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THE INTERACTION OF HEPATOCYTE PLASMA MEMBRANES WITH AN AZIDE DERIVATIVE OF PROCAINE

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Summary

A photoreactive derivative of procaine, *p*-azidobenzoyldiethylaminoethanol hydrochloride, has been synthesized and used as a site-directed probe to label hepatocyte plasma membranes. The procaine derivative was shown to have membrane binding and Ca^{2+} displacement characteristics quite similar to that of procaine. Photolysis of the derivative in the presence of hepatocyte plasma membranes resulted in the covalent incorporation of the probe into both the protein and lipid fractions. Analysis of the labeled membranes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis indicated that one membrane protein was significantly labeled with a molecular weight of 21 400 in addition to membrane lipids. Both binding and labeling could be inhibited in the presence of an excess of procaine. The labeled membrane components may be involved in the binding of Ca^{2+} to the membrane system.

Introduction

Local anaesthetics have been shown to interact with the plasma membranes of several cell systems resulting in a large number of effects. These compounds are known to induce membrane expansion [1], alter osmotic fragility [2], inhibit cell fusion [3] and adhesion [4,5] and to enhance the susceptibility of cells to lectin-stimulated agglutination [6]. In addition, the anesthetic, procaine, has been shown to affect lipolysis [7,8], activation of glycogen synthetase [9], and glucose transport in intact adipocytes [10]. Studies have suggested that anesthetics interact with membrane acidic phospholipids and are able to displace membrane associated Ca^{2+} [1,11], resulting in part in an increase in the fluidity of the membrane system.

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Abbreviation: N_3-Procaine, *p*-azidobenzoyldiethylaminoethanol hydrochloride.

Recent studies have also suggested that anesthetics can alter the structural organization of peripheral proteins (microfilaments and microtubules) on the inner surface of the plasma membrane, which are involved in the transmembrane regulation of surface receptors [12,13]. In this study we have utilized the technique of photocatalyzed labeling [14] with an azide derivative of procaine to characterize the binding sites of the anesthetic in hepatocyte plasma membranes. Upon photolysis, the highly reactive nitrene generated from the azido moiety should react with membrane components that are in close proximity to the bound anesthetic regardless of their specific chemical structure.

Materials and Methods

Reagents

Acrylamide and methylenebisacrylamide were obtained from Eastman Chemical Co. Sodium dodecyl sulfate, Coomassie Brilliant Blue and Bromphenyl Blue were obtained from Sigma Chemical Co.; [*carboxyl*-^{14}C]procaine and [*methoxy*-^{3}H]inulin were obtained from New England Nuclear; BBS-3 Biosolv and Butyl PBD-fluoralloy were obtained from Beckman.

Synthesis of p-azidobenzoyldiethylaminoethanol hydrochloride (N_3-procaine)

p-Aminobenzoyldiethylaminoethanol hydrochloride (procaine) (273 mg, 1.0 mmol) was dissolved in a mixture of concentrated HCl (1.5 ml) and water (0.5 ml) and cooled to −15° C. The solution was treated with $NaNO_2$ (21 mg, 1.2 mmol) in water (0.5 ml) over a period of 20 min. NaN_3 (33 mg, 2 mmol) was then added in water (0.5 ml) over a period of 10 min and the reaction permitted to proceed for an additional 20 min. To the clear solution was added 10 M NaOH and the pH adjusted to 7.0. The resultant precipitate was extracted with ether. After ether evaporation, the resultant oil was dissolved in water containing an equivalent amount of HCl to form the HCl salt. The product was crystallized from methanol/ether to afford a product of m.p. 146—147° C. Thin-layer chromatography on silica gel plates in $CHCl_3$/ethyl acetate (50 : 50 v : v) afforded a single spot with an R_F value of 0.6. In aqueous solution the product had a λ_{max} 276 nm (ϵ: $1.9 \cdot 10^4$). The infrared spectrum showed an azide peak at 4.7 μm. Elemental analysis gave the following results: $C_{13}H_{19}N_4O_2Cl$. Elemental analysis: C 52.07; H 6.65; N 18.43. Calculated: C 52.26; H 6.36; N 18.77.

[*Carboxyl*-^{14}C]procaine (50 μCi, 5.3 mg, 19.4 μmol) was dissolved in a mixture of concentrated HCl (0.6 ml) and water (0.2 ml) and treated with $NaNO_2$, followed by NaN_3, as described above, to afford a product identical to the unlabeled preparation, as assessed by thin-layer chromatography, melting point and ultraviolet spectrum.

Preparation of liver plasma membranes

Plasma membranes were isolated from the livers of male Sprague-Dawley rats fed ad libitum, according to the method of Neville [15], as modified by Ray [16]. The purified membranes were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The yield and purity of the membranes was followed by measuring protein content of the membrane fraction by the method

of Lowry et al. [17], as modified by Hartree [18] and the activities of marker enzymes for plasma membranes (5'-nucleotidase [19]), endoplasmic reticulum (glucose-6-phosphatase [20]) and mitochondria (succinate dehydrogenase [21]).

Assay for procaine and N_3-procaine binding to plasma membranes

Plasma membranes (200 μg) were incubated in 0.5 ml of 50 mM Tris · HCl, pH 7.4, containing [*carboxyl*-^{14}C]procaine or N_3-[*carboxyl*-^{14}C]procaine and the mixture shaken at 24°C. At the end of the incubation period the membranes were pelleted in a Brinkman microcentrifuge 3200 at 12 000 × *g* for 30 s, and the supernatant removed with a pipette. The amount of procaine in the pellet was determined by addition to 5 ml of liquid scintillation fluid containing 16% BBS-3 Biosolv and 0.33% Butyl-PBD fluorally in toluene. Estimation of the trapped procaine in the membrane pellet was made using [*methoxy*-^{3}H]-inulin, as previously described [25].

Calcium binding assay

Liver plasma membranes were incubated with $^{45}CaCl_2$ and the bound $^{45}Ca^{2+}$ determined by Millipore filtration, as previously described [22]. The effect of procaine and N_3-procaine on $^{45}Ca^{2+}$ binding was determined by the subsequent addition of the anesthetic to the Ca^{2+}-treated membranes. After a 10 min incubation at 37°C, the residual Ca^{2+} was determined by Millipore filtration.

Irradiation procedures

Photolysis of N_3-procaine (10 μCi) in the presence of liver plasma membranes was carried out in a glass water-jacketed vessel maintained at 24°C by means of a circulating pump. The plasma membrane preparation was stirred with a magnetic stirrer. Photolysis was carried out for 20 min with a General Electric 400-W medium pressure mercury arc lamp, 3 cm from the reaction vessel as previously described [23]. After photolysis, the membranes were washed several times with fresh incubation buffer and then prepared for gel electrophoresis and thin-layer chromatography.

Gel electrophoresis

Photolyzed and untreated liver plasma membranes were solubilized in 1% sodium dodecyl sulfate and 2% β-mercaptoethanol and the solution heated at 55°C for 45 min. Bromophenol Blue and sucrose were added, and 100-μl aliquots containing about 150 μg of protein were electrophoresed on 7.5% polyacrylamide cylindrical gels (90 × 6 mm) containing 0.1% sodium dodecyl sulfate and 0.5 M urea, as previously described [24]. Gels were stained for protein with Coomassie Brilliant Blue. The distribution of ^{14}C in the gel was determined by slicing the gel into 1-mm discs with a Misco 3015 gel slicer and counting the radioactivity in a Beckman LS-245 counter. The gel system was calibrated for molecular weights using thyroglobulin, phosphorylase *a*, bovine serum albumin, β-lactoglobulin, α-chymotrypsin and ribonuclease as standards.

Lipid extraction procedures

Lipids were extracted from the plasma membranes with 2 ml of $CHCl_3$/MeOH (1 : 1) and analyzed by thin-layer chromatography.

Additional procedures

Ultraviolet spectra were recorded on a Cary model 14 spectrophotometer. Infrared spectra were taken on a Perkin-Elmer spectrophotometer. Melting points were taken on a Fisher-Johns apparatus. Thin-layer chromatography was performed on pre-coated silica gel G plates (Brinkmann) which were run with $CHCl_3$/MeOH/H_2O (65 : 25 : 4) and ethyl acetate/MeOH (9 : 1). Elemental analysis was performed by Elek Laboratories, Los Angeles, Calif.

Results

A photoreactive derivative of procaine has been synthesized in an effort to covalently label the binding site(s) in liver plasma membranes. The binding characteristics of the photo-active derivative in the absence of light were measured in an effort to establish its biological integrity and, in turn, the significance of the photo-labeling results. The binding of procaine and N_3-procaine to liver plasma membranes as a function of concentration is shown in Fig. 1. Scatchard plot analysis indicated K_D values of 2.5 ± 0.1 mM and 3.8 ± 0.2 mM for procaine and N_3-procaine, respectively, and the number of binding sites to be 24 ± 1 nmol/mg membrane protein for both compounds. As shown in Fig. 2, a 20-fold excess of procaine resulted in an 80% inhibition of N_3-procaine binding. The binding of the anesthetics to the membranes was extremely rapid where maximum values were obtained in less than 15 s at 24°C. The ability of procaine to displace Ca^{2+} from the plasma membrane was also established by millipore filtration, as shown in Fig. 3. Similar values were obtained for N_3-procaine.

Purified hepatocyte plasma membranes were prepared and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. A densitometer scan of a gel stained with Coomassie Blue is shown in Fig. 4. Analysis of the appropriate enzymatic markers showed the membrane preparation to exhibit a 30-fold

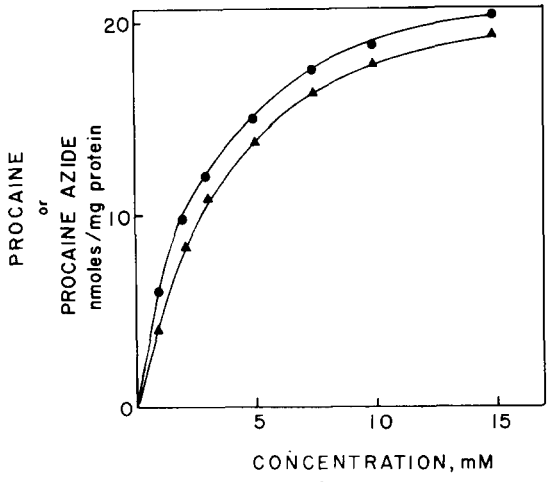
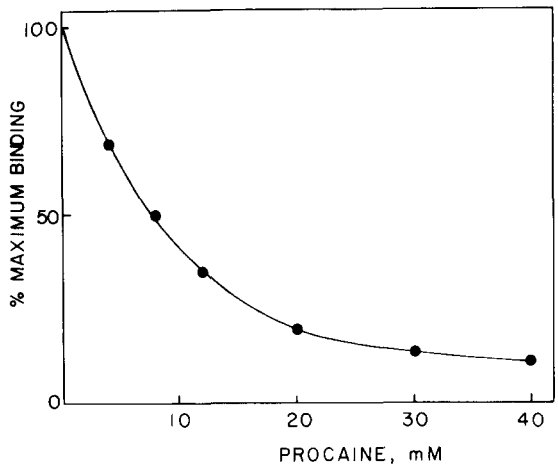


Fig. 1. Binding of [*carboxyl*-^{14}C]procaine (●———●) and N_3-[*carboxyl*-^{14}C]procaine (▲———▲) to hepatocyte plasma membranes. Samples containing 200 μg of membrane were incubated in the dark in the presence of the anesthetic. Binding was determined as described under Materials and Methods.



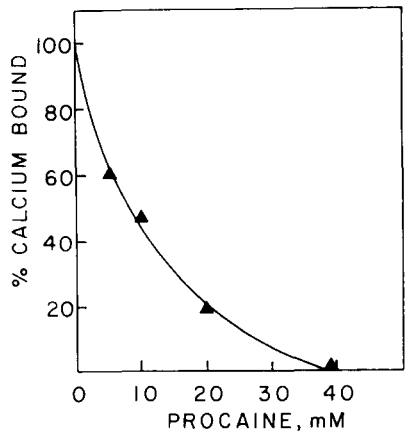


Fig. 2. The effect of procaine on the binding of N_3-procaine to hepatocyte plasma membranes. Plasma membranes were incubated with varying concentrations of procaine for 5 min prior to the addition of N_3-procaine. Binding was determined as described under Materials and Methods.

Fig. 3. The effect of procaine concentration on Ca^{2+} binding to hepatocyte plasma membranes. Membranes (100 μg) were incubated with $^{45}CaCl_2$ (10 μCi) in 0.1 M Tris · HCl (pH 7.5) at 37°C for 10 min followed by the addition of varying concentrations of procaine and the bound $^{45}Ca^{2+}$ determined by millipore filtration. In the absence of procaine a value of 14 ± 2 nmol Ca^{2+}/mg membrane protein was obtained.

purification, as measured by 5′-nucleotidase, and to be relatively free of contamination by endoplasmic reticulum and mitochondria, as measured by glucose-6-phosphatase and succinate dehydrogenase activity.

The membranes were irradiated with light of wavelength greater than 300 nm in the presence of N_3-procaine. Following irradiation, the membranes were

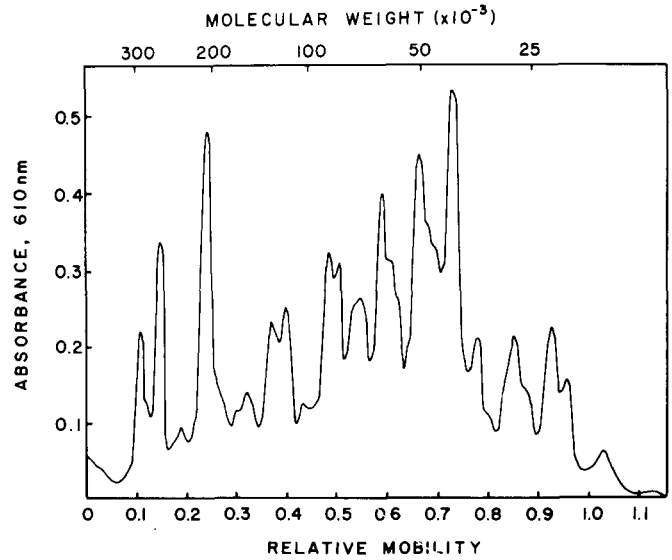


Fig. 4. Densitometer trace of a 7.5% sodium dodecyl sulfate-polyacrylamide gel of hepatocyte plasma membranes. Coomassie Blue-stained gel contained 120 μg of membrane protein.

TABLE I

COVALENT INCORPORATION OF N_3-PROCAINE INTO HEPATOCYTE PLASMA MEMBRANES

Hepatocyte plasma membranes (1 mg) were incubated for 20 min in 1.0 ml of 50 mM Tris · HCl, pH 7.4, containing N_3-[*carboxyl*-^{14}C]procaine (10 μCi). Membrane lipids were then extracted with $CHCl_3$/MeOH (1 : 1).

Incubation condition	N_3-Procaine bound to membranes (cpm)	% Label in	
		Protein	Lipid
N_3-procaine (irradiated)	30 500	35	65
N_3-procaine (unirradiated)	1 200		
N_3-procaine (preirradiated)	1 800		

thoroughly washed to remove unbound N_3-procaine and subjected to analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Membranes were also subjected to lipid extraction and the extract analyzed by thin-layer chromatography. As shown in Table I, the incorporation of radioactive label into plasma membrane components required irradiation of the incubation mixture. The amount of covalently bound label represents an insertion efficiency of 38%. Analysis of the photolyzed membranes showed that 65% of the total radioactivity was associated with the lipid fraction and 35% with the protein fraction. Negligible radioactive labeling of plasma membrane components occurred without photolysis. Furthermore, incubation of the membranes in the light or dark with pre-irradiated reagent also resulted in negligible incorporation of radioactivity into the plasma membranes. Thin-layer chromatography of the lipid fraction showed that N_3-procaine and the photo-products of N_3-procaine had been completely removed and that the remaining label was covalently bound to several membrane lipid components.

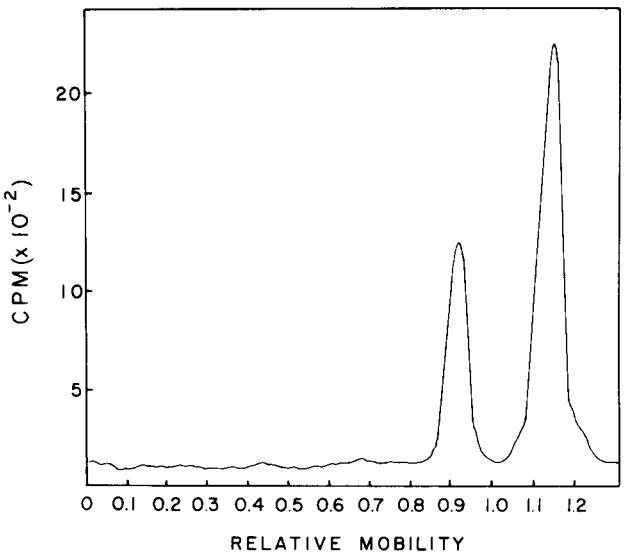


Fig. 5. Sodium dodecyl sulfate polyacrylamide gel electrophoresis of hepatocyte plasma membranes (140 μg) that had been irradiated in the presence of N_3-procaine (20 μCi).

The analysis of plasma membranes by sodium dodecyl sulfate-polyacrylamide gel electrophoresis is shown in Fig. 5. Photolysis results in the incorporation of radioactivity into a membrane protein component with a molecular weight of 21 400. Identical labeling patterns were obtained when the concentration of N_3-procaine was varied from 0.5 to 10 mM. Irradiation of the plasma membranes in the presence or absence of N_3-procaine for periods up to 30 min had no effect on the gel-staining patterns. When the irradiation of the plasma membranes was effected in the presence of N_3-procaine and a 20-fold excess of procaine an 80% decrease in the incorporation of radioactivity was observed.

Discussion

The results of this study have shown that N_3-procaine, a photoreactive derivative of procaine, is a useful probe to identify the binding sites of the anesthetic in hepatocyte plasma membranes. The binding of the azide derivative to the membrane system is quite similar to that of procaine and can be inhibited in the presence of excess procaine. Both compounds also exhibit a similar ability to displace membrane bound Ca^{2+}. These results suggest that the procaine derivative is functionally similar to the native compound.

Photolysis of N_3-procaine in the presence of hepatocyte plasma membranes resulted in the covalent incorporation of the probe into both protein and lipid membrane components. Sodium dodecyl sulfate-polacrylamide gel electrophoresis of the labeled membrane suggested that only one membrane protein was significantly labeled with a molecular weight of 21 400. Analysis of the lipids derived from membranes irradiated in the presence of N_3-procaine suggested that several lipid derivatives had also been formed. Photolysis in the presence of a 20-fold excess of procaine resulted in an 80% decrease in the incorporation of radioactivity into both protein and lipid fractions. The appropriate controls for photocatalyzed labeling studies showed that (a) photolysis was required for covalent incorporation of the radioactive label; (b) pre-irradiation of N_3-procaine followed by re-irradiation in the presence of hepatocyte plasma membranes resulted in negligible incorporation of radioactive label and (c) the incubation and photolytic conditions had no effect on the sodium dodecyl sulfate-polyacrylamide gel patterns. These studies thus suggest that in addition to interactions with membrane lipids there is at least one interaction with a membrane-associated protein. The direct interaction of procaine with cytoskeletal proteins such as microfilaments and microtubules has yet to be demonstrated by this method; however, the ability of anesthetics to displace membrane-bound Ca^{2+} [11] could have a significant regulatory effect on the organization of peripheral proteins involved in the trans-membrane control of surface topography. Iodination studies using lactoperoxidase have shown that this low molecular component is, at least in part, located on the exterior surface of intact hepatocytes (McQueen, H.M. and Levy, D., unpublished observations). The Ca^{2+} binding properties of this protein are under current investigation.

Acknowledgments

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