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Effects of three proposed inhibitors of adipocyte glucose transport on the reconstituted transporter

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Three compounds which inhibit glucose transport in rat adipocytes have been proposed to act directly on the glucose transporter protein. We tested these proposals by examining the effects of the compounds on the stereospecific glucose uptake catalyzed by adipocyte membrane proteins after reconstitution into liposomes. Effects on the transport activity reconstituted from human erythrocyte membranes were also examined. Glucose 6-phosphate, which was suggested to inhibit the transporter noncompetitively (Foley, J.E. and Hueckstead, T.P. (1984) Biochim. Biophys. Acta 805, 313–316), had no effect on either type of reconstituted transporter, even when present at 5 mM on both sides of the liposomal membranes. Thus, it is unlikely to act directly on the transporter. The metalloendoproteinase substrate dipeptide Cbz-Gly-Phe-NH_2, which inhibited insulin-stimulated but not basal glucose uptake in adipocytes (Aiello, L.P., Wessling-Resnick, M. and Pilch, P.F. (1986) Biochemistry 25, 3944–3950), inhibited the reconstituted erythrocyte transporter noncompetitively with a K_i of 1.5–2 mM. The inhibition of the erythrocyte transporter was identical in liposomes of soybean and egg lipid. Transport reconstituted using adipocyte membrane fractions was also inhibited by the dipeptide, with the activity from basal microsomes more sensitive than that from insulin-stimulated plasma membranes. These results indicate that the dipeptide interacts directly with the transporter, and may be a potentially useful probe for changes in transporter structure accompanying insulin action. Phenylarsine oxide, which was suggested to act directly on the adipocyte transporter (Douen, A.G., and Jones, M.N. (1988) Biochim. Biophys. Acta 968, 109–118), produced only slight (about 10%) inhibition of the reconstituted adipocyte and erythrocyte transporters, even when present at 100–200 μM and after 30 min of pretreatment. These results suggest that the major actions of phenylarsine oxide observed in adipocytes are not direct effects on the transporter, but rather effects on the pathways by which insulin regulates glucose transport activity (Frost, S.C. and Lane, M.D. (1985) J. Biol. Chem. 260, 2646–2652).

Introduction

Regulatory phenomena involving membrane transport proteins are commonly studied using intact cells. However, in such studies the mechanism by which a given effector alters transport is difficult to determine. It might act directly on a transport protein. Alternatively, it might exert its effect through intermediate components of a signalling pathway; as a secondary consequence of producing changes in metabolism; or by altering the structure of the membrane. The reconstitution of transport proteins in liposomes provides a means by which the direct interaction of agents with transport proteins can be tested.

In the experiments reported here, we examined three compounds which inhibit glucose transport in rat adipocytes, and which were suggested to produce their effects by interacting directly with the glucose transporter. The transporter was reconstituted using adipocyte membrane proteins, and the effects of these compounds on the reconstituted transport measured. In addition, the effects were also characterized using the transporter reconstituted from human erythrocyte membranes.

The first of these compounds is glucose 6-phosphate. Foley and Hueckstead [1] reported that incubation of adipocytes and membrane vesicles with glucose 6-phosphate produced inhibition of the uptake of glucose,

Abbreviations: Cbz, carbobenzoxy; PAO, phenylarsine oxide; DMSO, dimethylsulfoxide.

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3-*O*-methylglucose, and 2-deoxyglucose (though 2-deoxyglucose uptake was affected in vesicles but not intact cells). Effects were time- and concentration-dependent, which was attributed to the necessity for glucose 6-phosphate to penetrate the membrane and accumulate to inhibitory levels. It was estimated that intravesicular levels of about 2 mM were achieved when maximum inhibition was seen. The effect was specific for glucose 6-phosphate; several other sugar phosphates were ineffective. In intact cells, incubation with glucose 6-phosphate reduced V_{max} (by 50%), indicating noncompetitive inhibition. It was proposed that glucose 6-phosphate produced its effects by directly inhibiting the glucose transporter at a site on the intracellular surface of the membrane. A reduction in K_m (by 30%) was also observed in the presence of glucose 6-phosphate. This was interpreted in terms of a model having two transporters in series; in the presence of glucose 6-phosphate, the inner site (with a lower K_m) could become more rate-determining. Regulation by glucose 6-phosphate might be of physiological significance, reducing the uptake of glucose under conditions when metabolites arising from it accumulate.

The second compound we tested was the dipeptide Cbz-Gly-Phe-NH_2, a metalloendoproteinase substrate. Because such compounds inhibit certain membrane fusion events, their action on the insulin-dependent translocation of glucose transporters in adipocytes was investigated by Aiello et al. [2]. It was found that Cbz-Gly-Phe-NH_2, as well as another dipeptide substrate, inhibited insulin-stimulated glucose oxidation and 3-*O*-methylglucose transport in adipocytes, and glucose uptake in membrane vesicles from insulin-stimulated cells. The effects appeared to be independent of translocation. No significant effects were seen on basal cells and vesicles prepared from them. Nonsubstrate dipeptides were not inhibitors of transport or glucose oxidation in the insulin-stimulated cells. Cbz-Gly-Phe-NH_2 also inhibited glucose transport and cytochalasin B binding in human erythrocytes, but not in placental microsomes. It was suggested that the dipeptide might interact directly with the glucose transporter. However, it was also stated that an indirect effect on the structure of the membrane was possible; changes in erythrocyte shape were observed in response to treatment with the dipeptide. On the basis of its differential effects on basal and insulin-stimulated adipocytes, the dipeptide may be a useful probe for examining changes in the structure or the membrane environment of the glucose transporter which accompany activation by insulin.

The third compound tested was phenylarsine oxide (PAO), which reacts with vicinal thiol groups. Lane and coworkers have found that in 3T3-L1 adipocytes, PAO inhibits the insulin stimulation of deoxyglucose uptake without affecting basal uptake [3]. Changes in the pattern of protein phosphorylation were also observed [4,5]. It was proposed that PAO blocks a step in the pathway by which insulin stimulates glucose transport. However, in a recent paper by Douen and Jones [6], it was reported that PAO inhibits glucose uptake in both basal adipocytes and adipocytes which are already insulin stimulated. It was concluded that PAO most likely interacts directly with the glucose transporter, possibly via covalent reactions with sulfhydryl groups at or near the transport site, though effects on the signalling pathway of insulin were not excluded. Thus, there is some controversy concerning the effects and mechanisms of action of PAO.

The results of our studies indicate that glucose 6-phosphate does not inhibit the glucose transporter after reconstitution with liposomes, and thus is unlikely to act directly on the transporter. Cbz-Gly-Phe-NH_2, on the other hand, does inhibit reconstituted glucose transport, consistent with a direct effect on the transporter, although indirect effects on the membrane cannot be ruled out. Finally, PAO produces only a small degree of inhibition of the reconstituted transporter, indicating that its effects in adipocytes are most likely indirect.

Materials and Methods

Materials. D-[^{14}C]Glucose was obtained from ICN; L-[1-^{3}H]glucose from DuPont NEN; phenylarsine oxide from Aldrich; and glucose 6-phosphate, Cbz-Gly-Phe-NH_2, and phospholipids from Sigma. Membrane fractions from rat adipocytes were a gift of Dr. Samuel Cushman. Human erythrocyte membranes were prepared by the method of Dodge et al. [7] using outdated erythrocytes donated by the American Red Cross, Louisville, KY.

Procedures. Liposomes for reconstitution were prepared, unless otherwise noted, from soybean phospholipids (Sigma Type II-S); in some cases egg lipids (Type IX-E) were used. Erythrocyte membrane proteins were reconstituted without detergent treatment [8] at a ratio of 8 μg protein per mg lipid. Adipocyte membrane proteins were reconstituted either without detergent treatment (though after gel filtration to remove sucrose), or after dispersal with 0.3% cholate and 2 mg protein/ml followed by centrifugation at 15000 × g and gel filtration [9]. The adipocyte proteins were reconstituted at approximately 4 μg protein per mg lipid (in some cases the protein concentration was estimated based on typical recoveries from detergent treatment and gel filtration). In some experiments the final sonication step (after freezing and thawing) was performed using a probe sonicator (Branson Model 200 with microtip), at a setting of 3.5 for 5 s, rather than a bath sonicator. The volume in each tube (13 × 100 mm) used for the reconstitution was 0.3 ml, and the tip of the probe was placed midway between the top and bottom of the suspension.

Stock solutions of glucose 6-phosphate (monodium)

were brought to pH 7 with NaOH. Cbz-Gly-Phe-NH_2 was prepared as 100 and 300 mM solutions in dimethylsulfoxide, and PAO as a 10 mM solution in ethanol. When Cbz-Gly-Phe-NH_2 or PAO was used, all assays in an experiment contained the same amount of DMSO or ethanol, respectively.

Assays of glucose transport were performed as described [9], with 40 μl reconstituted liposomes (1 mg liposomal lipid) per assay in a total volume of 300 μl. Triplicate or quadruplicate assays were performed at each time point. In the case of assays using glucose 6-phosphate added only after reconstitution, the osmolarities of assay solutions containing 0 or 2 mM glucose 6-phosphate were adjusted to equal those of assay solutions containing 5 mM glucose 6-phosphate by addition of NaCl. For these assays, and for those using 5 mM external plus internal glucose 6-phosphate, the osmolarity of the stopping solution was also increased to equal that of the assay solutions.

Results

Effects of external glucose 6-phosphate on reconstituted glucose transport

We tested the effects of glucose 6-phosphate on the rate of glucose uptake into liposomes reconstituted with adipocyte membrane proteins. The samples used in these experiments were either plasma membranes from insulin-stimulated cells, or a microsomal fraction from basal cells. This fraction (often referred to as 'low-density microsomes') contains a high concentration of glucose transporters, some of which are translocated to the plasma membrane in response to insulin treatment [10]. Although it is possible that the translocation process results in modification of the properties of the transporter, it seems likely that a regulatory site for glucose 6-phosphate, if it existed in the plasma membrane form of the transporter, would also be found in the microsomal form. Because it is richer in glucose transporters, and thus gives higher and more reliable uptake data than the plasma membranes, we used the microsomes in several of the experiments in this study. The membrane proteins used to test effects of glucose 6-phosphate were reconstituted without prior detergent treatment [8].

Table I lists results from experiments in which the stereospecific glucose uptake at 30 s to 2 min was measured in the presence of 0, 2, or 5 mM glucose 6-phosphate. Using similar reconstituted samples, we had observed that the half-time for equilibration of the intraliposomal volume was about 2 min (data not shown), and therefore the uptakes at 30 s and 1 min give reasonable approximations of the initial rates of transport. The values of glucose 6-phosphate tested were equal to and 2.5-fold higher than the estimated intravesicular concentration (2 mM) producing maximal inhibition [1]. According to the results in Ref. 1, where a 30% decrease in K_m and 50% decrease in V_{max} were observed in the presence of glucose 6-phosphate, at very low glucose concentrations (such as used in our studies) the uptake rate should be decreased by 33%. However, as shown by the data in Table I, no inhibition of glucose transport (using either the microsomal or plasma membrane proteins) was produced by 2 or 5 mM glucose 6-phosphate. When the transport activity was reconstituted using human erythrocyte membranes (ghosts), 5 mM glucose 6-phosphate also produced no inhibition.

Effect of internal plus external glucose 6-phosphate

One possible complication in these experiments is that the orientation of the reconstituted transporter is unknown. We previously showed that the human

TABLE I

Effects of glucose 6-phosphate on reconstituted glucose transport activity

Assays contained 4 μg adipose membrane protein or 8 μg erythrocyte ghost protein, 1 mg liposome lipid, and 0.2 mM D-glucose. Stereospecific glucose uptake in the presence of 2 or 5 mM glucose 6-phosphate (Glc-6-*P*) was measured at the indicated times and compared to control assays in the absence of glucose 6-phosphate. Results are from single experiments, means from two experiments (with individual values in parentheses), or the mean (±S.E.) from three experiments.

Sample	Glc-6-*P* (mM)	Relative stereospecific uptake vs. control		
		30 s	1 min	2 min
External Glc-6-*P* only:				
Adipose (basal microsomes)	2	1.15	1.19	0.92
	5	1.10 (0.80, 1.39)	1.37	1.23
Adipose (insulin plasma membranes)	2	1.21 (1.05, 1.39)	1.12 (0.87, 1.45)	
	5	0.95 (1.10, 0.78)	1.02 (1.00, 1.05)	
Erythrocyte ghosts	5	1.18 (1.22, 1.14)		
Internal plus external Glc-6-*P*:				
Adipose (basal microsomes)	2	1.05 (1.21, 0.89)	1.04 (1.23, 0.86)	
	5	1.18 (1.31, 1.02)	1.12 (1.33, 0.92)	
Erythrocyte ghosts	5	1.07±0.12 (3)	1.20 (1.47, 0.92)	

erythrocyte transporter, either after purification [11] or using membranes without detergent treatment [8], was reconstituted with about half the transporters having each possible orientation in the membrane. The adipocyte transporter could be reconstituted similarly, or it could have a different distribution of orientations. Since the presumed regulatory site for glucose 6-phosphate would be on the cytoplasmic surface of the transporter, it would be accessible to external glucose 6-phosphate only in the case of those transporters reconstituted with an inside-out orientation. Thus, the inhibition expected for external glucose 6-phosphate, according to the proposal of Foley and Hueckstead [1], would be some fraction of 33%.

In order to compensate for the possible scrambling of orientations after reconstitution, we carried out additional experiments in which glucose 6-phosphate was present during the formation of the liposomes, during the reconstitution, and in the assays. Thus, it was present on both sides of the liposomal membrane, and potentially able to interact with all transporter regulatory sites. Using adipose microsomal proteins, again no inhibition of glucose uptake was observed (Table I). We also tested the effect of internal and external glucose 6-phosphate on the glucose transporter reconstituted from erythrocyte membranes; again, glucose 6-phosphate (5 mM) produced no inhibition of uptake.

These results indicate that glucose 6-phosphate does not interact directly with the glucose transporters of either adipocytes or erythrocytes.

Effects of dipeptide on reconstituted erythrocyte glucose transport

The effects on reconstituted glucose transport of the metalloendoproteinase substrate Cbz-Gly-Phe-NH_2 were first tested using human erythrocyte membranes. For initial experiments, we used the dipeptide at 1 mM, a concentration which produced near maximal effects in adipocytes and which inhibited glucose transport in erythrocytes by 48% [2], and a DMSO concentration of 1%. The effects of the dipeptide on glucose uptake into reconstituted liposomes are shown in Fig. 1. When added externally only (Fig. 1, open symbols) the dipeptide produced about a 35% inhibition in the initial rate of uptake (estimated at 30 s; the control liposomes equilibrated with a half-time of about 2 min). Since the dipeptide was added along the glucose at the initiation of the assays the inhibitory effect must be exerted relatively rapidly (within a few seconds). The apparent inhibition gradually decreased when measured at later times, with none being seen at 20 min. This indicates that the dipeptide has no effect on the internal volume of the reconstituted liposomes, so that even though the initial rate of uptake is inhibited the final extent of uptake is the same. For the ratio of protein to lipid used in these experiments, the transporters are predominantly reconstituted with at most one per liposome [8], so that the internal volume is a measure of the number of active transporters that have been reconstituted. The fact that the final equilibrated volume is unaffected by the dipeptide indicates that the decrease in the initial uptake rate does not come about by the complete inactivation of a fraction of the transporters; if that were the case, the liposomes containing these transporters would not achieve equilibrium in 20 min.

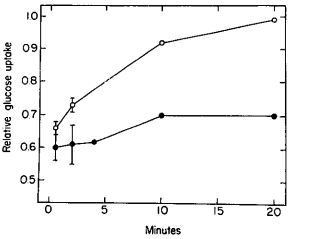

Fig. 1. Effect of Cbz-Gly-Phe-NH_2 on glucose uptake in liposomes reconstituted with erythrocyte membrane proteins. Stereospecific uptake of 0.2 mM glucose was measured at the indicated times. The ratio of uptake in the presence of 1 mM dipeptide to uptake in the absence of dipeptide is plotted. The dipeptide was either present during the formation of the liposomes, the reconstitution, and the assays (●), or was added only at the start of the assays (○). Data for 4 to 20 min are from single experiments, or are means of results from two experiments; data for 30 s and 2 min are means from four (●) or nine (○) experiments, with standard errors indicated.

When the dipeptide was included in the formation of the liposomes and in the reconstitution, as well as in the assays, it produced a somewhat greater inhibition (about 40%) in the initial rate of uptake (Fig. 1, solid symbols). However, there was about a 30% inhibition seen even at late times, when equilibration is nearly complete. This suggests that in the presence of the dipeptide the number of transporters reconstituted, and therefore the final equilibrated volume, is reduced by about 30%. However, if the reconstitution efficiency is reduced by about 30%, and those transporters that are reconstituted are inhibited by 35% (as seen when the dipeptide is added after reconstitution), then the combination of these two effects should result in $70\% \times 65\% = 45\%$ activity compared to controls, less than is observed. This discrepancy might arise if some or all of the effect of the dipeptide at late times is due to formation of smaller reconstituted liposomes in its presence rather than to a decrease in the number of transporters reconstituted.

Effects of assay volume and trypsin treatment on inhibition produced by dipeptide

We considered the possibility that the dipeptide, which can perturb membrane structure [2], binds to the

TABLE II

Effects of variations in assay conditions on inhibition of the reconstituted erythrocyte transporter by dipeptide

Assays contained 8 μg erythrocyte membrane protein, 1 mg liposome lipid and 0.2 mM D-glucose. Stereospecific uptake of 0.2 mM glucose at the indicated times was measured in the presence or absence of 1 mM Cbz-Gly-Phe-NH_2. The assay volume was 300 μl unless noted otherwise. For trypsin-treated liposomes, trypsin was added at 20 μg/ml after reconstitution. For both of the variations (assay volume and trypsin treatment), three experiments were performed in which the uptake was measured using both of the alternative conditions listed. Results are means (±S.E.) for the three experiments for both of the variations, and are expressed as ratios of uptake in the presence of the dipeptide to control uptakes.

Conditions	Relative uptake	
	30 s	2 min
300 μl assay volume	0.65±0.02	0.68±0.02
600 μl assay volume	0.70±0.01	0.70±0.01
Trypsin-treated	0.60±0.03	0.63±0.04
Untreated	0.61±0.01	0.63±0.03

liposomal membrane with sufficient affinity that its free concentration is significantly lower than 1 mM. Such binding of molecules to liposomes can lead to erroneous conclusions concerning the effectiveness of inhibitors in reconstitution experiments [11]. We tested this by comparing the effect of the dipeptide (1 mM) under our normal assay conditions to its effect in assays where the assay volume was doubled but the amount of liposomes left the same. Since the ratio of total inhibitor to lipid was 2-fold higher in the second situation, greater inhibition would be expected if the lipids were adsorbing a significant fraction of the total dipeptide. The results of three experiments comparing the two assay volumes are listed in Table II. No difference was seen, indicating that at 1 mM the bound dipeptide must be much lower than the total under the conditions of our assays.

Another consideration is the accessibility of the transporter to modulation by the dipeptide. The erythrocyte transporter is reconstituted with approximately half of the proteins oriented right-side out and half inside out [8]. If externally added dipeptide acted at only one surface of the transporter, and could not gain access to the inner surface of the liposomes, its effects would be diminished by the scrambling of orientations. We compared the effects of 1 mM dipeptide, added only externally, on control and trypsin-treated liposomes. Trypsin inactivates the transporter by acting at sites exposed only on the cytoplasmic surface of the transporter as it is found in erythrocytes [12,13]. Treatment of reconstituted liposomes with external trypsin inactivates the inside-out transporters, so the observed activity is due to transporters oriented as in the erythrocyte. Results of experiments using trypsin are listed in Table II. The dipeptide inhibited both trypsin-treated and untreated liposomes equally well. This indicates that the dipeptide can affect all transporters regardless of orientation, either because it exerts its effects in the membrane, or because it can penetrate the liposomal membrane and thereby gain access to inhibitory sites on either side.

Concentration dependence of inhibition by dipeptide

To determine the maximum concentration of the dipeptide which we could include in our assays, we first tested the effects of various concentrations of the solvent, DMSO, on stereospecific glucose transport by the reconstituted erythrocyte membrane proteins. The transport was inhibited about 20% by 3% (v/v) DMSO and about 30% by 5% DMSO; we chose to use the former as a level of DMSO which could be included in the assays without large effects on transport. We then found that with 3% DMSO in the assays, the dipeptide was soluble at 2.5 mM but not at 3 mM.

We measured the effects of various concentrations of the dipeptide (in 3% DMSO) on the initial rates of uptake (assayed at 20 s) by the reconstituted erythrocyte transporter. The results are shown in Fig. 2 (solid circles), which also includes some data obtained at 1 mM dipeptide in 1% DMSO. The inhibition was concentration-dependent, with 50% inhibition seen between 1.5 and 2 mM and 60% inhibition seen at 2.5 mM. Aiello et al. [2] reported 48% inhibition of glucose transport in erythrocytes by 1 mM dipeptide, which is greater than the inhibition we observed using the reconstituted transporter (about 35%).

Effects of glucose concentration on inhibition

The above experiments examining the inhibition by Cbz-Gly-Phe-NH_2 were all performed using a constant

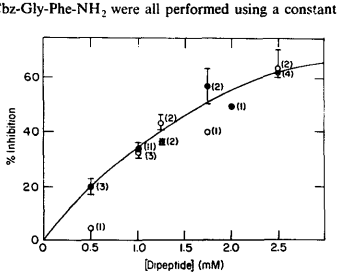

Fig. 2. Concentration dependence of dipeptide inhibition of reconstituted erythrocyte transporter. Stereospecific uptake of 0.2 mM glucose in 20 s or 30 s was determined for the indicated concentrations of Cbz-Gly-Phe-NH_2. Liposomes were prepared from soybean lipids (●) or egg lipids (○). The error bars indicate ranges ($n = 2$) or standard errors ($n \geq 3$), with the number of determinations in parentheses.

TABLE III

Inhibition of the reconstituted adipocyte transporter by dipeptide

Assays contained 4 μg adipocyte membrane protein. Two different preparations of each membrane fraction were tested. In the case of preparation 1 of plasma membranes from insulin-stimulated cells, the membranes were treated with cholate before reconstitution. For the second preparation, and for both preparations of microsomal membranes, the membranes were not treated with detergent. Stereospecific glucose uptake in the presence and absence of Cbz-Gly-Phe-NH_2 was determined at the indicated times. Assays with the first preparation of each type contained 1% DMSO; with the second preparation of each type, 3% DMSO. Results are from single experiments; means from two experiments, with individual values in parentheses; or means ± S.E. from three experiments. They are expressed as ratios of uptakes in the presence of the dipeptide to control uptakes. Negative uptakes refer to experiments in which L-glucose uptake exceeded D-glucose uptake; the differences were not statistically significant.

Sample	Preparation No.	Dipeptide (mM)	Relative uptake		
			30 s	1 min	2 min
Insulin plasma membranes	2	0.5	0.85	0.64 (0.59, 0.69)	
	1	1.0	0.58 (0.67, 0.50)		0.73 (0.62, 0.84)
	2	2.0	−0.46	0.32 (0.70, −0.07)	
Basal microsomes	2	0.5	0.40 ± 0.13 (3)		
	1	1.0	0.30 (0.30, 0.29)	0.29 (0.35, 0.23)	
	2	2.0	−0.06 ± 0.06 (3)		

(0.2 mM) glucose concentration. In order to explore the mode of inhibition, we performed three experiments in which the inhibition produced by 1.5 mM dipeptide (near the apparent K_i at 0.2 mM glucose, Fig. 2) was tested at various glucose concentrations. Results are shown in Fig. 3. At 0.5 1, and 2 mM glucose, the dipeptide produced 48 ± 3%, 49 ± 6%, and 49 ± 1% (mean ± S.E., $n = 3$) inhibition, respectively, indicating noncompetitive inhibition. Surprisingly, at 4 mM glucose, even greater inhibition (68 ± 4%) was observed.

Effect of lipid composition

One proposed mechanism of inhibition by the dipeptide was through effects on the membrane environment of the transporter [2]. Since insulin-stimulated aidpocytes were more sensitive to actions of the dipeptide, this suggests that the adipocyte membrane might be altered by insulin action, and that the effects of the dipeptide would be different in different types of membranes. We tested this possibility by comparing the inhibitory action of the dipeptide on the erythrocyte transporter reconstituted into liposomes prepared from egg lipids (Sigma Type IX-E) and into liposomes prepared from soybean lipids (Sigma Type II-S); the latter were used in all other experiments in this study. The two preparations differ in their contents of both lipid head groups and fatty acyl groups *. In cases in which the two types of liposomes were reconstituted in the same experiment, the egg liposomes gave a 1.8-fold higher transport activity. Fig. 2 (open circles) shows the degrees of inhibition produced by various concentrations of the dipeptide for the case of the erythrocyte transporter reconstituted into the egg liposomes. These were not significantly different than those observed with the soybean liposomes (solid circles).

This result argues against the dipeptide acting indirectly through the membrane in a manner that is sensitive to the membrane composition, and supports a direct interaction with the transporter. However, it is possible that changes in membrane structure which occur in response to insulin treatment, and which alter the effectiveness of the dipeptide in adipocytes, were

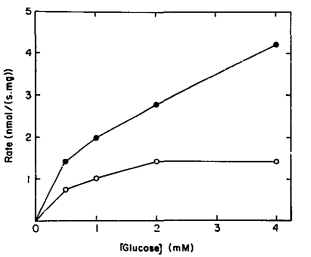

Fig. 3. Dipeptide inhibition at different glucose concentrations. Assays contained reconstituted erythrocyte membrane proteins, 0 (●) or 1.5 mM (○) Cbz-Gly-Phe-NH_2, and the indicated concentrations of glucose. Stereospecific uptake in 20 s or 30 s was determined. Results are means from three experiments.

* The egg lipids had 60% phosphatidylcholine and 20% phosphatidylethanolamine, the soybean lipids 15% phosphatidylcholine and 5 to 10% phosphatidylethanolamine; the soybean lipids had a greater content of linoleic residues, while the egg lipids had greater contents of palmitic, stearic, and oleic residues (data provided by Greg Wall, Technical Service, Sigma Chemical Co.).

not mimicked by the two different lipid environments we happened to test.

Effect of dipeptide on reconstituted adipocyte transporter

Having explored some aspects of the action of the dipeptide on the reconstituted erythrocyte transporter, we next tested its effect on the transporter from adipocytes, where the compound appears to discriminate between basal and insulin-stimulated glucose uptake. We initially tested a preparation of plasma membrane proteins from insulin-stimulated cells, reconstituted after treatment with low concentrations of cholate, and a preparation of microsomal proteins, reconstituted without detergent treatment. Effects of 1 mM dipeptide on the reconstituted transport are listed in Table III. In the case of the transporters from plasma membranes, the dipeptide inhibited about 40%. Using the microsomal fraction, a greater degree of inhibition (70%) was observed.

We later tested a second preparation of each type of membrane, this time reconstituting without detergent treatment in both cases, and testing the effects of 0.5 and 2 mM dipeptide (Table III). Again, the transporter from the basal microsomes was more sensitive to the dipeptide, being inhibited an average of 60% at 0.5 mM, compared to an average of 29% for the insulin-stimulated plasma membranes. At 2 mM dipeptide, the transport catalyzed by the microsomal proteins was completely inhibited. A comparison of the results of Table III to those of Fig. 2 indicates that the adipocyte transporters reconstituted from microsomal membranes and from plasma membranes were more sensitive and less sensitive, respectively, to the dipeptide than the erythrocyte transporter. In the case of the adipocyte plasma membrane transporters, the inhibition observed at 1 mM dipeptide (almost 40%) was less than observed by Aiello et al. [2] for insulin-stimulated cells (about 80%) and membrane vesicles (about 60%).

These results suggest that there might be different forms of the transporter, with the microsomal form being more sensitive to the inhibitory effect of the dipeptide. In any event, it is clear that the dipeptide does inhibit the adipocyte glucose transporter after reconstitution, and that its actions, observed here in membranes of soybean lipids, are not unique to the membranes of insulin-stimulated cells.

Since the dipeptide did not inhibit glucose transport in basal cells [2], it would be of interest to test whether the dipeptide inhibits the transporter reconstituted from plasma membranes of basal cells. However, the reconstituted activity of the transporter in the basal plasma membranes available to us was too low to allow a test of the effects of the dipeptide.

Effect of phenylarsine oxide on the reconstituted erythrocyte and adipocyte transporters

As with the dipeptide, we initially characterized the effects of phenylarsine oxide on the reconstituted erythrocyte glucose transporter. In experiments similar to those described above for the dipeptide, the transporter was reconstituted using erythrocyte membrane proteins without detergent treatment, and the stereospecific uptake of 0.2 mM glucose in 20 s determined. PAO at concentrations as high as 100 μM had no

TABLE IV

Effects of phenylarsine oxide on reconstituted glucose transport activity

Assays contained 8 μg erythrocyte or 4 μg adipocyte membrane protein (reconstituted without detergent treatment), 1 mg liposome lipid, and 0.2 mM D-glucose. Stereospecific uptake in 20 s (erythrocyte), 30 s (adipose microsomes), or 1 min (adipose plasma membranes) in the presence of the indicated concentrations of PAO was determined and compared to control uptakes. Results are single experiments, means from two experiments (with individual values given), or the mean (±S.E.) from four experiments. In some cases liposomes were preincubated with PAO for the indicated times before being assayed in the presence of the same concentration of PAO; otherwise liposomes were exposed to PAO only at the beginning of the assays.

[PAO] (μM)	Preincubation time (min)	Relative uptake		
		erythrocyte ghosts	adipose (insulin plasma membranes)	adipose (basal microsomes)
1	0	0.94		
2	0	1.05		
5	0	1.01		
10	0	1.08		
20	0	1.03	1.06 (0.94, 1.19)	0.83
50	0	1.08		0.77
100	0	1.01 (0.96, 1.06)	0.90 (0.75, 1.06)	0.75 (0.73, 0.77)
200	0	0.98		0.98 (1.08, 0.87)
20	30	1.01	1.08 (1.03, 1.12)	0.90
100	1	0.87 (0.72, 1.02)		
100	5	0.90 (0.75, 1.05)		
100	30	0.95 ± 0.05 (4)		0.74

significant effect on the uptake (Table IV). These concentrations extend to levels much higher than those (1–10 μM) at which significant effects were observed in the studies using adipocytes [3–6].

We next tested the effects of PAO on the adipocyte glucose transporter, using plasma membranes from insulin-treated cells and microsomes from basal cells. While inhibition of uptake was observed in some cases (Table IV), the effect was weak, with an average of only 10% inhibition seen in 10 determinations made at 20 μM PAO or higher. No trend toward increasing inhibition at higher PAO concentrations was apparent in the range of 20 to 200 μM.

In these experiments the liposomes were exposed to PAO only at the beginning of the assays. Since rapid (within a few second) effects of PAO were observed by Douen and Jones [6], such effects, if they occurred with the reconstituted transporters, should have become significant during the 30-second or 1 min uptake periods, especially at high concentrations of PAO. However, because time-dependent effects were also observed in these studies [6], and because the earlier reports of PAO effects [3–5] employed pretreatment of cells in many cases, we tested the effects of preincubating the liposomes with PAO. Preincubation of the reconstituted erythrocyte transporter with 100 μM PAO for 1 to 30 min before the initiation of the assays (which also contained 100 μM PAO) produced an average of 10% inhibition of the uptake rate; the effect was no greater with a 30 min incubation than with 1 min or 5 min incubations. In the case of the adipocyte transporter, the inhibition observed after a 30 min incubation with 20 or 100 μM PAO (an average of 5% inhibition) was no greater than that produced by PAO without preincubation.

Because PAO is hydrophobic, we considered the possibility that little inhibition was seen because most of it was binding to the lipids in the assays and not free to inhibit the transporter. Since the observed inhibition was so weak, we could not test this possibility by varying the ratio of total inhibitor to lipid at a constant concentration of inhibitor, as was described above for the dipeptide. However, the fact that the inhibition seen with the adipocyte transporter was no greater at 100–200 μM PAO than at 20–50 μM argues against a significant amount of binding of the inhibitor to the liposomes. We attempted to estimate the extent of binding of PAO at 50 and 100 μM to liposomes at 0.5 and 1 mg/ml by measuring the ultraviolet absorbance of the mixtures after filtration through Millipore filters. While the results indicated no significant binding, these are somewhat tentative because of the necessity to correct the data for elution of ultraviolet-absorbing material from the filters; for absorbing material in the filtrate derived from the liposomes; and for apparent binding of PAO to the filters. Nevertheless, it seemed clear that a significant portion of the PAO remained unbound under these conditions, and thus it would also be expected to be primarily unbound at the higher lipid concentrations (3.3 mg/ml) in the assays.

Another argument against the possibility that the lack of inhibition seen after reconstitution results from the binding of PAO to the liposomes is the fact that little or no added inhibition results from preincubation of the lipsoomes for up to 30 min before the assays. If PAO were inhibiting the transporter by reacting covalently with vicinal sulfhydryls, the resulting modification might be expected to be irreversible. With time a significant number of transporters should become modified even if the free concentration of PAO were relatively low; however, no such cumulative inhibition was observed.

It should also be noted that in the assays using adipocytes [6], the lipid concentration must have been much higher (on the order of 50–100 mg/ml) than in our assays, yet PAO effects were observed at micromolar levels. However, most of this lipid is intracellular fat rather than in the membranes, and possibly PAO adsorption to the fat would not occur immediately. Douen and Jones [6] noted the reversal of some effects of low concentrations of PAO and suggested this might be due to gradual partitioning of the PAO into the fat.

Discussion

In the experiments reported here we tested three compounds which were proposed, on the basis of their inhibitory effects on glucose transport in adipocytes, to act directly on the glucose transporter. Only one of the three compounds, the dipeptide Cbz-Phe-Gly-NH_2, produced significant inhibition of the reconstituted transporter.

Intracellular levels of glucose 6-phosphate in adipocytes incubated with 5 mM glucose have been estimated to be about 0.7 to 1.6 mM [14], though it is possible that higher levels could exist in a distinct intracellular pool, as proposed by Foley and Hueckstead [1]. The lack of inhibition of reconstituted glucose transport activity by glucose 6-phosphate at levels high as 5 mM (Table I), 2.5-fold higher than the level estimated to be maximally inhibitory in vesicles [1], argues against direct regulation of the glucose transporter by glucose 6-phosphate under physiological conditions. We do not know the explanation for the inhibitory effects of glucose 6-phosphate observed by Foley and Hueckstead [1], but it seems possible that the effects in intact cells could result from metabolism of the glucose 6-phosphate rather than from the sugar acting directly on the glucose transporter. Foley and Hueckstead [1] noted that "10 mM [^{14}C]glucose 6-phosphate is taken up and metabolized in adipocytes at rates that are near those observed for the simple diffusion of 20 mM glucose", which seems

surprising considering that a phosphorylated sugar should be much less permanent than its nonphosphorylated form. However, it was shown that little of the extracellular glucose 6-phosphate was hydrolyzed under the incubation conditions. In the case of the studies with membrane vesicles, metabolism of the glucose 6-phosphate presumably would not occur, and there must be some other basis for the inhibition which was observed.

Our studies using the dipeptide Cbz-Gly-Phe-NH_2 support the idea that the compound interacts directly with the glucose transporters of erythrocytes and adipocytes, as proposed by Aiello et al. [2]. Both types of transporters were inhibited by the dipeptide after reconstitution (Figs. 1–3; Tables II and III). Since the transport activity was reconstituted using membrane fractions rather than purified transport proteins, we cannot rule out inhibition occurring via interactions of the dipeptide with other membrane proteins which are reconstituted along with the transporters. However, such effects would be reduced, especially in the case of the aidpocyte proteins (which are much less enriched in glucose transporters), by the dispersal of the proteins into different liposomes, unless the proteins mediating the effects remained tightly asociated with the transporters.

It was suggested that the enhanced susceptibility of the adipocyte transporter after insulin stimulation might be due to changes in the membrane environment [2]. The inhibition was equally potent in membranes of egg and soybean lipids (Fig. 2), however, which argues against this possibility. As noted above for other protein components of the membranes, we cannot rule out the possibility that endogenous lipids carried through with the protein in the reconstitution may be involved in mediating the inhibition. However, because of the large excess of liposomal lipid, this appears unlikely, unless the endogenous lipid remained associated with the transporter.

Since the reconstitution results indicate that the dipeptide interacts directly with the transporter, the differences in susceptibility seen in adipocytes might be due to differences in the structure of plasma membrane transporters before and after insulin action. Alterations in the structure and intrinsic activity of plasma membrane transporters in response to insulin treatment, in addition to translocation between membrane fraction, have been supported by several recent studies [15–20]. The observation that the microsomal form of the transporter seems more sensitive to the dipeptide than the plasma membrane form (Table III), in addition to the results of Aiello et al. [2], indicates that the dipeptide might be a useful probe for the study of possible changes in transporter structure.

The transporter reconstituted from adipocyte membranes, as well as from erythrocyte membranes, showed little sensitivity to phenylarsine oxide, even when present at high levels and/or after extensive pretreatment. In agreement with our results, another group has found that 10 to 70 μM PAO had no effect on sorbose uptake in erythrocytes and on glucose transport by the reconstituted erythrocyte transporter [21]. Also in support of our results, a recent study of PAO effects in skeletal muscle found inhibition of insulin-stimulated, but not basal, 2-deoxyglucose uptake [24]. While the glucose transporter contains essential sulfhydryls, it is not surprising that PAO is not a potent inhibitor, since this compound is believed to be most effective when it can form a stable ring structure with vicinal sulfhydryls. The sequences determined for human [22] and rat [23] glucose transporters contain only six sulfhydryls, and it is likely that these are not properly aligned for the formation of such complexes, or else are not accessible to PAO action.

Our results suggest that the effects of PAO observed by Douen and Jones [6] were not due to direct actions of the compound on the transporter. Rather, the effects could be primarily on the pathway by which insulin stimulates the transport, as proposed by Frost and Lane [3]. In the case of inhibition of transport in cells that were already stimulated by insulin, it is possible that blocking the stimulatory pathway results in overall inhibition as the transporters return to their basal state despite the continued presence of insulin. This can be seen in Fig. 5 of Ref. 3, where similar decreases in deoxyglucose uptake in insulin-stimulated cells were produced by addition of PAO in the presence of insulin, or by withdrawal of insulin. The only clear inhibition of transport in basal cells by PAO reported by Douen and Jones is shown in Fig. 1 of Ref. 6. However, the stimulatory effect of insulin in these experiments, about 6-fold, was lower than in many other studies. Experiments with adipocytes are characterized by variability in basal rates, as several factors involved in the preparation of the cells can result in cells with elevated basal glucose uptakes [25]. It seems possible that PAO, in a manner similar to its inhibitory action in insulin-stimulated cells, could be lowering an elevated basal activity by blocking insulin-like actions of some other factors rather than by directly inhibiting the transporter.

In summary, the technique of reconstitution of the glucose transporter into liposomes has proven useful in testing proposed mechanisms of regulation of the transport protein, and should be useful in further studies aimed at identifying possible ways in which insulin may alter the structure of the transporter.

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