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The tertiary amine local anesthetic dibucaine binds to the membrane skeletal protein spectrin

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Abstract The quinoline-based tertiary amine dibucaine has been shown to bind the membrane skeletal protein spectrin with a dissociation constant of 3.5×10^{-5} M at 25°C. Such binding is detected by monitoring the quenching of the tryptophan fluorescence intensity with increasing concentrations of dibucaine only and not with the benzene-based local anesthetics procaine, tetracaine and lidocaine. Binding of dibucaine also indicated changes in the tertiary structure of spectrin indicated by a circular dichroism spectrum in the near-UV region due to absorption of the aromatic side chains. The thermodynamic parameters associated with the binding indicated the interaction of dibucaine and spectrin to be enthalpy-driven and insensitive to an increase in the ionic strength of the buffer.

Key words: Local anesthetic; Dibucaine; Erythroid spectrin; Fluorescence

1. Introduction

Tertiary amine local anesthetics cause anesthesia by blocking nerve transmissions via state-dependent binding of voltage-gated Na^+ channels. There are two major models of anesthetic action. The first of these, called the lipid hypothesis, ascribes the effect to physical alterations of the lipid bilayer upon binding of the anesthetic to it. The second, called the protein hypothesis, explains it in terms of direct specific interaction of local anesthetic molecules with proteins [1–5]. Local anesthetics have been shown to interact with many membrane-associated proteins other than the primary target Na^+ channel. Those include the acetylcholine receptor [6], cytochrome oxidase [7] and the F_1 ATPase [8,9] and band 3 of erythrocytes [10]. In addition it has been shown that the denaturation temperature of Ca^{2+}-ATPase of the sarcoplasmic reticulum is lowered slightly by dibucaine and tetracaine, indicating an interaction between local anesthetics and the membrane-bound Ca^{2+}-ATPase [11,12]. More studies have been done examining the effects of local anesthetics on protein structure and activity. Dibucaine and tetracaine inhibited the activation of mitogen-activated protein kinase mediated by calcium channels [13]. Procaine, tetracaine and dibucaine caused inhibitions of dog kidney Na^+,K^+-ATPase activity [14] and G protein-coupled receptor signaling [15]. Local anesthetics were also found to affect phospholipase D activity in differentiated human leukemic cells [16] and brain microtubule assembly [17].

Spectrin is the major constituent protein of the erythrocyte cytoskeleton that forms a filamentous network on the cytoplasmic face of the membrane. To establish the planar network, spectrin interacts with a large number of proteins such as actin, adducin, ankyrin and protein 4.1. In addition to those proteins spectrin binds to fatty acids, phospholipids and a few other hydrophobic ligands. Spectrin is composed of the two largest known, evolutionarily related polypeptide chains, α-subunit (~240 kDa) and β-subunit (~220 kDa), both of which are non-covalently associated in an antiparallel 'side-to-side' manner to form a two-stranded, highly elongated, worm-like heterodimer [18–20]. The heterodimers further self-associate in a 'head-to-head' fashion to form tetramers, the dominant form in vivo. In an earlier work we have studied the interaction of the quinoline-based local anesthetic dibucaine, with small unilamellar vesicles made of phospholipids containing different mole percentages of cholesterol indicating differences in the microenvironment of dibucaine in the presence and absence of cholesterol in the bilayer membranes [21]. As a part of our broad objective to understand the intracellular mode of action of the tertiary amine local anesthetics we have studied the interaction of dibucaine with the erythrocyte membrane skeletal protein spectrin, since such interactions of local anesthetics have been implied to have a functional association with the Na^+ channel [22] and the transbilayer movement of the anesthetic molecule to the cytoplasmic side of excitable membranes is a requirement for anesthetic action [23].

In the present study we show a moderately high-affinity binding between spectrin and the local anesthetic dibucaine using fluorescence spectroscopy. We also attempt to understand the thermodynamic basis of such a binding of dibucaine to erythroid spectrin. In addition to the evaluation of the binding affinities and associated thermodynamic parameters we have employed circular dichroism (CD) spectroscopy to throw light on the structural basis of recognition and conformational changes of the membrane skeletal protein spectrin.

2. Materials and methods

Dibucaine hydrochloride, Tris, phenylmethylsulfonyl fluoride (PMSF), dithiothreitol (DTT), and EDTA were purchased from Sigma (St. Louis, MO, USA). Deionized water, twice distilled on quartz, was used for preparing solutions and buffers. Stock solution of dibucaine was prepared in water and its concentration was determined by absorbance measurement on a Shimadzu UV-2101PC spectrophotom-

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eter, using a molar extinction coefficient of 4400 M^{-1} cm^{-1} at 326 nm [24]. The CD spectra were recorded in a Jasco 720 spectropolarimeter. CD spectra in the range 200–250 nm were recorded in a cylindrical cuvette of 1 mm pathlength. All spectra are the average of five runs. For measurements in the near-UV range (250–310 nm) a rectangular quartz cuvette of 1 cm pathlength was used. Each spectrum was subtracted from the buffer baseline and smoothed within the permissible limits using the in-built software of the instrument as described earlier [25].

White ghosts from goat blood were prepared by hypotonic lysis in 5 mM phosphate, 1 mM EDTA containing 20 μg/ml PMSF at pH 8.0 and spectrin dimers were purified following published protocols elaborated earlier [25,26]. Spectrin extraction was done by resuspending the erythrocyte ghosts in 20 vol of spectrin removal buffer (0.2 mM sodium phosphate, 0.1 mM EDTA, 0.2 mM DTT, 20 μg/ml PMSF, pH 8.0), and incubating at 37°C for 30 min. The crude spectrin was collected in the supernatant after centrifugation and was purified after concentration by 30% ammonium sulfate precipitation according to the method of Gratzer [26]. Spectrin was stored in the buffer containing 5 mM phosphate, 20 mM KCl, 1 mM EDTA, pH 8.0 containing 0.2 mM DTT. The protein was dialyzed extensively against the buffer containing 20 mM Tris–HCl, 50 mM NaCl, pH 7.4 to remove DTT before all fluorescence experiments. The purity of the preparation was checked by 7.5% SDS–PAGE under reducing conditions. The purified protein showed the characteristic spectrin dimer (α-chain of apparent molecular mass of ~240 kDa and β-chain of ~220 kDa) upon staining the gel with Coomassie blue. Spectrin concentrations were determined spectrophotometrically from an absorbance of 10.7 at 280 nm for 1% spectrin solution [26].

2.1. Fluorescence measurements

Steady-state fluorescence was measured in a Fluoromax 3 spectrofluorometer using a 1 cm pathlength quartz cuvette. The buffer used in the present study contained 20 mM Tris–HCl, 50 mM NaCl, pH 7.4. Small aliquots of an aqueous stock solution of dibucaine hydrochloride were added to 0.1 μM spectrin for fluorescence measurements using excitation at 295 nm and slits with bandpasses of 5 nm for both excitation and emission channels. Spectrin-free buffer containing different concentrations of dibucaine was used as reference blanks in all fluorescence measurements. Fluorescence intensities were corrected for the inner filter effect due to absorption of the spectrin and dibucaine, when absorbance, at both excitation and emission wavelengths, of the samples exceeded 0.05 [27]. All measurements were performed at 25°C, unless otherwise mentioned, with multiple sets (three to five) of samples.

2.2. Evaluation of binding constants

Any change in the fluorescence emission intensity of spectrin (λ_{ex} = 295 nm, λ_{em} = 340 nm) upon the progressive addition of dibucaine was measured to evaluate the binding constant. Results from fluorescence titrations were analyzed by the following methods. Apparent dissociation constant ($K_d = 1/K_{app}$) was determined using nonlinear curve fitting analysis (Eqs. 1 and 2) based on the following equilibrium:

$$\mathrm{Db} + \mathrm{Sp} = \mathrm{Db{-}Sp}$$

where Db represents dibucaine and Sp represents spectrin. All experimental points for binding isotherms were fitted by least-square analysis.

$$K_d = \frac{C_s - (\Delta F/\Delta F_{max})C_s]\cdot[C_d - (\Delta F/\Delta F_{max})\ C_s}{(\Delta F/\Delta F_{max})C_s} \quad (1)$$

$$C_s(\Delta F/\Delta F_{max})^2 - (C_s + C_d + K_d)(\Delta F/\Delta F_{max}) + C_d = 0 \quad (2)$$

ΔF is the change in fluorescence emission intensity at 340 nm (λ_{ex} = 295 nm) for each point of the titration curve, ΔF_{max} is the same parameter when the ligand is totally bound to spectrin, C_d is the concentration of dibucaine, C_s is the initial concentration of spectrin and K_d is the apparent dissociation constant. A double reciprocal plot was used for determination of ΔF_{max} using:

$$1/\Delta F = \frac{1/\Delta F_{max} + K_d}{\Delta F_{max}\cdot(C_d - C_s)} \quad (3)$$

The linear double reciprocal plot of $1/\Delta F$ against $1/(C_d - C_s)$ is extrapolated to the ordinate to obtain the value of ΔF_{max} from the intercept as described earlier [25]. The approach is based on the assumption that emission intensity is proportional to the concentration of the ligand and $C_d \gg C_s$, i.e. when ligand concentration is in large excess over the spectrin concentration. The apparent binding constant ($K_{app} = 1/K_d$) was also determined from the concentration of dibucaine corresponding to 50% of the total amplitude of change in fluorescence intensity of spectrin. An estimate of the stoichiometry of dibucaine binding to spectrin was obtained from the intersection of two straight lines obtained from the least-square fit plot of normalized increase in fluorescence intensity against the ratio of input concentrations of dibucaine and spectrin.

2.3. Evaluation of thermodynamic parameters

Thermodynamic parameters, ΔG (free energy), ΔH (heat content) and ΔS (entropy) were evaluated from the following equations:

$$\ln K_{app} = -(\Delta H/RT) + (\Delta S/R) \quad (4)$$

$$\Delta G = \Delta H - T\Delta S \quad (5)$$

where R and T are the universal gas constant and absolute temperature, respectively. The apparent binding constant ($K_{app} = 1/K_d$) was measured at four different temperatures to evaluate ΔH and ΔS from the slope and the intercept in a plot of $\ln K_{app}$ against $1/T$.

3. Results

The change in tryptophan fluorescence of spectrin indicates the association of the protein with dibucaine. Fig. 1 shows the progressive quenching of tryptophan fluorescence of spectrin upon the addition of increasing concentrations of dibucaine, thereby indicating the formation of a complex between spectrin and dibucaine. The inset of Fig. 1 shows characteristic fluorescence spectra of spectrin alone and in the presence of varying concentrations of dibucaine. Fig. 2 shows the plot of the extent of fluorescence quenching as the function of dibucaine concentration due to the association of dibucaine and spectrin. The nature of the curve shows a non-cooperative

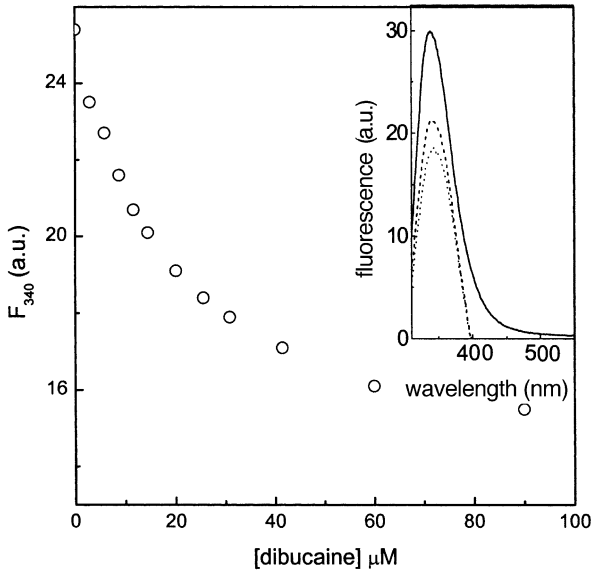

Fig. 1. Binding isotherm (fluorescence intensity at 340 nm versus ligand concentration) for the interaction of spectrin (0.1 μM) with dibucaine in 20 mM Tris–HCl, 50 mM NaCl buffer, pH 7.4 at 25°C. The excitation wavelength is 295 nm. Inset show the fluorescence emission spectra (λ_{ex} = 295 nm) of 0.1 μM spectrin alone (solid line), upon addition of 12 μM dibucaine (dashed line) and 30 μM dibucaine (dotted line).

Table 1
Dissociation constant (K_d) for the interaction of dibucaine with erythroid spectrin in 20 mM Tris–HCl, 50 mM NaCl, pH 7.4 at 25°C

Ligand	Salt	K_d (μM)
Dibucaine	0.05 M NaCl	32
	1.0 M NaCl	37
	2.0 M NaCl	35

type of binding. The dissociation constant (K_d) was determined by non-linear curve fit analysis described earlier [28]. The inset of Fig. 2 shows a representative double reciprocal plot of $1/\Delta F$ against $1/(C_d - C_s)$ to estimate the ΔF_{max}. A K_d of 35 μM was estimated for dibucaine binding to spectrin and was independent of ionic strength of the buffer, in the presence of both low and high concentrations of NaCl (Table 1).

The thermodynamic parameters, e.g. changes in free energy, enthalpy and entropy, were evaluated from the variation of the apparent binding constant with temperature, the Van't Hoff plot. Fig. 3 shows a representative Van't Hoff plot of $\ln K_{app}$ against $1/T$ for the interaction of dibucaine with spectrin. Table 2 summarizes the thermodynamic parameters associated with the binding of dibucaine and spectrin. The interaction is exothermic indicating the enthalpy-driven nature of the interactions between the local anesthetic and the skeletal protein. Fig. 4 shows representative CD spectra of spectrin in the presence and absence of the local anesthetic. It shows a significant change in the shape and intensity of the near-UV CD spectra of spectrin upon binding to dibucaine. However, the far-UV CD spectrum, shown in the inset of Fig. 4, indicated no significant change in the characteristic α-helical structure of spectrin upon association with dibucaine. The results from CD spectroscopic studies in the far-UV and near-UV regions indicate that there is a change in the tertiary structure of the protein upon binding of dibucaine without any significant alteration in the polypeptide backbone of the protein.

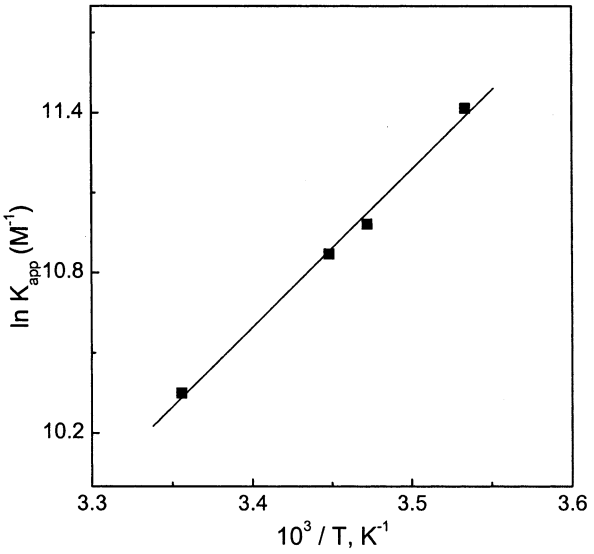

Fig. 3. Van't Hoff plot for the interaction of spectrin (0.1 μM) with dibucaine in 20 mM Tris–HCl, 50 mM NaCl buffer, pH 7.4.

4. Discussion

Spectrin has a number of tryptophan residues spread over the entire length of the dimer. It is noteworthy that the 106 amino acid long repeat units in spectrin have tryptophans strongly conserved at the 45th residue and partially conserved at the 11th residue [29,30]. The tryptophans in these positions represent more than 60% of the total tryptophans in the spectrin dimer. The fact that tryptophans are distributed over the entire molecule and yet are localized in the same position in each domain makes them convenient intrinsic fluorescence reporter groups for monitoring conformational changes in spectrin that contribute to its elastic deformability exhibited in physiological conditions [31]. Ovine erythroid spectrin, used

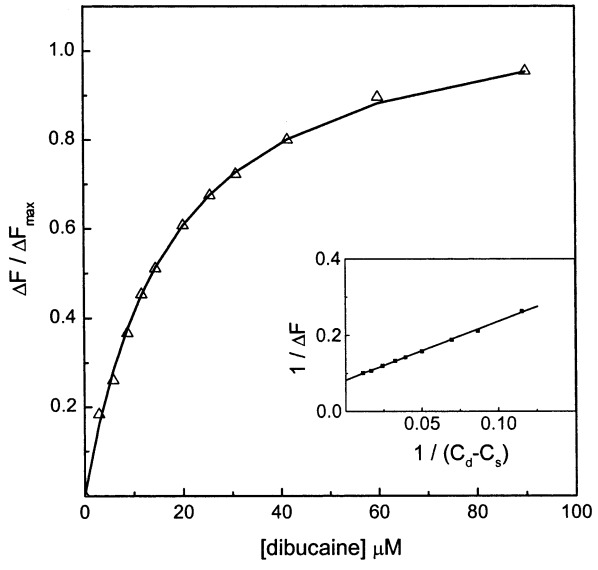

Fig. 2. Plot of the extent of dibucaine-induced quenching vs. concentration of dibucaine for the evaluation of dissociation constant of the association of dibucaine with spectrin (0.1 μM) in 20 mM Tris–HCl, 50 mM NaCl buffer, pH 7.4 at 15°C. Inset show the corresponding double reciprocal plot of $1/\Delta F$ against $1/(C_d - C_s)$ to evaluate the ΔF_{max}.

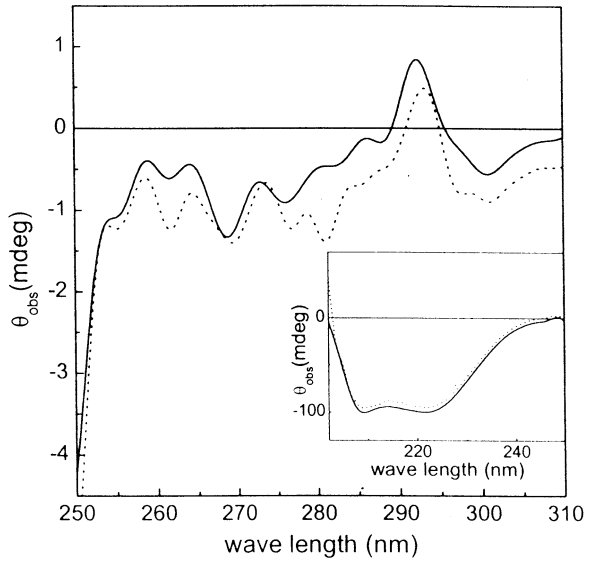

Fig. 4. Near-UV CD spectra (250–310 nm) of spectrin (3 μM) alone (solid line) and in the presence of 120 μM dibucaine (dotted line) in 20 mM Tris–HCl, 50 mM NaCl buffer, pH 7.4 at 25°C. Inset shows the far-UV CD spectra (200–250 nm) of spectrin (1.5 μM) alone (solid line) and in the presence of 40 μM dibucaine (dotted line) in 20 mM Tris–HCl, 50 mM NaCl buffer, pH 7.4 at 25°C.

Table 2
Thermodynamic parameters for the interaction of dibucaine with erythroid spectrin (0.1 μM) in 20 mM Tris–HCl plus 50 mM NaCl buffer, pH 7.4

Ligand	ΔH (kJ mol^{-1})	ΔS (eu)	ΔG (kJ/mol) at 25°C
Dibucaine	-11.9 ± 1.1	-18.8	-6.3

in this study, has structural and self-associating properties that are indistinguishable from those of human and bovine spectrin [32]. Spectrin contains a unique binding site for hydrophobic fluorescence probes such as Prodan and pyrene [33,34]. Spectrin also contains a large number of binding sites for fatty acids and its derivatives [35,36], phospholipids [37], ATP [28] and the DNA binding antitumor antibiotics chromomycin and mithramycin [25] along the length of the large worm-like protein molecule.

Fluorescence and CD studies indicate the binding of dibucaine to spectrin. An absence of change in the CD spectrum of spectrin in the wavelength region between 200 and 250 nm in the presence of dibucaine suggests that the association does not perturb the helical backbone structure of spectrin. Binding of dibucaine proximal to the spectrin tryptophan residues could lead to potential fluorescence energy transfer between dibucaine and tryptophan which is manifested in the quenching of tryptophan (donor) fluorescence. An estimate of the stoichiometry of binding showed about 200 dibucaine molecules to be associated with a spectrin dimer (Fig. 2), also indicated energy transfer to be one of the most plausible mechanisms through which the quenching takes place. However, alteration of the tertiary structure in spectrin also appears plausible when we compare the near-UV CD spectra of spectrin in the absence and presence of the local anesthetic since the bands in the near-UV CD spectrum of the protein originate from the absorption by the aromatic side chain of Phe, Trp, and Tyr. The linear nature of the Van't Hoff plot (Fig. 3) indicates that the association does not lead to any major reshuffling of the two spectrin subunits. Favorable non-covalent interactions leading to a negative change in enthalpy are a feature of the spectrin–dibucaine association. The specific nature of this interaction is indicated from the observation that the affinity constant for the association is insensitive to increases in ionic strength (Table 1).

One interesting aspect of the present work is that the quenching of spectrin tryptophan fluorescence and the change in the near-UV CD are only observed with the quinoline-based tertiary amine local anesthetic dibucaine. Similar changes were not observed in the benzene-based tertiary amine local anesthetics procaine, tetracaine and lidocaine indicating no binding of these local anesthetics to spectrin. The kinetic and electron microscopic studies of the effects of procaine, tetracaine and dibucaine on the polymerization and depolymerization of the microtubules indicated similar differential behavior of the benzene-based procaine and tetracaine with that of the quinoline-based dibucaine. While procaine and tetracaine were found to increase the rates of tubulin polymerization at 24°C and of microtubule depolymerization at 4°C as a linear function of the concentration of the local anesthetics, the quinoline-based dibucaine, on the other hand, showed a linear decrease in the tubulin polymerization and microtubule depolymerization rates [17]. The interaction of eight local anesthetics with cytochrome oxidase, chosen as a membrane model protein, has been earlier studied with fluorescence techniques using quinacrine as a fluorescent probe indicating the existence of hydrophobic interactions with a non-polar region of the cytochrome oxidase complex [7]. The stoichiometry and dissociation constants for the interaction of tetracaine with chloroform-released mitochondrial ATPase, determined from the enhancement of tetracaine fluorescence intensity upon binding, indicated a single class of a large number of approximately 60 thermodynamically equivalent binding sites on the ATPase for tetracaine with a dissociation constant of 4.9×10^{-4} M at 25°C [38]. The present work reports for the first time the binding of the quinoline-based local anesthetic, dibucaine to the membrane skeletal spectrin associated with the binding dissociation constant of 3.5×10^{-5} M at 25°C, an order of magnitude stronger than that of the benzene-based tetracaine with the ATPase. Our results also indicate a possible difference in the anesthetic potentials between the quinoline-based dibucaine and the benzene-based tetracaine, procaine and lidocaine in terms of binding to the membrane skeletal protein spectrin.

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