH_2, the original data for the slopes are contained in Epand (1986) and Epand et al. (1987). [b] Ratio of the rate of 2-deoxyglucose uptake in rat adipocytes with and without the addition of 10 μM peptide in the absence of 1 μM porcine insulin. % basal = 100(basal with peptide/basal). [c] % insulin = 100[(insulin with peptide − basal)/(insulin − basal)]. Rates of uptake for each case were calculated from the slope of a linear regression line for uptake vs time, including a point at the origin. Results are the average ± SEM of two to four experiments, each run in triplicate for three or four time points for each peptide.

Results

Z-D-Phe-L-Phe-Gly was originally designed as an antiviral peptide (Richardson et al., 1980). We have shown that this peptide is a bilayer stabilizer (Epand, 1986) and have suggested that this property may contribute to its antiviral activity. We find that this peptide inhibits insulin-promoted glucose uptake in rat adipocytes using 1 μM insulin and 10 μM Z-D-Phe-L-Phe-Gly. At 1 μM Z-D-Phe-L-Phe-Gly and 1 μM insulin, there is about 75% of the inhibition observed at 10 μM Z-D-Phe-L-Phe-Gly. The extent of inhibition of insulin-promoted glucose uptake at 1 μM Z-D-Phe-L-Phe-Gly is more variable with different batches of adipocytes than is the inhibition with 10 μM peptide. The IC_{50} of this peptide is therefore probably below 1 μM, but we have compared the action of several peptides at a fixed concentration of 10 μM to have more precise data. The insulin concentration was also varied. Again, the dose–response curve was somewhat dependent on the batch of adipocytes. At 0.1 and 1 nM insulin, there was substantial, but less than maximal, stimulation of 2-deoxyglucose uptake. The effect of 10 μM Z-D-Phe-L-Phe-Gly in inhibiting the insulin-promoted glucose uptake was similar with 1 nM or 1 μM insulin. To avoid batch to batch variation in insulin dose–response curves, we used the higher concentration of 1 μM insulin to measure the effects of the inhibitory peptides. Several other peptides that are bilayer stabilizers also inhibit insulin-promoted glucose uptake (Table I). We use the term bilayer stabilizer only to indicate that these substances stabilize the bilayer phase *relative to* the hexagonal phase. The bilayer-stabilizing activity of these peptides is measured by the extent to which they raise the bilayer- to hexagonal-phase transition temperature of dielaidoylphosphatidylethanolamine and is expressed by the slope parameter, which is the increase in the phase transition temperature per mole fraction of peptide added to the lipid suspension. The extent of inhibition of the insulin effect is directly related to the degree of bilayer stabilization promoted by these peptides. Little effect of these peptides was observed on the basal rate of glucose uptake (Table I). Among the peptides used, Z-D-Phe-L-Phe-Gly is the best bilayer stabilizer and is

Table II: Bilayer Destabilization and Glucose Uptake

compound	glucose uptake[a]	% basal[b]
none	5750 ± 350	100
hexane	9030 ± 610	157 ± 9
threo-dihydrosphingosine	8920 ± 350	155 ± 7

[a] Total counts of 2-deoxyglucose taken up by rat adipocytes with no added drug (none) or with the addition of 10 μM hexane or DL-*threo*-dihydrosphingosine after 15 min of incubation at 37 °C. Mean and SEM of a representative experiment run in triplicate. [b] Ratio of the uptake of 2-deoxyglucose at 15 min with and without the addition of 10 μM hexane or DL-*threo*-dihydrosphingosine.

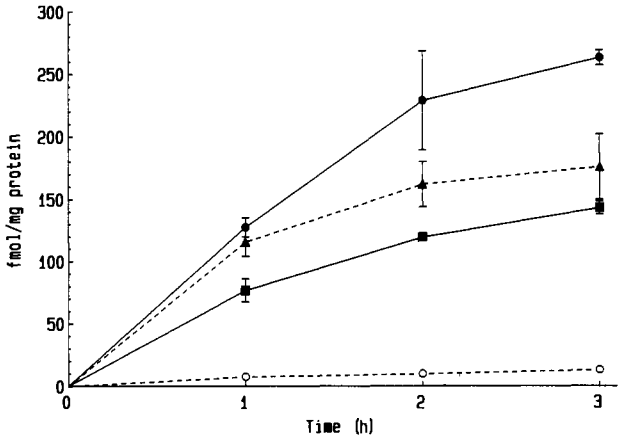

FIGURE 1: Effect of Z-D-Phe-L-Phe-Gly on the rate of uptake of leucine by rat adipocytes: basal, ■; 1 μM insulin, ●; 1 μM insulin + 10 μM Z-D-Phe-L-Phe-Gly, ▲; cycloheximide-inhibited control, ○. Points and error bars represent the mean and SEM of a representative experiment run in triplicate.

also the most effective in inhibiting insulin-promoted glucose uptake. This peptide was selected for several of the studies described below.

Membrane additives can destabilize as well as stabilize membrane bilayers toward hexagonal-phase formation. However, many promoters of the hexagonal phase are cytotoxic or are very insoluble in water and require a solvent carrier that has its own effects even at low concentration. However, we have been able to use hexane and *threo*-dihydrosphingosine to stimulate 2-deoxyglucose uptake into adipocytes (Figure 2). Both alkanes and *threo*-dihydrosphingosine promote hexagonal-phase formation. On the basis of the slope for eicosane (Epand, 1985), hexane should have a slope of −120 K per mole fraction if the hexane completely partitioned into the membrane while *threo*-dihydrosphingosine has a slope of −60 K per mole fraction (Epand, 1987). These agents that favor the hexagonal phase over the bilayer phase increase the basal uptake of 2-deoxyglucose into adipocytes (Table II). The effect of these agents on the insulin-stimulated activity showed great variability among different cell preparations that we were not able to account for.

To determine if the inhibition of insulin stimulation by Z-D-Phe-L-Phe-Gly was limited to glucose uptake, we tested its effect on the stimulation of protein synthesis by insulin. Leucine uptake by adipocytes is limited by the rate of protein synthesis (Marshall, 1989). Z-D-Phe-L-Phe-Gly inhibits insulin-promoted leucine uptake in adipocytes (Figure 1). Z-D-Phe-L-Phe-Gly at 10 μM has no effect on the basal rate of leucine uptake. Elimination of glucose from the incubation media decreased the rate of leucine uptake but did not alter the effects of insulin or Z-D-Phe-L-Phe-Gly (data not shown). This demonstrates that the stimulation of protein synthesis by insulin or the inhibition of that stimulation is not an indirect effect of a change in the rate of glucose uptake. DL-*threo*-

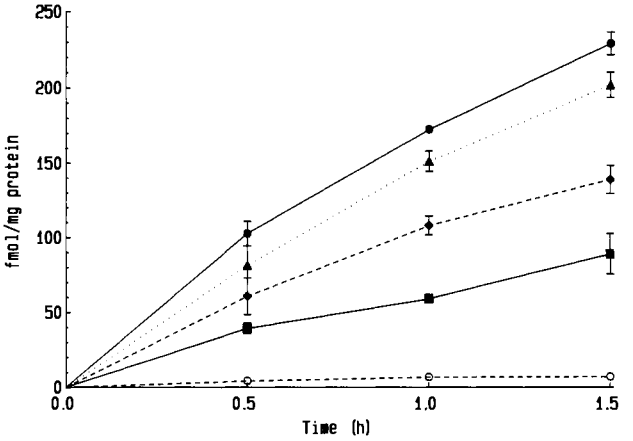

FIGURE 2: Effect of DL-*threo*-dihydrosphingosine on the rate of uptake of leucine by rat adipocytes: basal, ■, 1 μM insulin, ●; 1 μM insulin + 100 μM *threo*-dihydrosphingosine, ▲; 100 μM *threo*-dihydrosphingosine ◆; cycloheximide-inhibited control ○. Points and error bars represent the mean and SEM of triplicate determinations.

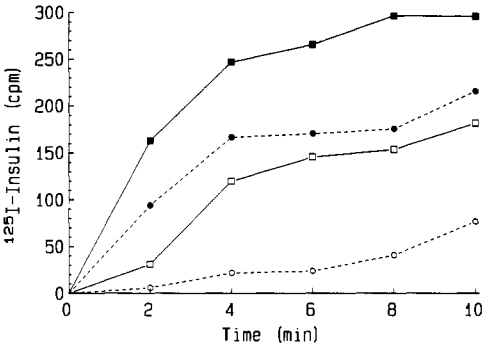

FIGURE 3: Effect of Z-D-Phe-L-Phe-Gly on the uptake of [^{125}I]-iodoinsulin by rat adipocytes: total insulin uptake with (□) or without (■) 10 μM Z-D-Phe-L-Phe-Gly; internalized insulin, not dissociable by acid wash with (○) or without (●) 10 μM Z-D-Phe-L-Phe-Gly.

Dihydrosphingosine has a stimulatory, insulin-like effect on leucine uptake (Figure 2). DL-*threo*-Dihydrosphingosine has little effect on the insulin-stimulated activity. It was not possible to test the effects of hexane because of its cytotoxic effects over the longer incubation times required for measuring leucine uptake.

Insulin promotes the internalization of insulin receptors. [^{125}I]Iodoinsulin was used to monitor the effects of Z-D-Phe-L-Phe-Gly on insulin binding to its receptor and subsequent internalization. Z-D-Phe-L-Phe-Gly inhibits the total uptake of insulin by adipocytes. The major effect was to inhibit the internalization of insulin as measured by the amount of insulin remaining in adipocytes after acid-promoted dissociation of surface-bound insulin (Figure 3).

The effect of Z-D-Phe-Phe-Gly on insulin-dependent protein phosphorylation was measured in intact adipocytes. One of the targets of insulin-dependent protein phosphorylation in the adipocyte is the 97-kDa β-subunit of the insulin receptor (Häring et al., 1982). Autoradiograms of the membrane fraction of ^{32}P-labeled adipocytes that had been purified by affinity chromatography showed a single major labeled band at 97 kDa, corresponding to the β-subunit of the insulin receptor (Figure 4). There is also a second band of lower molecular mass that may be a degradation product of the insulin receptor. Densitometry tracings show a 2-fold stimulation of the phosphorylation of the 97-kDa band by insulin and a 75% inhibition of this stimulation by Z-D-Phe-Phe-Gly. Comparable results were obtained with the anti-phosphotyrosine-immunoprecipitated fraction, although in this case there were several other bands in addition to the 97-kDa band.

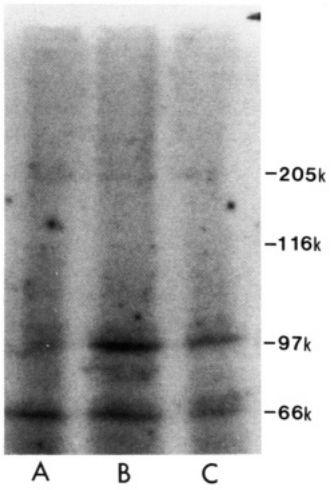

FIGURE 4: Autoradiogram of affinity-purified insulin receptors from sodium [^{32}P]phosphate labeled adipocytes: lane A, basal; lane B, 10^{-5} M insulin; lane C, 10^{-5} M insulin and 10^{-4} M Z-D-Phe-L-Phe-Gly.

Table III: Inhibition of Insulin-Dependent Protein Phosphorylation by Z-D-Phe-Phe-Gly

M_r × 10^{-3}	possible identification	% stimulation by insulin (10^{-5} M)	% inhibition of insulin stimulation by Z-D-Phe-L-Phe-Gly (10^{-4} M)
250	acetyl-CoA carboxylase	88	40
125	ATP citrate lyase	250	27
31	ribosomal S_6	350	70
21.5	phosphoprotein phosphatase inhibitor or pp24	100	100

Similar results were also obtained with affinity-purified membrane preparations from [γ-^{32}P]ATP-labeled permeabilized adipocytes. For this experiment, we again found a 2-fold stimulation of labeling of the 97-kDa band with 90% of this increased labeling being inhibited by 10^{-4} M Z-D-Phe-Phe-Gly. The intensity of labeled bands was weaker in this case compared with the intensity of the bands from the ^{32}P-labeled adipocytes.

There have been many studies identifying targets of insulin-dependent protein phosphorylation (Denton et al., 1981; Avruch et al., 1982; Klip & Douen, 1989; Del Vecchio & Pilch, 1989). A number of proteins are known to be phosphorylated in an insulin-dependent fashion. Using a lysate from cells that had been loaded with sodium [^{32}P]phosphate, we found that several of the insulin-dependent protein phosphorylations were inhibited by Z-D-Phe-Phe-Gly (Figure 5). Several of the bands that showed insulin-dependent phosphorylation had molecular masses coincident with known targets of insulin phosphorylation. Their putative identification and the stimulation of their phosphorylation by insulin as well as the inhibition of this effect by Z-D-Phe-Phe-Gly are summarized in Table III. The lower molecular mass 31- and 21.5-kDa bands show particularly large inhibition.

Discussion

The physical state of membrane lipids can modify glucose uptake and insulin action in adipocytes (Amatruda & Finch, 1979; Sandra & Fyler, 1981). Alcohols that increase membrane fluidity inhibit basal and insulin-stimulated glucose transport (Hutchinson et al., 1985). Aiello et al. (1986) had shown that several Z-dipeptide amides are inhibitors of insulin-promoted glucose uptake in adipocytes. They also showed that these peptides have many effects on membranes, including the inhibition of cytochalasin B binding to red cells and alteration of red cell shape (Aiello et al., 1986) as well as inhibition of myoblast fusion (Couch & Strittmatter, 1983), intracellular vesicular transport (Strous et al., 1988), and exocytosis (Baxter et al., 1983; Mundy & Strittmatter, 1985). It is possible that the Z-dipeptide amides affect so many diverse membrane-related properties because they alter the bulk biophysical properties of the membrane.

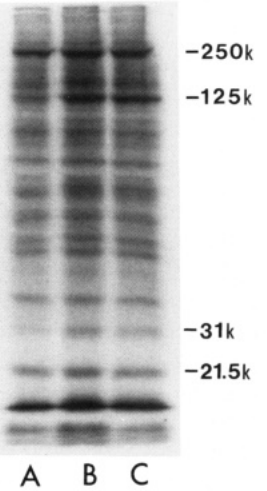

FIGURE 5: Autoradiogram of the cell lysate from sodium [^{32}P]-phosphate labeled adipocytes. Lanes A–C are as for Figure 4.

The extent to which peptides inhibit insulin action in adipocytes is correlated with their ability to stabilize model membranes against hexagonal-phase formation (Table I). In addition to the Z-dipeptide amides, Z-D-Phe-L-Phe-Gly and Z-L-Phe-Gly are also inhibitors of insulin-promoted glucose uptake (Table I). Thus peptides with different amino acid composition, sequence, and charge can affect this activity.

In addition to bilayer stabilizers inhibiting insulin action, several substances that promote hexagonal-phase formation have insulin-like effects. Sphingosine (Epand, 1987), sorbitol (Bryszewska & Epand, 1988), and diacylglycerols (Epand, 1985) are all hexagonal-phase promoters. Sphingosine, although it partially inhibits insulin-activated glucose uptake in adipocytes, has a stimulatory insulin-like action increasing the basal rate of glucose uptake (Robertson et al., 1989). High concentrations of sorbitol increase glucose transport by inducing the translocation of glucose transporters to the cell surface (Toyoda et al., 1986). Diacylglycerols also stimulate glucose transport in adipocytes and may be a physiological activator of this process by a mechanism independent of protein kinase C (Strålfors, 1988). *threo*-Dihydrosphingosine and hexane are two other hexagonal-phase promoters that increase the rate of glucose uptake in adipocytes (Table II). Thus, a number of different substances that lower the bilayer- to hexagonal-phase transition temperature in model membranes stimulate glucose uptake. This is in contrast to bilayer stabilizers that inhibit insulin-promoted glucose uptake. Because of the structural diversity of these compounds, the correlation of their effect on glucose uptake in adipocytes with their effects on model membranes, and their relatively low potency, it seems likely that they act by altering some bulk biophysical property of the membrane. However, we cannot exclude the possibility that the correlation is fortuitous and that they are binding to a specific site on a protein or proteins.

There are many possible sites of action of these agents that modulate insulin function. The effect of Z-D-Phe-L-Phe-Gly and DL-*threo*-dihydrosphingosine is not limited to effects on glucose uptake, but similar results are also found with protein synthesis (Figures 1 and 2). Previously, Z-Gly-Phe-NH_2 was

shown to inhibit protein synthesis in hepatoma cells (Strous et al., 1988). This suggests that one of the sites of action may be at the level of an insulin-signaling mechanism since two different targets of insulin action, i.e., translocation of glucose transporters from endosomes to the plasma membrane (Baly & Horuk, 1988; Kono, 1988) and increased transcriptional rates at the ribosome (Marshall, 1989), are affected. This would also explain why insulin-stimulated rates are decreased by the peptides but the basal rate is unaffected (Table I).

Insulin-signaling mechanisms are still not completely understood (Epand & Bryszewska, 1989), but there is much evidence that the tyrosine kinase activity of the insulin receptor is the first step in insulin signaling (Rosen, 1987; White & Kahn, 1988). Selective inhibitors of insulin receptor tyrosine kinase block insulin-promoted glucose uptake in adipocytes (Saperstein et al., 1989). The tyrosine kinase activity of the insulin receptor is modulated by its lipid environment (Ginsberg et al., 1981; Lewis & Czech, 1987). We find that the bilayer stabilizer Z-D-Phe-L-Phe-Gly inhibits insulin receptor autophosphorylation (Figure 4). The correlation between the lipid-phase preference of additives and their effect on insulin-dependent mechanisms is similar to the case of protein kinase C in which bilayer stabilizers are inhibitors and hexagonal-phase promoters are activators of enzymatic activity (Epand & Lester, 1990). However, with protein kinase C, the electric charge of the additive is also important, with positively charged amphiphiles being inhibitors and negatively charged amphiphiles being activators (Epand & Lester, 1990). This is not the case for the effect of the negatively charged Z-D-Phe-L-Phe-Gly on the autophosphorylation of the insulin receptor. *threo*-Dihydrosphingosine is an inhibitor of protein kinase C (Hannun et al., 1986) because of its positive charge (Bottega et al., 1989), but it has insulin-like effects on adipocytes (Table II and Figure 2) as would be expected for a hexagonal-phase promoter (Epand & Lester, 1990). Thus, for the modulation of insulin activity, only lipid-phase preference in model membranes, and not charge, is correlated with biological activity.

It is possible that insulin signaling results, at least in part, from an increased hexagonal-phase propensity in biological membranes. This could account for the observation that insulin activates protein kinase C and causes its translocation into membranes (Egan et al., 1990). However, this is likely to be a consequence of insulin signaling rather than the mechanism of signaling since protein kinase C inhibitors like sphingosine have insulin-like effects. Insulin may activate protein kinase C through the stimulation of diacylglycerol production (Cooper et al., 1990) or by additional mechanisms that make the membrane more prone to hexagonal-phase formation.

Activation of the insulin receptor promotes the phosphorylation of a number of protein substrates. Z-D-Phe-L-Phe-Gly inhibits the insulin-dependent phosphorylation of different target proteins to varying degrees. The pathways and functions of insulin-dependent protein phosphorylations are complex and have not been completely elucidated (Czech et al., 1988). It has been shown that there is not a linear relationship between the activation of the insulin receptor tyrosine kinase and the stimulation of glucose transport since an insulin analogue gives partial activation of the kinase but full activation of glucose transport (Joost et al., 1989).

There may also be other effects of the peptides on membrane properties independent of insulin signaling. Glucose transport is increased because of a recruitment of glucose transporters resulting from the fusion of endosomes to the plasma membrane (Kono, 1988). There are many examples where the propensity toward hexagonal-phase formation is increased together with an increase in the rate of membrane fusion [c.f. Siegel et al. (1989) and Tournois et al. (1990)]. It is thus reasonable to expect that bilayer stabilizers will inhibit the recruitment of glucose transporters. In addition, Z-D-Phe-L-Phe-Gly inhibits the internalization of the insulin receptor (Figure 3), a process independent of receptor autophosphorylation (Backer et al., 1989). The insulin receptor, like the glucose transporter, undergoes cycling between the plasma membrane and endosomes (Marshall, 1988). Bilayer stabilizers inhibit viral membrane fusion (Cheetham et al., 1990) and may also prevent the deformations required for both endocytosis and exocytosis. The receptor-mediated endocytosis of the asialoglycoprotein receptor was also shown to be inhibited by Z-Gly-Phe-NH_2 (Strous et al., 1988). A limited observation on the effects of 5 mM concentrations of Z-Gly-Phe-NH_2 and Z-Gly-Leu-NH_2 showed increased amounts of internalized insulin in adipocytes after 60 min (Jochen & Berhanu, 1986). This latter observation may be due to the high peptide concentrations and the long times used.

It has also been demonstrated that Z-Gly-Leu-NH_2 inhibits the elevation of intracellular Ca^{2+} levels and that this effect, rather than membrane fusion, may inhibit catecholamine secretion (Lelkes & Pollard, 1987). However, Z-Gly-Leu-NH_2 will inhibit catecholamine secretion even at elevated intracellular calcium levels (Lelkes & Pollard, 1987). Agents such as the bilayer-stabilizing peptides, which modify the physical properties of membranes, will affect a number of membrane-related functions. Therefore, it is not surprising that Ca^{2+} fluxes across membranes are also modulated. However, if the same effect occurred in adipocytes, it would result in Z-Gly-Leu-NH_2 stimulating, rather than inhibiting, glucose transport since elevated intracellular Ca^{2+} levels decrease insulin-promoted glucose uptake (Draznin et al., 1989).

It is also known that glucose transport is influenced by the lipid environment (Carruthers & Melchior, 1988). The glucose transporter from rat adipocytes has been reconstituted in lipid vesicles and found to be inhibited by Z-Gly-L-Phe-NH_2 (Wheeler, 1989). The concentration of peptide required for this inhibition is about 100-fold higher than that required to inhibit glucose transport in adipocytes. Nevertheless, this may be an additional site of action, partially contributing to the observed inhibition in the cellular system.

Thus, as might be expected for a series of compounds that were not designed for a specific target, they have several sites of action. Since the compounds tested are all hydrophobic and sparcely soluble in water, it is likely that much of their action will be on membranes. The effects can be explained by alterations in the physical properties of membranes and are correlated with effects observed in model membranes. It is thus possible to modulate cellular responses in a predictable manner by altering the bulk biophysical characteristics of the cell membrane.

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References

Aiello, L. P., Wessling-Resnick, M., & Pilch, P. F. (1986) *Biochemistry 25*, 3944–3950.

Amatruda, J. M., & Finch, E. D. (1979) *J. Biol. Chem. 254*, 2619–2625.
Avruch, J., Alexander, M. C., Palmer, J. L., Pierce, M. W., Nemenoff, R. A., Blackshear, P. J., Tipper, J. P., & Witters, L. A. (1982) *Fed. Proc. 41*, 2629–2633.
Backer, J. M., Kahn, C. R., & White, M. F. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3209–3213.
Baly, D. L., & Horuk, R. (1988) *Biochim. Biophys. Acta 947*, 571–590.
Baxter, D. A., Johnston, D., & Strittmatter, W. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4174–4178.
Bottega, R., Epand, R. M., & Ball, E. H. (1989) *Biochem. Biophys. Res. Commun. 164*, 102–107.
Bryszewska, M., & Epand, R. M. (1988) *Biochim. Biophys. Acta 943*, 485–492.
Carruthers, A., & Melchior, D. L. (1988) *Annu. Rev. Physiol. 50*, 257–271.
Cheetham, J. J., Epand, R. M., Andrews, M., & Flanagan, T. D. (1990) *J. Biol. Chem. 265*, 12404–12409.
Cooper, D. R., Hernandez, H., Kuo, J. Y., & Farese, R. V. (1990) *Arch. Biochem. Biophys. 276*, 486–494.
Couch, C. B., & Strittmatter, W. J. (1983) *Cell (Cambridge, Mass.) 32*, 257–265.
Czech, M. P., Klarlund, J. K., Yagaloff, K. A., Bradford, A. P., & Lewis, R. E. (1988) *J. Biol. Chem. 263*, 11017–11020.
Del Vecchio, R. L., & Pilch, P. F. (1989) *Biochim. Biophys. Acta 986*, 41–46.
Denton, R. M., Brownsey, R. W., & Belsham, G. J. (1981) *Diabetologia 21*, 347–362.
Douen, A. G., & Jones, M. N. (1989) *Biochim. Biophys. Acta 1010*, 363–368.
Draznin, B., Lewis, D., Houlder, N., Sherman, N., Adamo, M., Garvey, W. T., LeRoith, D., & Sussman, K. (1989) *Endocrinology 125*, 2341–2349.
Egan, J. J., Saltis, J., Wek, S. A., Simpson, I. A., & Londos, C. (1990) *Proc. Natl. Acad. Sci. U.S.A. 87*, 1052–1056.
Epand, R. M. (1985) *Biochemistry 24*, 7092–7095.
Epand, R. M. (1986) *Biosci. Rep. 6*, 647–653.
Epand, R. M. (1990) *Biochem. Cell Biol. 68*, 17–23.
Epand, R. M., & Bryszewska, M. (1989) in *Comprehensive Medicinal Chemistry* (Emmett, J. C., Ed.) Vol. 3, pp 901–923, Pergamon Books Ltd., Oxford, England.
Epand, R. M., & Lester, D. S. (1990) *Trends Pharmacol Sci. 11*, 317–320.
Epand, R. M., Lobl, T. J., & Renis, H. E. (1987) *Biosci. Rep. 7*, 745–749.
Epand, R. M., Stafford, A. R., Abbott, L., & Gorton, L. (1990) in *Peptides: Chemistry, Structure, and Biology* (Rivier, J. E., & Marshall, G. R., Eds.) pp 691–693, ESCOM, Leiden, The Netherlands.
Gibbs, E. M., & Lienhard, G. E. (1984) *J. Cell. Physiol. 121*, 569–575.
Ginsberg, B. H., Brown, T. J., Simon, I., & Spector, A. A. (1981) *Diabetes 30*, 773–780.
Hannun, Y. A., Loomis, C. R., Merrill, A. H., Jr., & Bell, R. M. (1986) *J. Biol. Chem. 261*, 12604–12609.
Häring, H.-U., Kasuga, M., & Kahn, C. R. (1982) *Biochem. Biophys. Res. Commun. 108*, 1538–1545.
Hui, S. W., & Sen, A. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 5825–5829.
Hutchinson, B. T., Hyslop, P. A., Kuhn, C. E., & Sauerheber, R. D. (1985) *Biochem. Pharmacol. 34*, 1079–1086.
Jochen, A. L., & Berhanu, P. (1986) *J. Cell Biol. 103*, 1807–1816.
Joost, H. G., Weber, T. M., Cushman, S. W., & Simpson, I. A. (1987) *J. Biol. Chem. 262*, 11261–11267.
Joost, H. G., Göke, R., Schmitz-Salue, C., Steinfelder, H. J., & Brandenburg, D. (1989) *Biochem. Pharmacol. 38*, 2269–2277.
Klip, A., & Douen, A. G. (1989) *J. Membr. Biol. 111*, 1–23.
Kono, T. (1988) *Vitam. Horm. 44*, 103–154.
Laemmli, U. K. (1970) *Nature 227*, 680–685.
Lelkes, P. I., & Pollard, H. B. (1987) *J. Biol. Chem. 262*, 15496–15505.
Lewis, R. E., & Czech, M. P. (1987) *Biochem. J. 248*, 829–836.
Marshall, S. (1985) *J. Biol. Chem. 260*, 13517–13523.
Marshall, S. (1988) in *Insulin Receptors, Part A: Methods for the Study of Structure and Function* (Kahn, C. R., & Harrison, L. C., Eds.) pp 59–82, A. R. Liss, Inc., New York.
Marshall, S. (1989) *J. Biol. Chem. 264*, 2029–2036.
Mooney, R. A., & Anderson, D. L. (1989) *J. Biol. Chem. 264*, 6850–6857.
Mundy, D. I., & Strittmatter, W. J. (1985) *Cell (Cambridge, Mass.) 40*, 645–656.
Richardson, C. D., Scheid, A., & Choppin, P. W. (1980) *Virology 105*, 205–222.
Robertson, D. G., Di Girolamo, M., Merrill, A. H., Jr., & Lambeth, J. D. (1989) *J. Biol. Chem. 264*, 6773–6779.
Rodbell, M. (1964) *J. Biol. Chem. 239*, 375–380.
Rosen, O. M. (1987) *Science 237*, 1452–1458.
Sandra, A., & Fyler, D. J. (1981) *Am. J. Physiol. 241*, E281–E290.
Saperstein, R., Vicario, P. P., Strout, H. V., Brady, E., Slater, E. E., Greenlee, W. J., Ondeyka, D. L., Patchett, A. A., & Hangauer, D. G. (1989) *Biochemistry 28*, 5694–5701.
Shinitzky, M. (1984) *Physiology of Membrane Fluidity*, Vols. I and II, CRC Press, Boca Raton, FL.
Strålfors, P. (1988) *Nature 335*, 554–556.
Strous, G. J., van Kerkhof, P., Dekker, J., & Schwartz, A. L. (1988) *J. Biol. Chem. 263*, 18197–18204.
Tournois, H., Fabrie, C. H. J. P., Burger, K. N. J., Mandersloot, J., Hilgers, P., van Dalen, H., de Gier, J., & de Kruijff, B. (1990) *Biochemistry 29*, 8297–8307.
Toyoda, N., Robinson, F. W., Smith, M. M., Flanagan, J. E., & Kono, T. (1986) *J. Biol. Chem. 261*, 2117–2122.
Wheeler, T. J. (1989) *Biochim. Biophys. Acta 979*, 331–340.
White, M. F., & Kahn, C. R. (1988) in *Insulin Receptors, Part A: Methods for the Study of Structure and Function* (Kahn, C. R., & Harrison, L. C., Eds.) pp 125–145, A. R. Liss, Inc., New York.
Yeagle, P. L. (1989) *FASEB J. 3*, 1833–1842.