Dipeptide Metalloendoprotease Substrates Are Glucose Transport Inhibitors and Membrane Structure Perturbants†

Lloyd P. Aiello, Marianne Wessling-Resnick, and Paul F. Pilch*,‡
Department of Biochemistry, Boston University School of Medicine, Boston, Massachusetts 02118
Received July 8, 1985; Revised Manuscript Received December 31, 1985

ABSTRACT: Dipeptide substrates for metalloendoproteases have previously been shown to block biological processes requiring membrane fusion. Thus, we employed such compounds as potential inhibitors of the insulin-dependent activation of glucose transport in fat cells. This event is thought to involve vesicle movement from an intracellular site to the cell surface and would therefore require membrane fusion during the activation step. We find that synthetic dipeptides which are metalloendoprotease substrates rapidly and reversibly inhibit insulin-activated glucose oxidation in a dose-dependent manner but exhibit essentially no effect on basal levels. A similar result is obtained when glucose transport is measured directly in intact fat cells, in metabolically poisoned cells, and in isolated membrane vesicles derived from insulin-activated or untreated fat cells. That is, the dipeptide substrates inhibit insulin-activated glucose uptake to a greater extent than basal transport, and they do so even when vesicle translocation and fusion have already taken place as in ATP-depleted cells and isolated vesicles. Onset of transport inhibition after dipeptide addition is rapid, but not instantaneous, with a $t_{1/2}$ of 15–30 s. The metalloendoprotease substrates also inhibit glucose uptake and cytochalasin B binding in human erythrocytes but not in human placental microsomes. Finally, light microscopic examination of substrate-treated red cells reveals marked cupping and/or echinolation of the cell membrane. We conclude the following from these observations: (1) Metalloendoprotease substrates are inhibitors of adipocyte glucose transport. This may be due to a direct effect on the transport protein or an effect on the membrane environment of the transporter. (2) The insulin-activated glucose transport system and/or its environment is more sensitive to the dipeptide substrates than the basal system. (3) The use of dipeptide metalloendoprotease substrates as presumptive specific inhibitors of membrane fusion events is problematical.

Membrane fusion is required for numerous constitutive and regulated cellular processes including endo- and exocytosis. The molecular details of membrane fusion are unknown, but it has been postulated in a recent review that proteolytic cleavage of membrane proteins may be required for fusion to occur (Lucy, 1984). Data supporting this hypothesis derive in part from the study of paramyxovirus (Scheid & Chopin, 1974) and influenza virus (Bosch et al., 1981) where proteolytic cleavage of a viral membrane glycoprotein is required for fusion of the virus with the host cell. Endogenous cellular proteins that could play a role in membrane fusion analogous to that of the viral glycoproteins have not been identified. However, the involvement of a cellular protease in myoblast fusion (Couch & Strittmatter, 1983), in mast cell degranulation (Mundy & Strittmatter, 1985), and in adrenal chromaffin granule secretion (Baxter et al., 1983; Mundy & Strittmatter, 1985) has recently been postulated by Strittmatter and colleagues. These investigators tested a variety of synthetic dipeptides as inhibitors of the biological processes just mentioned, all of which require membrane fusion. They found that only those peptides of the general structure Cbz-Gly-X-NH_2[1] (Cbz = carbobenzoxy, X = various aliphatic and aromatic amino acids) that inhibit metalloendoproteases in vitro (Feder, 1967; Morihara, 1974; Mumford et al., 1980; Kam et al., 1979; Orlowski & Wilk, 1981) will also inhibit granule secretion and myoblast fusion. These processes are known to require calcium ions (Douglas, 1977) as do the metalloendoproteases. Dipeptides that are not metalloendoprotease substrates in vitro are unable to affect membrane fusion events in vivo as demonstrated in the experiments cited above.

There is considerable evidence that suggests activation of glucose transport by insulin in fat cells (Cushman & Wardzala, 1980; Karnielli et al., 1981; Suzuki & Kono, 1980; Kono et al., 1981) and in muscle (Wardzala & Jeanrenaud, 1981, 1983) involves the movement of transport proteins to the cell surface from the cell interior. The model of transport activation that emerges from these studies would require the reversible fusion of intracellular vesicles with the plasma membrane in a process analogous to endo- and exocytosis. Exocytosis occurring in neurosecretory, endocrine, exocrine, and mast cells has been documented to require a calcium-dependent fusion of intracellular vesicles to plasma membrane (Douglas, 1977). Evidence for a calcium requirement in the insulin-dependent activation of glucose transport has been published (Shechter, 1984), and we have recently isolated from rat adipocytes a population of small intracellular vesicles enriched in glucose transporters (P. Pilch, unpublished observations). Thus, we reasoned that calcium-dependent metalloendoprotease activity might be required for the fusion of transporter-rich vesicles with the plasma membrane following exposure of cells to insulin. We found that the synthetic substrates of metalloendoproteases which were active as inhibitors of other cell fusion processes were also effective in inhibiting insulin-activated glucose transport. However, they appeared to act independently of membrane fusion events and may directly inhibit glucose transport. Our data demonstrated two biological activities of the relevant dipeptides other than their postulated role as metalloendoprotease substrates, namely,

† This research was supported by Grant AM-30425 from the National Institutes of Health.
‡ Recipient of Research Career Development Award AM-01352 from the U.S. Public Health Service.

[1] Abbreviations: Cbz, carbobenzoxy; EDTA, ethylenediaminetetraacetic acid; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/86/0425-3944$01.50/0

transport inhibition and effects on cell shape. Thus, caution is warranted in using these compounds as presumed specific inhibitors of membrane fusion events.

Materials and Methods

Preparation of Adipocytes. White fat cells were isolated from rat epididymal adipose tissue essentially by the method of Rodbell (1964). Briefly, fat pads, excised from 150–250-g male Sprague-Dawley rats, were finely minced in Krebs–Ringer phosphate buffer, pH 7.4, containing 3% bovine serum albumin and subjected to enzymatic digestion with 1.5 mg of collagenase/g of adipose tissue to a final concentration of 1 mg/mL. After incubation for 1 h at 37 °C, adipocytes were filtered through nylon mesh and washed twice with Krebs–Ringer phosphate, pH 7.4, also with 3% bovine serum albumin present, by 8-s centrifugation at 1000*g*. The cells were suspended in the same buffer and divided into two aliquots, one of which was treated with 3.5×10^{-8} M insulin, and both were incubated for 15 min at 37 °C. For every preparation, basal state, as well as insulin response, was monitored under appropriate experimental conditions as a control.

Preparation of Adipocyte Plasma Membrane Vesicles. Isolated adipocytes were washed with 20 mM Tris-HCl, 255 mM sucrose, and 1 mM EDTA, pH 7.4, with centrifugation at 1000*g* for 30 s, resuspended in the same buffer (1:1 v/v), cooled to 17 °C, and homogenized, employing a motor-driven Potter–Elvehjem tissue grinder. The homogenate was placed on a 1.12 M sucrose cushion in 20 mM Tris-HCl and 1 mM EDTA, pH 7.4. The membrane band including light and heavy microsomes as well as plasma membrane (Karnielli et al., 1981) was isolated on top of the 1.12 M sucrose cushion after centrifugation at 101000*g* for 1 h at 4 °C. The membrane vesicles were washed with 20 mM Tris-HCl, 255 mM sucrose, and 1 mM EDTA, pH 7.4, by centrifugation at 30000*g* for 15 min at 4 °C to pellet the plasma membrane, leaving microsomal membranes in the supernatant (Karnielli et al., 1981). Prior to glucose transport assays, vesicles were washed into Krebs–Ringer phosphate buffer, pH 7.4, and resuspended in this same buffer.

Glucose Oxidation Assays. The oxidation of D-[1-^{14}C]-glucose to $^{14}CO_2$ in isolated adipocytes was measured as described by Fain et al. (1967). Adipocytes were subjected to appropriate experimental conditions, $(1.5–3) \times 10^5$ cells were placed in an assay medium containing D-[1-^{14}C]glucose (0.2 mM, 0.15 μCi) in Krebs–Ringer phosphate buffer, pH 7.4, with 3% bovine serum albumin, and each tube was sealed. After incubation for 30 min at 37 °C, the assay was terminated by the addition of 200 μL of 1 N sulfuric acid, and phenethylamine-soaked filter paper suspended above the cells adsorbed released $^{14}CO_2$. Radioactivity was measured by liquid scintillation spectrophotometry.

3-O-Methyl-D-[^{3}H]glucose Uptake in Isolated Adipocytes. Transport rates for the nonmetabolizable glucose analogue 3-*O*-methyl-D-glucose were determined in fat cells, with or without prior exposure to insulin and after exposure to various dipeptides. Transport was initiated upon the mixing of 350 μL of adipocytes [$(3–6) \times 10^5$ cells] in Krebs–Ringer phosphate buffer, pH 7.4, containing 3% bovine serum albumin with 50 μL of buffer containing 2 μCi of 3-*O*-methyl-D-[^{3}H]glucose and 0.4 μCi of L-[^{14}C]glucose (0.1 mM final concentration of each hexose). At appropriate time points, uptake was terminated by the rapid addition of cytochalasin B, a specific inhibitor of transport, to a nominal final concentration of 0.37 mM. This high concentration of cytochalasin was used to ensure complete arrest of transport and, in fat cell preparations, showed no evidence of insolubility. Cells were separated from media by the oil flotation method as described (Foley et al., 1978), and radioactivity was measured by liquid scintillation spectrophotometry. Nonspecific uptake and cell-associated radioactivity for individual points were substracted as L-[^{14}C]glucose content to yield specific 3-*O*-methyl-D-[^{3}H]glucose uptake. Initial experiments determined the time course of 3-*O*-methyl-D-[^{3}H]glucose uptake under all experimental conditions. These conditions included uptake over a 60-min period to determine the equilibrium value for specific uptake in the presence and absence of insulin, and plus or minus each dipeptide. For all conditions on a given day, equilibrium glucose uptake was the same regardless of treatment. Time points of 5 s for insulin-treated cells, 10 s for insulin-activated, substrate-treated cells, and 20 s for preparations showing basal activity were selected as near one-third maximal uptake. Measurements of uptake at these specific times, as well as a 60-min equilibrium point, were subsequently made in order to calculate rates of 3-*O*-methyl-D-[^{3}H]glucose transport as described by Foley et al. (1978).

D-[^{3}H]Glucose Uptake in Adipocyte Plasma Membrane Vesicles. Fat cell plasma membrane vesicles were dispersed in Krebs–Ringer phosphate buffer, pH 7.4, and 90-μL aliquots were mixed with 10 μL of buffer containing 2 μCi of D-[^{3}H]glucose and 0.4 μCi of L-[^{14}C]glucose (final concentration 0.1 mM). At appropriate time points, transport was terminated by the addition of 3 mL of ice-cold buffer containing 2 mM $HgCl_2$ and rapid vacuum filtration of this medium through a membrane filter (Millipore, 0.45 μm), followed by two more 3-mL washes of stopping solution. Radioactivity on the filters was measured by liquid scintillation spectrophotometry, and nonspecific association and uptake were subtracted as L-[^{14}C]glucose content in order to determine D-[^{3}H]glucose uptake in individual points. Preliminary time course data, similar to those described for isolated adipocyte 3-*O*-methyl-D-[^{3}H]glucose transport experiments, determined that a 2-s time point for vesicles isolated from insulin-treated cells (3 s if these also were exposed to substrate peptides) and a 5-s time point for vesicles prepared from control cells were representative of one-third maximal uptake. Measurement of uptake at these times, along with a 60-min equilibrium time point, enabled rate calculations to be made as described above. Equilibrium uptake values were invariant regardless of conditions.

Human Placental Microsomes. Placental microsomal membranes and membrane vesicles were prepared exactly as described (Wessling & Pilch, 1984). Briefly, fresh term placenta was extensively washed in phosphate-buffered saline. The amnion and chorion were removed, and the remaining tissue was homogenized in a Tekmar "tissuemizer" in buffer consisting of 50 mM Hepes, 0.25 M sucrose, 1 mM benzamidine, and 1 mM phenylmethanesulfonyl fluoride, pH 7.6. The homogenate was centrifuged at 10000*g* for 30 min and the pellet discarded. The supernatant was adjusted to 0.1 M NaCl and 0.2 mM $MgCl_2$ and then spun for 45 min at 40000*g*. The resultant pellet was washed twice by centrifugation for 45 min at 40000*g* either in homogenization buffer (minus phenylmethanesulfonyl fluoride) or in 50 mM Hepes, pH 7.6. The former preparations were used for transport assays and the latter for cytochalasin B binding.

Uptake of D-[^{3}H]glucose into placental microsomal vesicles was performed exactly as for adipocyte membrane vesicles. The time course for uptake was first determined, and subsequent assay points approximated initial velocity as described above. Approximately 100 μg of protein [determined by the

method of Lowry et al. (1951)] was used for each triplicate assay point. [^{3}H]Cytochalasin B binding to placental microsomes was also performed exactly as previously described (Wessling & Pilch, 1984).

Human Red Blood Cells. Freshly drawn blood was made 3.8% in trisodium citrate and then washed 4 times in phosphate-buffered saline at 4 °C by centrifugation at 4500*g*. Red cells were suspended to a 20% hematocrit in the same buffer, and glucose uptake was performed at 4 °C. Uptake was initiated by the addition of 0.5 mM D-[^{3}H]glucose (2.5 μCi/assay point). At various times, 1-mL aliquots were withdrawn into 10 mL of stopping solution consisting of 1.25 mM KI, 1 mM $HgCl_2$, and 50 μM phloretin. The cells were pelleted as described above, washed once in stopping solution, and then suspended in isotonic saline solution made 6% in trichloroacetic acid. The subsequent precipitate was centrifuged for 10 min at 1800*g*, and a portion of the supernatant was assayed for radioactivity in a liquid scintillation counter. Data analysis was performed as described previously for adipocytes except that the presence at 50 μM cytochalasin B was used to correct for nonspecific uptake and trapping of label.

Cytochalasin binding to red cells, prepared as in the previous paragraph, was determined by a centrifugation assay (Wessling & Pilch, 1984; Axelrod & Pilch, 1983). Briefly, red cells at 10% hematocrit in phosphate-buffered saline were adjusted to 5.3 μM cytochalasin E and 4.7×10^{-8} M [^{3}H]cytochalasin B in the presence or absence of 4.2×10^{-5} M unlabeled cytochalasin B. After 30 min at 4 °C, the cells were spun for 15 min in a microfuge. The supernatant was carefully removed, and the pellet was extracted for 10 min with 1 mL of methanol. Radioactivity in the extract was determined by liquid scintillation counting.

Other Procedures and Reagents. Data analysis of the various assays was conducted on duplicate or triplicate measurements of individual points. All experiments were performed on at least three occasions to provide verification that mean values were accurate to within 2 standard deviations. Except for Table I, individual experiments are presented in the figures. Monocomponent porcine insulin was a gift of Dr. R. Chance, Eli Lilly Co. Protein determinations were accomplished by the method of Lowry et al. 3-*O*-Methyl-D-[^{3}H]glucose, L-[^{14}C]glucose, D-[1-^{14}C]glucose, D-[^{3}H]glucose, and [^{3}H]cytochalasin B were obtained from New England Nuclear. Altex MP scintillation fluid was used in radioactivity measurements. Fat cell isolations were done with bovine serum albumin obtained from Calbiochem and collagenase purchased from Worthington. The dipeptide compounds were purchased from Vega Biochemicals and were dissolved in dimethyl sulfoxide due to their poor solubility in aqueous buffers. At the concentration of peptides most commonly employed (1 mM), no precipitation of peptide was seen in the 1% dimethyl sulfoxide solution, and the peptides were therefore assumed to be soluble. Appropriate amounts of dimethyl sulfoxide were therefore added to control samples. The dansylated peptide substrate dansyl-D-Ala-Gly-*p*-nitro-Phe-Ala-Gly (see Results and Figure 4) was obtained from Calbiochem. All other chemicals were reagent grade and purchased from Sigma.

RESULTS

Neutral, metal chelator sensitive endoproteases implicated in membrane fusion events have been characterized by their sensitivity to inhibition by a series of synthetic dipeptide substrates as noted in the introduction. In general, this class of enzymes will only cleave synthetic dipeptide substrates containing either an aliphatic residue, leucine, for example,

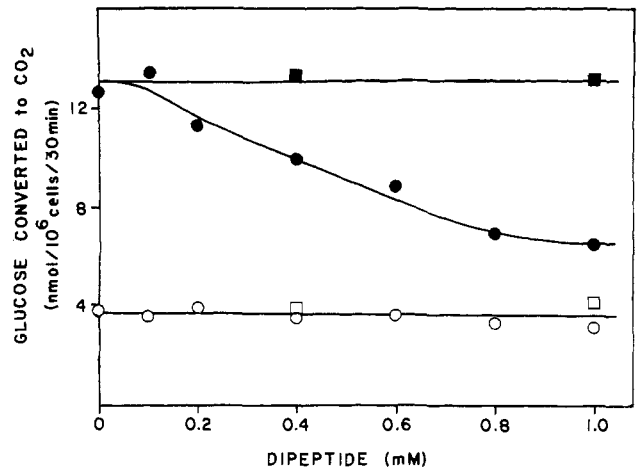

FIGURE 1: Metalloendoprotease substrate, Cbz-Gly-Phe-NH_2, inhibits insulin-activated glucose oxidation in a dose-dependent manner. Isolated adipocytes were incubated with the indicated concentrations of Cbz-Gly-Gly-NH_2 (squares) or Cbz-Gly-Phe-NH_2 (circles) at ambient temperature for 10 min and then treated with 3.5×10^{-8} M insulin (closed symbols) of left untreated (open symbols). After insulin exposure for 15 min., aliquots of 1.5×10^5 cells were mixed with 0.28 μCi of D-[1^{14}C]glucose (0.2 mM) in sealed test tubes. All additions contained dipeptides such that the indicated concentrations were maintained. Glucose oxidation was allowed to proceed at 37 °C for 30 min at which time the reaction was terminated. Released $^{14}CO_2$ was measured by scintillation counting, and the triplicate average is presented as individual points. Results are expressed as the rate of glucose conversion to $^{14}CO_2$ vs. concentration of dipeptide. Conditions were as follows: insulin stimulation in the presence of Cbz-Gly-Gly-NH_2 (■); insulin stimulation in the presence of Cbz-Gly-Phe-NH_2 (●); basal glucose oxidation in the presence of Cbz-Gly-Gly-NH_2 (□); basal glucose oxidation in the presence of Cbz-Gly-Phe-NH_2 (○).

or a noncharged aromatic, such as phenylalanine, as the amino-terminal group of the hydrolyzed peptide bond (Feder, 1947; Morihara, 1974; Mumford et al., 1980; Kam et al., 1979; Orlowski & Wilk, 1981). The presence of a free carboxyl or amino group prevents hydrolysis of the peptide. Hence, the dipeptide Cbz-Gly-Phe-NH_2 serves as a good substrate for metalloendoproteases, whereas the compounds Gly-Phe-NH_2, Cbz-Gly-Phe, and Cbz-Gly-Gly-NH_2 are not hydrolyzed by these enzymes. In vivo, the last three compounds have no biological activity whereas the first substance is a potent inhibitor of myoblast fusion and granular secretion. We tested the effect of the substrate compound Cbz-Gly-Phe-NH_2 on insulin-dependent glucose oxidation as a presumptive indicator of metalloendoprotease involvement in this process. Isolated adipocytes were incubated for 10 min with appropriate concentrations of dipeptide up to 1 mM. The cells were then treated with or without insulin for 15 min. Glucose oxidation was measured as described under Materials and Methods. This measurement indirectly assays glucose transport which is considered to be the rate-limiting step in the oxidation process. All incubations were performed at 37 °C. Figure 1 shows the result that Cbz-Gly-Phe-NH_2 (●) inhibits insulin-stimulated glucose oxidation in a dose-dependent manner with little alteration in the basal level (○). The presence of the nonsubstrate compound Cbz-Gly-Gly-NH_2 has no effect on insulin-stimulated (■) or on basal (□) glucose oxidation.

Further characterization of this phenomenon was accomplished by a similar experiment in which we measured the dose effect of a series of synthetic dipeptides on fat cell glucose oxidation. Metalloendoproteases have been distinguished by their affinities for different substrates which will vary for a particular enzyme and which are generally found to correspond with substrate concentrations in the millimolar range. For

Table I: Inhibition of Glucose Analogue Uptake by the Metalloendoprotease Substrate Cbz-Gly-Phe-NH_2 in Fat Cells and Isolated Membrane Vesicles[a]

addition[b]	(A)[c] 3-O-MG uptake (fmol cell^{-1} min^{-1})		(B)[d] 3-O-MG uptake (fmol cell^{-1} min^{-1})		(C)[e] glucose uptake (nmol mg^{-1} min^{-1})	
	−insulin	+insulin	−insulin	+insulin	−insulin	+insulin
control	0.097 ± 0.061	0.816 ± 0.093	0.120 ± 0.070	0.880 ± 0.095	1.04 ± 0.44	5.44 ± 0.44
Z-G-G-NH_2	0.063 ± 0.036	0.760 ± 0.095	0.085 ± 0.040	0.813 ± 0.033	0.89 ± 0.48	5.60 ± 1.30
Z-G-P-NH_2	0.048 ± 0.032	0.196 ± 0.057	0.068 ± 0.043	0.175 ± 0.075	1.39 ± 0.33	2.32 ± 0.39

[a] Results are expressed as the mean ± 1 standard deviation of eight independent experiments, and 60-min equilibrium uptake values were equivalent for all conditions within each column. [b] Abbreviations: Z-G-G-NH_2, Cbz-Gly-Gly-NH_2; Z-G-P-NH_2, Cbz-Gly-Phe-NH_2; 3-O-MG, 3-*O*-methyl-D-glucose. [c] Fat cells were incubated for 10 min with 1 mM concentrations of the appropriate dipeptide and then exposed for 10 min to 3.5 × 10^{-8} M insulin where indicated. 3-*O*-Methyl-D-glucose (3-*O*-MG) transport was assayed as described under Materials and Methods. [d] Fat cells were stimulated for 15 min with 3.5 × 10^{-8} M insulin where indicated and then ATP depleted by 10 mM NaN_3 treatment of 10-min duration. 3-*O*-MG transport was determined in the presence of the indicated dipeptide (1 mM) as outlined under Materials and Methods. [e] Plasma membrane vesicles isolated from insulin-stimulated or control adipocytes were utilized to determine D-[^{3}H]glucose (glucose) uptake in the presence of the indicated dipeptide (1 mM) as detailed under Materials and Methods.

example, the metalloendoprotease postulated to be involved in myogenesis (Couch & Strittmatter, 1983) has a higher affinity for substrates having the structure Cbz-Tyr-X-NH_2 and Cbz-Ser-X-NH_2, as the amino acid residue on the carboxy end of the hydrolyzed bond alters the interaction with the enzyme. Therefore, this particular metalloendoprotease appears to have a higher affinity for Cbz-Tyr-Leu-NH_2, less of an affinity for Cbz-Gly-Phe-NH_2, and no affinity for Cbz-Gly-Gly-NH_2. A somewhat similar pattern of sensitivity was observed with glucose oxidation in fat cells. In the experiment shown in Figure 2, there was some variability in dipeptide effects on the basal level of glucose oxidation. Cbz-Gly-Phe (○), Gly-Phe-NH_2 (□), and Cbz-Tyr-Leu (△) had no effect on insulin-stimulated values as would be expected for dipeptides having unblocked carboxy and amino termini and which therefore do not act as substrates for metalloendoproteases. These same compounds slightly depressed basal oxidation in this experiment. The nonsubstrate peptide Cbz-Gly-Gly-NH_2 (■) also slightly depressed basal glucose oxidation but did not alter insulin-dependent values, whereas Cbz-Gly-Phe-NH_2 (●) and Cbz-Tyr-Leu-NH_2 (▲) dramatically inhibited the insulin-dependent oxidation in a dose-dependent fashion. In contrast to the nonsubstrate dipeptides, substrates did not depress basal oxidation in this experiment nor in any of the large number of experiments we performed of this type ($n > 15$). Insulin-stimulated glucose oxidation is more sensitive to the presence of Cbz-Tyr-Leu-NH_2 (▲) than Cbz-Gly-Phe-NH_2 (●). Further experiments (results not shown) revealed a pattern of inhibition Cbz-Tyr-Leu-NH_2 > Cbz-Gly-Phe-NH_2 > Cbz-Ser-Leu-NH_2 > Cbz-Gly-Leu-NH_2. The fact that the K_i for these dipeptides is in the millimolar range correlates with the affinities of metalloendoproteases for these compounds as substrates observed in other systems (Morihara, 1974; Mumford et al., 1980; Kam et al., 1979; Orlowski & Wilk, 1981). Moreover, upon removal of the peptide from the media, the inhibitory effect was completely reversible (data not shown), as would be expected for a substrate acting to competitively inhibit a metalloendoprotease.

We determined that the preferential effect of the dipeptide substrates in insulin-activated glucose oxidation was not a direct effect on the insulin receptor. There was no effect of Cbz-Gly-Phe-NH_2 on equilibrium insulin binding to fat cells or to pure receptor as measured after 30 min at 23 °C, but the peptide did inhibit stimulation of glucose oxidation due to vitamin K5 (data not shown). The latter compound is thought to act independently of the insulin receptor, and subsequent data (see below) render a substrate peptide–receptor interaction unlikely. However, the results of Figures 1 and 2 do not rule out the possibility that the dipeptides alter adipocyte glucose metabolism. Thus, a direct measurement

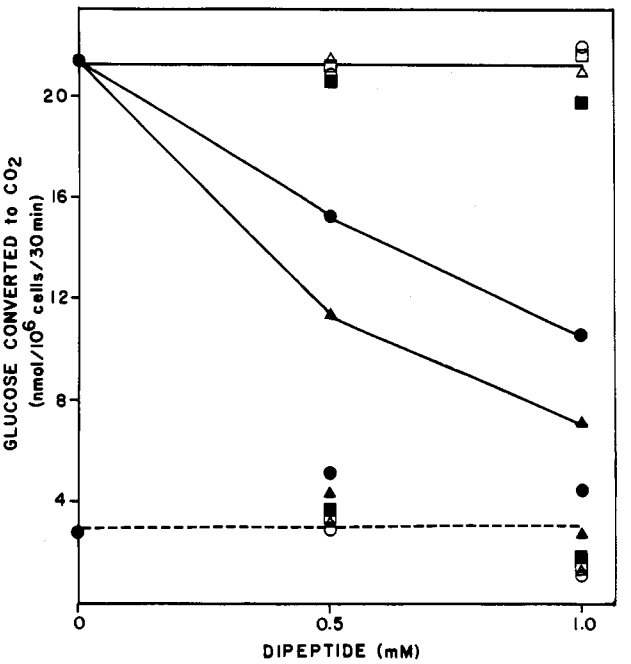

FIGURE 2: Dose dependency of glucose oxidation inhibition to various dipeptides. Isolated adipocytes were treated with appropriate concentrations of Cbz-Gly-Phe-NH_2 (●), Cbz-Gly-Phe (○), Gly-Phe-NH_2 (□), Cbz-Gly-Gly-NH_2 (■), Cbz-Tyr-Leu-NH_2 (▲), and Cbz-Tyr-Leu (△). Cells were exposed to 3.5 × 10^{-8} M insulin (solid line) or not exposed (dotted line). The rate of glucose oxidation was assayed with conditions identical with those described under Figure 1 and under Materials and Methods.

of glucose transport was necessary to determine the location of metalloendoprotease substrate action within the mechanism of insulin stimulation.

Fat cells were assayed for uptake of 3-*O*-[^{3}H]methyl-D-glucose, a nonmetabolizable glucose analogue after incubation with 1 mM concentrations of appropriate dipeptide and treatment with or without insulin. As shown in Table I, column A, the rate of 3-*O*-[^{3}H]methyl-D-glucose uptake is greatly stimulated by insulin as compared to untreated adipocytes. The presence of the nonsubstrate dipeptide Cbz-Gly-Gly-NH_2 does not significantly alter either basal or insulin-activated rates. However, the active substrate Cbz-Gly-Phe-NH_2 substantially suppresses insulin-stimulated glucose uptake and also lowers basal uptake to a lesser degree (see Discussion). This result is direct confirmation that the dipeptide's effect on the rate of insulin-dependent glucose oxidation is at least partially due to its action on insulin-activated glucose transport and not solely an alteration in subsequent glucose metabolism.

Since the metalloendoprotease substrate was present during

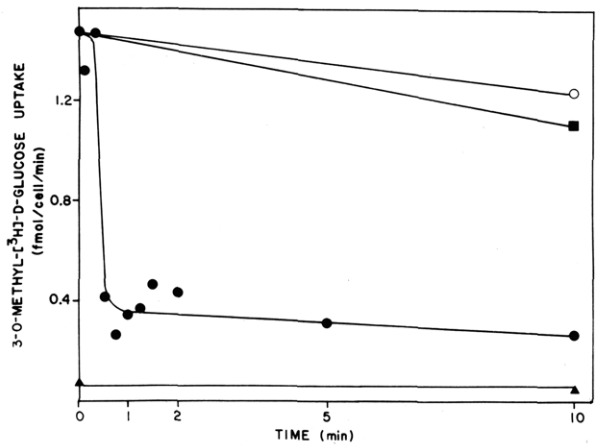

FIGURE 3: Time course of inhibition by Cbz-Gly-Phe-NH_2. Isolated adipocytes were treated with 3.5×10^{-8} M insulin for 15 min and then exposed to 1 mM Cbz-Gly-Phe-NH_2 (●) or Cbz-Gly-Gly-NH_2 (■) for indicated periods, at which time 3-*O*-methyl-D-[^{3}H]glucose uptake was initiated and performed as detailed under Materials and Methods. Rates of uptake are plotted vs. incubation time with dipeptide. The basal rate of 3-*O*-methyl-D-[^{3}H]glucose (▲) was measured in cells not exposed to insulin. Insulin-stimulated rates in the absence of dipeptide (○) are also presented.

the entire experiment shown in Table I, column A, either it could conceivably have blocked vesicle translocation due to insulin or it could be affecting the transporters themselves. Therefore, fat cells were treated with insulin (or not) to activate transport, and then further vesicle movement was blocked by using azide to deplete intracellular ATP. ATP depletion by uncouplers such as azide prevents deactivation of insulin-stimulated transport (Kono et al., 1982; Vega et al., 1980) and inhibits intracellular vesicle movement (Silverstein et al., 1977). As shown in Table I, column B, substrate dipeptide dramatically inhibited 3-*O*-methyl-D-glucose uptake in activated, ATP-depleted fat cells and had minimal effects on basal values. The nonsubstrate had no appreciable effect in either case. The same pattern was observed when the peptides were used as probes of glucose uptake in plasma membrane vesicles isolated from untreated or insulin-treated cells (Table I, column C). Translocation of transporters due to insulin was verified by a [^{3}H]cytochalasin B binding assay (data not shown). Again, only substrate dipeptides altered glucose uptake into adipocyte membrane vesicles, and only when the latter was insulin-activated. These data suggest that metalloendoprotease substrates act directly on the glucose transporter or its environment.

The fat cell glucose transport assay was also employed to study the time course over which metalloendoprotease substrates exert their action. Insulin-stimulated fat cells were treated with 1 mM Cbz-Gly-Phe-NH_2, and after an appropriate incubation time, the rate of 3-*O*-methyl-D-[^{3}H]glucose uptake was measured. The effect of dipeptide on insulin-activated transport was very rapid, as observed in Figure 3. Cbz-Gly-Phe-NH_2 (●) inhibited to a maximal extent within 1 min of exposure time. Control basal (▲), insulin-stimulated (○), and Cbz-Gly-Gly-NH_2-treated cells (■) exhibited rates of uptake unaltered by incubation time. The immediate action of the dipeptide on insulin-activated transport also suggests that the metalloendoprotease substrates may be acting directly on the insulin-activated glucose transport system, rather than at a step such as membrane fusion. The latter may be expected to have a half-time of 3–5 min (Karnielli et al., 1981) whereas the peptide's effect has a 15–30-s half-time (Figure 3).

Taken together, the results shown in Figures 1–3 and Table I suggest a preferential, direct inhibitory effect of metalloendoprotease substrates on the insulin-activated glucose transport system rather than an inhibition of membrane fusion. The inhibitory mechanism of the relevant dipeptides could be acting directly on the transporter or on its membrane environment. Thus, it was of interest to determine if the metalloendoprotease substrates possess a general inhibitory effect on glucose transport. Two systems in which glucose transport is not hormonally regulated were assayed for cytochalasin B binding and glucose uptake. Our findings were that human red blood cells demonstrated 51% inhibition of cytochalasin B binding and 48% inhibition of glucose uptake in the presence of 1 mM dipeptide substrate with a nonsubstrate having no effect on these parameters. Glucose uptake and cytochalasin B binding in human placental microsomes, however, remained unaffected by either compound under similar conditions (data not shown). Thus, in three cell types exhibiting similar cytochalasin B binding parameters, and therefore presumably similar glucose transporters, we observed variable action of the metalloendoprotease substrates on the transport process. These results are indirectly suggestive that the dipeptides act on the membrane environment of the transport protein.

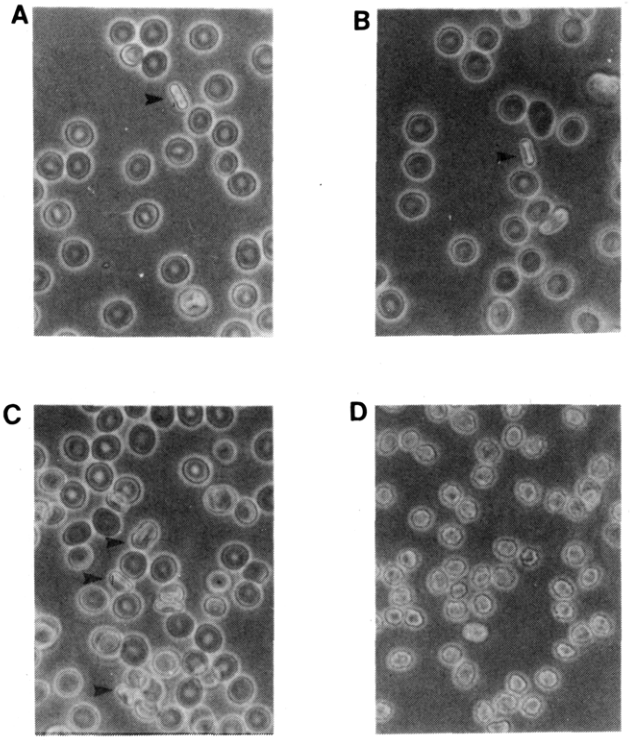

FIGURE 4: Metalloendoprotease substrates change red cell shape. Red cells were prepared as described under Materials and Methods and were treated with Cbz-Gly-Gly-NH_2 (panel B), Cbz-Gly-Phe-NH_2 (panel C), or fluorescent substrate (panel D, see text), or were not treated (panel A). They were then fixed with glutaraldehyde (0.5%) for 15 min and photographed at 32× magnification with phase-contrast optics.

Red cells are known to undergo shape changes under a variety of conditions, and we therefore examined them by light microscopy after dipeptide exposure exactly as when transport was measured. We fixed the red cells in 0.5% glutaraldehyde after peptide exposure and before microscopy. Figure 4 shows cells untreated (A) and treated (B) with the control peptide Cbz-Gly-Gly-NH_2. The cells look normal, and arrows mark the biconcave disk shape of cells observed on edge. Panels C and D of Figure 4 show cells treated with Cbz-Gly-Phe-NH_2 and dansyl-D-Ala-Gly-*p*-nitro-Phe-Gly, respectively. The latter fluorescent compound is also an inhibitor of insulin-dependent glucose transport (data not shown), apparently due to the Gly-Phe dipeptide in the middle of the molecule. It causes

100% crenolation of the red cells (D). Also apparent is that Cbz-Gly-Phe-NH_2 causes cupping and/or additional shape changes in those red cells observed on edge (Figure 4C, arrows). We examined four different preparations of red cells on four separate days. The qualitative results on each occasion were that the fluorescent substrate always produced a complete population of echinocytes and the other metalloendoprotease substrate caused an increase in aberrant red cell shapes compared to nonsubstrates and untreated cells. These results document that metalloendoprotease substrates can dramatically alter red cell shape and therefore also membrane environment.

Discussion

It seems reasonable to postulate a membrane fusion event would be required for insulin to activate glucose transport based on our current understanding of this system (Gliemann & Rees, 1983; Lienhard, 1983; Karnielli et al., 1981; Kono et al., 1982). That is, in the basal state, glucose transporters are mostly in the cell interior, and upon insulin exposure of cells, they redistribute such that they are increased 3–5-fold in the plasma membrane with a concomitant depletion of the intracellular pool. The kinetics (Karnielli et al., 1981) and energy dependency (Kono et al., 1981; Vega et al., 1980) of fat cell transporter translocation are consistent with these parameters of vesicle movement in general (Silverstein et al., 1977), and vesicles arriving at their destination must fuse with the target membrane. The data of Strittmatter and co-workers (Baxter et al., 1983; Couch & Strittmatter, 1983; Mundy & Strittmatter, 1985) suggested the strategy we employed here to study vesicle movement in fat cells, namely, the use of metalloendoprotease substrates as specific inhibitors of membrane fusion. A recent review suggests that Ca^{2+}-dependent proteolysis of membrane proteins may be a very general event in membrane fusion including insulin-activated glucose transport (Lucy, 1984). Additional recent evidence implicates a Ca^{2+} requirement for insulin-activated glucose transport (Shechter, 1984). However, it is clear from the present data that dipeptide metalloendoprotease substrates can have multiple biological activities that complicate their use as "specific" tools to study membrane fusion.

Our data provide additional indirect evidence that there are differences between the basal and insulin-activated glucose transport systems. In intact cells, we always observe marked inhibition of insulin-dependent glucose oxidation by metalloendoprotease substrates and see essentially no inhibition of the basal state by these same compounds (Figures 1 and 2). We do see some inhibition of basal glucose transport by substrate peptides in certain cases (Table I, columns A and B). This inhibition is always a lesser percentage than that seen for inhibition of the insulin-stimulated state. Moreover, this inhibition of basal transport is of marginal statistical significance whereas inhibition of the insulin-stimulated state is clearly statistically significant. We see no inhibition of transport in adipocyte membrane vesicles from fat cells (Table I, column C). These data suggest differences in the transporter and/or its environment and are in general agreement with previous studies that have also indirectly implicated differences between basal and insulin-activated transport (Pilch et al., 1980; Simpson et al., 1983). In the former study, cis-unsaturated fatty acids were shown to stimulate glucose uptake into adipocyte plasma membrane vesicles derived from untreated adipocytes with no effect on transport in vesicles from insulin-treated cells. Detailed examination of cytochalasin B binding parameters in the insulin-activated vs. basal state in fat cell membranes also revealed slight but significant differences in K_d (Simpson et al., 1983). Thus, there is justification for postulating that the insulin-activated glucose transporter, and/or its membrane environment, differs from the transporter in the basal state. Direct proof awaits purification and characterization of the protein and the presumptive vesicles that mediate its translocation.

The fact that the metalloendoprotease substrates inhibit glucose transport (Table I), inhibit cytochalasin B binding to red cells (not shown), and alter red cell shape (Figure 4) indicates that considerable caution must be exercised in interpreting their effects on biological processes in living cells. The observation that these substrate compounds inhibit myoblast fusion (Couch & Strittmatter, 1983) and exocytosis in mast cells and adrenal cells (Baxter et al., 1983; Mundy & Strittmatter, 1985) cannot be used as unequivocal evidence that metalloendoprotease activity is essential to these processes, although this may well be the case. The use of specific inhibitors or stimulators can only give indirect data on biological processes, and this indirect data can be very helpful but not definitive in determining the involvement of a specific protein or enzyme. Thus, direct data identifying the enzymes, structural proteins, and vesicular organelles involved in membrane fusion are ultimately necessary to understand this complex process. The cellular location of the metalloendoprotease activity that may be involved in myoblast fusion has recently been determined (Couch & Strittmatter, 1984) as a partial achievement of this goal.

Acknowledgments

We thank Dr. David Albertini of Tufts University Medical School for assistance with the light microscopy.

Registry No. Z-G-G-NH_2, 6422-35-1; Z-G-P-NH_2, 68385-08-0; 3-O-MG, 146-72-5; D-glucose, 50-99-7; insulin, 9004-10-8; mammalian metalloproteinase, 80498-20-0; cytochalasin B, 14930-96-2.

References

Axelrod, J. D., & Pilch, P. F. (1983) *Biochemistry 22*, 2222–2227.

Baxter, D. A., Johnston, D., & Strittmatter, W. J. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 4174–4278.

Bosch, F. X., Garten, W., Klenk, H.-D., & Rott, R. (1981) *Virology 113*, 725–735.

Couch, C. B., & Strittmatter, W. J. (1983) *Cell (Cambridge, Mass.) 32*, 257–265.

Couch, C. B., & Strittmatter, W. J. (1984) *J. Biol. Chem. 259*, 5396–5399.

Cushman, S. W., Wardzala, L. J. (1980) *J. Biol. Chem. 255*, 4758–4762.

Douglas, W. W. (1977) *Neurosci, Res. Program Bull. 15*, 591–602.

Fain, J. N., Reed, N., & Saperstein, R. (1967) *J. Biol. Chem. 242*, 1887–1894.

Feder, J. (1967) *Biochemistry 6*, 2088–2093.

Foley, J. E., Cushman, S. W., & Salans, L. B. (1978) *Am. J. Physiol. 234 (2)*, E113–E119.

Gliemann, J., & Rees, W. D. (1983) *Curr. Top. Membr. Trans. 18*, 339–379.

Kam, C. M., Nishina, N., & Powers, J. C. (1979) *Biochemistry 18*, 3032–3038.

Karnielli, E., Zarnowski, M. J., Hissin, P. J., Simpson, I. A., Salans, L. B., Cushman, S. W. (1981) *J. Biol. Chem. 256*, 4772–4777.

Kono, T., Suzuki, K., Kansey, L. E., Robinson, F. W., & Blevins, T. L. (1981) *J. Biol. Chem. 256*, 6400–6407.

Kono, T., Robinson, F. W., Blevins, T. L., & Esaki, O. (1982) *J. Biol. Chem. 257*, 10942–10947.

Lienhard, G. E. (1983) *Trends Biochem. Sci. (Pers. Ed.) 8*, 125-128.
Lowry, O. H. Rosebrough, N. J., Farr, A. L., & Randall, R. J. (1951) *J. Biol. Chem. 193*, 265-275.
Lucy, J. A. (1984) *FEBS Lett. 166*, 223-231.
Morihara, K. (1974) *Adv. Enzymol. Relat. Areas Mol. Biol. 41*, 179-243.
Mumford, R. A., Strauss, A. W., Powers, J. C., Pierzchala, P. A., Nishino, N., & Zimmerman, M. (1980) *J. Biol. Chem. 255*, 2227-2230.
Mundy, D. I., & Strittmatter, W. J. (1985) *Cell (Cambridge, Mass.) 40*, 645-656.
Orlowski, M., & Wilk, S. (1981) *Biochemistry 20*, 4942-4950.
Pilch, P. F., Thompson, P. A., & Czech, M. P. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 915-918.
Rodbell, M. (1964) *J. Biol. Chem. 239*, 375-380.
Scheid, A., & Choppin, P. W. (1974) *Virology 57* 475-490.
Shechter, Y. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 327-331.
Silverstein, S. C., Steinman, R. S. M., & Cohn, Z. (1977) *Annu. Rev. Biochem. 46*, 669-722.
Simpson, I. A., Yver, D. R., Hissin, P. J., Wardzala, L. J., Karnielli, E., Salans, L. B., & Cushman, S. W. (1983) *Biochim. Biophys. Acta 763*, 393-407.
Suzuki, K., & Kono, T. (1980) *Proc. Natl. Acad. Sci. U.S.A. 77*, 2542-2545.
Vega, F. V., Key, R. J., Jordan, J. E., & Kono, T. (1980) *Arch. Biochem. Biophys. 203*, 167-173.
Wardzala, L. J., & Jeanrenaud, B. (1981) *J. Biol. Chem. 256*, 7090-7093.
Wardzala, L. J., & Jeanrenaud, B. (1983) *Biochim. Biophys. Acta 730*, 49-56.
Wessling, M., & Pilch, P. F. (1984) *Biochim. Biophys. Acta 777*, 123-132.

Structure and Dynamics of a Glyceroglycolipid: A ^2H NMR Study of Head Group Orientation, Ordering, and Effect on Lipid Aggregate Structure†

Harold C. Jarrell,* Jerzy B. Giziewicz, and Ian C. P. Smith
Division of Biological Sciences, National Research Council of Canada, Ottawa, Ontario, Canada K1A 0R6
Received November 5, 1985; Revised Manuscript Received March 6, 1986

ABSTRACT: The head group orientation and the motional characteristics of 1,2-di-*O*-tetradecyl-3-*O*-β-D-glucopyranosylglycerol selectively ^2H-labeled on the glucose moiety have been investigated by differential scanning calorimetry and ^2H NMR. The glycolipid undergoes a major endothermic transition at 52 °C, which is attributed to the gel to liquid-crystal phase transition. The nature of a less energetic endothermic transition at 58 °C, determined to be a lamellar to hexagonal mesophase transition by ^2H NMR, is confirmed by X-ray diffraction. In the lamellar phase, the glycolipid head group undergoes axially symmetric motion and has an orientational order parameter S_{mol} of 0.45, which is significantly larger than that (0.31) reported for an analogous glucosylcerebroside. The head group is extended away from the bilayer surface. On entering the hexagonal mesophase, the orientational order parameter for the sugar ring is reduced slightly to 0.38, but the local rotation axis undergoes a large reorientation with respect to the carbohydrate ring. In a phospholipid matrix, the orientation of the carbohydrate head group of the glycolipid is affected by the greater extension of the surface residues of the host lipid. Two orientations of the exocyclic hydroxyl group of the carbohydrate moiety were detected by ^2H NMR and are shown to have unequal populations.

Glycolipids constitute a class of lipid that occurs in plants, microorganisms, and animals (Gigg, 1980). Glycolipids are most frequently composed of a carbohydrate head group anchored to the membrane through a diacyl- (or dialkyl-) glycerol or a sphingosine residue. The carbohydrate head group can be relatively simple (e.g., a single sugar residue) or very complex and may be neutral or charged (Gigg, 1980). Carbohydrates at cell surfaces have been implicated in important cellular events such as cell–cell recognition, ligand–receptor interaction [e.g., cholera toxin receptor (Critchley, 1979)], and ion transport (Karlsson, 1977). The involvement of carbohydrates in such biologically important functions is dependent upon the primary sequence of the surface component but most certainly is also dependent upon the spatial relationship of the constituent residues. Glycolipids may also be the major constituent lipid of membranes such as in *Acholeplasma laidlawii* (Rottem, 1980; Razin, 1978) and in such cases must play a major role in defining the physical properties of the cellular membrane. In view of the major but diverse roles that glycolipids can assume, the elucidation of the orientation of the carbohydrate residues relative to the membrane surface and the dynamical behavior of the head group are of considerable interest. In addition, the response of the head group orientation and motion to perturbations such as ion binding, ligand–receptor interactions, and other surface components is of fundamental importance to the understanding of cell surface phenomena.

Deuterium (^2H) NMR[1] is a powerful technique for the elucidation of orientational and motional properties of mole-

† Issued as NRCC Publication No. 25827.

[1] Abbreviations: PC, phosphatidylcholine; PE, phosphatidylethanolamine; DTGL, 1,2-di-*O*-tetradecyl-3-*O*-β-D-glucopyranosyl-*rac*-glycerol; GC, glucocerebroside; DSC, differential scanning calorimetry; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography.

0006-2960/86/0425-3950$01.50/0