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Involvement of Membrane Sulfhydryls in the Activation and Maintenance of Nutrient Transport in Chick Embryo Fibroblasts

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At 5 μg/ml, insulin stimulates hexose, A-system amino acid, and nucleoside transport by serum-starved chick embryo fibroblasts (CEF). This stimulation, although variable, is comparable to that induced by 4% serum. The sulfhydryl oxidants diamide (1–20 μM). hydrogen peroxide (500 μM), and methylene blue (50 μM) mimic the effect of insulin in CEF.

PCMB-S,[1] a sulfhydryl-reacting compound which penetrates the membrane slowly, has a complex effect on nutrient transport in serum- and glucose-starved CEF. Hexose uptake is inhibited by 0.1–1 mM PCMB-S in a time- and concentration-dependent manner, whereas A-system amino acid transport is inhibited maximally within 10 min of incubation and approaches control rates after 60 min. A differential sensitivity of CEF transport systems is also seen in cells exposed to membrane-impermeant glutathione-maleimide I, designated GS-Mal. At 2 mM GS-Mal reduces the rate of hexose uptake 80–100% in serum- and glucose-starved CEF; in contrast A-system amino acid uptake is unaffected. D-glucose, but not L-glucose or cytochalasin B, protects against GS-Mal inhibition. These results are consistent with the hypothesis that sulfhydryl groups are involved in nutrient transport and that those sulfhydryls associated with the hexose transport system and essential for its function are located near the exofacial surface of the membrane in CEF.

Key words: **transport, sulfhydryl oxidants, p-chloromercuribenzenesulfonate, glutathione maleimide I**

When insulin binds to its receptors, the resulting mitogen-receptor complexes generate an array of temporally distinct changes in the cell, commencing with a rapid increase in the flux of ions, sugars, amino acids, and nucleosides across the plasma membrane and culminating in replication. Kinetic analysis of the insulin-induced increase in nutrient transport establishes it as due mostly to an increase in V_{max} rather than to a decrease in K_m (1). This has been interpreted as reflecting the recruitment and/or activation of transport components.

[1]Abbreviations: α-AIB – α-aminoisobutyric acid; α-meAIB – α-methylaminoisobutyric acid; 2-dG – 2-deoxy-D-glucose; DTP – dithiopyridine; NEM – N-ethylmaleimide; PCMB – p-chloromercuribenzene; PCMB-S – p-chloromercuribenzenesulfonate; DMSO – dimethyl sulfoxide.

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How the formation of mitogen-receptor complexes triggers the increased activity of transport proteins remains obscure, but membrane sulfhydryls may play a key role in this event. This idea stems from the fact that low concentrations of sulfhydryl oxidants can mimic insulin stimulation of hexose transport. Czech (2) and his colleagues have observed that 20 mM diamide, 4 mM hydrogen peroxide, and 50 μM methylene blue stimulate the uptake of 3-O-methylglucose by isolated brown fat cells to rates comparable to those achieved by 0.1 μg/ml of insulin. Furthermore, compounds which react with sulfhydryl groups inhibit hexose, amino acid, and nucleoside transport. For example, 30 μM PCMB inhibits 2-dG uptake in Novikoff cells (3) and a more hydrophilic derivative, PCMB-S, inhibits 2-dG uptake in mouse embryo cells (4). PCMB (3) and DTP (4) also inhibit uridine transport in cultured cells. Hare (4) observed that PCMB-S inhibits System L amino acid transport to a greater extent than System A in mouse embryo cells. His observations, together with those of Kwock and his colleagues (5, 6) on the effects of γ-ray irradiation on amino acid transport in thymocytes suggest that System L transport components, like those mediating glucose transport, have sulfhydryl groups lying near the exofacial membrane surface whereas the sulfhydryl reactant-sensitive sites of System A transport components lie near the endofacial surface or in hydrophobic regions of the membrane (7).

Recent evidence supporting the idea that some part of the glucose transport system is located in the exterior face of the plasma membrane is based on the observation by Batt et al. (8) that a membrane-impermeant maleimide (GS-Mal) inhibits glucose efflux in human red blood cells. The extent of inhibition was directly related to the binding of GS-Mal to the membrane (9). We have synthesized GS-Mal and have observed that it is a potent inhibitor of glucose transport in CEF while having little or no effect on amino acid transport. These results are communicated in this report along with our observations on the effects of sulfhydryl oxidants and inhibitors on nutrient transport.

MATERIALS AND METHODS

Cell Cultures

The procedures used for culturing chick embryo fibroblasts were as described previously (1). In general, cells were plated at 5×10^5 cells per 60-mm diameter Lux plastic plate or at 1.5×10^5 cells per 35-mm plate, sometimes fed on day 3 and used on day 4 or 5. For serum starvation experiments, CEF were rinsed in either Tris-dextrose or serum-free medium and maintained at 37°C for 6–8 h in serum-free medium prior to the measurement of transport. In some experiments, the serum-free medium was supplemented with 0.15% tryptose-phosphate.

For glucose starvation experiments, cells were rinsed in glucose-free medium and maintained in glucose-free medium containing 4% dialyzed serum for 20–24 h. In certain experiments, cells were starved for both glucose and serum. Under these conditions, although numerous cells often detach from the plate, the majority appear to tolerate glucose and/or serum starvation without incurring ostensible damage.

Experiments With Sulfhydryl Oxidants

Triplicate plates were each rinsed twice with approximately 5 ml warm (37°C) Dulbecco's phosphate-buffered saline (PBS), pH 7.4, and then incubated for 10 min at 37°C in 1 ml PBS containing 5 μg of porcine insulin (Schwarz-Mann, Orangeburg, New York) or various amounts of oxidizing agent. After 10 min, 0.5 ml reaction mixture con-

taining 3 mM α-[1-^{14}C] AIB acid (New England Nuclear Corporation, Boston, Massachusetts; specific activity 0.3 μCi/μmol) and 3 mM [G-^{3}H] 2-dG (New England Nuclear Corporation; specific activity 1.3 μCi/μmol) or 0.75 μM [5,6-^{3}H]-uridine (New England Nuclear Corporation; specific activity 5.2 μCi/μmol) in PBS was added; the final concentration of sugar and of amino acid was 1 mM, while that of uridine was 0.25 μM. Under these conditions, transport was the rate-limiting step for uptake of sugar for at least 10 min. After 5 min incubation at 37°C, uptake of the radiolabeled nutrients by the cells was arrested by rapidly removing the radioactive medium and rinsing each plate 3–5 times with 5 ml ice-cold PBS. The cells were digested in 1 ml 0.2 N NaOH; the digest was transferred to tubes and combined with 0.5 ml H_2O rinse. Aliquots were then taken for protein estimation (10) and for determination of radioactivity using dioxane-toluene scintillation fluid and an Intertechnique (Canatech, Incorporated, Montreal, Canada) liquid scintillation counter.

Experiments With PCMB-S

A solution of PCMB-S (Sigma Chemical Co., St. Louis, Missouri) in PBS was prepared just prior to use. Cells grown in 35-mm diameter plates were rinsed twice in warm PBS and incubated in 0.5 ml PBS containing various concentrations of PCMB-S for designated intervals at 37°C. This solution was removed, the cells washed twice in warm PBS, and uptake of 2-dG and α-me AIB was measured over 2 min as in experiments with GS-Mal.

Experiments With GS-Mal

Bismaleimidoethyl ether was prepared by the procedure of Tawney et al. (11) and was used in the synthesis of GS-Mal I according to the method of Abbott and Schachter (9). This species differs from GS-Mal II and III in its relatively short "arm" that joins the reactive maleimide with glutathione. Just before use, GS-Mal was dissolved in cold 50 mM sodium phosphate, pH 7.4, 100 mM NaCl, to a concentration that was twice that of the final concentration designated in the legends to the Figures or Tables. Depending on the concentration of GS-Mal, the pH shifted to 6.8 or was adjusted to this value. Confluent cultures of glucose-starved CEF in 35-mm plates were rinsed twice with warm 50 mM sodium phosphate, pH 6.8, 100 mM NaCl, and then incubated with 0.5 ml phosphate buffer, pH 6.8, containing the substance to be tested for various times at 37°C. Uptake of sugar and amino acid by CEF was measured either directly after this treatment or after 2 rinses in warm PBS and a 30-min incubation in 1 ml PBS. In either case, plates were rinsed with warm PBS and 0.5 ml reaction mixture containing 1 mM α-[1-^{14}C] meAIB (New England Nuclear Corporation; specific activity 0.3 μCi/μmol) and 1 mM [G-^{3}H] 2-dG in PBS (specific activity 1.3 μCi/μmol) was added. Under these conditions, uptake of sugar and amino acid in glucose-starved cells is linear for at least 5 min. The uptake assay was terminated after 2 min by removing the reaction mixture, placing the culture plates on ice, and washing the cells 5 times with ice-cold PBS. The protection against GS-Mal-induced inhibition of 2-dG transport by competing hexoses and competitive inhibitors of glucose transport was examined. Cytochalasin B (Aldrich Chemical Co., Milwaukee, Wisconsin) and phloretin (Sigma) were prepared as stock solutions (2 mg/ml) in DMSO. Controls were treated with equivalent amounts of DMSO during incubation and/or during the transport assay. Cell protein was hydrolyzed in 0.4 ml 0.5 N NaOH, the digest was transferred to tubes and combined with a 0.4 ml H_2O rinse. Samples were processed as described above, except Triton X-100:toluene (1:2) was employed as scintillant.

RESULTS

Stimulation of Nutrient Transport by Insulin

Preliminary experiments were conducted to determine the optimal conditions for stimulation of sugar transport in CEF by insulin. In most of the experiments reported here, the fibroblasts were serum-starved for 6–18 h before treatment with various agents and measurement of transport. Raizada and Perdue (12) have shown that although serum-starvation depresses the basal rate of sugar uptake by CEF, the response of these cells to insulin is relatively greater than that of serum-fed CEF.

Confluent cells were incubated in PBS containing 5 μg/ml insulin for varying times up to 60 min and then 2-dG uptake was measured over 5 min. Insulin stimulated CEF to transport sugar at rates averaging 20–30% and up to 70% greater than the rate of sugar uptake in controls. In general, the percent stimulation of transport by insulin was comparable to that induced by 4% serum. Transport was maximally stimulated by 7.5 min exposure of cells to insulin, although in some experiments, this occurred as early as 2 min (data not shown).

The stimulation of sugar and amino acid uptake by insulin was highly variable in that out of 53 experiments, only 50% showed a significant increase in the rates of transport of insulin-treated cells. Similarly, out of 12 experiments involving serum-stimulation of transport, 5 indicated that CEF took up sugar and amino acids at higher rates than controls. The reason for this variability remains obscure.

Stimulation of Nutrient Transport by Sulfhydryl Oxidants

The effect of diamide and hydrogen peroxide on nutrient transport by CEF was studied by exposing cells to various concentrations of these reagents for 10 min and measuring the uptake of labeled 2-dG, α-AIB and/or uridine during 5 min. Figure 1 and 2 show that both of these oxidizing agents stimulate transport and that this effect is concentration-dependent. Maximum response of CEF in these experiments was at 1–20 μM diamide and 500 μM hydrogen peroxide. As seen in Fig. 3, 50 μM methylene blue stimulates sugar transport. In this experiment, methylene blue also stimulated α-AIB uptake, but only by 10%. The magnitude of the stimulation of transport activity induced by diamide and methylene blue was maximum by 10 min treatment of the cells. High concentrations of all 3 reagents inhibit sugar transport whereas they have little or no effect on the basal rate of amino acid transport.

As in studies of the response of CEF to insulin, the stimulation of transport by the chemical oxidants was variable with respect to whether the response occurred, its magnitude and the optimal concentration of reagent required to elicit a response. Approximately half of the 15–19 experiments in which the effect of diamide, hydrogen peroxide, or methylene blue on nutrient transport was tested showed stimulation of uptake by these reagents. In some experiments the sulfhydryl oxidants stimulated transport while insulin failed to do so, and in other experiments the converse was observed.

Effect of PCMB-S

The effect of PCMB-S on nutrient transport in CEF is complex. At very low concentrations (0.01 mM) and short treatment times, PCMB-S causes a slight stimulation of hexose and also amino acid uptake (Table I). At higher concentrations, however, this sulfhydryl reactant inhibits hexose uptake in a concentration- and time-dependent manner.

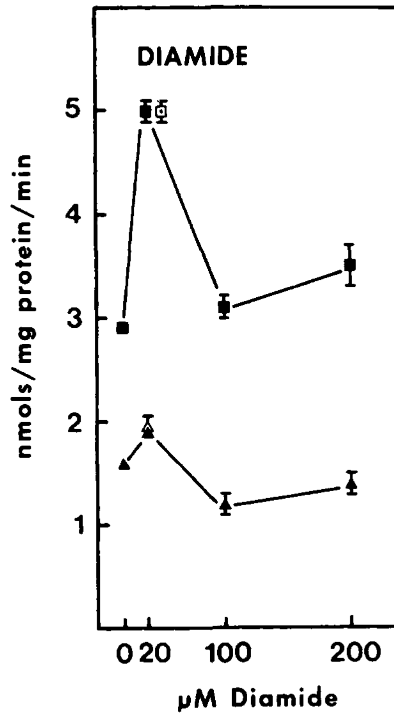

Fig. 1. Stimulation of nutrient transport by diamide. Serum-starved CEF were treated with various concentrations of diamide for 10 min and then uptake measured over 5 min. ▲—▲) uridine uptake; ■—■) α-AIB uptake. Open symbols denote uridine (△) and α-AIB (□) uptake in the presence of 5 μg/ml insulin. Bars represent standard error.

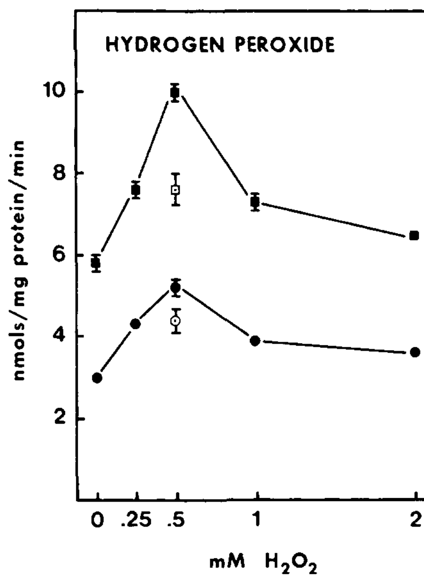

Fig. 2. Stimulation of nutrient transport by hydrogen peroxide. Serum-starved CEF were treated with various concentrations of hydrogen peroxide for 10 min and then uptake measured over 5 min. ●—●) 2-dG uptake; ■—■) α-AIB uptake. Open symbols denote 2-dG (○) and α-AIB (□) uptake in the presence of 5 μg/ml insulin. Bars represent standard error.

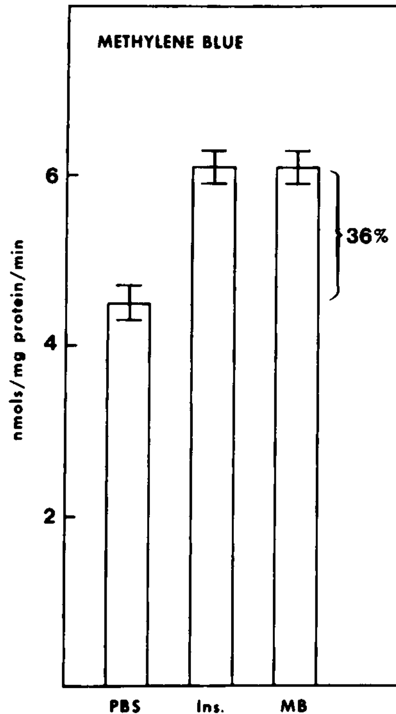

Fig. 3. Stimulation of hexose transport by methylene blue. Serum-fed CEF were treated with 5 μg/ml insulin or 50 μM methylene blue for 10 min and uptake of 2-dG measured over 5 min. Bars represent standard error. The uptake of α-AIB was elevated by insulin and methylene blue 20% and 10% respectively in this experiment.

TABLE I. Effect of PCMB-S on Sugar and Amino Acid Uptake as a Function of Time and Concentration*

	2-dG uptake (% of control)			α-meAIB uptake (% of control)		
PCMB-S (mM)	10 min	30 min	60 min	10 min	30 min	60 min
0.01	129	74,90	–	120	62	–
0.1	88,76	59,80,90	56	50,29	84,77	79
0.5	47	34,47	–	40	77	–
1	50,58	24,30,24	17	32,60	95,85	91
10	–	15	–	–	–	–

*CEF starved for serum and glucose for approximately 24 h were incubated in PBS, pH 7.4, containing various concentrations of PCMB-S for 10, 30, or 60 min. Uptake of sugar and amino acid was measured over 2 min. Data is from 3 separate experiments.

Table I shows that inhibition is rapid; about 50% inhibition is achieved within 10 min at 1 mM PCMB-S. Maximum inhibition is 85% and results from 30 min treatment with 10 mM PCMB-S. Thus 15% of sugar transport activity appears resistant to PCMB-S.

The effect of PCMB-S on amino acid transport is distinct from its effect on sugar transport. Low concentrations (0.01–0.1 mM) or 10 min treatment with higher concentrations (0.1–1 mM) of PCMB-S inhibit α-meAIB uptake greater than 50%. However, this inhibition

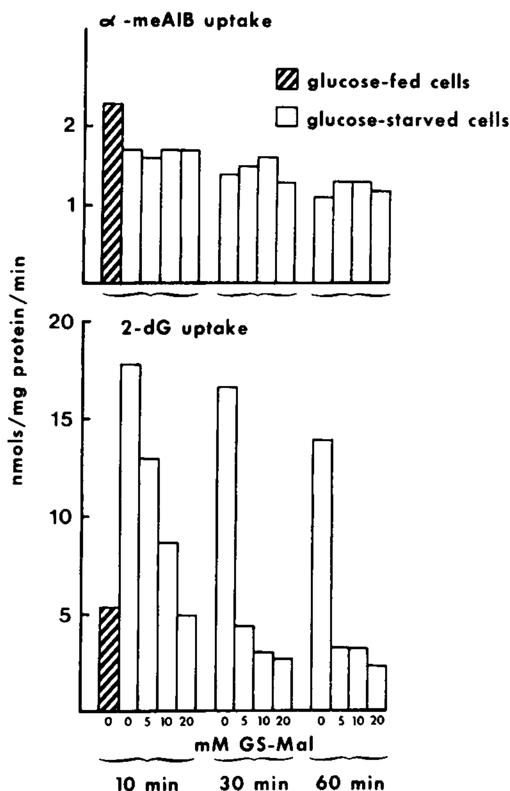

Fig. 4. Effect of GS-Mal on nutrient transport as a function of inhibitor concentration and time of treatment. Glucose-starved (24–27 h) CEF were exposed to 0–20 mM GS-Mal in 50 mM sodium phosphate, pH 6.8, containing 100 mM NaCl for 10, 30, or 60 min. Glucose-fed and glucose-starved controls were treated with pH 6.8 sodium phosphate buffer for comparable intervals. Cells were rinsed twice in PBS and the uptake of α-meAIB and 2-dG was measured over 2 min. The standard error in this experiment averaged less than 5% for sugar uptake and 10% for amino acid uptake.

is transient and when cells are exposed to high concentrations of PCMB-S for 30 or 60 min, amino acid transport is no longer as severely inhibited.

Experiments With GS-Mal

Since GS-Mal breaks down more rapidly at pH 7.4 than in acid conditions (9), treatment of CEF with this compound was carried out in isotonic 50 mM sodium phosphate buffer, pH 6.8. The effect of low pH is to decrease the basal rate of both sugar and amino acid uptake but these transport systems still remain sensitive to the addition of stimulatory and inhibitory agents. To elevate the basal rate of glucose uptake, cells were either glucose-starved or serum- and glucose-starved for approximately 24 h prior to treatment. Under these conditions, glucose uptake by starved CEF is enhanced about fivefold when measured in PBS and twofold after cells are incubated in pH 6.8 buffer. Figure 4 shows the effect of treatment with 5, 10, or 20 mM GS-Mal for 10, 30, or 60 min on 2-dG and α-me AIB uptake in glucose-starved CEF; the latter uptake reflects amino acid transport by System A. A maximum 85% inhibition of 2-dG uptake resulted from exposure of cells to 20 mM GS-Mal for 30 min in this experiment. Experiments were conducted to see if lower con-

TABLE II. Effect of Glucose, Cytochalasin B (CB), and Phloretin on the Inhibition of Hexose Transport by GS-Mal*

Treatment of cells	Uptake 2-dG nmols/mg protein/min	% of control
Expt. 1		
glucose-fed	6.1 ± 0.48	60
glucose-starved	10.2 ± 0.72	100
glucose-starved + 20 mM GS-Mal	3.1 ± 0.13	30
glucose-starved + 50 mM glucose	12.0 ± 1.20	118
glucose-starved + GS-Mal + 50 mM glucose	4.1 ± 0.30	34
glucose-starved + 100 mM glucose	14.1 ± 0.27	138
glucose-starved + GS-Mal + 100 mM glucose	4.1 ± 0.12	29
Expt. 2		
glucose-fed	12 ± 0.22	54
glucose-starved	22.4 ± 0.53	100
glucose-starved + 20 mM GS-Mal	6.0 ± 0.57	27
glucose-starved + 10 μg/ml CB	1.4 ± 0.22	6
glucose-starved + 10 μg/ml CB, reversal	21.6 ± 0.90	96
glucose-starved + GS-Mal + CB, reversal	4.8 ± 0.10	21
"Trapping" control	2.7 ± 0.67	
Expt. 3		
glucose-starved	9.6 ± 0.52	100
glucose-starved + 10 mM GS-Mal	2.7 ± 0.03	28
glucose-starved + 10 μg/ml phloretin	2.0 ± 0.23	14
glucose-starved + 10 μg/ml phloretin, reversal	5.4 ± 0.47	56
glucose-starved + GS-Mal + phloretin, reversal	3.1 ± 0.13	32
"Trapping" control	2.2 ± 0.20	

*Glucose-starved cells (24 h) were incubated in 50 mM sodium phosphate buffer, pH 6.8, containing 100 mM NaCl with or without GS-Mal. Glucose, cytochalasin B, and phloretin were made up double-strength in pH 6.8 buffer and combined with twice-concentrated GS-Mal to give the appropriate final concentration of each compound. In the case of experiments involving cytochalasin B and phloretin, all incubations were carried out in the presence of 0.5% DMSO. After treatment with GS-Mal and/or "protective" agents for 30 min, the cells were rinsed twice with PBS and incubated in PBS for 30 min before a 2 min assay for uptake of 2-dG and α-meAIB. In general, the rate of amino acid transport was not affected by GS-Mal, glucose, cytochalasin B, or phloretin. Values for "trapping" were determined by adding the radioactive assay mixture to rinsed cells on ice and immediately removing this solution.

centrations of GS-Mal might achieve as dramatic an inhibition of 2-dG transport as 20 mM GS-Mal if the duration of treatment were extended. It was found that if cells are exposed to 1 or 2 mM GS-Mal for 60 min, glucose uptake is reduced by greater than 60% or 85%, respectively (see Table III for GS-Mal at 2 mM). Although 20 mM GS-Mal occasionally affects α-meAIB transport, 2 mM GS-Mal never inhibits this transport system. Uridine transport is also unaffected by 2 mM GS-Mal, but preliminary results indicate that leucine, an L-system substrate, may be inhibited.

Experiments were carried out to determine whether substances known to compete with glucose for the hexose-binding site could protect against inhibition by GS-Mal. Since 30 min treatment with 20 mM GS-Mal resulted in maximal inhibition of 2-dG transport,

TABLE III. Effect of D-Glucose and L-Glucose on the Inhibition of Hexose Transport by 2 mM GS-Mal*

Treatment of starved cells	Uptake 2-dG (nmoles/mg protein/min)	% of control
control	4.7 ± 0.20	100
2 mM GS-Mal	2.0 ± 0.06	27
2 mM D-glucose	5.2 ± 0.55	114
GS-Mal + 2 mM D-glucose	4.7 ± 0.10	100
2 mM L-glucose	5.2 ± 0.45	114
GS-Mal + 2 mM L-glucose	2.4 ± 0.60	38
50 mM D-glucose	5.1 ± 0.50	111
GS-Mal + 50 mM D-glucose	5.0 ± 0.10	108
50 mM L-glucose	4.9 ± 0.20	105
GS-Mal + 50 mM L-glucose	1.8 ± 0.25	22
"Trapping" control	1.0 ± 0.05	

*Cells were starved for both serum and glucose for 24 h prior to treatment with GS-Mal in the presence or absence of D- or L-glucose for 60 min. After 2 rinses in PBS, the cells were incubated in PBS for 30 min and then uptake of 2-dG and α-meAIB measured over 2 min. The rate of amino acid transport was constant under all these conditions.

this condition was selected for "protection" experiments. D-Glucose, cytochalasin B, and phloretin were added alone or together with GS-Mal to CEF and incubated for 30 min at 37°C. After 2 washes, the cells were incubated in PBS for another 30 min before the measurement of transport was carried out. The results in Table II show that even 50 or 100 mM D-glucose do not alleviate the inhibitory effect of GS-Mal. Maltose (10 mM) was also tested; it failed to protect against GS-Mal inhibition, but this finding was later explained by the fact that maltose does not compete with 2-dG for uptake by CEF (data not shown). Cytochalasin B (10 μg/ml) inhibits 2-dG uptake in glucose-starved CEF nearly 100%. The inhibition is almost completely reversed 30 min following removal of cytochalasin B from the incubation medium. Although α-meAIB uptake also appears to be reduced in this experiment, in most cases amino acid transport was unaffected in cells treated with cytochalasin B. When cytochalasin B is included in the buffer containing GS-Mal, the rate of sugar transport remains as low as in cells treated with GS-Mal alone after both inhibitors are removed. Similar results were obtained when 10 μg/ml phloretin was added simultaneously with GS-Mal in an attempt to protect the hexose transport component.

The possibility that D-glucose might protect against inhibition of low amounts of GS-Mal was tested and the results are shown in Table III. When D-glucose is present during 60 min treatment of CEF with 2 mM GS-Mal and both glucose and inhibitor are removed for 30 min, the rate of sugar uptake is substantially higher than that in cells exposed to GS-Mal alone. The protective effect of D-glucose ranged from 30 to 100% in several experiments. Equimolar (2 mM) concentrations of D-glucose protect as efficiently as extremely high amounts. Table III also shows that L-glucose fails to protect the hexose transport system against GS-Mal-induced inhibition. The possibility that cytochalasin B might block the inhibition of low amounts of GS-Mal was examined. Unlike D-glucose, 10 μg/ml cytochalasin B does not have any effect on the degree of inhibition of 2-dG uptake by 2 mM GS-Mal.

DISCUSSION

The results of our studies on the effects of sulfhydryl-reacting compounds on nutrient transport in CEF are consistent with the hypothesis that membrane sulfhydryl groups are involved in the activation and maintenance of transport components. In agreement with Czech's observations on isolated fat cells (2), diamide, hydrogen peroxide, and methylene blue stimulate nutrient uptake by CEF to a degree comparable to the increase in transport induced by insulin. Avian fibroblasts appear to be more sensitive than fat cells to diamide and hydrogen peroxide since the concentrations of these sulfhydryl oxidants required to optimally stimulate transport in CEF are much lower than those needed to stimulate hexose uptake in fat cells. In fat cells, it has been shown that diamide, hydrogen peroxide, and methylene blue exert their effect on the transport step, rather than on the metabolism of glucose. Previous work indicates that whereas diamide (13) and hydrogen peroxide (14) primarily oxidize intracellular glutathione, methylene blue oxidizes NADH and NADPH (14) which may secondarily oxidize glutathione. Presumably, reduced glutathione maintains membrane sulfhydryls in the reduced state.

A differential in the sensitivity of nutrient transport systems was also underscored in these studies. At relatively high concentrations of diamide, hydrogen peroxide, and methylene blue, 2-dG uptake, but not α-AIB uptake, was substantially inhibited. The cause for this inhibition is not clear, but the hexose transport component is selectively affected. PCMB-S, a hydrophilic sulfhydryl-reacting compound which does not readily penetrate the membrane (15), has a complex effect on nutrient transport in CEF. At 10 μM, PCMB-S stimulates both hexose and amino acid transport slightly. With 10- to 100-fold greater concentrations of PCMB-S and/or longer exposure of cells to this compound, hexose uptake is progressively diminished. However, complete inhibition of 2-dG uptake is not achieved even at 10 mM PCMB-S. These results are consistent with the idea expressed by Hare (4) that sulfhydryl groups lying near the exterior face of the membrane are associated with hexose transport. In contrast to hexose transport, A-system amino acid transport is transiently inhibited by PCMB-S after brief exposure to the sulfhydryl reactant, but with longer treatment, the cells resume close to control rates of α-meAIB transport. This recovery is not due to the inactivation of PCMB-S since it simultaneously inhibits 2-dG uptake. These results contrast with those of Hare (4) who found that uptake of phenylalanine by mouse embryo cells was rapidly and irreversibly inhibited by PCMB-S over a period of 1 h. These conflicting observations may be accounted for by differences in the disposition of A- and L-system amino acid transport components in the membrane or differences between CEF and mouse embryo cells. An explanation is being sought by extending uptake determinations to L-system amino acids as well as to cells grown under different culture conditions.

By using the membrane-impermeant GS-Mal, which reacts irreversibly with free sulfhydryl groups lying on or very close to the exofacial surface of the membrane, we have demonstrated the importance of these groups for hexose transport in CEF. The same sulfhydryl groups which react with maleimide are involved in the binding of D-glucose, since this hexose but not L-glucose protects against GS-Mal inhibition. Two competitive inhibitors of glucose transport in CEF, cytochalasin B and phloretin (16, 17), failed to protect against GS-Mal-induced inhibition and thus presumably bind to a site distinct from that which reacts with maleimide. By inference, this site must also be different from that which binds glucose. Perhaps the cytochalasin B- and phloretin-binding site(s) is close enough to the glucose-binding site to sterically hinder the binding of the sugar and thus

display competitive kinetics for hexose uptake. However, their interaction with the site is then not so tight as to block the binding of GS-Mal. On the other hand the apparent competitive inhibition of glucose uptake by compounds whose structure does not resemble that of hexoses must be interpreted with caution. In this regard, Plagemann and Erbe (18) in their studies of the activation energies of 2-dG transport in cultured Novikoff cells suggested that cytochalasin B competitively inhibits hexose uptake by altering the fluidity of the lipid associated with this transport system.

It is evident that the hexose transport system in cultured cells differs from that present in human erythrocytes. For example, maltose which is recognized by the glucose transport system in the red blood cell, although it is not transported, protects against GS-Mal-induced inhibition (8). In CEF, maltose does not compete with 2-dG for uptake and does not protect against the inhibitory effect of GS-Mal in fibroblasts. Whereas in the erythrocyte, both glucose and cytochalasin B protect against inhibition of glucose transport induced by high concentrations of GS-Mal (10–20 mM) (8), only glucose protects against the impermeant-maleimide inhibition in CEF and only when the concentration of the inhibitor is reduced to 1–2 mM. In erythrocytes glucose also inhibits the binding of radiolabeled cytochalasin B, whereas in 3T3 cells glucose fails to compete for the cytochalasin B-binding site (19). Finally, evidence from reconstitution studies of red blood cell membranes strongly suggests that the 55,000–60,000 dalton glycopeptides present in zone 4.5 of sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels are components of the hexose transport system (20, 21). Recent studies by Banjo and Perdue (22) suggest that polypeptides of 75,000 and 95,000 daltons may comprise the transport system in CEF. This conclusion is based on studies of radiolabeled amino acid incorporation into plasma membrane polypeptides of glucose-starved cells. Future studies will be directed toward the identification and characterization of the hexose transport component(s) in CEF by analyzing extracted membrane peptides from cells exposed to radiolabeled GS-Mal in the presence or absence of glucose.

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