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Lidocaine, Carbamazepine, and Imipramine Have Partially Overlapping Binding Sites and Additive Inhibitory Effect on Neuronal Na^+ Channels

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ABSTRACT

Background: Despite the structural differences, local anesthetics, anticonvulsants, and tricyclic antidepressants exert similar use-dependent actions against voltage-gated Na^+ channels, which may be contributory to pain control. The authors explore whether these drugs could doubly occupy the channel and exert synergic clinical effect.

Methods: The authors performed electrophysiologic recordings and quantitative analyses in mutant and native neuronal Na^+ channels to investigate molecular interactions between different drugs.

Results: The authors demonstrate significant interactions between F1764 and W1716, two residues reported for local anesthetic binding, indicating uncertainties to conclude a common drug-binding site by mutation data. Therefore, the authors performed detailed functional studies in native neurons. Quantitative analyses of the inactivation curve shift argue against effective double occupancy of different drugs. For example, the shift of 20.9 ± 1.3 mV in the simultaneous presence of 10 μM imipramine, 100 μM lidocaine, and 100 μM phenytoin is consistent with the one-site (21.5 mV) rather than the two-site (30.5–33.8 mV) or three-site (42.7 mV) predictions. However, there is a deviation from the recovery courses predicted by one site if lidocaine or imipramine coexists with anticonvulsants. Moreover, gating state dependence of macroscopic-binding rates markedly differs between imipramine and carbamazepine.

Conclusions: Carbamazepine, lidocaine, and imipramine bind to a common site with the common aromatic motif. External to the aromatic site, there is another weaker and less gating-dependent site for the tertiary amine chain in the latter two drugs. Concomitant clinical use of these drugs, thus, should have at most a simple additive but not a synergistic inhibitory action on Na^+ currents.

What We Already Know about This Topic

- Antiepileptics, local anesthetics, and antidepressants all contribute to pain control and also inhibit sodium channels in a similar way, but their interactions at the channel are unclear

What This Article Tells Us Is New

- By using dissociated cells from the rat hippocampus and quantitative analyses, we found that all three drugs bind to a common aromatic site, but the latter two also bind to a tertiary amine site on sodium channels
- Theoretically, combinations of these drugs should produce additive rather than synergistic actions at this site

LOCAL anesthetics (*e.g.*, lidocaine), prototypical anticonvulsants (*e.g.*, carbamazepine, phenytoin, and lamotrigine), and tricyclic antidepressants (*e.g.*, imipramine), in oral or transdermal forms, have been prescribed widely for the treatment of neuropathic or even nociceptive pain. The mechanisms of action are not fully clear, but they may involve modulation of central transmitters (*e.g.*, inhibition of norepinephrine and/or serotonin reuptake) and ion channels (*e.g.*, inhibition of Na^+ or Ca^{2+} channels) related to pain transmission.[1–4] Interestingly, although the mechanisms underlying the analgesic effect remain unsettled and could involve multiple factors, all three groups of drugs seem to inhibit Na^+ currents, and thus cellular discharges, in a characteristic use-dependent fashion.[5–14] We have shown that the anticonvulsants bind to a common binding site in the Na^+ channel with the diphenyl motif in their structure,[15] which may be also the case for the tricyclic antidepres-

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sants and other compounds containing a similar motif.[16] Conversely, lidocaine does not contain a diphenyl structure but one aromatic group with an amine chain. Interestingly, a similar amine chain is also present in the tricyclic antidepressants but not in the anticonvulsants. Pharmacologically, it would be desirable to clarify whether the binding site(s) for these structurally dissimilar but interrelated drugs actually overlap to make the very similar inhibitory effect on Na^+ currents and whether the functional role is different for different structural components. From a clinical point of view, if the drugs do bind to different sites in the channel, then concomitant use of two or more different drugs might be more advocated for the possibility of double occupancy and consequent superadditive use-dependent inhibition of the Na^+ channel.

A common binding site for local anesthetics and anticonvulsants at the intracellular pore-lining parts of the sixth transmembrane segments in the Na^+ channel (*e.g.*, F1764 in Nav1.2) has been proposed based on the mutation studies.[17–20] However, most of these mutations are also found to alter gating[17,19] or abolish drug action only in certain gating states,[21,22] arguing against a direct damage of the binding site. Conversely, there is also evidence for an external drug-binding site. For example, point mutations of the external residues in the sixth transmembrane segment of domain 4 and in the pore loop external to the presumable DEKA selectivity filter (*e.g.*, mutation on the residue homologous to W1716 in Nav1.2) significantly alter local anesthetics binding to the pore.[17,23–26] Moreover, binding studies and functional data also suggest an external binding site for the anticonvulsants.[15,27,28] The apparent discrepancy in the extracellular or intracellular location of the drug-binding site strengthens the concern of a significant allosteric effect on the binding site because of mutation. In this study, we demonstrate that there is a significant interaction between point mutations in F1764 and W1716 in terms of lidocaine binding. The significant interactions of F1764 with the other parts of the channel protein indicate that one should be cautious in the interpretation of the mutation data to conclude whether there is a common drug-binding site. To spare the possible confounding factors because of mutation, we then carried out detailed quantitative investigations on the action of coexisting local anesthetic (or tricyclic antidepressant) and anticonvulsant drugs in native neuronal Na^+ channels. We show that local anesthetics, tricyclic antidepressants, and anticonvulsants bind to a common site, possibly with the aromatic motif in their structure. In addition to this aromatic site, there is another site accommodating the tertiary amine chain in the Na^+ channel. Compared with the aromatic site, however, the amine site undergoes much less gating conformational changes but may serve to expedite local anesthetics and tricyclic antidepressants binding to the channel.

Materials and Methods

Cell Preparation

The coronal slices of the brain were prepared from 7- to 14-day-old Wistar rats. Animals were maintained and handled under the supervision of National Taiwan University College of Medicine and College of Public Health Institutional Animal Care and Use Committee, Taipei, Taiwan. The CA1 region was dissected from the slices and cut into small chunks. After treatment for 5–10 min at 34°C in dissociation medium (82 mM Na_2SO_4, 30 mM K_2SO_4, 3 mM $MgCl_2$, 5 mM HEPES, 0.001% phenol red indicator, and pH = 7.4) containing 0.5 mg/ml trypsin (type XI; Sigma, St. Louis, MO), tissue chunks were moved to dissociation medium containing no trypsin but 1 mg/ml bovine serum albumin (Sigma) and 1 mg/ml trypsin inhibitor (type II-S; Sigma). Each time when cells were needed, two to three chunks were picked and triturated to release single neurons.

Whole Cell Recording

The dissociated neurons were put in a recording chamber containing Tyrode's solution (150 mM NaCl, 4 mM KCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, and pH = 7.4). The whole cell voltage clamp recordings were obtained using pipettes pulled from borosilicate micropipettes (OD 1.55–1.60 mm; Hilgenberg GmbH, Malsfeld, Germany), fire polished, coated with Sylgard (Dow-Corning, Midland, MI), and filled with the standard internal solution containing 75 mM CsCl, 75 mM CsF, 2.5 mM $MgCl_2$, 5 mM HEPES, 5 mM EGTA, and pH adjusted to 7.4 by CsOH. Seal was formed, and the whole cell configuration was obtained in Tyrode's solution. The cell was then lifted from the bottom of the chamber and moved in front of an array of flow pipes (Microcapillary from Hilgenberg GmbH; content 1 μl, length 64 mm) emitting external recording solutions, which were Tyrode's solution with or without different concentrations of drugs. Lidocaine, phenytoin, carbamazepine, and lamotrigine were dissolved in dimethyl sulfoxide, and imipramine was dissolved in water to make 100 mM stock solutions, which were then diluted in Tyrode's solution to attain the desired final concentrations. The final concentration of dimethyl sulfoxide (0.4% or less) was not found to have a significant effect on the Na^+ channel inactivation curve or recovery kinetics of the inhibited Na^+ currents. Currents were recorded at room temperature (~25°C) with an Axoclamp 200A amplifier (MDS Analytical Technologies, Sunnyvale, CA), filtered at 5 kHz with a four-pole Bessel filter, digitized at 20–50-μs intervals, and stored using a Digidata-1200 analog/digital interface along with the pCLAMP software (MDS Analytical Technologies). Residual series resistance was generally smaller than 1 MΩ after partial compensation (typically >80%). Lidocaine, phenytoin, carbamazepine, and imipramine were obtained from Sigma, and lamotrigine was a kind gift from Wellcome Foundation Ltd. (Kent, England).

Molecular Biology and Expression of Na^+ Channels

Side-directed mutagenesis was performed in the Nav1.2 Na^+ channel clone (the rat brain type IIA Na^+ channel clone kindly provided by Alan L. Goldin, M.D., Ph.D., Professor, Department of Microbiology and Molecular Genetics,

University of California, Irvine, California) using the QuikChange mutagenesis system (Agilent Technologies, Santa Clara, CA). The RNA transcripts from the mutation-containing clone were synthesized using the T7 mMESSAGE mMACHINE transcription kit (Applied Biosystems, Foster City, CA). The synthesized complementary RNA was then injected into the oocytes of *Xenopus* and incubated at 18°C for 1–4 days before electrophyiologic recording.

Two-electrode Intracellular Recording

Macroscopic Na^+ currents in the oocytes were recorded by a standard two-microelectrode voltage clamp method. During recording, the injected oocyte was continuously perfused with modified ND-96 solution (96 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 0.3 mM $CaCl_2$, 5 mM *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonic acid, and pH = 7.6). The recordings were performed at room temperature of ~25°C with the electrodes having serial resistance of 0.1–0.4 MΩ. Data were acquired by a two-electrode voltage clamp amplifier (model OC-725C; Warner Instrument, Hamden, CT) with a Digidata-1200 analog/digital interface and the pCLAMP software (digitized at 20–100 μs interval).

Analyses and Statistics

To obtain the inactivation curves, the cell was held at −120 mV and stepped every 15 s to the indicated inactivating pulse for 9 s. The channels that remained available were assessed by a short test pulse to 0 mV for 10 ms immediately after each inactivating pulse. The fraction available is defined as the normalized peak current (normalized to the peak current with an inactivating pulse at −120 mV) and is plotted against the voltage of the inactivating pulse (V) to make the inactivation curves. The inactivation curves are fitted with a Boltzmann function $1/(1 + \exp[\{V - V_{1/2}\}/k])$, where $V_{1/2}$ is the half-inactivated potential in micro Volts and k is the slope factor. Two sets of control data were obtained before and after the experiments to demonstrate the lack of significant voltage drift during this long experimental period. The recovery courses from inhibition by drugs were obtained with the following protocols. A cell was held at −120 mV and then prepulsed to −40 mV for 9 s to ensure maximal (steady state) block of the Na^+ current. The cell was then stepped back to a recovery gap potential at −120 mV for 10 ms to 5 s before being stepped again to a short test pulse at 0 mV for 5 ms to assess the available current. The pulse protocol was repeated for every 15 s. After a long recovery period (~5 s), the peak currents at the test pulse reached a "steady-state" level, which was slightly reduced in drug than in control because of mild inhibition of the resting channels by high concentrations of drugs. The fraction recovered is defined as the normalized peak current at the test pulse (relative to the peak current with the recovery gap at −120 mV) and is plotted against the length of the recovery gap period. Basic results are expressed as mean ± SEM, and the statistical tests were performed using Excel (Microsoft, Redmond, WA) and SigmaPlot software (version 9.0, Systat Software Inc., San Jose, CA). The comparisons were made using paired (when indicated) or unpaired Student *t* tests where appropriate. Two groups of results are considered as significantly different if $P < 0.05$ using *P* value method and two-tailed tests. Curve fittings were performed using pCLAMP software (version 10.2) and SigmaPlot software (version 9.0). A model is considered rejected if the predicted value based on that model is outside the 95% CI of the experimental data.

Results

Lidocaine Exerts Use-dependent Inhibitory Effects on Hippocampal Neuronal Na^+ Currents

Figure 1A shows the chemical structure of the drugs used in this study. We first characterized the actions of local anesthetic lidocaine on the Na^+ channels in the acutely dissociated rat hippocampal CA1 neuron, the same preparation where the effects of the anticonvulsant phenytoin, carbamazepine, and lamotrigine have been documented thoroughly.[10–12] Resembling the cases of anticonvulsants, 30–100 μM lidocaine has only negligible effect on the Na^+ currents elicited from a holding potential of −120 mV, but it has a much stronger inhibitory effect with a more positive holding potential (−70 mV), where more channels tend to occupy the inactivated state (fig. 1B). Figure 1C plots the concentration-dependent effect of lidocaine with different holding potentials. Each set of data can be fit by a one-to-one binding curve, which gives the apparent dissociation constant of lidocaine binding to neuronal Na^+ channels. (One-to-one binding of lidocaine to the inactivated Na^+ channel was also confirmed by the linear relationship between the macroscopic binding rate and the lidocaine concentration, giving a binding rate constant of $\sim 5 \times 10^4\ M^{-1} \cdot s^{-1}$, data not shown.) At a holding potential of −60 mV, lidocaine has a apparent dissociation constant of ~21 μM, whereas the apparent dissociation constant increases to ~2,000 μM when the holding potential is −120 mV (fig. 1C). Because most channels occupy the inactivated and the resting state at −60 and −120 mV, respectively (see the control inactivation curve in fig. 2A), the dissociation constants of lidocaine for the resting and inactivated Na^+ channel (K_R and K_I) would be approximately 2,000 and 21 μM, respectively. We also examined the dissociation constant of lidocaine by the shift of the inactivation curve.[7,29] The inactivation curve can be approximated by a Boltzmann function, $1/(1 + \exp[\{V - V_{1/2}\}/k])$ (fig. 2A), where V is the membrane potential, $V_{1/2}$ is the half-inactivated potential, and k is a slope factor. When lidocaine is added, the shape (k) of the curve remains the same, but the curve is shifted leftward. Lidocaine shows concentration-dependent shift (ΔV) of the inactivation curve, consistent with that lidocaine binds more favorably to the inactivated state than to the resting state (figs. 2A and B). The ΔV value could be defined by the following equation:

$$exp(\Delta V/k) = (1 + (D/K_I))/(1 + (D/K_R)) \qquad (1)$$

where D is a lidocaine concentration,[7,29] which gives a K_I value of 23 ± 2.3 μM when K_R is set at 2000 μM (fig. 2C).

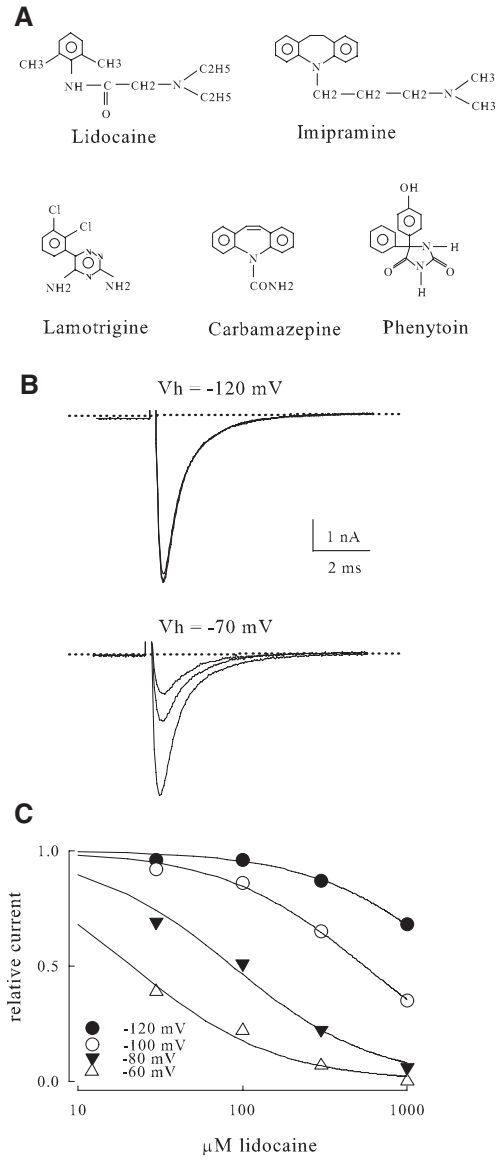

Fig. 1. (*A*) Chemical structure of the drugs. Lidocaine contains a phenyl group and a tertiary amine chain, whereas imipramine contains a diphenyl motif and a tertiary amine chain. The other three drugs contain a diphenyl structural motif but not the amine chain. (*B*) Inhibition of Na^+ currents by 30–100 μM lidocaine. The cell was held at −120 or −70 mV and stepped to 0 mV for 10 ms every 2 s. The inhibitory effect is dramatically different with different holding potentials. The *dotted lines* indicate zero current level. (*C*) Dose-response curves for inhibition of Na^+ currents by lidocaine at different holding potentials (−60 to −120 mV). The test pulse protocol is the same as that described in *B*. The peak current in drug is normalized to the peak current in control to give the relative current. The SE of each data point (n = 5, 6, 3, and 3 for 30, 100, 300, and 1000 μM, respectively) is in general smaller than 5% of the mean value and is omitted for simplicity. The lines are the best fits to each set of the mean values with the form: relative current = 1/(1 + [lidocaine]/*Kapp*), where lidocaine is the lidocaine concentration and the apparent dissociation constant (*Kapp*) values (in micromolar) obtained from the best fit are 2096, 550, 86.5, and 21.3 at holding potentials of −120, −100, −80, and −60 mV, respectively.

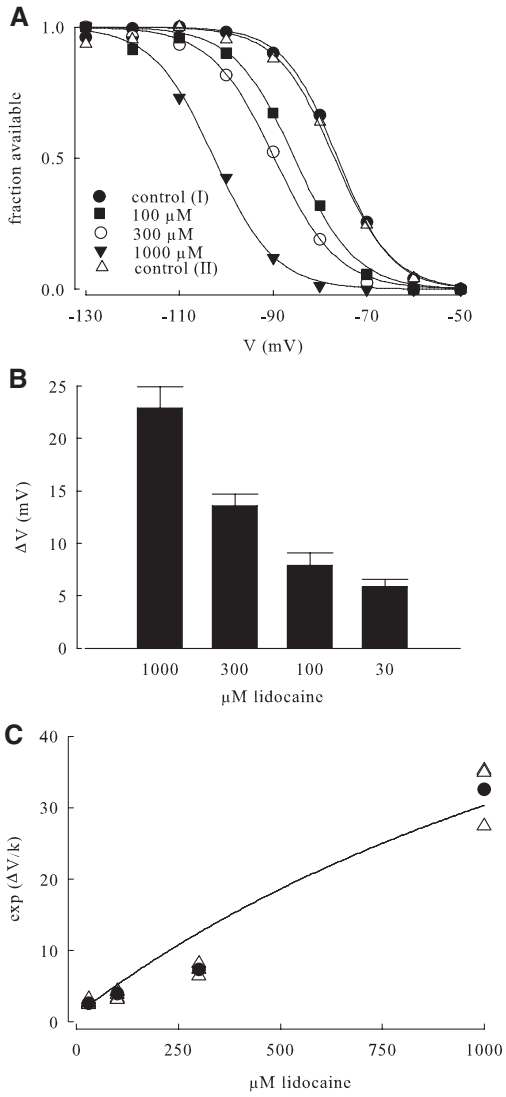

Fig. 2. Shift of the inactivation curve by lidocaine. (*A*) The inactivation curves in the presence or absence of lidocaine. See Materials and Methods for the pulse protocol and the definition of the fraction of the available channel. The *lines* are the fits with a Boltzmann function $1/(1 + \exp[\{V - V_{1/2}\}/k])$, with $V_{1/2}$ values ($V_{1/2}$ is the half-inactivated potential in mV) of −76.2, −76.9, −85.4, −89.7, and −102.6, and k values of 5.9, 6.2, 6.5, 6.8, and 6.5 for control I (before drugs), control II (after drugs), 100, 300, and 1000 μM lidocaine, respectively. (*B*) Because lidocaine shifts the inactivation curve without significantly altering the slope of the curve (*A*), we fix the slope factor k at 6.7 (the average value of k from 18 determinations in different cells and different drug concentrations in the fitting processes is 6.7 ± 0.3) to determine ΔV (the difference between $V_{1/2}$ in control and that in drug). n = 5, 6, 3, and 3 for 30, 100, 300, and 1000 μM lidocaine, respectively. (*C*) The averaged (*closed circles*) and individual (*open triangles*) values of exp(ΔV/k) calculated from all 17 determinations in different drug concentrations (*B*) are plotted against lidocaine concentration. The *line* is the best fit to the averaged data with the form: exp(ΔV/k) = (1 + [D/23.9])/(1 + [D/2000]), where D denotes lidocaine concentration in micromolar and K_R (the dissociation constant of lidocaine for the resting Na^+ channel) is set at 2,000 μM (according to equation 1 and Fig. 1C).

This is very much consistent with the K_I of 21 μM estimated from a different approach in figure 1C.

Different Point Mutations of W1716 and/or F1764 Reveal Interactive Influences of These Two Residues on Lidocaine Binding to the Inactivated Na^+ Channel

The findings of lidocaine (figs. 1 and 2) are very similar to those reported previously for anticonvulsants in the same preparation (*e.g.*, The K_I values are ~25 μM for carbamazepine and ~9 μM for both phenytoin and lamotrigine and are all much smaller than K_R values[10–12]), demonstrating very similar use-dependent inhibitory effects of these drugs on hippocampal neuronal Na^+ channels. We then studied whether these very similar effects are ascribed to an overlapped binding site for these two groups of drugs. W1716 (presumably located in the external pore loop) and F1764 (presumably located in the internal pore-lining part of S6) are two residues reported to be involved in the binding of local anesthetics and/or anticonvulsants.[17–18,20,26] Thus, we compared the possible change in local anesthetic binding affinity by mutation of W1716 and/or F1764 (fig. 3). We chose to mutate the aromatic residues to alanine and cysteine (both with small side chains) to minimize the chance of inadvertent introduction of new steric properties into the mutant site with the elimination of the aromatic side chain. We used 100 μM lidocaine, which is ~4-fold of K_I (fig. 2), to examine the shift of the inactivation curve. Mutation of W1716 or F1764 into either alanine or cysteine evidently decreases the relative affinity (the shift of the inactivation curve) of lidocaine to the inactivated Na^+ channel (fig. 3A). However, double mutation of W1716 and F1764 does not significantly decrease the affinity furthermore in most cases (fig. 3A), implying a significant interaction between the two aromatic residues. Thus, we explored the possible interaction energy (the coupling energy) between a W1716 mutation and a F1764 mutation by making corresponding double mutations. Based on the double-mutant cycle analysis developed by Carter *et al.*,[30] the energetic coupling of two mutations could be characterized by an interaction energy ($\Delta\Delta G_{inter}$), which is equal to the free energy changes in the double mutants minus the summation of the free energy changes in each component single mutant. The calculated interaction energy $\Delta\Delta G_{inter}$ between a W1716 mutant and a F1764 mutant are quite consistently at ~3 kcal/mol in all cases (fig. 3). The results reveal that all the double mutations (W1716A/F1764A, W1716A/F1764C, W1716C/F1764A, and W1716C/F1764C) cannot have additive effects in terms of drug affinity changes (figs. 3A and B). We also found similar nonadditive effects and significant interaction energies between these two residues if probed by carbamazepine.[31] Moreover, the susceptibility of W1716C to Cd^{2+} and methanethiosulfonate reagents is significantly decreased by concomitant mutation F1764C. Also, W1716E plus F1764R double mutation significantly alters the ion selectivity of the pore. These findings suggest that F1764 and W1716 are located in proximity and interact to make a binding site for both anticonvulsants and local anesthetics. It is plausible that these two aromatic residues work together to constitute an appropriate conformation for the binding of the

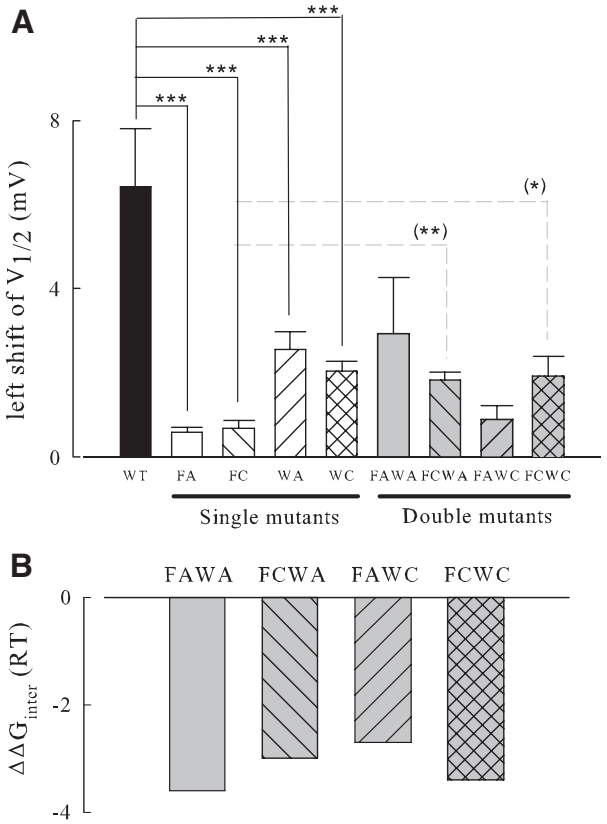

Fig. 3. Decrease of binding affinity of lidocaine to the inactivated Na^+ channel by W1716 and/or F1764 mutations. (*A*) The comparison of $V_{1/2}$ shift by 100 μM lidocaine (n = 3–5) in the wild-type and single and double (*i.e.*, F1764A [FA], F1764C [FC], W1716A [WA], W1716C [WC], F1764A + W1716A [FAWA], F1764A + W1716C [FAWC], F1764C + W1716A [FCWA], and F1764C + W1716C [FCWC]) mutant channels (n = 5, 4, 5, 3, 5, 3, 4, 3, and 3, respectively). The data are fits with a Boltzmann function $1/(1 + \exp[\{V - V_{1/2}\}/k])$, and the average values of $V_{1/2}$ shift are plotted. $V_{1/2}$ is the half-inactivated potential in microvolts. The $V_{1/2}$ shift of each of the four single mutant channel is significantly different from that of the wild-type channel (*** $P < 0.005$). Interestingly, the $V_{1/2}$ shift of the double mutant channel is not significantly different from each of its component single mutants in most cases ($P = 0.09$, 0.77, 0.12, 0.34, 0.08, and 0.89 for the cases of FAWA *vs.* FA, FAWA *vs.* WA, FCWA *vs.* WA, FAWC *vs.* FA, FAWC *vs.* WC, and FCWC *vs.* WC, respectively), except for the cases of FCWA *versus* FC and FCWC *versus* FC (** $P = 0.006$ and * $P = 0.03$, respectively. However, it should be noted that FCWA is still not significantly different from WA, and FCWC is not significantly different from WC). (*B*) The comparison of the interaction energies ($\Delta\Delta G_{inter}$) probed by 100 μM lidocaine in different mutant channels. The free energy change ($\Delta\Delta G$) is directly derived from the product of the averaged value of $V_{1/2}$ shift (*A*) and the apparent equivalent gating charges (derived from RT/Fk or 25 mV/k). The $\Delta\Delta G_{inter}$ are −3.6, −3.0, −2.7, and −3.4 RT for FAWA, FCWA, FAWC, and FCWC mutant channels, respectively. All the $\Delta\Delta G_{inter}$ values are significantly smaller than 0, strongly suggesting the nonadditive nature for W1716 and F1764 mutations to alter the binding affinity of lidocaine.

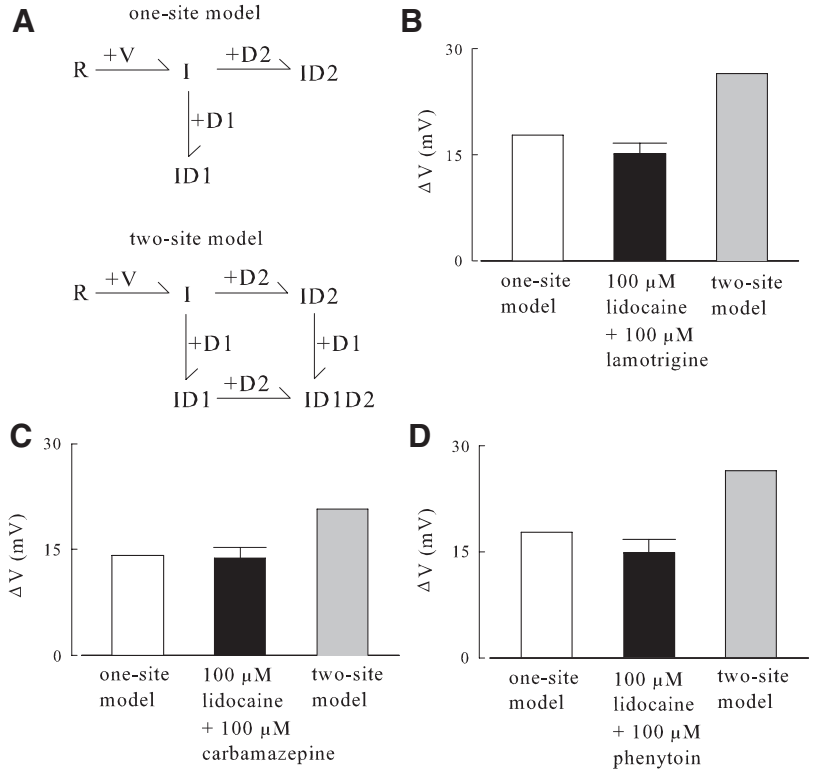

Fig. 4. Shift of the inactivation curve by lidocaine plus an anticonvulsant. (*A*) Simplified gating schemes illustrating the possible actions of lidocaine and a coexisting anticonvulsant (phenytoin, carbamazepine, or lamotrigine). R and I represent the resting and inactivated states of the Na^+ channel, respectively. During depolarization (+V), most channels are moved to the inactivated state. D1 and D2 represent two different drugs, both of which selectively bind to the inactivated state rather than the resting state. State RD, the drug-bound resting states, is, thus, neglected for simplicity. If D1 and D2 share the same binding site in the inactivated channel (*top*, the "one-site" model), then the channel can be bound by either D1 or D2 but not both. If D1 and D2 have separate binding sites (*bottom*, the "two-site" model), then the inactivated Na^+ channels may be significantly occupied by both D1 and D2 (*i.e.*, state ID1D2), especially when high concentrations of D1 and D2 are present. (*B–D*) The experimental data on the inactivation curve shift in different drug combinations and the predictions based on different models in *A* (n = 3 for each part). The predicted values of shift by the one-site model are 17.8, 14.2, and 17.8 mV, which always fall into the 95% CI of the experimental data (12.2–18.0, 10.9–16.7, and 10.9–18.7 mV, respectively), for 100 μM lidocaine + 100 μM carbamazepine (*B*), 100 μM lidocaine + 100 μM lamotrigine (*C*), and 100 μM lidocaine + 100 μM phenytoin (*D*), respectively. Conversely, the predicted values of shift based on the two-site model are 26.5, 20.8, and 26.8 mV for *B*, *C*, and *D*, respectively, and are all outside the 95% CI of the experimental data.

aromatic motif in these drugs. W1716 may be the primary binding counterpart for the aromatic motif, whereas F1764 may be very influential in the spatial orientation of W1716 and in the meanwhile responsible for the classic hydrophobic pathway of local anesthetic binding to the Na^+ channel.[31] Together with the gating changes frequently associated with mutations of either of the two residues, it is clear that allosteric effect is an essential confounding factor that should be carefully taken into consideration when probing local anesthetic or anticonvulsant-binding site in the Na^+ channel with mutation studies.

The Shift of Inactivation Curve in Different Drug Mixtures Argues Against Effective Double Occupancy of the Channel by Two Different Drugs

Because there are essential concerns that mutations may cause allosteric effects rather than direct disruption of the drug-binding site, we turned to more detailed and in-depth functional studies in a native preparation (acutely-dissociated rat hippocampal CA1 neurons). According to equation 1, because K_R is generally in the millimolar range and is much larger than K_I for both carbamazepine and lidocaine (fig. 1),[12] item D/K_R should be much smaller than D/K_I. Drop item D/K_R and equation 1 becomes:

$$exp(\Delta V/k) \cong 1 + (D/K_I) \quad (2)$$

If two drugs have the same binding site in the channel, then the binding of one drug to the inactivated channel would preclude the binding of the other (*i.e.*, the one-site model, fig. 4A), and ΔV would be given by the following equation:

$$exp(\Delta V/k) \cong 1 + (D1/K_{I,1}) + (D2/K_{I,2}) \quad (3)$$

where D1 and D2 are the concentrations of the two drugs, and $K_{I,1}$ and $K_{I,2}$ are the K_I of drug 1 and drug 2, respectively. Conversely, if the inactivated channel can be doubly occupied by the drugs (*i.e.*, there are different binding sites for

different drugs; the two-site model, fig. 4A), then there would be a new double-occupancy state ID1D2, and ΔV is thus given by the following equation:

$$exp(\Delta V/k) \cong 1 + (D1/K_{I,1}) + (D2/K_{I,2}) + (D1/K_{I,1}) \times (D2/K_{I,2}) \quad (4)$$

The shift of inactivation curve predicted by equation 3 would be different from that by equation 4 especially in the presence of high concentration of drugs. Therefore, we documented the shift of inactivation curve (ΔV) in the presence of lidocaine plus an anticonvulsant (both in 100 μM, figs. 4B–D). Consistent with the previous data,[10–12] we found that the K_I values of the anticonvulsants are ~25 μM for carbamazepine and ~9 μM for both phenytoin and lamotrigine in this preparation (estimated from the shift of inactivation curve, data not shown). We used a relatively high drug concentration (*i.e.*, 100 μM, ~4- to 10-fold of K_I) to assure that no possible doubly occupancy of the channel by two different drugs would be overlooked. (We have also repeated the experiments with lower and more "physiologic" concentrations of the drugs, for example, 10 μM lidocaine plus 10 μM carbamazepine, and reached the same conclusion to make sure that no inadvertent artifact happened with the simultaneous existence of high concentrations of multiple drugs, data not shown.) With the K_I values for each drug and a slope factor k of 6.7 (fig. 2), the predicted ΔV value in the coexistence of 100 μM lidocaine and 100 μM carbamazepine would be 17.8 mV by the one-site model (equation 3) and 26.5 mV by the two-site model (equation 4). The experimental data (15.1 ± 1.5 mV, middle column of fig. 4B) are consistent with the former rather than the latter. The combination of 100 μM lidocaine and 100 μM lamotrigine (fig. 4C) and the combination of 100 μM lidocaine and 100 μM phenytoin (fig. 4D) also shift the inactivation curve by a ΔV value consistent with the prediction of the one-site model than the two-site model. Quantitative analyses of the inactivation curve shifts thus argue against significant existence of a doubly occupied state (ID1D2) by two different drugs.

The Recovery Kinetics of the Na^+ Current Inhibited by a Combination of Lidocaine and an Anticonvulsant Are Slower Than the Prediction by the One-site Model

The double occupancy of the inactivated channel by drugs can also be examined from a kinetic point of view. As a first approximation, we assume in figure 4A that the dissociation rate of each drug (D1 and D2) from the doubly occupied state (ID1D2) is the same as that from the singly occupied state (ID1 and ID2) and that the inactivated channel cannot recover to the resting state (R) unless all the bound drug molecules have dissociated from the channel. Also, from our previous work on the recovery of inactivated Na^+ channel in the absence or presence of an anticonvulsant,[10,12] the rate limiting step of recovery of the drug-bound inactivated channel is drug dissociation rather than conversion of the inactivated to the resting forms of the channel (*i.e.*, the rate from ID1 or ID2 to I is much slower than that from I to R). Thus, if the rates from ID1 to I and ID2 to I are k1 and k2, respectively, then simplistically the mean time for ID1 and ID2 (the single drug-bound inactivated channel) to recover to the resting channel should roughly be 1/k1 and 1/k2, respectively (disregarding the forward reactions I to ID1 or ID2, and the time for the reaction I to R). In this regard, the recovery time for ID1D2 would roughly be 1/k1 + 1/k2, no matter if it recovers *via* ID1 or ID2. The recovery of ID1D2, thus, would necessarily be longer than that of ID1 or ID2, as if it is one step farther away from the R state. Because the amount of the double occupancy state (ID1D2) would be very different with the one-site and the two-site models, especially in high concentrations of drugs (D1 and D2), one may have a rough estimate of the extent of double occupancy *via* the comparison between the experimental and the predicted (based on the one-site or the two-site model) macroscopic recovery courses. Figures 5A and B show the recovery courses of inactivated Na^+ channels in the presence of two different anticonvulsants (carbamazepine plus lamotrigine) and lidocaine plus an anticonvulsant (carbamazepine). A 100 μM concentration (*i.e.*, ~4- to 10-fold of K_I) of these drugs was used to assure significant (nearly saturating) binding of the drugs to the inactivated channel. In either case (fig. 5A or B), the recovery course in the presence of both drugs lies in between the recovery courses in the presence of each single drug. The results are consistent with competition for a common binding site by two different drugs. These findings strongly indicate that the faster-recovery drug in the mixture actually has an antagonistic rather than a synergistic action on the slower-recovery drug in terms of the recovery kinetics, again arguing against effective double occupancy of the channel in both combinations of drugs. Nevertheless, the mutual antagonizing effect between lidocaine and an anticonvulsant (fig. 5B) looks weaker than that between two anticonvulsants (fig. 5A). To examine this subtle difference in more detail, we construct theoretical recovery courses based on the one-site and the two-site models (fig. 5C). According to the one-site model, the proportion of the carbamazepine-bound inactivated channel at steady state in the presence of 100 μM carbamazepine and 100 μM lamotrigine is 4/(1 + 4 + 11.1) or 4/16.1 (D/K_I for carbamazepine = 100/25 = 4; D/K_I for lamotrigine = 100/9 = 11.1), and the proportions of the lamotrigine-bound and the drug-free inactivated channels are 11.1/16.1 and 1/16.1, respectively. Conversely, according to the two-site model, the proportions of each states should be given by (the chance of a drug-free carbamazepine site or the chance of an occupied carbamazepine site) × (the chance of a drug-free lamotrigine site or the chance of an occupied lamotrigine site) or (1/[1 + 4] or 4/[1 + 4]) × (1/[1 + 11.1] or 11.1/[1 + 11.1]), which yields 1/60.5, 4/60.5, 11.1/60.5, and 44.4/60.5 for the drug-free, the carbamazepine-bound, the lamotrigine-bound, and carbamazepine and lamotrigine doubly bound inactivated channels, respectively. The recovery courses predicted by the one-site

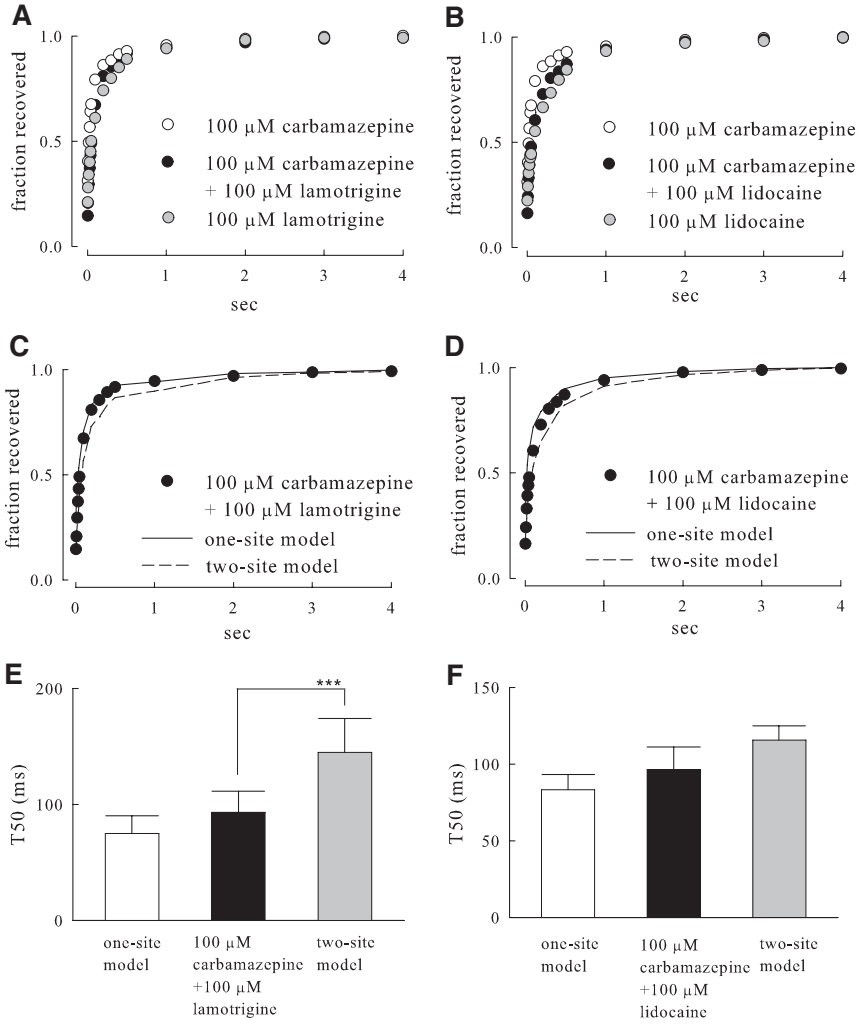

Fig. 5. Recovery courses from inhibition by carbamazepine, lamotrigine, and lidocaine. (*A*) Recovery courses in the continuous presence of 100 μM carbamazepine or 100 μM lamotrigine, or both. See Materials and Methods for the pulse protocol and the definition of the fraction of the recovered channel. (*B*) The recovery time courses in 100 μM carbamazepine and/or 100 μM lidocaine. (*C*) The predicted recovery courses in 100 μM carbamazepine plus 100 μM lamotrigine are calculated according to the one-site model and the two-site model (see Results, equations 5 and 6). The experimental data (*black circles*, *A*) coincide well with the course predicted by the one-site model (*solid line*) rather than the two-site model (*dashed line*). (*D*) The experimental and simulated recovery courses in 100 μM carbamazepine plus 100 μM lidocaine are compared. Given a K_I value (the dissociation constant between a drug and the inactivated Na^+ channel) of 24 μM for lidocaine, the recovery course predicted by the one-site model (*solid line*) would be $1/9.2 + (4/9.2) \times CBZ + (4.2/9.2) \times LID$, and the recovery course predicted by the two-site model (*dashed line*) would be $1/25.9 + (4/25.9) \times C + (4.2/25.9) \times LID + (16.7/25.9) \times CBZ \times LID$, where CBZ and LID denote the recovery courses in the same cell when only 100 μM carbamazepine or only 100 μM lidocaine is present, respectively (*B*). The experimental data (*black circles*, *B*) are close to but not as well fitted with the prediction of the one-site model as the case in *C*. (*E*) The half-recovery time (T50) is defined as the length of the recovery gap potential to recover half of the Na^+ current. The T50 values for 100 μM carbamazepine + 100 μM lamotrigine are 74.8 ± 15.4, 93.2 ± 18.2, and 144.6 ± 29.3 ms (all n = 5) for the calculated result based on the one-site model, the experimental data, and the calculated result based on the two-site model courses, respectively. The model prediction of T50 is also expressed as mean ± SEM, because it is derived from the average of the estimate for each single cell, which is in turn derived from the kinetics of each single drug in each particular cell as described earlier. *** $P < 0.005$ (comparison between the experimental data and the one-site model prediction) by paired Student *t* test. $P = 0.06$ for the comparison between the experimental data and the two-site model prediction. (*F*) The T50 values for 100 μM carbamazepine plus 100 μM lidocaine are 83.5 ± 9.7, 96.5 ± 14.8, and 115.8 ± 9.2 ms (all n = 4) for the calculated result based on the one-site model, experimental data, and the calculated result based on the two-site model courses, respectively. $P = 0.1$ by paired Student *t* test for the comparison between the experimental data and the predictions of the one-site and the two-site model predictions, respectively.

and the two-site models would then be simplistically given by the following equations 5 and 6, respectively.

$$recovery\ course = 1/16.1 + (4/16.1) \times C + (11.1/16.1) \times Lm \quad (5)$$

$$recovery\ course = 1/60.5 + (4/60.5) \times C + (11.1/60.5) \times Lm + (44.1/60.5) \times C \times Lm \quad (6)$$

where C and Lm denote the recovery courses when only 100 μM carbamazepine or 100 μM lamotrigine is present, respectively. Similar approaches were repeated for 100 μM carbamazepine plus 100 μM lidocaine (fig. 5D). It is evident that the experimental data exactly coincide with the predicted course by the one-site model rather than the two-site model for carbamazepine plus lamotrigine (fig. 5C). The exact coincidence may be viewed as a strong support for the validity of the foregoing simplistic approach for the prediction of the recovery course. In this regard, it is interesting to note that the experimental data are subtly deviated from, although still stay closer to, the one-site model prediction for carbamazepine plus lidocaine (fig. 5D). Because the recovery courses cannot be exactly fitted by a monoexponential function, we first try to compare the recovery speed in more detail with the time of 50% recovery in each course (T50, figs. 5E and F). It is evident that the T50 is consistent with the prediction of the one-site rather than the two-site model in the presence of two anticonvulsants (the experimental data are significantly different from the two-site, rather than the one-site, model prediction, fig. 5E), but it is always longer than the prediction of the one-site model (although still much shorter than the prediction of the two-site model) in the presence of lidocaine and an anticonvulsant (the experimental data are not significantly different from the prediction of either model, fig. 5F). It seems that although the fast-recovery anticonvulsant can still partially "antagonize" the slow-recovery lidocaine, detailed quantitative analyses do not support absolute mutual exclusion of lidocaine and anticonvulsant binding to the Na^+ channel.

The Recovery Kinetics Gets More Deviated from the Prediction by the One-site Model in Higher Concentrations of Lidocaine

To confirm the deviation from the prediction by the one-site model in the presence of lidocaine (fig. 5), we performed another quantitative approach of the recovery kinetics by forced fit of the recovery courses like those in figure 5 with monoexponential functions. The monoexponential functions may not perfectly fit those experimental data points but may suffice to give another rough estimate of the apparent recovery speed. According to the one-site model, the recovery speed in the coexistence of any two drugs should be a weighted average of the recovery speed in each component drug (weighted according to the relative occupancy of the channel by each drug). For example, the recovery course in 100 μM carbamazepine plus 100 μM lamotrigine should have a predicted recovery speed as: $(4 \times [CBZ] + 11.1 \times [LMT])/15.1$, where 4/15.1 and 11.1/15.1 are the relative occupancy by carbamazepine and lamotrigine (given K_I of 25 and 9 μM, respectively), and CBZ and LMT are the recovery speeds (inverses of the time constants obtained from the monoexponential fits to the recovery courses) in the presence of 100 μM carbamazepine and 100 μM lamotrigine, respectively. The experimental data are then divided by the predicted speed to obtain the percentage of the one-site model prediction (% predicted speed). Figure 6A shows that the recovery speeds in the drug mixtures that contain lidocaine and an anticonvulsant are less "accurately" predicted by the one-site model (as slow as 72% of the predicted speed) than the cases in the drug mixtures containing only different anticonvulsants. These findings support a finite possibility of double occupancy of the Na^+ channel by lidocaine and anticonvulsants. If this is true, then the deviation from the prediction of the one-site model should be even more manifest in the presence of higher concentration of drugs. Therefore, we examined the recovery courses with higher concentration (fig. 6B). In this case, the comparison between lamotrigine and lidocaine may be especially informative as these two drugs have similar recovery speeds. It is clear that the recovery speed in 200 μM carbamazepine plus 200 μM lamotrigine is still well predicted by the one-site model. Conversely, the mean deviation from the prediction by the one-site model is more manifest in 200 μM carbamazepine plus 200 μM lidocaine or 200 μM lamotrigine plus 200 μM lidocaine compared with the cases in mixtures of 100 μM drugs.

The Effect of Imipramine Plus an Anticonvulsant Is Analogous to That of Lidocaine Plus an Anticonvulsant

We have demonstrated that double occupancy of the Na^+ channel by an anticonvulsant and a local anesthetic is discernible when examined by the recovery kinetics but not by the shift of the steady-state inactivation curve. Because the anticonvulsants contain a diphenyl structure but no amine side chain, whereas lidocaine contains a phenyl group and an amine chain (fig. 1A), the aforementioned findings would suggest that the phenyl group-binding region (*i.e.*, in the common anticonvulsant-binding site)[14–16] but not the tertiary amine chain-binding region most likely undergoes significant conformational changes during the gating processes of the channel. To characterize the functional role of the tertiary amine chain further, we examined the inactivation shift and recovery kinetics in the existence of carbamazepine and imipramine (both having the dibenzazepine tricyclic structure but only the latter contains a tertiary amine chain, see fig. 1A). Consistent with the previous data,[14] we found that the K_I value of imipramine is approximately 1.3 μM. According to the gating scheme shown in figure 4A and the equations elaborated from the scheme, the magnitude of inactivation curve shift is again well predicted by the one-site rather than the two-site model, suggesting an overlapped binding region for imipramine and carbamazepine (figs. 7A and B). Figure 7C furthermore confirms that imipramine could not simultaneously occupy the inactivated channel with a local anesthetic or

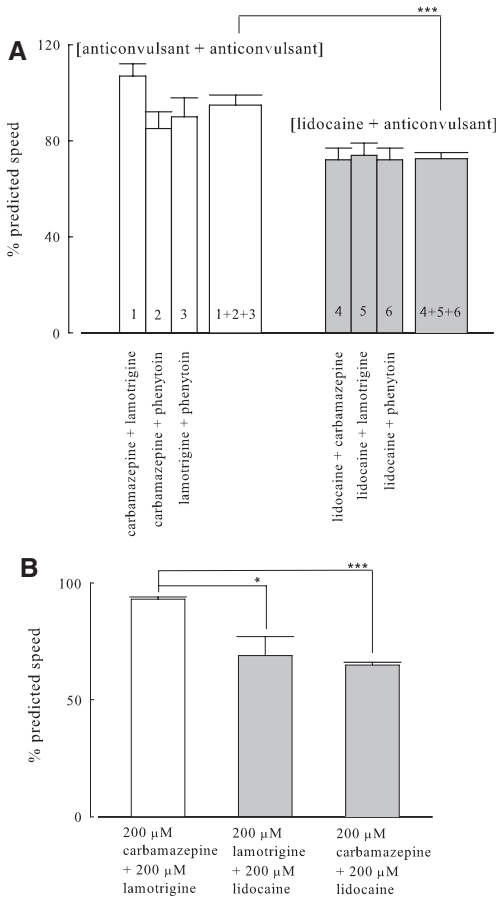

Fig. 6. Slower recovery than the prediction of the one-site model when lidocaine is present in the drug mixture. (*A*) In drug mixtures containing 100 μM drug, it is evident that the actual (experimental) recovery speed is well predicted by the one-site model when there are two anticonvulsants but no lidocaine in the mixture (*thin white bars*; 107 ± 5%, 90 ± 8%, and 85 ± 7% of prediction, and n = 7, 6, and 5 for carbamazepine plus lamotrigine, lamotrigine plus phenytoin, and phenytoin plus carbamazepine, respectively). For the derivation of predicted recovery speed, see Results. In contrast, the actual recovery speed is always slower than the one-site model prediction when lidocaine is present (*thin gray bars*; 72 ± 5%, 74 ± 5%, and 72 ± 5% of prediction, and n = 5, 5, and 4, for carbamazepine plus lidocaine, lamotrigine plus lidocaine, and phenytoin plus lidocaine, respectively). All data from the experiments where only the anticonvulsants are present are together to give an average "% predicted speed" of 94.8 ± 4.2% (n = 18, *thick white bar*), whereas pooled data from the experiments with lidocaine in the drug mixture give an evidently smaller "% predicted speed" (72.6 ± 2.6%, n = 14, *thick gray bar*). *** $P < 0.005$ by Student *t* test (compared between the pooled data of the two-anticonvulsants experiments and the anticonvulsant + lidocaine experiments). (*B*) Larger deviation from the one-site model prediction with higher concentration of lidocaine in the drug mixtures. The percentage of the predicted speeds are 93 ± 1%, 69 ± 8%, and 65 ± 1% for 200 μM carbamazepine plus 200 μM lamotrigine, 200 μM lamotrigine plus 200 μM lidocaine, and 200 μM caramazepine plus 200 μM lidocaine, respectively. n = 3 for each column. * $P < 0.05$ and *** $P < 0.005$ by Student *t* test (when compared with the data of the two-anticonvulsants, that is, carbamazepine + lamotrigine, experiments).

another anticonvulsant (*i.e.*, phenytoin). For simplicity, we assume that there is just one specific binding site in the channel for each drug. In this regard, the predicted inactivation curve shift (ΔV) by the two-site model would be given by the following equation (assuming D1 and D2 bind to a common site and D3 binds to the other site, so that double occupancy of the channel by D1 and D3 or by D2 and D3 is possible, but not by D1 and D2):

$$exp(\Delta V/k) \cong 1 + (D1/K_{I,1}) + (D2/K_{I,2}) + (D3/K_{I,3}) + (D1/K_{I,1}) + (D3/K_{I,3}) + (D2/K_{I,2}) \times (D3/K_{I,3}) \quad (7)$$

If all three different drugs have different binding sites (the three-site model), then in the simultaneous presence of three different drugs, ΔV would be predicted by the following equation:

$$exp(\Delta V/k) \cong 1 + (D1/K_{I,1}) + (D2/K_{I,2}) + (D3/K_{I,3}) + (D1/K_{I,1}) + (D2/K_{I,2}) + (D1/K_{I,1}) \times (D3/K_{I,3}) + (D3/K_{I,3}) \times (D2/K_{I,2}) + (D1/K_{I,1}) \times (D2/K_{I,2}) \times (D3/K_{I,3}) \quad (8)$$

In comparison, if there is a common binding site for all the three drugs (the one-site model), then in the simultaneous presence of three different drugs, ΔV would be predicted by the following equation:

$$exp(\Delta V/k) \cong 1 + (D1/K_{I,1}) + (D2/K_{I,2}) + (D3/K_{I,3}) \quad (9)$$

One may readily see that the deviation (ΔV) would be the largest with the three-site model and the smallest with the one-site model. The experimental data are consistent with the prediction of the one-site model (fig. 7C). We then examined the possible double occupancy of the inactivated channel by imipramine and carbamazepine from a kinetic point of view (figs. 7D and E). Consistently, the recovery in the simultaneous existence of both drugs is faster than that in just the slower-recovery drug (*i.e.*, imipramine), strongly suggesting that carbamazepine (the faster-recovery drug) competes and, thus, antagonizes the binding of imipramine (fig. 7D). The recovery course, however, does not exactly coincide with the prediction of the one-site model (fig. 7E). This is very much analogous to the case of lidocaine plus carbamazepine and once more substantiates the existence of a tertiary amine chain binding region external to binding region for the phenyl group. We again documented the time of 50% recovery (T50, fig. 7F). Probably, because of the dramatically different recovery rates with imipramine and with carbamazepine, the recovery course in the presence of both drugs seems to be more deviated from the two-site model in the late phase than in the earlier phase (fig. 7E). Thus, T50 could only be viewed as a rough index. Nevertheless, the data here are clearly more analogous to the case of lidocaine plus carbamazepine than to that of two anticonvulsants (figs. 5E and F).

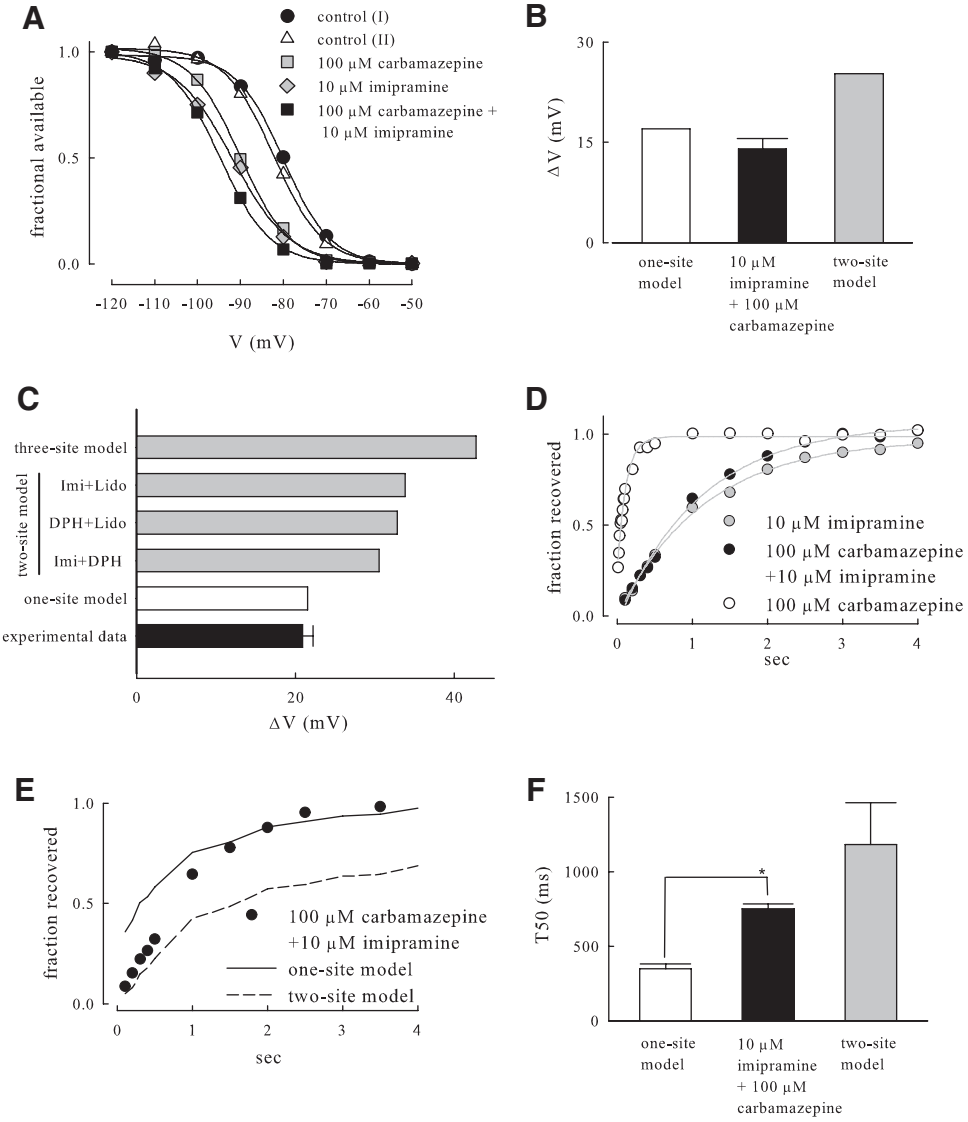

Fig. 7. Shift of the inactivation curve and recovery courses from inhibition by imipramine plus an anticonvulsant and/or lidocaine. (*A*) Shift of the inactivation curve by 10 μM imipramine, 100 μM carbamazepine, or 10 μM imipramine plus 100 μM carbamazepine is compared in one cell. The *lines* are fits with a Boltzmann function $1/(1 + \exp[\{V - V_{1/2}\}/k])$, with $V_{1/2}$ values ($V_{1/2}$ is the half-inactivated potential in mV) of −80.0, −82.2, −91.8, −90.1, and −94.8, and k values of 5.4, 5.7, 6.7, 5.8, and 5.9 for control I (before drugs), control II (after drugs), 10 μM imipramine, 100 μM carbamazepine, and 10 μM imipramine plus 100 μM carbamazepine, respectively. (*B*) The experimental data (n = 4) and predictions of the inactivation curve shift (shown in mV in the y-axis) by 10 μM imipramine plus 100 μM carbamazepine based on the models in Figure 4A. The shift of the inactivation (ΔV) predicted by the one-site model is 17.0 mV, which falls within the 95% CI of the experimental data (10.9–17.1 mV). The shift predicted by the two-site model is 25.3 mV and is clearly outside the 95% CI of the experimental data. (*C*) The shift of the inactivation curve (ΔV, shown in microvolts in the x-axis) in the presence of 10 μM imipramine (Imi), 100 μM lidocaine (Lido), and 100 μM phenytoin (DPH). The experiments and plots are analogous to those described in Figure 4 according to a gating scheme elaborated from Figure 4A. Imi + Lido, DPH + Lido, and Imi + DPH indicate the two drugs that are presumed to bind to a common site in the simultaneous presence of three different drugs when the two-site model is taken into consideration. The experimental data (95% CI, 18.4–23.5 mV; n = 3) are consistent with the prediction of' the one-site (21.5 mV) rather than the two-site (33.8, 32.8, and 30.5 mV for Imi + Lido, DPH + Lido, and Imi + DPH, respectively) or the three-site (42.7 mV) models (see text for details). (*D*) The recovery time courses in the presence of 10 μM imipramine and/or 100 μM carbamazepine. (*E*) The predicted recovery courses in 100 μM carbamazepine plus 10 μM imipramine are calculated according to the one-site model and the two-site model (the same approaches as those in Fig. 5D). According to the one-site model, the predicted recovery course would be 1/12.7 + (4/12.7) × CBZ+ (7.7/12.7) × IMI, where CBZ and IMI denote the recovery courses in the same cell when only carbamazepine or imipramine is present, respectively (*D*). Conversely, according to the two-site model, the predicted recovery courses would be 1/43.5 + (4/43.5) × CBZ + (7.7/43.5) × IMI + (30.8/43.5) × CBZ × IMI. (*F*) The T50 values are 367 ± 44, 750 ± 33, and 1183 ± 278 ms (all n = 3) for the one-site model, experimental (100 μM carbamazepine + 10 μM imipramine), and two-site model courses, respectively. * $P < 0.05$ by paired Student *t* test (comparison between the experimental data and the model predictions).

The Binding Kinetics of Carbamazepine but Not Imipramine Is Much Faster in the Inactivated Than in the Resting States

To characterize the functional role of the tertiary amine chain further, we compare the binding kinetics of carbamazepine and imipramine at different gating states. Figures 8A and B show that carbamazepine but not imipramine has evident gating state-dependent binding kinetics onto the channel. This finding supports the idea that significant gating changes occurs only in the phenyl group but not in the tertiary amine chain binding region. Conversely, the binding of the tertiary amine chain is relatively independent of the gating states of the channel and may, therefore, help "docking" of the drug onto the Na^+ channel irrespective of the membrane potential.

Discussion

Lidocaine and Imipramine Bind to the Common Anticonvulsant Binding Site to Facilitate Na^+ Channel Inactivation

F1764 and W1716 are two residues presumably located externally and internally in the $Na_v1.2$ Na^+ channel pore, respectively, and are foci of previously reported binding ligands for local anesthetics and anticonvulsants by mutational studies. In this study, we showed that the mutations in F1764 and W1716 are significantly coupled energetically in terms of local anesthetic binding, making it difficult to have a straightforward interpretation of the mutation data. Therefore, we focused on functional studies and quantitative analyses in native neurons and showed that the effects of lidocaine on neuronal Na^+ currents are exactly analogous to those of the anticonvulsant (carbamazepine, phenytoin, and lamotrigine) or tricyclic antidepressant (imipramine) previously characterized in the same preparation.[10–12,14] Most importantly, the shift of inactivation curve by drug mixtures suggests that the binding of lidocaine, anticonvulsants (carbamazepine, lamotrigine, phenytoin), and imipramine to the inactivated Na^+ channel essentially precludes each other. These findings strongly argue that lidocaine and imipramine should also bind to the common anticonvulsant-binding site because it would be extremely unlikely that two drugs favoring the same channel conformations would so much precludes the binding of each other with an allosteric mechanism. We have demonstrated that the structural determinant for binding of the anticonvulsants to their common binding site in the Na^+ channel is a diphenyl motif.[14–16] Because the binding counterparts of the phenyl groups are usually also phenyl groups, and also because there is no amine chain in the anticonvulsants, lidocaine and imipramine most likely bind to the common anticonvulsant-binding site with the phenyl group rather than the tertiary amine side chain (fig. 9). This proposal is reminiscent of the finding that only phenol (a compound mimicking the phenyl group of lidocaine) but not diethylamide (a compound mimicking the tertiary amine end of lidocaine) could produce long blocking event in batrachotoxin-activated Na^+ channels.[32]

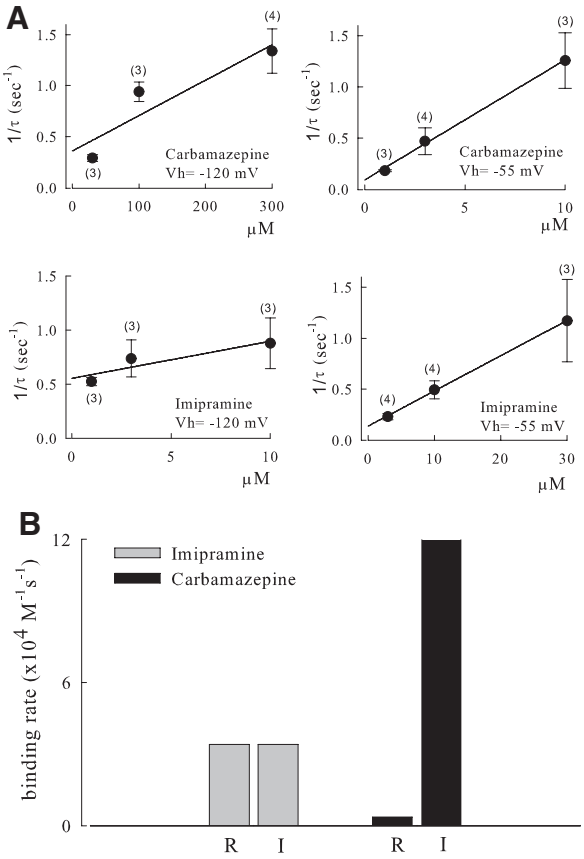

Fig. 8. Comparison of the state-dependent binding rates of imipramine and carbamazepine. (*A*) The macroscopic binding rates of imipramine or carbamazepine are examined at holding potentials (Vh) of −120 and −55 mV, where most Na^+ channels stay at the resting and the inactivated states, respectively. The neuron was stepped to 0 mV for 17 ms after the designated holding potential every 1 or 0.5 s to continuously elicit Na^+ currents and quickly moved between the control (Tyrode's solution) and the drug (carbamazepine or imipramine)-containing solutions. When a neuron was exposed to carbamazepine or imipramine, the elicited current was reduced in a time-dependent manner. We plot the time-dependent reduction of the current amplitude and fit the time courses with a monoexponential function. Only raw data that are readily reversible are collected. The number of measurement is given beside each data point in the figure. The inverse of the time constant form the monoexponential fitting is then plotted against drug concentration. The data are fitted with a regression line with the assumption of one-to-one binding to obtain the macroscopic binding rates, which are $0.35 \pm 0.15 \times 10^4$, $11.7 \pm 0.47 \times 10^4$, $3.4 \pm 1.6 \times 10^4$, and $3.5 \pm 0.08 \times 10^4$ $M^{-1} \cdot s^{-1}$ for carbamazepine (Vh = −120 mV), carbamazepine (Vh = −55 mV), imipramine (Vh = −120 mV), and imipramine (Vh = −55 mV), respectively. (*B*) Comparison between the macroscopic binding rates of imipramine and carbamazepine to the resting (R, Vh = −120 mV) and inactivated (I, Vh = −55 mV) states from the experiments described in *A*. The binding rates to the resting and inactivated states are similar for imipramine but are apparently dissimilar for carbamazepine.

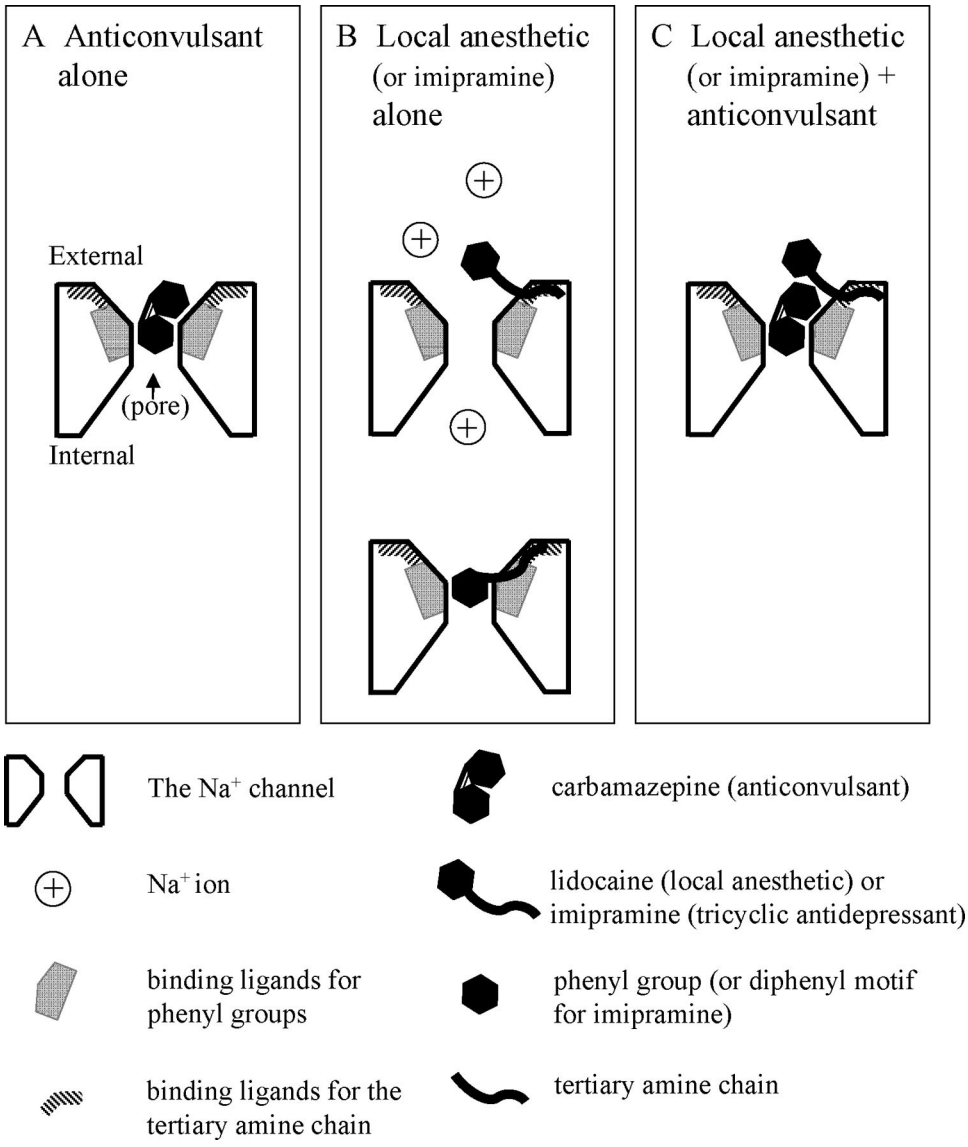

Fig. 9. A schematic model illustrating that lidocaine and imipramine bind to part of the binding site of carbamazepine in the Na^+ channel. (*A*) Carbamazepine binding site probably is located at the junction of the widened external vestibule and the narrow part of the pore, and it is composed of binding ligands for the two phenyl groups in the drug.[15,27] Carbamazepine binding to this site would modify channel gating by stabilization of the open and inactivated conformation and also block Na^+ ion passage through the pore. (*B*) Lidocaine (or imipramine) may bind to some more externally located ligands with its tertiary amine chain with very fast kinetics but low affinity. Because of the insignificant gating conformational changes in the binding ligands for the tertiary amine chain, lidocaine (or imipramine) binding in this configuration would have a negligible effect on channel gating but could still slightly jeopardize Na^+ ion permeation through the pore (and consequently the flickery blocking phenomenon in the single channel currents[32,35,36]). Lidocaine (or imipramine) may also reorient itself to get to the binding ligands for the phenyl groups in the common anticonvulsant binding site. Lidocaine binding in this configuration would have much slower binding kinetics, but it exert a significant effect on channel gating and blockade of Na^+ ion permeation. (*C*) When the ligands for the phenyl group have been taken by an anticonvulsant drug, lidocaine (or imipramine) could still bind to the channel with its tertiary amine chain. In this case, the binding affinity of lidocaine is much lowered, and thus, the channel could be doubly occupied by lidocaine and an anticonvulsant drug (*e.g.*, carbamazepine) only when the drug concentrations are high. Because of the less significant gating conformational changes in the binding site for the tertiary amine chain, the existence of this doubly occupied channel is not discernible by study of the inactivation curve shift but by close examination of the recovery kinetics of the drug-bound channel (as unbinding of the anticonvulsant would be deterred by the simultaneously bound lidocaine or imipramine).

The Amine Site Is Located at the Entry Zone of the Common Anticonvulsant Binding Site but Shows Little Gating Conformational Changes

Although lidocaine and anticonvulsant binding to the Na^+ channel preclude each other, it is intriguing that there is still a small chance of double occupancy of the channel by the two drugs according to the kinetic data (figs. 5–7). The double occupancy state is discernible only when lidocaine or imipramine is present in the drug mixtures with an anticonvulsant and still gets more manifest with even higher lidocaine concentration (~200 μM). Therefore, lidocaine and imipramine very likely have a second and low-affinity binding configuration in the Na^+ channel. This second configuration seems to be achieved with the amine chain, a structural motif not present in the anticonvulsants. This proposal is consistent with the two very different time constants (1–6 and 100–900 ms) of lidocaine block of cardiac Na^+ currents[33] and the fast flickering block of unitary Na^+ currents by high concentration of lidocaine, transcainide, or diethylamide.[32,34,35] Because the double occupancy state is discernible only when examined by the recovery kinetics but not by the shift of the steady-state inactivation curve, the phenyl group binding region (*i.e.*, in the common anticonvulsant-binding site) but not the tertiary amine chain binding region should undergo significant conformational changes during the gating processes of the channel. This idea is further supported by the findings that the binding kinetics of carbamazepine are much more correlated with the gating states of the Na^+ channel than that of imipramine (fig. 8) and that there is a very similar difference between the apparent dissociation constants to the resting and inactivated Na^+ channels for lidocaine and carbamazepine (fig. 1).[12] In this regard, the deterred (slowed) recovery kinetics in drug mixtures containing lidocaine may indicate that the amine site is located directly at the entry zone of the phenyl site (fig. 9). Conversely, the slowed kinetics may also provide an additional support for direct competition rather than allosteric interaction between lidocaine (or imipramine) and anticonvulsant binding. If lidocaine (or imipramine) and anticonulsant binding precludes each other *via* an allosteric mechanism, the binding of lidocaine (or imipramine) to its own site should profoundly disrupt the binding ligands in the anticonvulsant site to abolish concomitant binding of anticonvulsants, the unbinding rates of anticonvulsants thus should be markedly increased rather than decreased. In this case, it would take extraordinary fortuity that lidocaine binding also narrows the doorway of the anticonvulsant site even more profoundly to have an ultimate slowing effect on the escape of the bound anticonvulsant.

The Amine Site May Serve to Facilitate Drug Binding to the Na^+ Channel Relatively Irrespective of the Gating State

When the anticonvulsant binding site is occupied by an anticonvulsant (with the diphenyl motif), the aromatic group of lidocaine could not be suitably coordinated, and thus, the amine site may only bind the tertiary amine chain with low affinity. That is why the deterring effect of lidocaine on unbinding of the bound anticonvulsant would be manifest only with high concentrations of lidocaine. This molecular picture is consistent with or even well explains the previous observations that diethylamide profoundly decreased the effect of R13Q (a polycationic peptide blocking the external pore mouth of the Na^+ channel[36]), that lidocaine binding did not affect the chemical accessibility to the internal inactivation lid,[37] and that external Na^+ concentration significantly affects the slow but not the fast (flickering) blocking effect of transcanide.[34] It is an interesting possibility that lidocaine or imipramine binding to the amine site would be much easier if the contiguous aromatic site is not occupied. In this case, binding to the amine site may even help docking the drug molecule (or more precisely, the aromatic group in the drug molecule) to a favorable configuration, so that imipramine would tend to have less gating state-dependent binding rates than carbamazepine.[14] These findings not only lend further support for the direct interaction between the binding parts in the aromatic and the amine sites but also shed light on the molecular steps of drug binding to these important binding sites in the Na^+ channel.

Combined Clinical Use of Local Anesthetics, Anticonvulsants, and Tricyclic Antidepressants Should Give Additive but Not Synergistic Inhibitory Effect on Na^+ Currents

Tricyclic antidepressants, anticonvulsants, and local anesthetics have been the major categories of medications prescribed against neuropathic pain. The comparison of different monotherapies has been done. For example, tricyclic antidepressants and the Na^+ channel-blocking anticonvulsants seem to have comparable effect against pain in polyneuropathy, slightly better than gabapentin (a Ca^{2+} channel-blocking agent) and even tramadol (a mixed opioid and monoaminergic drug), and much better than the selective serotonin reuptake inhibitors.[2] In this regard, it is interesting to note that although the selective serotonin reuptake inhibitor antidepressants do not affect the norepinephrine and histamine systems as the tricyclic antidepressants do, they are also different from the tricyclic antidepressants in the lack of an diphenyl motif and tertiary amine chain arranged in an "appropriate" configuration to bind to and inhibit the Na^+ channel in a use-dependent fashion. On the other hand, there has been no detailed clinical study to evaluate the clinical efficacy of combination therapy with different groups of drugs. On the basis of this study, we would argue that combined use of local anesthetics, anticonvulsants, and tricyclic antidepressants should give no synergic inhibitory effect on Na^+ currents because of the essential overlapping of drug binding sites and preclusion of effective double occupancy of the channel. However, because the free plasma concentrations of the drugs in therapeutic conditions are usually quite lower than (roughly <30%) the dissociation constants of the drugs to the inactivated channel, the binding of these drugs to the common site is unlikely to be saturating in clinical

conditions. One may, therefore, expect an additive, and only an additive, effect in terms of use-dependent inhibition of Na^+ currents and cellular discharges with combined use of local anesthetics, anticonvulsants, and tricyclic antidepressants in clinical conditions.

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