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DIFFERENTIAL INACTIVATION OF THE "L" AND "Ly^+" AMINO ACID TRANSPORT SYSTEMS BY A SULFHYDRYL REAGENT AND A PHOTOAFFINITY PROBE

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SUMMARY

Using a substrate-stimulated amino acid efflux system, it has been shown that the "Ly^+" and "L" amino acid transport systems of mouse embryo cells in culture are differentially inhibited by parachloromercuribenzene sulfonate (PCMB-S) and the photoaffinity probe 4-fluoro-3-nitrophenylazide (FNPA). Three types of evidence support the conclusion that these transport systems are mediated by separate carrier proteins. (1) The specificity of substrate-stimulated efflux is high for each system; (2) PCMB-S inhibits L-phenylalanine and L-leucine stimulated L-[^{3}H]phenylalanine efflux with no effect on L-lysine stimulated L-[^{3}H]lysine efflux, and (3) the photoaffinity probe FNPA inhibits L-lysine efflux with little effect on the L-phenylalanine-stimulated efflux.

INTRODUCTION

Chemical reagents have been utilized frequently to probe the function of a variety of carrier-mediated transport systems. In combination with careful kinetic analysis, reagents such as 2,4-dinitrofluorobenzene (DNFB) have led to a better understanding of the complex kinetic behavior of "carrier" systems such as that involved in the amino acid transport system "L" [1, 2] and the sugar transport system [3].

A second type of approach utilizing chemical reagents which react with specific chemical groups has proven useful for the purpose of recognizing potential differences in the presence or accessibility of specific reactive groups among several transport systems. This has been exploited by Cabantchik and Rothstein [4] to identify and isolate a membrane protein associated with the anion transport mechanisms in red blood cells. More recently, studies in our laboratories have utilized this approach, in which differential effects of sulfhydryl reactive reagents on sugar, nucleoside and

Abbreviations: PCMB-S, parachloromercuribenzene sulfonate; FNPA, 4-fluoro-3-nitrophenylazide; DNFB, 2,4-dinitrofluorobenzene.

two amino acid transport systems [5] have been demonstrated. In addition, we have begun the development of photoaffinity reagents which behave as either site-specific analogs of a transported substrate or as chemicals which react more nonspecifically yet differentially in hydrophobic or hydrophilic regions of the membrane.

This paper illustrates the use of several such reagents which react differentially with the "L" and the "Ly+" systems for amino acid transport. These studies suggest that there are fundamental structural differences between the proteins which function in the "L" and "Ly+" transport systems of mouse embryo cells.

MATERIALS AND METHODS

Mouse embryo cell cultures were prepared from 14- to 18-day old embryos by trypsinization followed by growth in Eagle's minimal essential medium (Auto-Pow, Flow Laboratories) containing 5–10 % fetal calf serum. Experiments were carried out with first passage cells growing as a confluent monolayer in 35 mm plastic Petri dishes.

The transport system studied was the stimulated efflux of an intracellular labeled amino acid brought about by the counter-transport of an extracellular, non-labeled amino acid. Cells were pre-labeled with 0.01 mM L-[^{3}H]phenylalanine or L-[^{3}H]lysine for 10 min at 26 °C. The cell monolayers were rinsed three times with iced Tris buffered saline, buffer A [6] and kept on ice for no more than 5–10 min until the beginning of the efflux period. At that time, 2 ml of buffer A at 20 °C containing combinations of 1.0 mM parachloromercuribenzene sulfonic acid (PCMB-S) and the amino acid (0.4 mM) to be used for the trans-stimulation, were added to dishes which had been pre-equilibrated 1–2 min at 20 °C on a perforated aluminum tray on a circulating constant-temperature water bath. Efflux was allowed to proceed for between 1 and 20 min at which time the efflux was removed and counted in an Isocap 300 scintillation spectrometer in PCS (Amersham-Searle) diluted 1 : 1 with toluene. Each experiment was carried out 2 or 3 times and demonstrated identical results. Each experimental point was carried out in duplicate and duplicate counts of radioactivity measured. All experimental results showed standard errors of 5–10 % or less.

Photolysis procedure

Preliminary experiments were carried out to evaluate the rate of efflux from cells held at 5 °C. These studies demonstrated that over a period of at least 20 min there was a negligible efflux of either [^{3}H]lysine or [^{3}H]phenylalanine but that efflux could be stimulated 3-fold by the counter-transport of the homologous substrate. The low level of spontaneous efflux at 5 °C made it possible for the cells to be pre-loaded with [^{3}H]amino acid, rinsed on ice with iced buffer A and to be exposed to the photoaffinity probe, 4-fluoro-3-nitrophenyl azide (FNPA) at 0.8 mM in buffer A for 5 min at 5 °C during photolysis. FNPA was prepared as described by Fleet et al. [7]. Photoactivation was carried out with a Model 457 micropulser (Xenon Corp) equipped with a xenon flash lamp delivering 11 pulses per s. Controls monitored in the dark showed no effect of FNPA on the transport system studied and cells photolyzed in the absence of the photoprobe showed no loss of transport capacity.

Following photolysis, the buffer containing FNPA was removed, the cell monolayer rinsed three times, and 2 ml of efflux buffer with and without the amino acid substrate was added for time periods indicated. The radioactivity was measured as described above.

Radioactive substrates purchased from New England Nuclear Corp. had specific activities as follows: L-[*alanine* 3-^{3}H(N)] phenylalanine 15.1 Ci/mmol, and L-[4,5-^{3}H(N)]lysine 38.9 Ci/mmol. Parachloromercuribenzene-sulfonic acid and aminoisobutyric acid were from Sigma Chemical Co., and all other chemicals were reagent grade.

RESULTS

Specificity of the transport systems

The transport of L-lysine and L-phenylalanine in mouse embryo cells in culture is mediated by systems which may be distinguished on the basis of the specificity of efflux stimulation. As shown in Fig. 1, if cells are pre-loaded with 0.01 mM L-[^{3}H]-lysine, efflux of the intracellular amino acid is stimulated only by extracellular L-lysine (0.4 mM) and by none of the other amino acids representative of substrates transported by the "L", "A", or "ASC" systems of Christensen [1]. On the other hand, L-[^{3}H]phenylalanine efflux is stimulated only by L-leucine and L-phenylalanine but not by L-lysine or other representative substrates. These results are compatible with the interpretation that L-lysine is transported predominantly by the "Ly$^+$" and L-phenylalanine by the "L" systems in the mouse embryo cells.

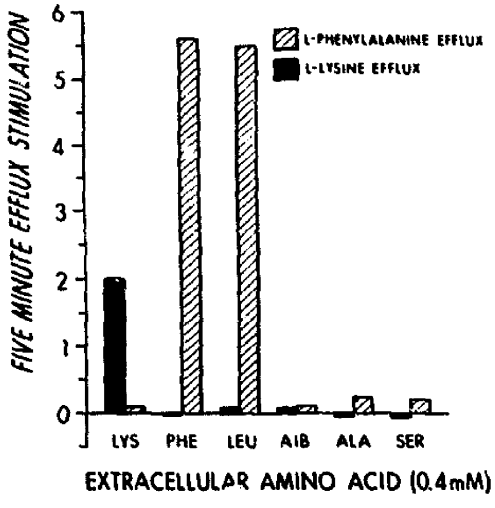

Fig. 1. Stimulated efflux of L-lysine or L-phenylalanine by various amino acids. Mouse embryo cells were pre-loaded with 0.01 mM L-[^{3}H]phenylalanine or L-[^{3}H]lysine for 10 min at 26 °C and rinsed three times on ice with cold Tris buffered saline. Cells were exposed to either buffer alone or to buffer with each amino acid (0.4 mM) in Tris for 5 min at 26 °C. This efflux medium was removed and counted. Stimulation is the ratio of the cpm in the efflux medium in the presence of the amino acid divided by that in the buffer control. Lys, lysine; Phe, phenylalanine; Leu, leucine; AIB, α-amino isobutyric acid; Ala, alanine; Ser, serine.

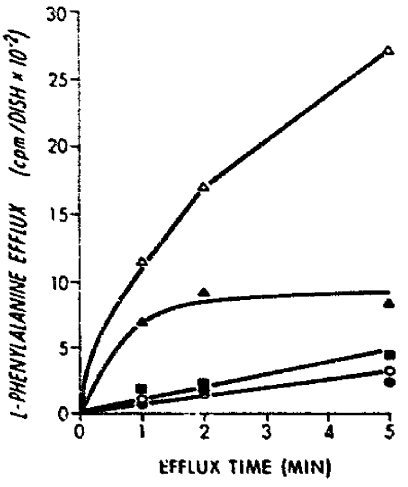

Fig. 2. Effect of parachloromercuribenzene sulfonate (PCMB-S) on phenylalanine stimulated efflux of [^{3}H]phenylalanine. Cells were pre-loaded with 0.01 mM [^{3}H]phenylalanine as in Fig. 1. At time zero, buffer containing either 0.1 mM PCMB-S or 0.4 mM phenylalanine or a mixture of both was added to dishes and the buffer removed at the indicated times for counting. Another set was pre-treated with PCMB-S for 5 min at 20 °C, removed, rinsed once and the 5 min efflux in the presence of 0.4 mM phenylalanine measured. ○, control; ●, 0.1 mM PCMB-S; △, 0.4 mM phenylalanine; ▲, phenylalanine plus PCMB-S; ■, PCMB-S pre-treatment, then phenylalanine.

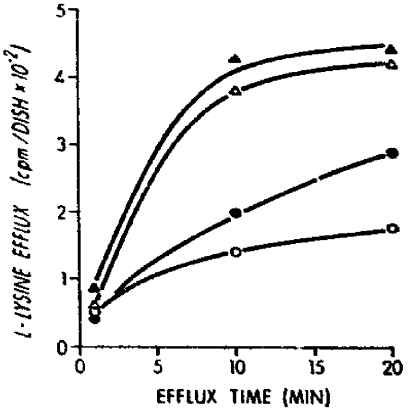

Fig. 3. Effect of PCMB-S on [^{3}H]lysine efflux stimulated by lysine. See Fig. 2 for details. Cells were pre-loaded with 0.01 mM [^{3}H]lysine. ○, control; ●, 0.1 mM PCMB-S; △, 0.4 mM lysine; ▲, PCMB-S plus lysine.

Inhibition of the "L" system by PCMB-S

A recent study [5] demonstrated that L-phenylalanine influx is rapidly inhibited by PCMB-S with little effect on L-lysine influx. The ability of this slowly penetrating sulfhydryl reagent to modify stimulation of L-phenylalanine efflux by extracellular substrate was then studied. As shown in Fig. 2, control efflux of L-phenylalanine at 20 °C is slow whether in the presence or absence of PCMB-S. Extracellular L-phenylalanine stimulates a massive efflux of intracellular substrate, a process rapidly inhibited by PCMB-S. Pre-treatment of cells with PCMB-S completely inhibits

TABLE I

EFFECT OF SODIUM-FREE MEDIUM AND OUABAIN ON L-PHENYLALANINE STIMULATED EFFLUX

Cell monolayers were pre-loaded with 0.01 mM L-[^{3}H]phenylalanine for 10 min at 26 °C, rinsed three times on ice with Tris buffered saline and once with the efflux medium. The cells were then pre-incubated on ice with each efflux medium without L-phenylalanine for 5 min prior to the start of the efflux period. For the efflux, each medium with and without L-phenylalanine (0.4 mM) was pre-equilibrated at 20 °C and added to the petri dishes for 5 min. This efflux medium was then harvested and counted to give the amount of labeled L-phenylalanine effluxed during that time period. The stimulation factor is the stimulated efflux divided by the control efflux.

Efflux medium	5 min efflux (cpm/dish · 10^{-2})		Stimulation factor
	Control	L-Phenylalanine (0.4 mM)	
Tris buffered saline	111	707	6.4
Tris buffered saline+ouabain (1 mM)	85	575	6.8
Mannitol (300 mM)	119	775	6.5
Mannitol+ouabain (1 mM)	114	544	4.8

stimulated efflux. On the other hand, L-lysine efflux stimulated by extracellular L-lysine is not inhibited by PCMB-S at similar concentrations (Fig. 3). On the contrary, control efflux is actually enhanced by PCMB-S. These experiments demonstrate that the function of the "L" system is quite sensitive to inhibition by the hydrophilic mercurial reagent PCMB-S when the function of the carrier is measured by its response to extracellular substrate. The "Ly^+" system, however, is not sensitive to PCMB-S whether measured by influx directly [5] or by the stimulated efflux system studied here.

Efflux of L-phenylalanine was also measured in Na^+-free medium with and without ouabain to study the possible requirement for Na^+ and ($Na^+ + K^+$) activated ATPase function in the stimulation process. As shown in Table I, the efflux of L-phenylalanine was stimulated equally well in medium lacking Na^+ although with the addition of ouabain there was a modest reduction in the rate of efflux. These data indicate that the stimulation of efflux by extracellular L-phenylalanine is not dependent on the presence of a fully functional membrane ATPase and Na^+.

Inhibition of the "Ly^+" system by FNPA

Previous work has shown that FNPA is a useful photoaffinity probe capable of photoinactivating enzymes in vitro [8]. Fig. 4 demonstrates the inhibition of L-lysine influx by FNPA following photoactivation and that FNPA in the dark results in no loss of transport capacity. Furthermore, this inhibitory activity is partially reversed by the simultaneous presence of 1 mM dinitrophenol. On the other hand, if cells are pre-treated with dinitrophenol and this is then removed before FNPA is added, there is no reduction in the photoinactivation of L-lysine uptake by FNPA. These data suggest that there is a site associated with the "Ly^+" system with an affinity for both dinitrophenol and FNPA.

Experiments were then carried out to evaluate the action of photoactivated FNPA on the stimulated efflux of both L-lysine and L-phenylalanine. As shown in

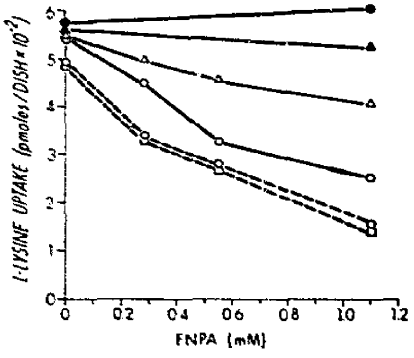

Fig. 4. The photolytic inactivation of [^{3}H]lysine uptake by FNPA and the protective effect of dinitrophenol. The uptake of 0.01 mM [^{3}H]lysine over a 2-min period at 20 °C, was measured in cells treated for 5 min either in the dark or under the Xenon lamp (see Materials and Methods) in the presence of various concentrations of FNPA with and without 1 mM dinitrophenol (solid lines). In another experiment (broken lines), cells were pre-treated with 1 mM dinitrophenol, rinsed thrice with buffer, followed by photolysis with various concentrations of FNPA for 5 min, after which the uptake of [^{3}H]lysine was measured as above. ○, photolysis for 5 min; ●, dark for 5 min; △, photolysis plus 1 mM dinitrophenol; ▲, dark plus 1 mM dinitrophenol; □, 1 mM dinitrophenol pre-treatment.

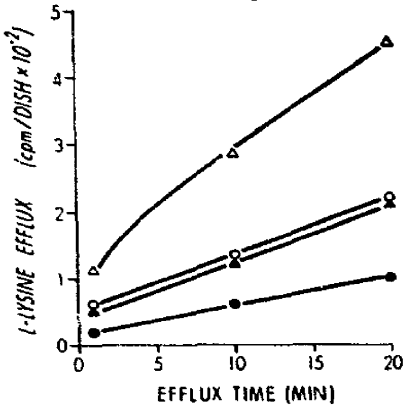

Fig. 5. The photolytic inactivation of the [^{3}H]lysine efflux by FNPA. Cells were pre-loaded with 0.01 mM [^{3}H]lysine and rinsed as in Fig. 2. The cells were then photolyzed for 5 min at 5 °C in the presence or absence of FNPA (0.8 mM), rinsed once with buffer, following which buffer pre-equilibrated at 20 °C with and without 0.4 mM lysine added. The efflux buffer was harvested at various times and counted for radioactivity. ○, control; ●, 0.8 mM FNPA; △, 0.4 mM lysine; ▲, FNPA plus lysine.

Fig. 5, the control efflux as well as the stimulated efflux of L-lysine are both significantly inhibited. In contrast to this, the stimulated efflux of L-phenylalanine (Fig. 6) is not inhibited although there is a modest reduction in the control rate of L-phenylalanine efflux. This latter effect might indicate that there is some minor alteration of the "L" system activity which is overcome by the high levels of L-phenylalanine used to stimulate efflux.

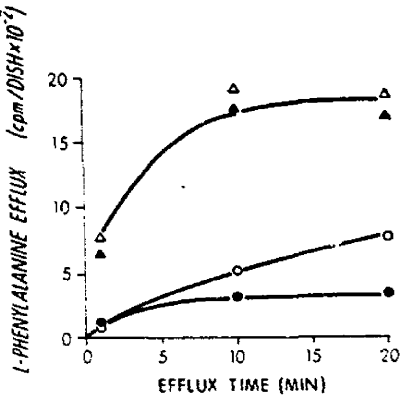

Fig. 6. The effect of FNPA on [^{3}H]phenylalanine efflux. Cells pre-loaded with 0.01 mM [^{3}H]-phenylalanine and rinsed as in Fig. 2 were treated as in Fig. 5 except that efflux in the presence and absence of 0.4 mM phenylalanine was measured. ○, control; ●, 0.3 mM FNPA; △, 0.4 mM phenylalanine; ▲, phenylalanine plus FNPA.

DISCUSSION

The existence of membrane proteins which function as carriers to bring about an increase in the rate of translocation of substrate molecules across the semipermeable plasma membrane is based on several types of indirect evidence. Features such as specificity of the transport process for substrates with closely related structure, as well as saturability, temperature dependence and exchange diffusion have been proposed as criteria for carrier-mediated transport [9]. Studies of the mechanism by which these postulated carrier proteins bring about trans-membrane translocation have focused on the kinetics and specificity of the translocation mechanism. Other more recent approaches have included differential inactivation of the function of carrier proteins by chemicals which react at either the substrate acceptor site or at secondary sites involved in the translocation function. It has recently been demonstrated by Cabantchik and Rothstein [4] that the putative anion transport protein of the red blood cells can be labeled and identified. Using this general approach, several sulfhydryl-reactive reagents have been shown to inactivate in a differential manner the transport of nucleosides, sugars and amino acids in mouse embryo cells in culture [5]. For instance, the hydrophilic organic sulfhydryl PCMB-S rapidly inhibits L-phenylalanine transport while both nucleoside and L-lysine transport are inhibited only after a lag period.

The studies presented here and those reported previously [5] provide evidence that the transport of L-lysine and L-phenylalanine are mediated by different proteins. This conclusion is based on several types of evidence. In the first place, there is essentially no cross-stimulation of the two efflux systems by extracellular substrate in mouse embryo cells. Secondly, inhibition of transport by reagents which recognize unique aspects of the structure of each transport system provides more evidence for structural dissimilarity. The sensitivity of L-phenylalanine exchange diffusion to the slowly penetrating reagent PCMB-S parallels the findings that the influx of L-phenylalanine is rapidly inhibited by PCMB-S. This supports the postulate that the extracellular substrate (L-phenylalanine) must be capable of interacting successfully

with the external portion of the carrier system in order to participate in exchange diffusion with substrate at the internal surface of the membrane. Furthermore, recent studies have demonstrated that several sulfhydryl-reactive reagents produce differential effects in the transport of amino acids [10] and water [11]. These data have been interpreted as evidence for distinct populations of sulfhydryl groups associated with the membrane structures which play decisive roles in transmembrane translocation of a variety of small molecules. Thus, the failure of PCMB-S to modify L-lysine exchange diffusion is further evidence that the effect of PCMB-S is restricted to certain membrane functions (i.e., the "L" transport system) and are not due solely to generalized, non-specific effects on membrane structure such as elution of membrane proteins [12].

One possible mechanism by which a sulfhydryl-reactive reagent could alter selectively the activity of one amino acid transport system, however, would be to inhibit a secondary function required by one system and not another. Such a possibility would be the $(Na^+ + K^+)$ activated ATPase. It has been shown that an organic mercurial covalently bound to a high molecular weight non-penetrating dextran inhibits this plasma membrane enzyme [13]. Furthermore, cytochalasin A has been shown to inhibit a variety of enzymes including membrane ATPases [14]. Since this inhibitory effect is reversed by L-cysteine and dithiothreitol, it was suggested that the mechanism was through an interaction with sulfhydryl groups.

Several considerations suggest that the inactivation of $(Na^+ + K^+)$ activated ATPase by PCMB-S is not the probable mechanism for the differential inhibition of the "L" system. In the first place, this transport system is usually considered to be Na^+-independent and that energy coupling for active transport occurs through an ATP-dependent process not coupled with Na^+ flux [1]. Phenylalanine uptake has been shown to be an active process in hamster cells [15] as well as in mouse cells using a similar method (unpublished data). Thus, the demonstration that L-phenylalanine successfully stimulates L-phenylalanine efflux from cells in an isotonic mannitol medium containing 1 mM ouabain (Table I) supports the conclusion that the PCMB-S effect is not mediated by an alteration in $(Na^+ + K^+)$ activated ATPase. Preliminary data also suggest that treatment of cells with oligomycin does not interfere with L-phenylalanine stimulated efflux. These findings indicate that interference by PCMB-S with an energy coupling system would not lead to the rapid, differential inactivation of the "L" system as observed in this study.

The differential activity of the photoprobe, FNPA, on the two transport systems is further evidence for the unique structure of the proteins responsible for the "L" and "Ly^+" transport functions. The competition between dinitrophenol and FNPA for the inactivation of L-lysine stimulated efflux is of particular interest since a similar effect is also observed with mitochondrial ATPase where dinitrophenol and FNPA activate the enzyme in the dark while FNPA following photolysis inactivates the enzyme [16]. The full significance of the existence of a dinitrophenol-FNPA reactive site related to the "Ly^+" transport function must await further characterization of the proteins reacting with this photoprobe.

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