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Electron spin resonance investigation of the interaction of the anion and glucose transport inhibitor, *p*-azidobenzylphlorizin, with the human red cell membrane

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The membrane perturbations caused by the interaction of *p*-azidobenzylphlorizin (*p*-AzBPhz), a potential photoaffinity labeling agent of the anion and D-glucose transporters in the human erythrocyte, have been studied using electron spin resonance (ESR) spectrometry. Two lipid-specific spin labels have been employed; one of these agents, a hexadecyl-quarternary amine with the nitroxide reporter group covalently attached to the cationic nitrogen, (CAT-16), has been used to monitor changes in the physical state of the membrane's extracellular phospholipid/water interface. The other spin label, 5-doxylstearic acid (5-NS), is designed to examine the order and motion of the lipid bilayer near the cell surface. In separate experiments, intact human red cells labeled with these lipid-specific spin labels were exposed to small amounts of the phlorizin azide. A dose-dependent alteration in CAT-16 motion was observed, but the *p*-AzBPhz interaction with the membrane had no effect on the spectrum of 5-NS. The half-maximal effect of the phlorizin derivative on the CAT-16 spectrum occurred when about 2 million molecules were bound to each cell. This is also the combined amount of band 3 and band 4.5 present in the red cell membrane and represents the concentration necessary to inhibit both anion and glucose transport. Our results suggest that the first *p*-AzBPhz molecules binding to the red cell membrane interact with the anion and sugar transporters, and not with the bulk lipid bilayer.

Introduction

The photoactive membrane probe *p*-AzBPhz (Fig. 1) possesses a high affinity for both the glucose and anion transporter in human erythrocytes [1]. In subdued light, this β-glucoside is a strictly competitive inhibitor of 3-methoxylglucose equilibrium exchange; conversely, it blocks zero-trans-efflux noncompetitively, as would be expected if the compound reacted only with the external face of the sugar transport system. The K_i of *p*-AzBPhz is about 1–2 μM at pH 7.6, but its potency increases ($K_i = 0.3–0.5$ μM) when more of the 4′-hydroxyl group ($pK_a = 7.2$) is protonated by adjusting the transport medium to pH 6.2 [1]. Results from the following kinds of experiment to be published elsewhere indicate that this glucoside, like phlorizin, binds to but does not penetrate cell membranes: the red cell membrane can be photo-labeled while the intracellular hemoglobin remains unmodified; binding of [^{3}H]azide to intact cells shows saturation, but not if the cells are lysed; and finally, the azide-induced cell-shape changes are rapidly reversible by simple dilution. Some early photoaffinity labeling results indicated that *p*-AzBPhz has a high affinity for a second integral protein in the erythrocyte membrane, namely the anion exchanger [1]. The available data (some to be presented here) suggest that the

Abbreviations: 5-NS, 5-doxylstearic acid; *p*-AzBPhz, *p*-azidobenzyl-5′-phlorizin; CAT-16, 4-(*N′*, *N′*-dimethyl-1-*n*-hexadecyl)ammonium-2,2,6,6-tetramethylpiperidine-1-oxyl iodide; DIDS, stilbene disulfonic acid; PBS, phosphate-buffered saline.

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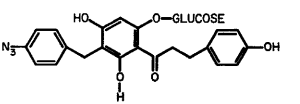


Fig. 1. The structure of *p*-AzBPhz.

derivative shares a part of the DIDS binding site perhaps in a way similar to that found for phloretin by Fröhlich and Gunn [2,3]. Both phloretin and its derivative glucoside (i.e., *p*-AzBPhz) appear to act as mixed or non-competitive inhibitors of chloride exchange and their half-maximal effective concentration (ED_{50}) are about 0.5–1.5 μM.

In the present study, we have investigated the effect of *p*-AzBPhz interaction with the erythrocyte membrane on the order and motion of the lipid bilayer as well as the physical state of the intracellular lipid/water interface. ESR experiments have been performed using two lipid-specific spin labels: CAT-16, which reports on the motion of the local micro-environment of the extracellular phospholipid/water interface [4–7] and 5-NS, which monitors the order and motion of the lipid bilayer at a depth of approx. 5 Å [8,9]. It is important to realize that the effects of *p*-AzBPhz in the present study have been examined at nominal concentrations that are two orders of magnitude lower than those known to cause an increase in salt and water influx in normal and sickled red cells [10].

Materials and Methods

Cell preparation

Blood was obtained from normal volunteers by venipuncture into heparinized tubes which were immediately placed on ice. Cells were washed three times with freshly prepared 150 mM sodium chloride, buffered to pH 7.4 with 5 mM (sometimes 15 mM) sodium phosphate (hereafter called PBS buffer) and the buffy coat was carefully removed after each centrifugation at $2500 \times g$ in the cold.

Chloride flux

These experiments were performed in collaboration with Dr. Otto Fröhlich, Emory University. Red cells were washed four times with the efflux buffer (150 mM NaCl, 27 mM glycylglycine (pH 7.0 at 0°C). After the final spin, cells were resuspended to a hematocrit of about 40% and $Na^{36}Cl$ was added. The cells were kept on ice for 5 min to allow tracer equilibrium with intracellular chloride. The mixture was then transferred to narrow nylon tubes and the cells were packed by centrifugation. Flux measurements were conducted essentially as described by Gunn and Fröhlich [11]. The tracer-loaded cells were injected into a well-stirred efflux medium (final hematocrit, 1–2%) maintained at 0–4°C in subdued light, and containing the desired concentration of *p*-AzBPhz (added as an aqueous ethanol solution; final ethanol concentration 0.02%). At intervals of several seconds, aliquots of the cell suspension were obtained by syringe through prefilters and Millipore filters mounted in a Swinnex holder, to obtain cell-free, extracellular fluid and the time-course of radioactivity appearing in the filtrate was recorded.

ESR studies

The CAT-16 spin label was obtained from Molecular Probes (Eugene, OR). A stock solution was prepared by dissolving 1 mg CAT-16 in 40 μl 95% ethanol and an equal volume of PBS. An aliquot of this stock mixture (40 μl) was subsequently diluted to 100 ml with PBS at room temperature to prepare the labeling solution. 1 ml of washed, packed cells (about 75% hematocrit) was treated with 20 ml of the CAT-16 labeling solution at room temperature for 30 min. The labeled cells were regained by centrifugation at $2500 \times g$ at 4°C, stored on ice and used within 4 h.

The 5-NS label was obtained from Aldrich Chemical Company (Milwaukee, WI). 1 ml of washed, packed cells, diluted in 20 ml of PBS (final hematocrit of about 3.5%) were treated with 4 μl of a freshly prepared 5-NS stock solution (1 mg in 50 μl ethanol) for 30 min at room temperature; the cells were then re-isolated and stored as described above for the CAT-16 experiments.

Packed, intact cells labeled with either spin label were suspended in 20 ml PBS (20°C) to make a final hematocrit of 1.5%, and the suspension was treated with 6 μl of a 70% ethanol solution containing from 6 to 100 mM *p*-AzPBhz. After a few minutes, the cells were pelleted and the supernatant was used to adjust the hematocrit to 50%. The ESR spectrum was then recorded at 20 ± 1°C on a Varian E-109 ESR spectrometer with computerized data acquisition. Instrumental settings are indicated in the legends to Figs. 3 and 5.

Data analysis

The Student's *t*-test was used in all cases to judge the level of significance.

Results

p-AzBPhz as an anion transport inhibitor

Some of the results obtained by Dr. Otto Fröhlich illustrating the effectiveness of *p*-AzBPhz as a chloride exchange blocker are displayed in Fig. 2. The efflux process under these conditions in the absence of azide has a pseudo-first-order rate constant of about 0.14 s^{-1}; at a nominal concentration of 1 μM *p*-AzBPhz, this rate is reduced to 0.056 s^{-1} and 50 μM blocks flux almost completely. At the lowest azide concentrations and at this hematocrit, approx. 15% of the inhibitor is bound. The apparent K_i appears to be about 0.5 μM, assuming that the inhibition is non-competitive (efflux was determined at only one chloride concentration). It is interesting that the derivative inhibits the glucose transporter competitively at this same low concentration, but presumably as the uncharged (protonated) molecule [1].

Effect of p-AzBPhz on ESR spectra

Since *p*-AzBPhz inhibits both anion and glucose transport at extremely low levels, we hypothesized that

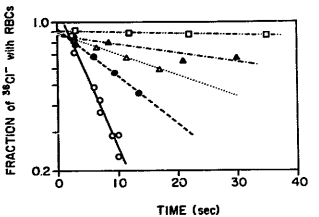
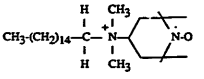


Fig. 2. Inhibition of chloride efflux (equilibrium exchange) by *p*-AzBPhz. Washed human erythrocytes were incubated with 150 mM $^{36}Cl^-$, buffered with 27 mM glycylglycine (pH 7.0, 0°C) to allow equilibration of the tracer anion. The appearance of radioactivity in the efflux buffer containing varying concentrations of *p*-AzBPhz are plotted as a pseudo-first-order reaction. Chloride efflux in the absence of the azide (○) has a rate constant of 0.141 s^{-1}; flux was blocked by the azide at nominal concentrations (μM) of 1(●), 2(△), 5(▲) and 50(□). The apparent K_i of *p*-AzBPhz is 0.5 μM (corrected for 15% binding at this final hematocrit of 1–2%, see text).

at these concentrations, the drug may bind these transport proteins without affecting the bulk lipid. ESR was used to test this hypothesis. The structure of CAT-16 is given in Fig. 3. When *p*-AzBPhz is added to a solution of CAT-16 in buffer, no change in the ESR spectrum is observed over a period of several hours, suggesting that



CAT-16

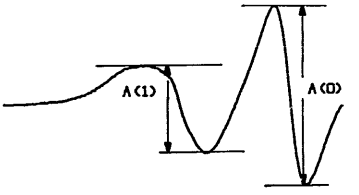


Fig. 3. The structure of CAT-16 and a representation of the low-field ($M_I = +1$) and center ($M_I = 0$) resonance lines of a typical spectrum of CAT-16 labeled intact erythrocytes. τ, the apparent rotation time, was calculated by Eqn. 1 employing the peak-to-peak heights of the center ($A(0)$) and low-field ($A(+1)$) resonance lines and the peak-to-peak width of the former. The instrument settings were: 3360 G magnetic-field set, 50 G scan width, a modulation amplitude of 0.32 G at 100 kHz, and a time constant of 0.128 s.

the azide does not directly react with the spin label. A representative spectrum of this label in the erythrocyte membrane is shown in Fig. 3. This cationic, lipid-specific label is intercalated into the lipid bilayer of intact erythrocytes, such that the nitroxide reporter moiety probes only the external phospholipid/water interface. The relevant spectral parameter is an apparent rotational correlation time, τ, which can be conceptualized as the time required for the nitroxide moiety of the spin label to rotate one radian through space. It is calculated from the spectral data according to the following Eqn. 1 [7]:

$$\tau = (6.5 \cdot 10^{-10}\ \text{s}) \left(\frac{A(0)}{A(+1)}\right)^{1/2} (H_{pp}(0)) \qquad (1)$$

where $A(0)$, $A(+1)$, and $H_{pp}(0)$ are respectively, the amplitudes of the $M_I = 0$ and $+1$ resonance lines and the peak-to-peak width of the former. An increase in τ is indicative of decreased motion of the micro-environment of the nitroxide probe at the lipid/water interface. Two of us recently demonstrated that the primary determinants of CAT-16 motion in erythrocyte membranes at 20°C appear to be both the motion and order of the lipid bilayer and the interactions of CAT-16 with the bilayer regions of transmembrane proteins [7]. Due to a number of theoretical considerations, including an M_I-dependent unresolved hydrogen coupling [12], as well as the spectrum itself which shows considerable motional line broadening, absolute values of τ calculated by Eqn. 1 should be regarded as estimates. However, changes in τ caused by the drug's interaction with the membrane are accurately given; consequently, trends shown by the concentration dependence of the drug on τ are valid.

Accordingly, the results presented in Fig. 4, illustrate that *p*-AzBPhz, over the nominal concentration range of 2–28 μM, caused a concentration-dependent decrease in the motion (increase in τ) of CAT-16 embedded in the erythrocyte membrane. These results were statistically different from control at concentrations of 4-, 7- and 14 μM *p*-AzBPhz. The ED_{50} is about 2.5 μM (corrected for about 20% binding, see below), which is only slightly higher than that needed to half-maximally inhibit either anion and D-glucose transport. However, it is more informative to equate the concentration necessary to cause these effects with the number of *p*-AzBPhz molecules bound to each red cell. Binding studies employing [^{3}H]*p*-AzBPhz with intact cells at 0.2% hematocrit [10] and employing bioassay methods at higher hematocrits show that azide binding to intact cells varies with hematocrit. In the present experiments, of the total amount of azide added, about 20% is bound to the 1.5% red cell suspension under our conditions. Thus, the half-maximal change in ESR signal occurs when about 2 million molecules of *p*-AzBPhz are bound

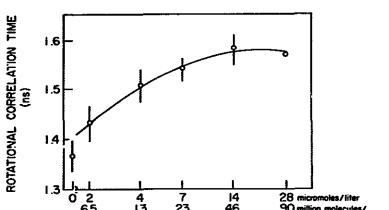


Fig. 4. The effect of *p*-AzBPhz on the rotational motion of CAT-16 in intact erythrocytes. Plots of log nominal [azide]·μM vs τ. Error bars are one S.D. for each concentration of drug used. Statistically significantly different values of τ from control were found at the following azide concentrations (μM): 4 ($P < 0.01$, $N = 2$); 7 ($P < 0.001$, $N = 4$) and 14 ($P < 0.001$, $N = 3$).

per cell *. This is about the number of copies of band 3 (1.5 million) plus band 4.5 (0.5 million) thought to be present in normal, human red cell membranes [13]. It is therefore conceivable that these two transport proteins are titrated by *p*-AzBPhz and, as binding to these membrane proteins occurs, changes in the physical state of the membrane lipid/water interface are induced which restrict the motion of the CAT-16 reporter group.

Comparable experiments with the lipid-specific doxyl spin label, 5-NS, yielded results that indicate *p*-AzBPhz does not distrub the depths of bulk membrane lipid and are consistent with the preferential binding sites of *p*-AzBPhz being located on the transport proteins, band 3 and band 4.5. The motion of the nitroxide moiety in the membrane lipid bilayer was assessed by measuring the half-width at half-height (HWHH) of the low-field $M_I = +1$) line of the resulting spectrum. This parameter, discussed in previous papers from our laboratory [8,9,14,15], is a polarity-independent monitor of lipid motion and order that is reflective of the rate of reorientation of the 5-NS spin label between parallel and perpendicular orientations relative to the normal to the membrane surface. Alterations in the lipid bilayer will be signaled by the membrane-bound 5-NS agent. For example, an increase in the HWHH of the spectral peak is observed when erythrocyte membranes are exposed to benzyl alcohol, a known lipid-fluidizing agent (HWHH: control, 3.78 ± 0.02; with 100 mM benzyl alcohol, 3.94 ± 0.01; $N = 2$, $P < 0.01$). The results presented in Table I suggest that the addition of *p*-AzBPhz to 5-NS-labeled erythrocyte membranes did not alter the motion of the nitroxide moiety. The azide caused no detectable change in the HWHH measurement over the same concentration range where anion and glucose transport were inhibited and the rotational motion of CAT-16 at the lipid/water interface was decreased. This result is consistent with the idea that at these low levels examined in this study, minor amounts of *p*-AzBPhz penetrate into the lipid portion of the bilayer.

TABLE I

The effect of p-AzBPhz on the motion of 5-NS-labeled intact cells

Azide concentration (μM)	HWHH [a] (G)
Vehicle	3.56 ± 0.02
2	3.57 ± 0.07
4	3.56 ± 0.05
7	3.60 ± 0.07
14	3.59 ± 0.05
28	3.58 ± 0.06

[a] Mean ± S.D. ($N = 2$) in gauss. No significant difference between vehicle and azide addition was observed by Student's *t*-test.

Discussion

The *p*-AzBPhz molecule is clearly amphipathic; its highly polar β-glucosidic moiety can be sharply distinguished from the lipophilic aromatic azide group. One might naturally assume that when the derivative is presented to the membrane, the *p*-azido-aromatic ring would sink into the lipid bilayer, its depth restricted to about 12 Å by the glucose moiety which remains in the aqueous environment. Our results do not support this view if the azide concentration is kept low. Its interaction with the membrane caused no detectable alteration in 5-NS motion, even at the highest nominal concentration tested in these studies (28 μM; Table I). This finding suggests that the nitroxide reporter group, which is assumed to reside in the bilayer at a depth comparable to that projected for the azide moiety, did not experience an altered environment. The intercalation of sufficient *p*-AzBPhz molecules into the bilayer would have presumably altered the physical state of the lipid bilayer, that is, changed the order or motion of the lipid components.

In contrast to the absence of changes in the lipid bilayer monitored by 5-NS, *p*-AzBPhz appeared to

* An example of the calculation using the half-maximum effect of *p*-AzBPhz on the apparent rotational correlation time, τ, of CAT-16 in erythrocytes is:

$$1.7 \cdot 10^6 \frac{\text{molecules bound}}{\text{red cell}} = \frac{(2.5\ \mu\text{M};\text{ED}_{50})(6 \cdot 10^{23})(85 \cdot 10^{-15}\text{l};\text{MCV})(0.2;\text{FB})}{(0.015;\text{hematocrit})}$$

ED_{50} = concentration required to yield half-maximal effect; MCV = mean corpuscular volume; FB = fraction of *p*-AzBPhz bound.

cause alterations in the physical state of the local micro-environment probed by CAT-16. The primary motional determinants of CAT-16 at 20°C have recently been discussed by two of us [7]. Among the membrane alterations which were found to have no effect on the ESR spectrum of this label are the following: perturbation of the glycocalyx with phytohemataglutinin, wheat germ agglutinin, or concanavalin-A lectins, proteolytic cleavage of membrane glycoconjugates and alterations in cytoskeletal protein–protein interactions when the CAT-16 label was present in erythrocyte membranes. In contrast, the motion of the CAT-16 was significantly increased in isolated protein-free lipid membranes, compared to ghost membranes in which protein was present. In addition, treatment of CAT-16-labeled intact cells with the lipid-fluidizing agent, benzyl alcohol, caused a significant increase in the motion of the CAT-16 reporter group at the lipid/water interface [7]. From these studies, it was concluded that the motion of the CAT-16 label was influenced primarily by the nature of the bilayer region surrounding the transmembrane proteins. Furthermore, it appeared that the lipid–protein interactions at the extracellular lipid/water interface were being monitored [7]. We are presently unable to explain how the *p*-AzBPhz binding to either anion of glucose transporters caused such dramatic changes in the physical state of the membrane interface that the motion of the micro-environment of the nitroxide moiety of CAT-16 became restricted (Fig. 4). Preliminary results (to be published elsewhere) suggest that drastic red cell-shape changes occur at these same low levels of azide (about 2 million molecules/cell).

Acknowledgements

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