Interactions between Local Anesthetics and Na⁺ Channel Inactivation Gate Peptides in Phosphatidylserine Suspensions as Studied by ¹H-NMR Spectroscopy

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Received March 29, 2000; accepted May 26, 2000

Interactions between local anesthetics and a sodium channel inactivation gate peptide (Ac-GGQDIFM-TEEQK-NH₂, MP-1A), which was dissected from the cytoplasmic linker between domains III and IV of the sodium channel α -subunit (G1484—K1495 in rat brain type IIA), have been studied by ¹H-NMR spectroscopy. Changes in ¹H-NMR chemical shifts of the aromatic proton resonances of dibucaine (pH 7.0) and lidocaine (pH 6.0 and 9.0) in phosphatidylserine (PS) suspensions were observed. The effects of substitution of glutamine (F1489Q; MP-2A) or D-phenylalanine (MP-1A') for L-phenylalanine (F1489) in MP-1A and the effects of substitution of neutral amino acid residues for the corresponding acidic amino acid residues (D1487N, MP-1NA; E1492Q, MP-1QEA; E1493Q, MP-1EQA) in MP-1A, on the aromatic ¹H-NMR chemical shift changes of dibucaine and lidocaine were also investigated. From these results it was concluded that: the aromatic ring of phenylalanine of MP-1A and the aromatic ring of the cationic form of dibucaine or lidocaine are interacting by π - π stacking; the tertiary amine nitrogen of dibucaine is interacting electrostatically with D1487, whereas that of lidocaine is interacting with E1492.

Key words ¹H-NMR; dibucaine; lidocaine; phosphatidylserine; Na⁺ channel; inactivation gate peptide

Local anesthetics are chemicals that block action potentials in excitable membranes.^{1,2)} Their receptor sites are now considered to be within a sodium channel α -subunit.^{3,4)} This hypothesis is called a specific receptor theory.⁴⁾ However, there still remains the hypothesis that considers the receptor site to be located within the lipid membrane,^{5,6)} because a wide variety of different chemicals which include neutral and both negatively and positively charged molecules can act as local anesthetics.^{5,7)} There are good correlations between the membrane concentrations of anesthetics and their potencies,⁸⁾ suggesting that local anesthetics are binding at a hydrophobic region of the lipid membrane. This hypothesis is called a non-specific general perturbation theory.⁴⁾ Since both theories have their own rationalizations, a new concept which involves both theories is necessary for elucidating the molecular mechanisms of local anesthesia.

The sodium channel α -subunit consists of four homologous domains, I—IV, each with six transmembrane segments, S1—S6, (Fig. 1).⁹⁾ The cytoplasmic linker between domains III and IV of the sodium channel α -subunit (III—IV linker) is known to play a decisive role in a fast inactivation process.^{10,11)} The inactivation gate is considered to close by hydrophobic interactions between the three adjacent hydrophobic amino acids, Ile-Phe-Met (I1488-F1489-M1490, Fig. 1), and their receptors which are considered to be composed of the short S4—S5 loops of both domains III¹²⁾ and IV.^{13—15)} Bennett *et al.* have demonstrated that removal of the fast inactivation by mutations in the IFM motif to QQQ in human heart sodium channels (hH1) results in loss of a high-affinity inactivated-state block of the channel by lidocaine.¹⁶⁾ Their results suggest that the III—IV linker, especially the IFM motif, is a determinant of the local anesthetic binding site. In our previous paper,¹⁷⁾ we addressed the III—IV linker, especially the three adjacent hydrophobic amino acids (IFM) moiety as a docking site of charged amine type local anes-

thetics, because there exist negatively charged amino acids only on both sides of the IFM motif (D1487, E1492, E1493) in the III—IV linker (Fig. 1). We have proposed a new concept that can satisfy the requirements from both the specific receptor theory and the non-specific general perturbation theory: a local anesthetic molecule, located at the polar head-group region of the so-called boundary lipids in the vicinity of the Na⁺ channel pore, is also interacting by π - π stacking interactions with the phenylalanine residue in the III—IV linker, and by electrostatic interactions with one of the negatively charged amino acid residues.¹⁷⁾ We have studied interactions between a model peptide MP-1 (Ac-GGQDIFM-TEEQK-OH), which was dissected from the III—IV linker (G1484—K1495, Fig. 1), and dibucaine (Fig. 2) by ¹H-NMR spectroscopy. We found that the quinoline ring of dibucaine can interact with the aromatic ring of Phe by π - π stacking of the rings. However, we could not specify which negatively charged amino acid residues are interacting electrostatically with the tertiary amine nitrogen of dibucaine. In the present study, in order to specify the negatively charged amino acid residue, we have synthesized MP-1A (Ac-GGQDIFMTEEQK-NH₂), which was amidated at the C-terminus of MP-1, and some related peptides in which some acidic amino acid residues (Asp, Glu) are substituted by the corresponding neutral amino acid residues (Asn, Gln), and investigated the interactions with dibucaine in sonicated phosphatidylserine (PS) suspensions by ¹H-NMR spectroscopy. In order to obtain information on the role of the cationic charge at the tertiary amine nitrogen of a drug, we also studied the interactions between the peptides and lidocaine at both pH 6.0 (cationic form) and pH 9.0 (neutral form), since we could not use dibucaine under alkaline conditions on account of its low solubility.

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Fig. 1. Schematic Representation for the Sodium Channel α Subunit

The amino acid sequence of the cytoplasmic linker between domains III and IV of the rat brain type IIA sodium channel is shown at the bottom using one-letter symbol; the amino acid sequence corresponding to MP-1A is between G1484 and K1495.

Fig. 2. Structures of Dibucaine and Lidocaine

Experimental

Materials Dibucaine hydrochloride, lidocaine hydrochloride and bovine brain L- α -PS were obtained from Sigma and used without further purification. All the peptides were synthesized automatically by the solid phase method using 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 433A peptide synthesizer; their N-termini were acetylated (denoted by Ac-) and their C-termini were amidated (denoted by-NH₂). They were purified on a reverse-phase C₁₈ high-performance liquid chromatography column using a gradient of 90% A, 10% B to 70% A, 30% B, where A is 0.1% trifluoroacetic acid (TFA) in water and B is 0.1% TFA in acetonitrile; the rate of decrease in A was 20%/40 min. They were characterized by ion spray mass spectrometry. The amino acid sequences of the peptides were: MP-1A, Ac-GGQDIFMTEEQK-NH₂; MP-1A', Ac-GGQDIFMTEEQK-NH₂ (F means D-phenylalanine); MP-2A, Ac-GGQDIQMTEEQK-NH₂; MP-1NA, Ac-GGQNIQMTEEQK-NH₂; MP-1QEA, Ac-GGQDIFMTQEQQK-NH₂; MP-1EQA, Ac-GGQDIFMTQEQQK-NH₂.

Preparation of Sample Solutions Single bilayer vesicles (liposomes) were prepared by ultrasonic irradiation of an isotonic (310 mOsm, 150 mM) phosphate buffer (in D₂O) suspension of dried PS for 20 min, cooling in an ice/water bath and bubbling with nitrogen gas. A weighed amount of a drug (3 mM) dissolved in the buffer and/or a peptide (3 mM) was added to the suspension of the pre-formed vesicles (15 mM).

Measurements The ¹H-NMR experiments were carried out on a Bruker AM-600 (600.13 MHz) spectrometer with a digital resolution of 0.25 Hz per

Fig. 3. ¹H-NMR Spectra of (a) Dibucaine in a Phosphate Buffer (pH 7.0), (b) Dibucaine in PS Suspensions (pH 7.0), and (c) Dibucaine and MP-1A in PS Suspensions (pH 7.0)

point. Ambient probe temperature was 27 °C. Chemical shifts were referenced to internal TSP (3-trimethylsilylpropionic acid-*d*₄).

Results and Discussion

Figure 3 shows a typical example of ¹H-NMR spectra of (a) dibucaine in a phosphate buffer (pH 7.0), (b) dibucaine in PS suspensions (pH 7.0), and (c) dibucaine and MP-1A in PS suspensions (pH 7.0), respectively. At pH 7.0, most of the dibucaine molecules exist as a cationic form ($pK_a = 8.0$).¹⁸ Since the peaks due to the drug, PS, and the peptide overlapped with one another at a lower frequency region than that of the HDO resonance, we followed the changes in the chemical shifts of the aromatic proton resonances of the drug and the phenylalanine residue of the peptide. The assignments for the quinoline ring proton resonances were 3, 6, 7, 8, and 5, respectively, from low to high frequency.^{17,19} The spin-coupled sharp resonances at around 7.2–7.3 ppm in Fig. 3c

Fig. 4. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Quinoline Ring Proton Resonances (at 3, 5, 6, 7, and 8 Positions) of Dibucaine as Caused by the Peptides

(a) MP-1A, (b) MP-2A, (c) MP-1A', (d) MP-1NA, (e) MP-1QEA, and (f) MP-1EQA.

were due to the aromatic ring protons of the phenylalanine residue of MP-1A; the assignments were *ortho*, *para*, and *meta* protons from low to high frequency, respectively. The quinoline ring proton resonances were broadened by the presence of PS suspensions (Fig. 3b), indicating that dibucaine partitioned into the lipid bilayer. The resonance at the 5 position shifted to higher frequency, whereas all the remaining resonances (8, 7, 6, and 3 positions) shifted to lower frequency in PS suspensions. All these quinoline ring proton resonances shifted slightly to lower frequencies when MP-1A was added (spectrum c). Since in the PS suspensions, the resonance at the 5 position and those at the 8, 7, 6, and 3 positions shifted to the opposite direction with each other as compared to those in the phosphate buffer solution, the changes in the chemical shifts from spectrum b to spectrum c are not due to the change in the partition of dibucaine into the PS bilayers which might be caused by the peptide. Observed changes in chemical shifts (in units of Hz at 600.13 MHz) by MP-1A are summarized graphically in Fig. 4 together with the results by the other peptides.

It can be seen that: 1) the quinoline ring proton resonances of dibucaine were shifted to a low frequency by MP-1A (Fig. 4a), whereas to a high frequency by MP-2A (Fig. 4b); 2) MP-1A' (Fig. 4c) caused smaller chemical shift changes than did MP-1A; 3) the magnitudes of the changes in chemical shifts were the smallest by MP-1NA (Fig. 4d), while the

largest were by MP-1EQA (Fig. 4f); 4) MP-1EQA caused chemical shift changes more than twice as much as those done by MP-1A (Fig. 4a) or by MP-1QEA (Fig. 4e). Observation 1 suggests that the aromatic ring of the phenylalanine residue of MP-1A and the quinoline ring of dibucaine are interacting by π - π stacking with each other. Observation 2 supports this view and suggests that to interact by the π - π stacking, an appropriate stereochemical configuration is required. In our previous paper,¹⁷⁾ we also deduced this π - π stacking interaction by comparing the changes in chemical shifts of the quinoline ring proton resonances caused by the peptides, MP-1 and MP-2; MP-2 is the F1489Q substituted peptide of MP-1. In the case of MP-1, all the quinoline ring proton resonances are shifted to a low frequency in a similar manner as in MP-1A, but to a much larger extent than by MP-1A. Moreover, MP-2 also caused a low frequency shift to the quinoline ring proton resonances, although the magnitudes were smaller than those caused by MP-1.¹⁷⁾ Since the C-termini of both MP-1 and MP-2 are not amidated, the C-terminal carboxyl group appears to induce the low frequency shift to the quinoline ring proton resonances of dibucaine. Observation 3, especially for MP-1NA is rather dramatic and means that D1487 is indispensable for the interaction with dibucaine, while observation 4 means that E1493 is interfering with the binding of dibucaine.

In Fig. 5, we summarized the changes in chemical shifts of

Fig. 5. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of F1489 as Caused by Dibucaine

m: meta, p: para, and o: ortho positions. (a) MP-1A, (b) MP-1A', (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA.

the aromatic proton resonances of the phenylalanine residues of (a) MP-1A, (b) MP-1A', (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA in PS suspensions as caused by dibucaine. The aromatic proton resonances were shifted to a low frequency by dibucaine in MP-1A, MP-1A', and MP-1NA, whereas to a high frequency in MP-1QEA and MP-1EQA. All these chemical shift changes may include those due to changes in the strength of the interaction of the peptides with PS suspensions as caused by dibucaine. The changes in chemical shifts due to the interaction of a peptide with PS suspensions were found to be as large as -1.8 Hz for all the peptides. Thus, the observed high frequency shifts of MP-1QEA (Fig. 5d) and MP-1EQA (Fig. 5e), especially of MP-1EQA can be considered to be due to relatively strong interaction of dibucaine with the peptide, which is also seen in Fig. 4f.

Figure 6 shows a typical example of ^1H -NMR spectra of a) lidocaine in a phosphate buffer (pH 9.0), b) lidocaine in PS suspensions (pH 9.0), and c) lidocaine and MP-1A in PS suspensions (pH 9.0), respectively. Aromatic proton resonance regions are shown here for brevity. At pH 6.0, the overall spectral features were the same as in Fig. 6 except that spectrum c contained some amide proton resonances due to the peptide. The lidocaine molecules exist as a cationic form at pH 6.0 and a neutral form at pH 9.0 ($pK_a = 7.86$).²⁰ The meta and para ring proton resonances of lidocaine (Fig. 2), which were observed as sharp spin-coupled peaks at around 7.2 ppm (spectrum a), became a broad single peak and were shifted to a low frequency by the interaction with PS liposomes. The broad peak in spectrum b shifted to a high fre-

Fig. 6. ^1H -NMR Spectra of (a) Lidocaine in a Phosphate Buffer (pH 9.0), (b) Lidocaine in PS Suspensions (pH 9.0), and (c) Lidocaine and MP-1A in PS Liposomes (pH 9.0)

Fig. 7. Changes in Chemical Shifts (in units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of Lidocaine as Caused by the Peptides

(a) MP-1A, (b) MP-2A, (c) MP-1A', (d) MP-1NA, (e) MP-1QEA, and (f) MP-1EQA.

quency in the presence of MP-1A (spectrum c). Thus in the case of lidocaine, the changes in chemical shifts of the aromatic proton resonances from spectrum b to spectrum c may include changes in the partitioning of lidocaine into PS liposomes which are caused by the peptide. All the observed changes in chemical shift of lidocaine by MP-1A and also by the other peptides at pH 6.0 and 9.0 are summarized in Fig. 7. It is seen that: 1) in all the cases (Figs. 7a–f) and at both pH 6.0 and 9.0, the aromatic proton resonances of lidocaine were shifted to a high frequency by the peptides; 2) these high frequency shifts were much larger than those observed

in dibucaine (Fig. 5); 3) MP-1NA (Fig. 7d) caused the largest chemical shift changes, whereas MP-1QEA (Fig. 7e) caused the least changes at both pH 6.0 and 9.0; 4) MP-1A' (Fig. 7c) caused smaller chemical shift changes than did MP-1A (Fig. 7a) at pH 6.0; 5) in MP-1A, MP-1NA, and MP-1QEA, each of the low frequency shifts was larger at pH 6.0 than the corresponding shift change at pH 9.0; 6) at pH 9.0, the shift changes by MP-2A (Fig. 7b) and MP-1A' (Fig. 7c) were larger than by MP-1A (Fig. 7a). Although it is difficult to rule out the possibility that the large high frequency shift (observations 1 and 2) was due to the decrease in the partition of lidocaine into PS liposomes, observed variations in the magnitude of the changes in chemical shifts appear to indicate the differences in the strength and the mode of the interaction between lidocaine and the peptides. Observation 3 can thus be considered to mean that D1487 interfered with the interaction, while E1492 played an important role in the interaction with lidocaine. Moreover, observation 4 appears to be a manifestation of the importance of the stereochemical configuration around F1489 for the interaction. Observation 5 suggests that the electrostatic interaction between the positive charge at the tertiary amine nitrogen of lidocaine and the negative charge at E1492 strengthened the binding of lidocaine with the peptide. Although both MP-1A (Fig. 7a) and MP-2A (Fig. 7b) caused a high frequency shift, the magnitude was larger in MP-1A than in MP-2A at pH 6.0. Thus the π - π stacking interaction may be present between the aromatic ring of F1489 and that of the cationic form of lidocaine. However at pH 9.0, since observation 6 indicates that the presence of F1489 and the stereochemical configuration around F1489 are not important for the binding, the π - π stacking interaction is not operating or weak, if any, in the interaction for the neutral form of lidocaine. The ^2H -NMR quadrupole splittings of the aromatic ring of tetracaine indicate that charged and uncharged tetracaine occupy different sites in the PS bilayer;²¹ the latter binds at a more ordered environment than the former. In analogy with tetracaine, uncharged lidocaine may also be binding with PS at a more ordered environment than its charged counterpart, probably a little deeper into the PS bilayer. This may be the reason why the π - π stacking interaction was weakened in the interaction with the peptide for the neutral form of lidocaine.

Figure 8 shows changes in the chemical shifts of the aromatic proton resonances of the phenylalanine residues of (a) MP-1A, (b) MP-1A', (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA in PS suspensions as caused by lidocaine and at pH 6.0 and 9.0. It is seen that: 1) except for MP-1QEA (Fig. 8d), the aromatic protons of the phenylalanine residues shifted to a high frequency at pH 6.0 and oppositely at pH 9.0; 2) the magnitudes were smaller than those observed with interactions for dibucaine (Fig. 5); 3) at pH 6.0, MP-1NA showed the largest high frequency shift; 4) at pH 9.0, in all the cases (Figs. 8a–e), the phenylalanine ring protons showed a very small low frequency shift. Observation 3) again means that at pH 6.0, D1487 is interfering with the interaction for lidocaine. The fact that MP-1QEA showed an opposite chemical shift change as compared to the other peptides means that E1492 had been playing a key role in the interaction. Finally observation 4) implies that the neutral form of lidocaine does not interact with F1489 as discussed above.

In conclusion, the cationic forms of dibucaine and lido-

Fig. 8. Changes in Chemical Shifts (in Units of Hz at 600.13 MHz) of the Aromatic Ring Proton Resonances of F1489 as Caused by Lidocaine at pH 6.0 and pH 9.0

(a) MP-1A, (b) MP-1A', (c) MP-1NA, (d) MP-1QEA, and (e) MP-1EQA.

caine interact differently with MP-1A. The tertiary amine nitrogen of dibucaine interacts electrostatically with the negative charge of D1487, while its quinoline ring interacts with the aromatic ring of F1489. On the other hand, the tertiary amine nitrogen of lidocaine interacts electrostatically with the negative charge of E1492, while its aromatic ring interacts with the aromatic ring of F1489.

Finally, it should be added that since the absolute magnitudes of the presently observed changes in chemical shifts of the aromatic proton resonances of the local anesthetics and those of the phenylalanine residues of the peptides were small, other explanations not including the π - π stacking interactions cannot be excluded for explaining the differences in the magnitudes of the chemical shift changes among the peptides. Differences in the magnetic anisotropy effects between the side-chains of the amino acids and/or the conformational changes due to substitution of one of the amino acids in a peptide can be a candidate for explaining the reason for the differences in the observed chemical shift changes. To verify these hypotheses and for a more thorough discussion, determination of the structures of the peptides in solution is required. Structural studies on the peptides including the IMF motif in trifluoroethanol solutions and in micelles are currently being undertaken and some results have been reported elsewhere.²²⁾

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