Biochimica et Biophysica Acta, 550 (1979) 245—258

BBA 78242

PHOTOAFFINITY LABELING OF PROCAINE-BINDING SITES IN NORMAL AND SICKLE CELL MEMBRANES

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(Received June 15th, 1978)

Key words: Procaine binding; Photoaffinity labeling; (Sickle cell)

Summary

A photoaffinity probe, procaine azide, was employed to determine the sites of interaction of procaine in normal and sickle cell erythrocytes. Studies show that the number of binding sites and affinity of procaine to membranes derived from normal and sickled cell erythrocytes were similar, although procaine retards the in vitro formation of irreversibly sickled cells from cells. The results show that procaine azide, a photoaffinity analogue of procaine, is covalently incorporated into both protein (60—70%) and lipid (40—30%) components of the membrane. Sodium dodecyl sulfate-gel electrophoresis of the labeled ghosts show that procaine binds specifically to band 3 and periodic acid-Schiff staining bands in membranes derived from labeled erythrocytes. Binding of procaine or covalent incorporation of procaine azide into membrane proteins does not affect the phosphate transport. Moreover, pre-treatment of intact erythrocytes with 4,4′-diisothiocyano-2,2′-stilbene disulfonate, an anion transport inhibitor, did not affect either the binding or covalent incorporation of procaine azide into erythrocytes. These results indicate that the binding of procaine azide to Band 3 protein occurs at a locus different than that involved in anion translocation process.

Introduction

Studies have shown that binding of cationic anaesthetics to erythrocyte membrane increases the membrane area, protects against osmotic hemolysis and increases the membrane fluidity [1]. Further, it has been shown that these

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Abbreviations: DIDS, 4,4′-diisothiocyano-2,2′-stilbene disulfonate; AA, normal human erythrocytes; SS, sickle cells.

cationic anesthetics compete with calcium for binding sites on the intracellular side of the membrane [2—5]. It has been shown that reversible change in shape from biconcave to cup shape occurs when cationic agents are added, while anionic agents cause crenation [6]. Sheetz and Singer [7] proposed a bilayer couple hypothesis to explain the changes induced by amphipathic drugs in cell shape of erythrocytes. According to this hypothesis, anionic drugs intercalate mainly into the lipid in the exterior half of the bilayer, expand that layer relative to the cytoplasmic half, and thereby induce the cell to crenate, while the cationic drugs interact with the lipids on the inner half of the lipid bilayer and cause the cells to form cup shapes.

Recent studies by Baker et al. [8,9] have shown that procaine hydrochloride, a cationic anesthetic, retards the in vitro formation of irreversible sickle cells obtained from homozygous sickle cell anemic individuals. It was also observed that procaine hydrochloride minimizes the loss of deformability associated with metabolic depletion of normal erythrocytes in vitro [8]. Elevated levels of cell-associated calcium have been implicated in erythrocyte shape changes [9], a decrease in membrane deformability [10] and changes in sodium and potassium flux [11]. In vivo high calcium levels have been observed in sickle cell anemia [12] and in hereditary spherocytosis [13]. Studies of Baker et al. [14] have shown that pretreatment of normal erythrocytes with procaine hydrochloride causes a reduced uptake of calcium. Moreover, cell-associated calcium was lower by 45% in deoxygenated sickle cells pre-exposed to procaine hydrochloride as compared to untreated cells [14]. However, elevated levels of calcium may not be the decisive factor in the formation of irreversibly sickled cells, since they can also be formed in the absence of calcium [15]. In an effort to gain insight into the mechanism of interaction of procaine with normal and sickle cells, procaine azide was used as a photoaffinity label to probe specific binding sites for procaine in these membranes. The technique of photoaffinity labeling [16—21] has been found to be useful in delineating the sites of interaction of a variety of biological molecules with the membranes. The results presented in this communication show that procaine azide binds to Band 3 and periodic acid-Schiff staining bands in intact erythrocytes and Mg^{2+}-sealed ghosts. The labeling profiles of membrane proteins in ghosts prepared from normal human (AA) and sickle (SS) cells were similar. Moreover, it is shown that the covalent binding of procaine azide to Band 3 in intact erythrocytes does not affect the anion transport.

Materials and Methods

Blood. Normal blood was collected by venipuncture from human volunteers with heparin as anticoagulant and was used within 4 h. Sickle cell blood was drawn from patients with homozygous sickle cell anemia. Diagnosis of sickle cell anemia was based on the hematological parameters of hemoglobin electrophoresis, anemia and demonstration of the sickling phenomenon. Hemoglobin F, as determined by the Singer alkali denaturation method, was less than 10% in all patients studies. Percentages of irreversibly sickled cells were estimated by phase contrast microscopy of wet mounts of oxygenated whole blood.

Preparation of irreversibly sickled cells. The freshly drawn SS blood (5 ml)

was mixed with 0.5 ml Tris buffer, pH 7.6, containing 147 mM NaCl, 2 mM $CaCl_2$ and 12 mM Tris-hydroxyethylaminomethane. 1 vol. of this blood was mixed with 2 vols. 2% sodium metabisulfite (in distilled water) and the contents were incubated in sealed test tubes at 37°C for 4 h under sterile conditions. At the end of the period of incubation the contents were centrifuged at 1400 $\times g$ for 10 min. The pellet was washed three times with 5 ml cold phosphate-buffered saline (5 mM sodium-phosphate buffer, pH 8.0, containing 150 mM NaCl). At the end of the period of incubation 95—100% of the cells were observed to be sickled.

In an alternate procedure in vitro sickling was carried out by flushing N_2 for 60 min through a capillary tube in a two-holed rubber stopper, tightly fitted into a test tube containing SS blood, essentially as described by Shen et al. [22]. The test tube was sealed in a humidified atmosphere of 5% CO_2 in nitrogen under sterile conditions and incubated overnight at 37°C. The contents were centrifuged and washed with phosphate-buffered saline as described above. Under these conditions the percentage of irreversibly sickled cells formed was 30—45%.

Red blood cell membranes. Unsealed erythrocyte ghosts were prepared from freshly drawn AA blood, essentially according to the procedure of Fairbanks et al. [23]. A similar procedure was adopted for the preparation of membrane from SS cells. Resealed ghosts were prepared like unsealed ghosts except the hemolysis and subsequent washes were performed in 40 to 100 vols. of 5 mM sodium-phosphate buffer, pH 8.0, containing 1 mM $MgSO_4$, as described by Kant and Steck [24]. The percentage of inside out (IO) and right side out (RO) oriented vesicles in the membrane preparations was ascertained by assaying the marker enzymes, acetylcholine esterase [25] and glyceraldehyde-3-phosphate dehydrogenase [26] activity in the presence and absence of Triton X-100.

Preparation of p-azidobenzoyl diethylamino ethanol hydrochloride (N_3-procaine). N_3-procaine was synthesized essentially according to the procedure of Levy et al. [20]. [*carboxy*-^{14}C]Procaine (50 μCi, 5.3 mg) was dissolved in a mixture of concentrated HCl (0.5 ml) and water (0.2 ml) and contents cooled to —15 to —20°C. 50 mg $NaNO_2$ dissolved in 0.2 ml water was added in 50-μl aliquots to the stirred solution over a period of 20 min. This was followed by the addition of 50 mg of NaN_3 dissolved in 0.2 ml water over a period of 10 min. After an additional 20 min of stirring, the reaction mixture was neutralized by the addition of NaOH and then extracted with ether. The ether layer was washed with water and evaporated to dryness. The residue was dissolved in 0.5 ml of concentrated HCl and lyophilized. Further purification of [^{14}C]procaine azide was carried out on silica gel plates using 2.5% MeOH in $CHCl_3$ as solvent system. The procaine azide band was eluted with methanol. The yield of the product was 80—90% and the purity of the product was assessed by ultraviolet spectrum and co-chromatography with the standard sample.

Assay for procaine and procaine azide binding to membranes. Erythrocyte membranes (200 μg protein) were incubated in 0.2 ml of 5 mM sodium-phosphate buffer, pH 8.0, containing [^{14}C]procaine or [^{14}C]procaine azide at 25°C for 5 min. At the end of the incubation period, the contents were centrifuged in a Beckman airfuge at 100 000 $\times g$ for 30 s. The pellet was dissolved in Bray's solution containing 20% protosol for estimation of radioactivity. The super-

natant was assayed for the radioactivity by adding Bray's solution.

Assay for procaine and procaine azide binding to intact erythrocytes. Erythrocytes (10 mg protein) were incubated in 0.5 ml phosphate-buffered saline containing various concentrations of [^{14}C]procaine or [^{14}C]procaine azide at 25°C for 5 min and centrifuged in a Beckman centrifuge for 60 s. The pellet thus obtained was dissolved in 1.0 ml protosol, and 0.05 ml H_2O_2 was added to decolorize the solution. The radioactivity was counted after the addition of 5 ml Bray's solution. The radioactivity in the supernatant was assayed using Bray's solution.

Photolysis of N_3-procaine with ghosts and erythrocytes. To the erythrocyte membranes (0.5 mg protein) suspended in 0.5 ml of 5 mM sodium-phosphate buffer, pH 8.0, was added 5 μmol [^{14}C]procaine azide (1 μmol containing $2 \cdot 10^6$ dpm) and 5 mM eserine sulfate. The contents were magnetically stirred in a 3 ml quartz cuvette and photolysis was done for 30 min with a General Electric 400-W medium pressure mercury arc lamp placed at approximately 3 inches distance from the cuvette. The solutions being photolyzed were kept cold by being partially immersed in ice. After photolysis the ghosts were washed twice with 5 mM sodium-phosphate, pH 8.0. The photolysis of intact cell with procaine azide was carried out in phosphate-buffered saline.

Phosphate transport. To 0.9 ml of erythrocytes (30% suspension of cells in the citrate buffer, pH 6.4) was added a solution of sodium-phosphate containing sodium-[^{32}P]orthophosphate ($5 \cdot 10^6$ dpm). The cell suspension was gently agitated on a shaker at 23°C and samples of 0.2 ml were withdrawn at 20, 60, 120 and 300 s timed intervals. The cells were spun down in a Beckman microfuge B and 0.1 ml of the supernatant solution was added to 0.4 ml of 5% trichloroacetic acid. At the end of the experiment, the solutions containing trichloroacetic acid were centrifuged and 0.1 ml supernatant solution was added to 5 ml Bray's solution for counting. When inhibitors were used, they were added to the cell suspension in phosphate-buffered saline and incubated for the indicated time. The cells were washed to remove the unreacted ligand and assayed for phosphate transport as described above.

Extraction of lipids. Lipids were extracted from intact erythrocytes and membranes with 10 vols chloroform/methanol (1 : 1, v/v). The chloroform/methanol layer was evaporated to dryness. Incorporation of radioactivity into lipid was determined by adding Bray's solution.

Gel electrophoresis. Electrophoresis was performed on 5.6% acrylamide gels containing 0.2% SDS following the procedure of Fairbanks et al. [23]. The erythrocyte ghosts were solubilized by adding the following (to the stated final concentrations): 1% SDS, 10% sucrose, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 40 mM DTT and bromophenol dye (10 mg/ml) and heating the samples at 100°C for 3 min. The electrophoresis was carried out in cylindrical tubes in a Bio-Rad electrophoresis cell. The electrophoresis was carried out for 6 h using a current of 5 mA/tube. The gels were stained with Coomassie Blue, fixed and destained in 7.0% acetic acid. The gels were scanned using a Gilford gel scanner. The gels were sliced into 1.6 mM discs using a gel slicer. The radioactivity in the sliced gel was determined after solubilization of the gel in Econofluor containing 5% Protosol and incubating overnight on a rotary shaker. The radioactivity was counted using Beckman Mark I scintillation counter.

Protein estimation. Protein was estimated according to the method of Lowry et al. [27].

Materials. [^{14}C]Procaine (46.8 mCi/mmol), sodium-[^{32}P]phosphate, Protosol and Econofluor were obtained from New England Nuclear, Mass. Diphenylhexatriene and perylene were obtained from Pfaltz-Bauer, Inc. Procaine was obtained from Matheson-Coleman and Bell, Ohio. Acrylamide and SDS were obtained from Bio-Rad, Richmond, Calif. 4,4′-Diisothiocyano-2,2′-stilbene disulfonate (DIDS) was obtained from Pierce Chemical Co.

Results

Binding of procaine to unsealed and sealed erythrocyte ghosts. The interaction of procaine with unsealed ghosts, Mg^{2+}-resealed ghosts and SS ghosts exhibited saturation isotherms, as shown in Fig. 1. The capacity and affinity for the ghost-procaine interaction were estimated by plotting equilibrium binding data according to Scatchard [28]. The binding of procaine to unsealed ghost showed 250 nmol procaine bound/mg membrane protein with a K_d of $2.82 \cdot 10^{-3}$ M, while binding to Mg^{2+}-released ghost exhibited 233 mol procaine bound/mg membrane protein with a K_d of $2.77 \cdot 10^{-3}$ M (Fig. 2). It is pertinent to mention that the percentage of unsealed ghosts in membrane population was 15—20%, as determined by accessibility of glyceraldehyde-3-phosphate to cytoplasmically loacted glyceraldehyde-3-phosphate dehydrogenase in the absence and presence of Triton X-100. The binding of procaine to ghost prepared from irreversibly sickled cell exhibit 242 mol procaine bound/

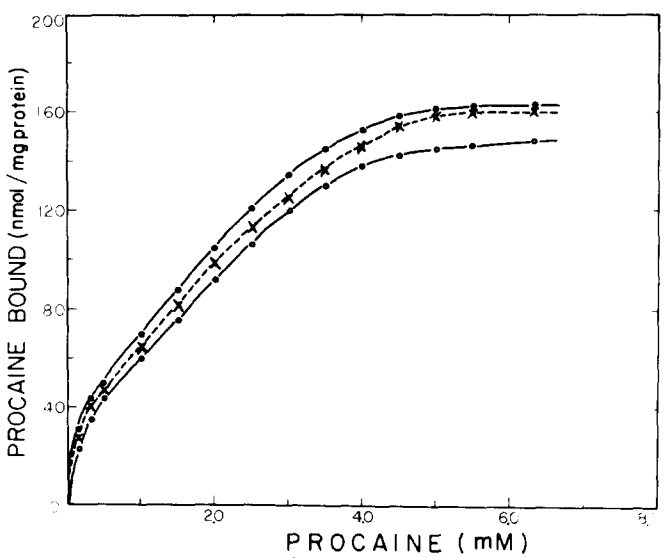

Fig. 1. Binding of [*carboxy*-^{14}C]procaine to unsealed, Mg^{2+}-resealed ghosts and SS ghosts. Ghost protein (200 μg protein) in 0.2 ml of 5 mM sodium-phosphate buffer, pH 8.0, were incubated with varying concentrations of [^{14}C]procaine (spec. act. 10 mCi/mmol) at 25° C for 5 min. The samples were then centrifuged at 100 000 × *g* for 30 s in a Beckman Airfuge. The pellet was dissolved in Bray's solution containing 20% protosol for assay of radioactivity. The radioactivity in the supernatant was determined using Bray's solution. X———X, unsealed ghosts; ○———○, Mg^{2+}-sealed ghosts; ●———●, SS ghosts.

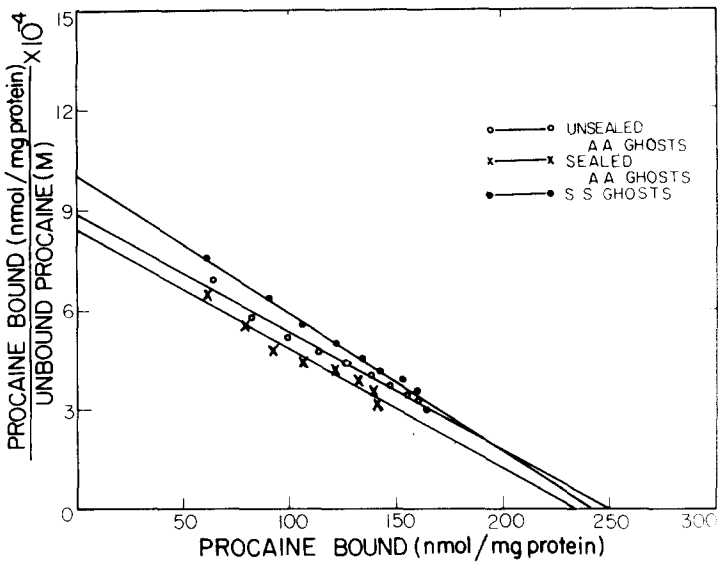

Fig. 2. Scatchard plot of binding of [^{14}C]procaine to ghosts derived from AA and SS cells. Binding of [^{14}C]procaine to unsealed and Mg^{2+}-sealed ghosts from AA and irreversibly sickled cells was carried out as described in legends to Fig. 1. The amount of bound and unbound procaine to ghosts was determined and the data plotted according to Scatchard [28] using regression analysis programme and Tektronix plotter. o———o, unsealed ghosts; X———X, sealed ghost from AA cells; •———•, ghosts from irreversibly sickled cells.

mg membrane protein with a K_d of $2.40 \cdot 10^{-3}$ M (Fig. 2). These results indicate that the number of binding sites and affinity of procaine are similar for the membranes from normal and sickle cell erythrocytes.

Effect of procaine azide on the binding of procaine to membranes. Studies were carried out to determine whether procaine azide, a structural analogue of procaine could affect the binding of procaine to erythrocyte membranes. As shown in Fig. 3, the binding of [^{14}C]procaine to erythrocyte membrane was inhibited by 80% using a 60-fold excess of procaine azide. Similarly, the binding of [^{14}C]procaine azide to membranes was abolished by 75—80% with a 60-fold excess of procaine (data not shown). The binding of procaine azide to membranes from AA cell (Fig. 4) showed 264 nmol bound/mg protein with a K_d of $2.53 \cdot 10^{-3}$ M as compared to membranes from SS cells which showed binding of 278 nmol bound/mg protein with a K_d of $2.30 \cdot 10^{-3}$ M, as estimated by plotting equilibrium binding data according to Scatchard [28].

Time course for the covalent incorporation of [^{14}C]procaine azide into membranes. The erythrocyte membranes were incubated with [^{14}C]procaine azide and photolyzed for various lengths of time (10—45 min). Following irradiation, the membranes were washed to remove unbound procaine azide. As shown in Fig. 5, the maximum incorporation of procaine azide into membranes occurred when the photolysis was carried out for 30 min.

Covalent incorporation of procaine azide into AA and SS mebranes. Irradiation of AA cell membranes with [^{14}C]procaine azide resulted in the covalent incorporation of the label into the membranes (Table I). If procaine azide was

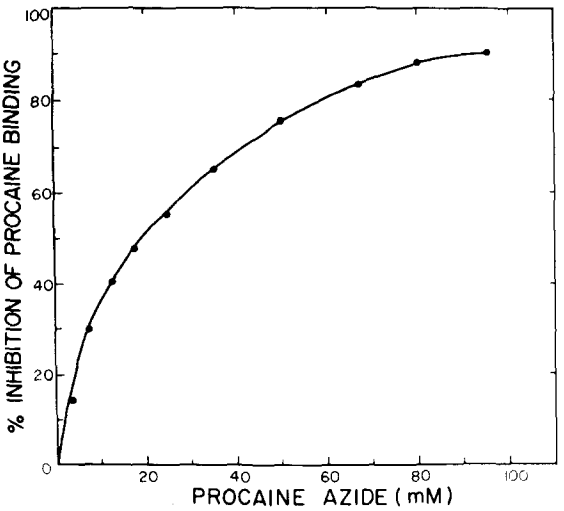

Fig. 3. The effect of varying concentrations of procaine azide on the binding of procaine to erythrocyte membranes. Erythrocyte membranes (200 μg protein) were incubated in 0.2 ml of 5 mM sodium phosphate buffer, pH 8.0, with various concentrations of procaine azide (10—100 mM) for 5 min at 25°C prior to the addition of procaine. The binding of procaine to membranes was determined as described in legend to Fig. 1.

preirradiated with light and then mixed with membranes and then irradiated for an additional 30 min, the extent of incorporation was 10—12% as compared to non-irradiated procaine azide. The covalent incorporation into erythrocyte

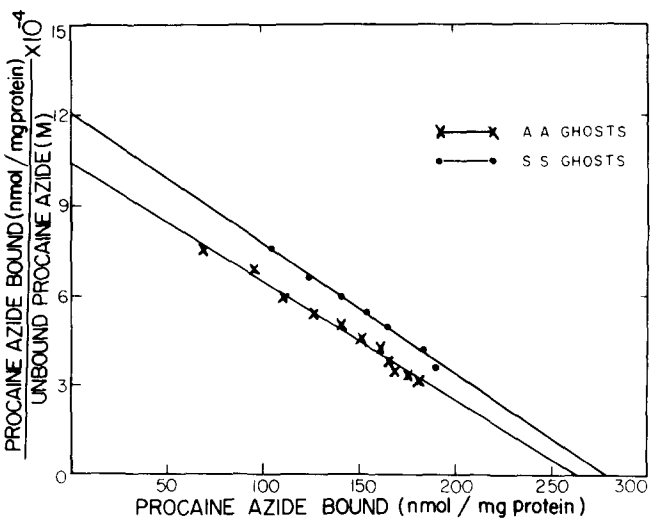

Fig. 4. Scatchard plot for the binding of [*carboxy*-^{14}C]procaine azide to membranes from AA and irreversibly sickled cells. Erythrocyte membrane (200 μg protein) in 5 mM sodium-phosphate buffer, pH 8.0, were incubated with various concentrations of [^{14}C]procaine azide ($2 \cdot 10^6$ dpm/μmol) for 5 min at 25°C. The contents were centrifuged in a Beckman Airfuge at 100 000 × *g* for 5 min. The amount of bound and free procaine azide in the pellet and supernatant were determined and the data plotted according to Scatchard. The data was analyzed using linear regression analysis and plotted using Tecktronix Programmable Plotter. X———X, ghosts from normal cells; ●———●, ghosts from in vitro formed irreversibly sickled cells.

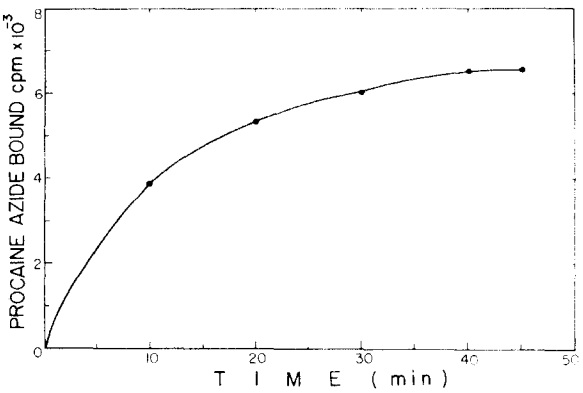

Fig. 5. Covalent incorporation of [^{14}C]procaine azide into erythrocyte membranes as a function of time. The erythrocyte membranes (200 μg protein) from AA cells were suspended in 0.5 ml of 5 mM sodium-phosphate buffer, pH 8.0. [^{14}C]Procaine azide ($2 \cdot 10^6$ dpm/μmol) and 0.5 μM serine sulfate were added and the contents magnetically stirred in a 3 ml quartz cuvette. The photolysis was carried out for varying lengths of time period as described in Materials and Methods. The contents were centrifuged at 100 000 × *g* for 5 min in a Beckman Airfuge and the incorporation of radioactivity into pellet was assayed as described in legend to Fig. 1.

membranes in the absence of photolysis was negligible (Table I). Of the total radioactivity associated with membrane 60—70% was associated with the protein fraction and 30—40% with the lipid fraction.

The covalent incorporation of procaine azide into AA and SS membranes was reduced by 76% and 80%, respectively, upon preincubation of the membranes with an 80-fold excess of procaine (Table II). This indicates that procaine can effectively compete with procaine azide and, thus, the difference in the incorporation of radioactivity observed in the presence and absence of procaine represents the specific binding of procaine. The extent of covalent incorporation of procaine azide in normal and sickle cell membrane was 4.0—6.6% of the labeled procaine azide added.

Covalent labeling of procaine binding sites in AA and SS membranes with procaine azide. Photolysis of Mg^{2+}-resealed ghosts with procaine azide followed

TABLE I

COVALENT INCORPORATION OF [^{14}C]PROCAINE AZIDE INTO ERYTHROCYTE GHOST MEMBRANES

Erythrocyte membranes (0.5 mg protein) were photolyzed as described under Materials and Methods for 30 min at 37°C in 0.5 ml, 5 mM sodium-phosphate buffer, pH 8.0, containing [^{14}C]procaine azide, $5 \cdot 10^6$ cpm. Where indicated, procaine azide was irradiated for 30 min prior to addition to membranes. The contents were centrifuged and pellet washed twice with phosphate buffer. The pellet was extracted with $CHCl_3$-MeOH (2 : 1). The aqueous phase and lipid phase were counted for radioactivity.

Incubation conditions	Radioactivity incorporated (cpm/mg protein)	% Label in	
		Lipid	Protein
Irradiated with procaine azide	11 800	38	62
Preirradiated procaine azide	1 440	—	—
Non-irradiated procaine azide	1 200	—	—

TABLE II

COVALENT INCORPORATION OF [^{14}C]PROCAINE AZIDE INTO NORMAL AND SICKLE CELL MEMBRANE

The incubation mixture in a total volume of 0.5 ml contained ghost protein (0.5 mg protein) obtained from AA or SS cells, 400 μl of 5 mM sodium-phosphate buffer, pH 8.0, $1.8 \cdot 10^6$ dpm of [^{14}C]procaine azide (spec. act. 10 mCi/mmol) 80 mM procaine hydrochloride for 10 min at 37°C. The contents were photolyzed for 30 min as described in Materials and Methods. The ghosts were washed and aliquot portions were assayed for incorporation of radioactivity in lipid and protein fraction. % Radioactivity refers to the percentage of total radioactivity incorporated.

Specimen	Azide incorporated (nmol/mg protein)	Radioactivity incorporated in		Added label incorporated into membranes (%)
		Lipid (%)	Protein (%)	
AA ghosts	130	28.9	71.1	4.4
AA ghosts + procaine	31	35.7	64.3	1.0
SS ghosts	147	25.1	74.9	6.6
SS ghosts + procaine	29	27.5	72.5	0.8
Resealed AA ghosts	65.3	—	—	2.3
Resealed AA ghosts + 80 mM procaine	23	—	—	0.6

by washing the membranes with buffer, in order to remove unreactive ligand, resulted in the incorporation of label into membranes. The incorporation of radioactive label into the membranes was analyzed by SDS polyacrylamide gel electrophoresis (Fig. 6). It was observed that bands corresponding to periodic acid-Schiff staining (glycoprotein) and band 3 were labeled (nomenclature according to Fairbanks et al. [23]). However, the procaine azide labeling was not proportional to the relative staining of the band. As shown (Fig. 6), Band 3 carries label at a relatively higher level than other proteins present in the membrane. Preincubation of membranes with procaine prior to photolysis with procaine azide resulted in the reduction of 80% of the covalent incorporation into all protein components. This indicates that the binding of procaine azide to various membrane proteins reflects the binding of procaine. The unsealed ghost preparation exhibited labeling of spectrin, Bands 4, 5 and 6 in addition to Band 3 and periodic acid-Schiff staining bands (Fig. 6B). A similar pattern of labeling was found in the case of sickle cell membranes (Fig. 6B).

In contrast to the reactivity that procaine azide exhibits towards proteins and glycoproteins of unsealed ghosts, the labeling pattern obtained upon labeling intact erythrocytes with procaine azide is quite selective (Fig. 7). Significantly, no label is observed in Bands 1 and 2 or in Bands 4, 5 or 6 (lysis during the photolysis was negligible, less than 2% of the cells, as determined by measurement of A_{540nm} of the supernatant). Bands 3, PAS-1, PAS-2.1, PAS 2.2, PAS 3 and Bands 2.1 to 2.6 were labeled. As observed in sealed AA ghosts, Bands 3 and PAS-1 were predominantly labeled.

Effect of procaine and procaine azide on the phosphate transport in erythrocytes. Since Band 3 protein is predominantly labeled with procaine azide and has been shown to be involved in anion transport [29—31] studies were undertaken to determine whether binding of procaine azide affects the anion trans-

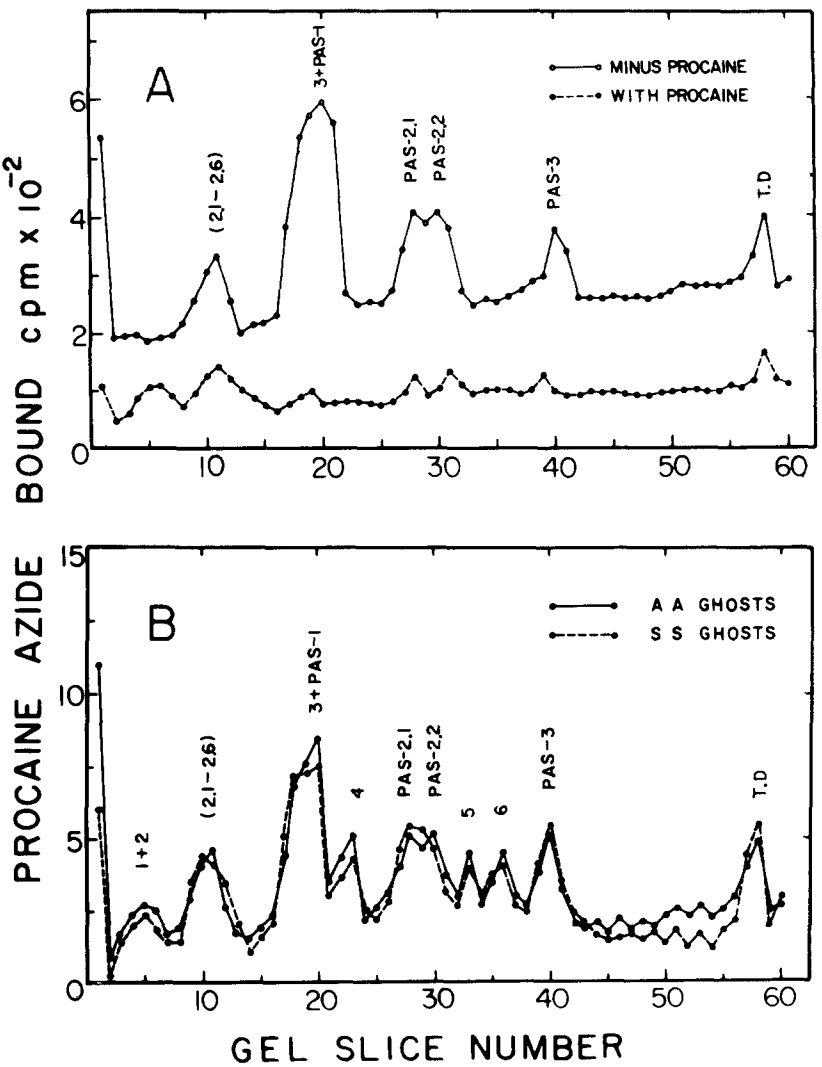

Fig. 6. Sodium dodecyl sulfate-gel electrophoresis of ghosts labeled photocatalytically with [^{14}C]procaine azide. Mg^{2+}-resealed ghosts of AA cells were photolyzed with procaine azide in the presence and absence of 80 mM procaine and treated as given in the legend to Table II. An aliquot (50—100 μg protein) of the photolyzed ghost were electrophoresed on acrylamide gel (5.6%) and stained with Coomassie Blue. The bands are numbered as suggested by Fairbanks et al. [23]. A corresponding unstained gel was cut into 60 slices by a gel slicer and the radioactivity incorporated into each slice was determined as described in Materials and Methods. In A the trace with solid lines represents the incorporation of radioactivity in the absence of procaine while broken lines show incorporation in the presence of procaine. B. Covalent incorporation of [^{14}C]procaine azide in •———•, unsealed ghosts from AA cells; •- - - - -•, ghosts from SS cells.

port in erythrocytes. As shown in Fig. 8, procaine (2 mM) and procaine azide (2 mM) did not affect the transport of phosphate in normal erythrocytes. Photolysis of erythrocytes with procaine azide (2 mM) did not alter the phosphate transport. This data suggests that presumably procaine azide covalently binds to a site different from that involved in phosphate transport in Band 3,

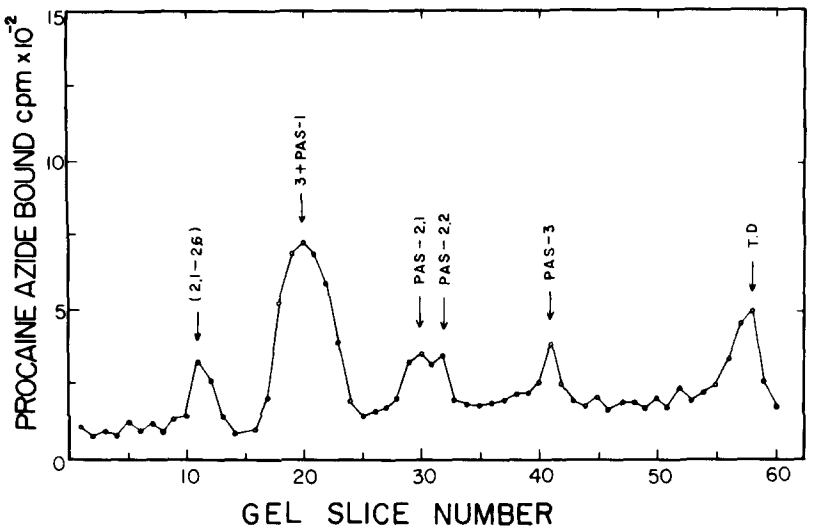

Fig. 7. SDS-gel electrophoresis of ghosts isolated from procaine azide-labeled intact AA cells. AA cells (10 mg protein) were incubated in phosphate-buffered saline, pH 8.0, and photolyzed for 30 min in the presence of [^{14}C]procaine azide ($2 \cdot 10^6$ dpm; spec. act. 10 mCi/mmol). The cells were washed with buffer and ghosts were isolated as described in Materials and Methods. Gel electrophoresis and incorporation of radioactivity in each gel slice was ascertained as described in legend to Fig. 6.

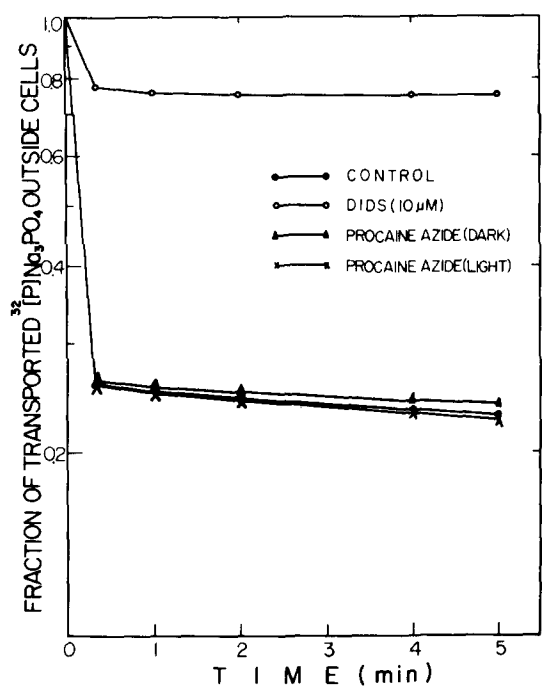

Fig. 8. Effect of procaine, procaine azide and DIDS on phosphate transport in normal erythrocytes. A 30% suspension of erythrocytes in 0.3 ml of phosphate-buffered saline, pH 8.0, was allowed to react with procaine (2 mM), procaine azide (2 mM; dark or photolytic conditions) or DIDS (10 μM) for 30 min. The cells were washed with phosphate-buffered and suspended in 0.9 ml of 0.3 M sodium citrate. To this was added 100 μl of sodium orthophosphate (100 mM) containing $5 \cdot 10^6$ dpm of ^{32}[P]orthophosphate. Aliquots of sample were withdrawn and processed as described in Materials and Methods. The fraction of transported radioactivity remaining outside the cells is plotted as a function of time.

since it has been shown that covalent binding of Band 3 in erythrocytes with DIDS [29] and sodium sulfanilate [30] inhibits phosphate transport. These results were further substantiated by the observation that the binding of procaine azide under dark and photolytic conditions to erythrocytes was not reduced upon pretreatment of erythrocytes with DIDS (data not shown). It is pertinent to mention that treatment of erythrocytes with DIDS results in its covalent incorporation into Band 3 of erythrocyte membranes.

Discussion

In the present report we have examined the binding and site of interaction of procaine to normal and irreversibly sickled cells by employing procaine azide, a photoaffinity derivative of procaine. The results show that the number of binding sites and affinity of procaine to membranes derived from normal and in vitro-formed irreversibly sickled cells were similar (AA membranes, 250 nmol procaine bound/mg membrane protein, $K_d = 2.82 \cdot 10^{-3}$ M; SS membranes, 242 nmol procaine bound/mg protein, $K_d = 2.40 \cdot 10^{-3}$ M). This indicates that the number of binding sites and affinity of procaine to erythrocyte membranes is presumably not related to the observed retardation of in vitro formation of irreversible sickle cells of SS blood effected by this drug. No significant differences in the binding of procaine to intact erythrocytes from AA and SS blood were observed (data not shown).

Procaine azide, was found to effectively compete with the binding of procaine to the erythrocyte membranes. The binding of procaine azide to the erythrocyte membranes of AA cells (264 nmol procaine azide bound/mg protein, $K_d = 2.53 \cdot 10^{-3}$ M) was quite similar to that of procaine and can be displaced by excess of procaine, indicating that procaine azide can be used as a probe to investigate into the binding sites of procaine as has been observed in hepatocyte plasma membranes [20]. Photolysis of procaine azide with erythrocyte membranes resulted in the covalent incorporation of label into both protein (60—70%) and lipid (30—40%) components of the membrane. The labeling of protein and lipid components appear to be specific for procaine binding, since the preincubation of membrane with procaine prior to photolysis reduced the covalent incorporation of the probe by 80%. Studies showed that photolysis was essential for covalent incorporation of the label into the membrane since the control experiment carried out in dark revealed negligible incorporation. Moreover, preirradiation of procaine azide prior to photolysis with membranes resulted in less than 5—12% incorporation of the label. The covalent incorporation of the label increased linearly with increased time of photolysis.

Solubilization of the labeled, sealed ghosts followed by SDS-gel electrophoresis revealed the labeling of Band 3 and bands corresponding to periodic acid-Schiff staining glycoproteins. A similar labeling profile of membrane proteins was observed in ghosts isolated from procaine azide-treated intact erythrocytes. The absence of label in the gel of hemoglobin isolated from labeled erythrocytes suggests that the reagent does not penetrate into the cell. Moreover, our studies show that the labeling pattern of normal cells was similar to that observed in irreversible sickle cells. However, when unsealed ghosts were photolysed with procaine azide, labeling of most of the membrane proteins

present was observed; this is expected since the membrane is leaking, and photoreactive nitrene generated as a result of photolysis can insert into C-H bonds of residues that are usually considered to be inert [16,32]. Since the extent of labeling in Band 3 was relatively high compared to the other bands, it was of interest to ascertain whether the binding of procaine azide to membranes specifically affected the anion transport. The results show that phosphate transport in erythrocytes is not affected by procaine azide. Moreover, covalent incorporation of procaine azide into intact erythrocytes did not affect the transport of phosphate anion, suggesting that procaine azide binds to Band 3 protein at a site different to that involved in anion translocation. The anion transport shown to be mediated by Band 3 protein [29], can be specifically inhibited by DIDS, which covalently interacts with this protein component in the membrane. In our studies, we observed that pretreatment of erythrocytes with DIDS did not affect either the binding or the covalent incorporation of procaine azide into erythrocytes. These results suggest that binding of procaine azide to Band 3 protein is at a locus different to that involved in anion translocation process. Further studies are in progress to delineate the nature of lipid components involved in the interaction of procaine or procaine azide.

Acknowledgement

We are indebted to Dr. Darleen Powars for help in procuring sickle cell blood. This work was supported by NIH grant HL-15162. The assistance of Ms. Sharon Vonderohe in typing of the manuscript is gratefully acknowledged.

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