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PHLORETINYL-3′-BENZYLAZIDE: A HIGH AFFINITY PROBE FOR THE SUGAR TRANSPORTER IN HUMAN ERYTHROCYTES

II. IRREVERSIBLE TRANSPORT INHIBITION IS INDUCED BY PHOTOLYSIS

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In the dark, phloretinyl-3′-benzylazide (PBAz), at a nominal concentration of 10 μM, will inhibit the transport of D-glucose in human erythrocytes by more than 90%. This inhibition can be completely reversed by percolating the cell suspension through a small column of Sephadex G-10; cells recovered after this treatment, and then loaded with 100 mM D-glucose, possess a transport capacity (glucose efflux) equal to untreated cells. The Sephadex matrix completely removes non-covalently bound inhibitor even though, under these conditions (subdued light, 0.2% hematocrit, 0°C, pH 6.2 or 7.8), from 70 to 80% of the PBAz added is bound to the cells (mostly non-specifically to hemoglobin). However, when erythrocytes exposed to 10 μM inhibitor are irradiated with long wavelength ultraviolet light, the glucose transporter is irreversibly inhibited; after 1 min irradiation, about 50% of transporter activity cannot be restored by Sephadex treatment. Under identical conditions, control cells (no PBAz, but irradiated and treated with Sephadex) retain over 90% of carrier activity. The photolytic conversion of the inhibition to an irreversible form is directly dependent on PBAz concentration. The results reaffirm our earlier conclusions that PBAz is a potentially useful photoaffinity labeling agent for the glucose transporter in erythrocyte membranes.

Introduction

We have recently described the synthesis of a new phloretin derivative, phloretinyl-3′-benzylazide (PBAz), and presented evidence that, in subdued light and under four different conditions to determine sugar transport in the human erythrocyte, it is from 6- to 20-times more potent an inhibitor than phloretin [1]. PBAz acts like the parent phloretin; it is a reversible inhibitor with a K_i ranging from 40 to 120 nM and competes with hexose for the transporter site on the outside of the cell membrane. If given time to equilibrate with the cells, both phloretin and its azide derivative penetrate the membrane and then act as mixed inhibitors (i.e., both competitive and non-competitive) of sugar uptake. Evidence is now presented that, upon photolysis, the azide forms a reactive intermediate which covalently binds to and irreversibly inactivates the sugar transporter in this cell.

Experimental procedures

Materials. PBAz was synthesized as previously described [1]. The Amersham Corporation, Arlington Heights, IL, used their TR-7 procedure

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Abbreviation: PBAz, phloretinyl-3′-benzylazide.

to incorporate tritium into the precursor amine from which we prepared radiochemically pure azide (80 mCi/mmol). Sephadex G-10 obtained from Pharmacia Fine Chemicals, Piscataway, NJ, was equilibrated in the appropriate buffer for at least a day before it was used. Human blood was either freshly drawn or obtained from local blood banks and used within 2 weeks. After removal of the plasma and buffy coat ($3000 \times g$, 5 min), the red cells were washed in the appropriate buffer. Buffer A; 310 mosM sodium phosphate, pH 7.4. Buffer B; 150 mM NaCl, 27 mM glycylglycine, pH 7.8 (10°C). Buffer C; 150 mM NaCl, 20 mM sodium phosphate, pH 6.2.

PBAz binding studies in dim light. Freshly drawn cells were washed and finally resuspended in the appropriate buffer to make a 50% hematocrit. At zero time, 20, 50, 100 or 200 μl of this cell suspension were added to 5 ml of either Buffer B or Buffer C containing [^{3}H]PBAz at varying concentrations (added as an ethanol solution; final alcohol level was less than 0.5%). All incubations and aliquot transfers were in glass vessels and pipets because the azide is extensively bound to plastic. Samples were taken at about 20 and 60 s and at equilibrium (15 min) and then centrifuged in 6×50 mm glass tubes in an Eppendorf 3200 centrifuge for 7 s. Ligand binding was assessed as loss of radioactivity from the supernatant.

Sugar transport assay. Transporter activity was assessed by the Ørskov technique [2] in which zero-*trans* D-glucose efflux was measured. Transport-dependent changes in turbidity of the cell suspension were monitored at 700 nm with an Aminco DW-2a Recording Spectrophotometer. The optical-cell compartment was adapted to hold a small stirring paddle connected to the shaft of a variable speed motor through a flexible cable to keep erythrocytes suspended in the cuvette. Addition of preloaded cells was made through a permanently attached, light-tight needle adapter. Usually, erythrocyte suspensions were equilibrated with 100 mM D-glucose in buffer A. The cells were then centrifuged and the supernatant was removed to yield a 40% Ht. Efflux was initiated by the rapid addition of 10 μl of this preloaded cell suspension into 4 ml of buffer A (20°C) containing 100 mM mannitol to prevent abrupt cell swelling which would dilute intracellular glucose. A continuous tracing of absorbance increase was obtained from which the initial rate of D-glucose efflux could be determined [3].

Photolysis conditions. In subdued light, cells were mixed with appropriate buffer volumes (containing variable amounts of PBAz) to make a final hematocrit of 0.2%. Glass vessels were chosen so that the incubation medium attained a depth of about 3–4 mm. At zero time, the vessel was placed in a Dubnoff shaking incubator (0°C) under a Sylvania sun lamp (120 V, 250 W) positioned 15 cm above the solution surface. After varying periods of light exposure, the cells were centrifuged and resuspended in 0.5 ml buffer before further treatment.

Results

Non-covalent interaction of PBAz with red blood cells

Early in our studies, we observed that PBAz was extensively bound to erythrocytes. To determine its K_i, the free concentration of the inhibitor has to be known. We therefore determined some binding characteristics of the tritiated azide in the dark with intact red cells. The results of one of these studies is shown in Fig. 1. The amount of PBAz bound at equilibrium (15 min) is linearly related to the concentration of ligand added and the process exhibits no saturation over the range tested (0.5 to 10 μM). A greater fraction of ligand was bound as hematocrit was increased, as temperature was lowered (but the difference was not significant) and in the more acidic medium.

Sephadex G-10 reverses transport inhibition by PBAz

To determine whether photolysis would convert the azide to an irreversibly bound inhibitor, a means to remove unreacted agent from the cells had to be developed. The procedure had to be rapid and non-destructive so that afterwards, residual transporter activity could be assessed. Although PBAz inhibition in the dark is reversible, extensive binding to the hemoglobin reservoir prohibited its removal by simply washing the cells with azide-free medium. Since we had earlier found Sephadex G-10 to be a useful adsorbent for other

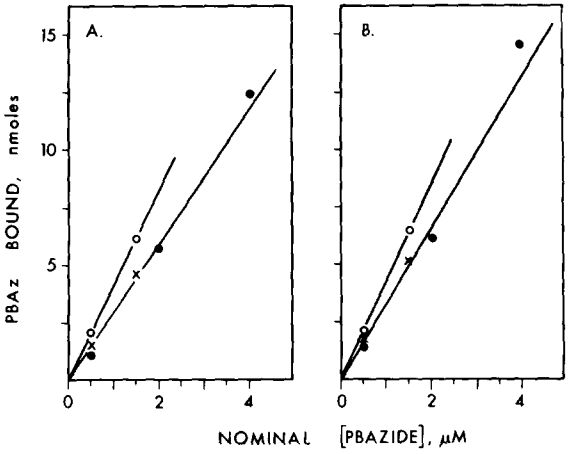

Fig. 1. PBAz interaction with intact erythrocytes in subdued light. Washed cells were incubated with varying nominal concentrations of [^{3}H]PBAz in 5 ml of buffer B (pH 7.8) at 10°C (×) or 15°C (●) or in buffer C (pH 6.2) at 10°C (○). Panel A, 0.2% hematocrit; Panel B, 0.5% hematocrit. The points are the means of duplicate measurements of tritiated ligand loss from the medium at equilibrium (15 min). Values for 10 μM PBAz at pH 7.8 and 15°C are not included to conserve space in the figure but were included in the construction of the regression lines.

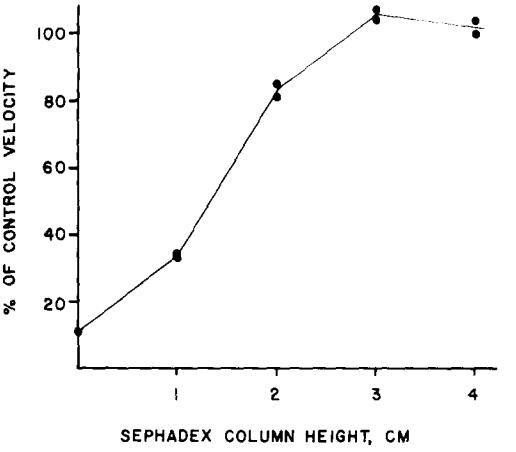

Fig. 2. PBAz inhibition of glucose transport is reversed by Sephadex G-10. Washed erythrocytes (0.2% Ht) were incubated in buffer A containing 10 μM PBAz (added in ethanol to make the final alcohol level 3% or less) for 10–15 min at 0°C in the dark. The suspension was then centrifuged at 1000×*g* for 5 min; the packed cells were resuspended in 0.5 ml of buffer A (containing 3% ethanol) and were then passed through 0.7 cm diameter Sephadex G-10 columns of varying heights which had been equilibrated in buffer A containing 100 mM (or 150 mM) D-glucose. Manual intermittent slow stirring of the top half of the column bed with a thin rod minimized physical entrapment of the cells and over 90% were recovered in 4 ml of the equilibration buffer used as a wash solution. The cells were then collected by centrifugation, equilibrated in 100 mM D-glucose and then assayed for transporter activity. Control cell incubations were also passed through columns of appropriate size but contained no PBAz. The results from two separate studies are shown.

phloretin-like compounds [4], we tested the cross-linked dextran as a PBAz scavenger. Suspensions of red cells that had been exposed to PBAz in the dark were passed through small Sephadex columns of varying length and the recovered cells (>90%) were then assayed for transporter activity. Our results (Fig. 2) show that cells incubated in subdued light with 10 μM azide without column treatment are about 90% (usually >95%) inhibited but percolation through 3 cm of the adsorbent is sufficient to reactivate the carrier system to control levels. Thus, even when more than 75% of the azide was associated with the cells (0.2% Ht, 15 min, 0°C), it was removed to noninhibitory levels if a sufficient time of exposure to Sephadex G-10 (but not G-25) was allowed.

In another series of experiments, PBAz in buffer A (no cells) was irradiated for 5–10 min as described in methods; a major portion of the azide was converted to a mixture of products (TLC analysis) under these photolysis conditions. The solution was then passed through a 4 cm Sephadex column and washed with buffer A. About 2 column volumes were collected, pooled and then used as the efflux medium in a transport experiment. The efflux rate of D-glucose from untreated red cells preloaded with 100 mM sugar into this Sephadex effluent was identical to the rate found with buffer A alone. The results indicate that not only was free PBAz removed by Sephadex but also any light-generated inhibitory products.

Transport inhibition becomes irreversible after photolysis

Under our photolytic conditions, we found that unless PBAz was present, irradiation of erythrocytes followed by their passage through a Sephadex G-10 column had little effect on glucose transport activity. Even after 12 min of light exposure, the glucose efflux rate from cells recovered from the column was still 80–90% of that mea-

sured in non-irradiated control cells (Fig. 3). In the presence of 10 μM PBAz (nominal concentration), but before irradiation (zero time), Sephadex treatment restored transporter activity to control levels (Fig. 3). However, light exposure led to a time-dependent loss of carrier activity which could not be reversed by passage through the Sephadex column. The half time for this photolytic reaction is approx. 1 min.

Irreversible inactivation of transport is dependent on PBAz concentration

After 4 min irradiation, the difference in loss of transporter activity between cells with and without PBAz was maximal (Fig. 3). This exposure time was therefore chosen to determine if the irreversible inactivation by photolyzed PBAz was dose-dependent. Results shown in Fig. 4 indicate that when cells (0.2% Ht) were irradiated for 4 min in the presence of varying concentrations of PBAz, an irreversible inhibition of 50% occurred when

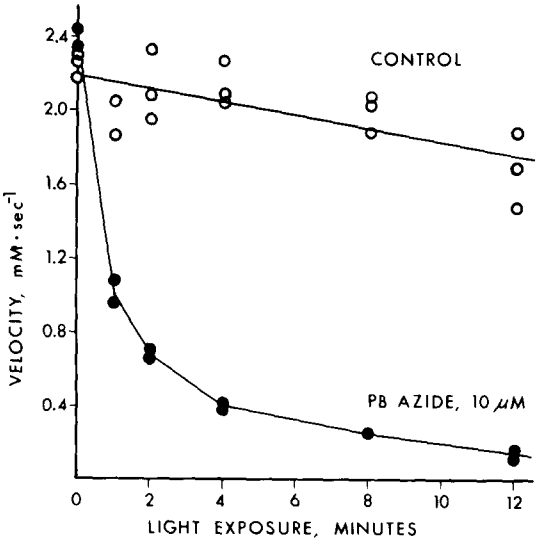

Fig. 3. PBAz inhibition of glucose efflux becomes irreversible with light exposure. Erythrocyte suspensions (0.2% Ht) containing 10 μM PBAz (●) or with no inhibitor (○) were exposed to light for varying periods at 0°C as indicated in Experimental Procedures. Cells were then centrifuged, resuspended in 0.5 ml buffer A and then passed through a 3.5–4.0 cm column of Sephadex G-10 to remove non-covalently bound azide plus any light-generated products. The recovered cells were then equilibrated with 100 mM D-glucose and assayed for transport capacity.

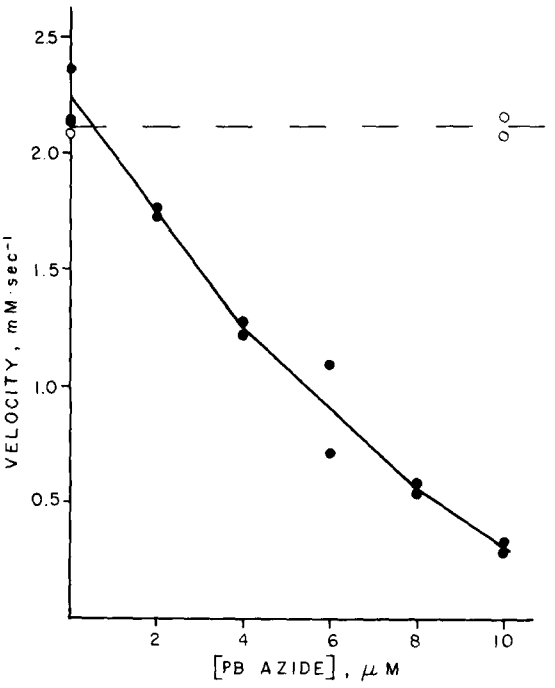

Fig. 4. Concentration dependence of PBAz inactivation of glucose transport. Cells (0.2% Ht) were incubated with PBAz at varying concentrations at 0°C in the dark (○) and with a 4 min light exposure (●). The cell suspensions were then percolated through Sephadex G-10 and assayed for glucose efflux capacity. The PBAz concentrations have not been corrected for the approx. 75% non-covalent binding which occurs in the dark under these conditions.

nominal concentration was about 5 μM. Before illumination (at zero time) about 75% of the azide was bound non-covalently to the cells at this pH and temperature. Transporter activity of cells exposed to 10 μM PBAz for 4 min but not irradiated and then treated with Sephadex was identical to that found for control cells (Fig. 4).

Discussion

The interaction of PBAz with the intact erythrocyte is multifaceted and complex. Although our transport inhibition studies indicate that reaction with the transporter is instantaneous, the overall binding is time-dependent at each temperature we have tested (0, 10 and 15°C). At the earliest time we have sampled the mixture (20 s), the fraction bound is about 94% of the equilibrium value (at 15 min).

More PBAz is bound at pH 6.2 than at pH 7.8. We have previously found that compounds of this type with a free 4′-hydroxy group have a pK_a of about 7.2 [4]. The unionized form of the azide preferentially binds to the membrane and a majority of it apparently penetrates and is sequestered by the hemoglobin which acts as a binding sink [1]. Results of some limited tests [5] suggest that the effectiveness of PBAz as a competitive inhibitor of 3-*O*-methylglucose self-exchange is not much greater at pH 6.2 than at 7.8, even though more of the compound is in the unionized, active form at the lower pH. This would be expected if the azide concentration in the medium decreased further as a result of converting it to the membrane-permeable, hemoglobin-bound species. In comparison, the inhibitory effectiveness of the corresponding 2′-β-glucoside of PBAz, which we assume penetrates the membrane less readily than the aglycone, is much greater at pH 6.2 than at pH 7.8; (the glycoside's K_i is about 0.5 μM at the low pH).

It seems reasonable to conclude that the photocatalyzed irreversible inhibition by PBAz is attributable to the generation of reactive intermediates (mainly the nitrene) which bind covalently to (a part of) the protein assembly constituting the hexose transporter. It is less likely that photolysis of the azide simply leads to a photocatalyzed destruction of the carrier (without binding) since the reaction is directly related to PBAz dose. Furthermore, in preliminary photolysis experiments conducted with [^{3}H]PBAz, several protein components of white ghost membranes were found to be labeled (SDS-PAGE) including those migrating in the Band 3 and 4.5 area. Hemoglobin recovered from these lysed cells was also extensively labeled; this supports our earlier argument that the azide penetrates the membrane and is sequestered intracellularly [1].

These experiments with radiolabeled PBAz were conducted as described in the legends to Figs. 3 and 4; tritiated ligand (4 μM) was photolyzed with cells (0.2% Ht) for 4 min (after 10 min of preincubation). After removal of non-covalently bound ligand and by-products, 30 μl of the recovered cells were lysed [6] and the white ghosts were isolated; these membranes contained 1.3% of the total [^{3}H]PBAz originally added to the incubation medium. If we take the red cell volume to be $9 \cdot 10^{-11}$ cm^3, we can calculate that $1.3 \cdot 10^6$ molecules of ligand were bound to the membrane of each cell. Estimates of the number of copies of D-glucose transporters have ranged from $2 \cdot 10^5$ to $7 \cdot 10^5$ per erythrocyte [7–12]. Thus, the amount of ligand bound to each cell was from 4 to 13 times that necessary to irreversibly inhibit the functioning of 50% of the carriers under our conditions (Fig. 4), assuming a 1:1 stoichiometry of binding to inhibition. Labeling of membrane components other than the glucose transporter, especially membrane lipids, must of course also be expected with this agent in spite of its high affinity for the sugar transporter. More discriminating [^{3}H]PBAz photolysis experiments are being conducted in the presence of ligands known to interact with (and perhaps protect) the sugar transporter (e.g. D-glucose, maltose, cytochalasin B).

Acknowledgements

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