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Inhibition of Na^+ Current by Diphenhydramine and Other Diphenyl Compounds: Molecular Determinants of Selective Binding to the Inactivated Channels

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ABSTRACT

Diphenhydramine is an H1 histamine receptor antagonist, yet it also has a clinically useful local anesthetic effect. We found that diphenhydramine inhibits the neuronal Na^+ current, and the inhibition is stronger with more positive holding potentials. The dissociation constant between diphenhydramine and the inactivated Na^+ channel is ~10 μM, whereas the dissociation constant between diphenhydramine and the resting channel is more than 300 μM. The local anesthetic effect of diphenhydramine thus is ascribable to inhibition of Na^+ current by selective binding of the drug to the inactivated channels. Most interestingly, many other compounds, such as the anti-inflammatory drug diclofenac, the anticonvulsant drug phenytoin, the antidepressant drug imipramine, and the anticholinergic drug benztropine, have similar effects on neuronal Na^+ current. There is no apparent common motif in the chemical structure of these compounds, except that they all contain two phenyl groups. Molecular modeling further shows that the two benzene rings in all these drugs have very similar spatial orientations (stem bond angle, ~110 degrees; center–center distance, ~5 Å). In contrast, the two phenyl groups in phenylbutazone, a drug that has only a slight effect on Na^+ current, are oriented in quite a different way. These findings strongly suggest that the two phenyl groups are the key ligands interacting with the channel. Because the binding counterpart of a benzene ring usually is also a benzene ring, some aromatic side chain groups of the Na^+ channel presumably are realigned during the gating process to make the very different affinity to the aforementioned drugs between the inactivated and the resting channels.

Diphenhydramine and many other H1 histamine receptor antagonists, such as chlorpheniramine, cyproheptadine, and tripelennamine, have long been known for their significant and clinically useful local anesthetic effect (Steffen et al., 1957; Meyer and Jakubowski, 1964; Singh et al., 1975; Howard et al., 1984). For example, diphenhydramine is only slightly inferior to lidocaine in the duration and depth of anesthesia in a double-blind study (Dire and Hogan, 1993) and has been successfully used as a substituting local anesthetic agent in "caine"-sensitive patients (Munsey, 1966; Pollack and Swindel, 1989). Despite that the local anesthetic effect of antihistamines is well documented, the molecular events underlying such an effect are not fully characterized. It has been shown that diphenhydramine and cyproheptadine exerted a frequency-dependent blocking effect on neural discharges (Neto, 1979). In addition, diphenhydramine, chlorpheniramine, and cyproheptadine significantly inhibited binding of [^{3}H]batrachotoxin to voltage-sensitive Na^+ channels in vesicular preparations from guinea pig cerebral cortex (McNeal et al., 1985). More recently, it is demonstrated in ventricular myocytes that 3 μM terfenadine (a newer, nonsedating H1 receptor antagonist) potently blocked Na^+ current when the holding potential was −40 mV, whereas the inhibitory effect became insignificant if the holding potential was −90 mV (Ming and Nordin, 1995). These data imply that antihistamines may be similar to lidocaine or other classic local anesthetics, which produce use-dependent block of neuronal Na^+ current because of much higher affinity to the inactivated than to the resting Na^+ channels (Bean et al., 1983; for a review, see Butterworth and Strichartz, 1990).

Other than classic local anesthetics, the anticonvulsants phenytoin, carbamazepine, and lamotrigine constitute another major group of drugs showing significant voltage- or use-dependent inhibition of Na^+ current (Matsuki et al., 1984; Willow et al., 1985; Lang et al., 1993; Kuo and Bean, 1994a; Xie et al., 1995; Kuo et al., 1997; Kuo and Lu, 1997). It has been shown that these anticonvulsants in general have a 100-fold higher affinity to the inactivated state than to the resting state of the Na^+ channel, and all bind to the channel via a simple bimolecular reaction (a one-to-one binding process; Kuo and Bean, 1994a; Kuo et al., 1997; Kuo and Lu, 1997). Quantitative analysis of the steady-state effect and

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reaction kinetics in mixtures of different anticonvulsants further argues that these anticonvulsants bind to the same binding site in the inactivated Na^+ channel (Kuo, 1998), suggesting the same molecular determinants underlying the drug-channel interactions. Because the only common structural motif shared by these anticonvulsants is two phenyl groups separated by one to two C—C or C—N bonds, such a diphenyl structure seems to involve the major ligands interacting with the inactivated Na^+ channel. In this regard, it is interesting to note that the diphenyl structural motif is also present in many aforementioned H1 antagonists, for which there is indirect evidence suggestive of inhibition of Na^+ current by selective binding to the inactivated channels. We therefore explored the effect of diphenhydramine and other diphenyl compounds on neuronal Na^+ currents in detail. We found that diphenhydramine blocks neuronal Na^+ current via selective binding to the inactivated Na^+ channel, and its binding affinity to the resting channels is at least 30-fold lower than that to the inactivated channels. We also found that many other diphenyl compounds have a similar effect on neuronal Na^+ currents. Molecular modeling further shows that the two phenyl groups in these compounds have very similar spatial orientations. We conclude that the local anesthetic effect of diphenhydramine and other H1 antagonists is ascribable to selective binding of these drugs to the inactivated Na^+ channel, with the diphenyl structure playing an essential role in such drug-channel interactions.

Materials and Methods

Cell Preparation. Coronal slices of the whole brain were prepared from 7- to 14-day-old Long-Evans rats. CA1 region was dissected from the slices and cut into small chunks. After treatment for 5 to 10 min 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 0.5 mg/ml type XI trypsin, pH 7.4, 37°C), tissue chunks were transferred to dissociation medium containing no trypsin but 1 mg/ml BSA and 1 mg/ml type II-S trypsin inhibitor (Sigma Chemical Co., St. Louis, MO). Every time cells were needed, two or 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 $CaCl_2$, and 10 mM HEPES, pH 7.4). Whole-cell voltage-clamp recordings were obtained using pipettes pulled from borosilicate micropipettes (o.d. 1.55–1.60 mm; Hilgenberg Inc., Malsfeld, Germany), fire polished, and coated with Sylgard (Dow-Corning, Midland, MI). The pipette resistance was 1 to 2 MΩ when filled with the internal solution (75 mM CsCl, 75 mM CsF, 2.5 mM $MgCl_2$, 5 mM HEPES, 2.5 mM EGTA, 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; Hilgenberg Inc.; content, 1 μl; length, 64 mm) emitting either control or drug-containing external recording solutions. Diphenhydramine, tripelennamine, benztropine, imipramine, and phenylephrine were dissolved in water, and the other drugs were dissolved in dimethyl sulfoxide to make 100 mM stock solutions, which were then diluted into Tyrode's solution to attain the final concentrations desired. The final concentration of dimethyl sulfoxide (0.1% or less) was not found to have detectable effect on Na^+ currents. All drugs were purchased from Sigma Chemical Co. or Research Biochemicals Inc. (Natick, MA). Currents were recorded at room temperature (~25°C) with an Axoclamp 200A amplifier, filtered at 5 kHz with four-pole Bessel filter, digitized at 50- to 200-μs intervals, and stored using a Digidata-1200 analog/digital interface along with the pCLAMP software (Axon Instruments, Foster City, CA). All statistics are given as mean ± S.D.

Molecular Modeling. Models of the tertiary structure of the drugs were built by the Macromodel version 4.5 program (Department of Chemistry, Columbia University, 1994), which was followed by searches for the minimum energy conformations. Typically more than 200 conformations were picked, and energy minimization using the Monte Carlo method and MM2 force-field parameters was exercised to obtain the minimum energy conformations.

Results

Different Inhibitory Effect of Diphenhydramine on Na^+ Currents Elicited from Different Holding Potentials. Figure 1 shows the effect of diphenhydramine on neuronal Na^+ currents. Diphenhydramine (10 μM) has only a slight inhibitory effect on the Na^+ current elicited from a holding potential of −110 mV, and even 100 μM diphenhydramine produces no more than ~30% inhibition of the Na^+ current (Fig. 1A). On the other hand, diphenhydramine has much stronger inhibitory effect on the Na^+ current elicited from more positive holding potentials (e.g., −70 mV). The effect of diphenhydramine on Na^+ currents elicited from different holding potentials are plotted in Fig. 1B, where the

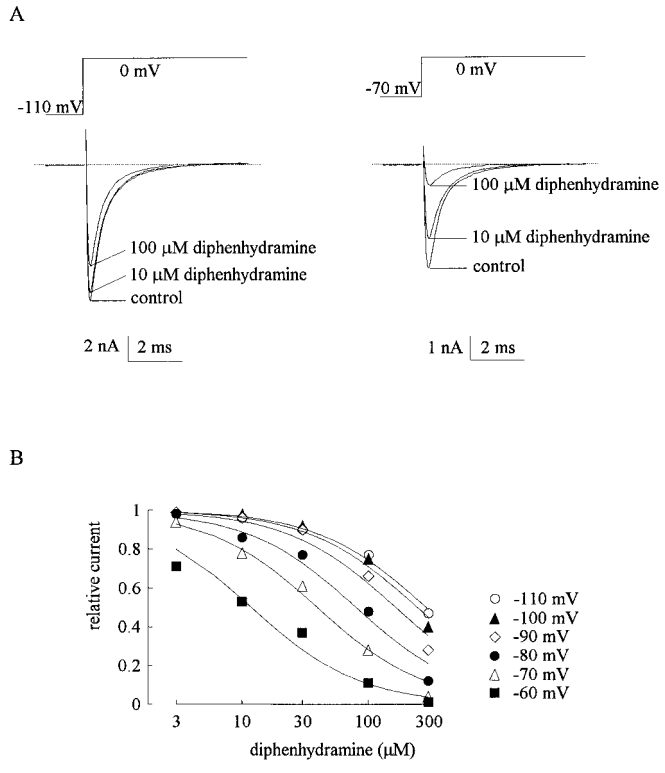
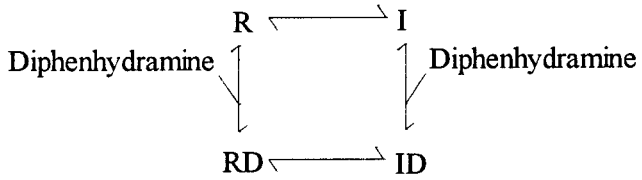


Fig. 1. Inhibition of Na^+ currents by diphenhydramine. A, Na^+ currents in control or 10 or 100 μM diphenhydramine. The cell was held at −110 or −70 mV and stepped to 0 mV for 6 ms every 2 s. The dashed lines indicate zero current level. B, dose-response curves for inhibition of Na^+ currents by 3 to 300 μM diphenhydramine at different holding potentials (−60 to −110 mV). The data are from one cell, and the test pulse protocol is the same as that in A. The peak currents in the presence of diphenhydramine are normalized to the peak current in control at each holding potential to give the relative currents, which are plotted against the concentration of diphenhydramine ([diphenhydramine]) in a semilogarithmic scale. The lines are fits to each set of data of the form: relative current = $1/(1 + ([\text{diphenhydramine}]/K_{app}))$, where K_{app} stands for the apparent dissociation constant and is 287, 244, 165, 80.1, 39.4, and 11.8 μM at holding potentials −110, −100, −90, −80, −70, and −60 mV, respectively.

data could be reasonably fit by one-to-one binding curves. Diphenhydramine inhibits neuronal Na^+ currents with an apparent dissociation constant (K_{app}) of ~12 μM if the holding potential is −60 mV, whereas the K_{app} increases to ~290 μM if the holding potential is −110 mV.

Because Na^+ channels would be mostly in the inactivated and resting states at holding potentials of −60 and −110 mV, respectively (see the control inactivation curves in Fig. 2A), the above finding is consistent with the notion that diphenhydramine binds to the inactivated Na^+ channel with high affinity but to the resting channels with low affinity. This point can be illustrated in a more quantitative manner in Scheme 1.



Scheme 1.

where R and I are the resting and inactivated states of the channel, and RD and ID are the diphenhydramine-bound (and inhibited) resting and inactivated states, respectively. The activated (open) state is omitted here because most activated Na^+ channels would be quickly inactivated, and thus for simplicity one may just consider R and I states for a steady-state condition. According to this scheme, at any particular holding potential the fraction of channels in state R (the channels that can be activated, or therefore the current that is elicited on membrane depolarization) would be reduced by diphenhydramine with a K_{app} value defined by (Bean, 1984; Kuo and Bean, 1994a):

$$K_{app} = \frac{1}{(h/K_R + (1-h)/K_I)}$$

where h is the fraction of channels in state R in the absence of drug ("fraction available" in the control condition in Fig. 2A), and K_R and K_I are the dissociation constants for the resting and inactivated channels, respectively. This is as if the overall affinity of diphenhydramine toward the channel ($\sim 1/K_{app}$) is a weighted average of the affinity toward each state of the channel ($h/K_R + (1-h)/K_I$). According to this equation and a K_{app} value of ~12 μM at −60 mV, where h is ~0.15 (Figs. 1B and 2A), K_I should be ~10 μM. With a K_I value of ~10 μM and a K_{app} value of ~290 μM at −110 mV where h is close to 1 (Figs. 1B and 2A), K_R must be somewhat larger than 290 μM (K_R = 410 μM if h = 0.99 or = 690 μM if h = 0.98 at −110 mV).

Measurement of Affinity between Inactivated Na^+ Channels and Diphenhydramine by Shift of the Inactivation Curve. One may also estimate K_I with another approach based on the foregoing scheme. In the control condition, the inactivation curve can be approximated by a Boltzmann distribution, $1/\{1 + \exp[(V - V_h)/k]\}$ (Fig. 2A), where V is the membrane potential, V_h is the half-inactivated potential (at which half of the channels are in state R and the other half are in state I), and k is the slope factor. When diphenhydramine is added, the shape of the curve should remain the same, but the midpoint (V_h) would be shifted by

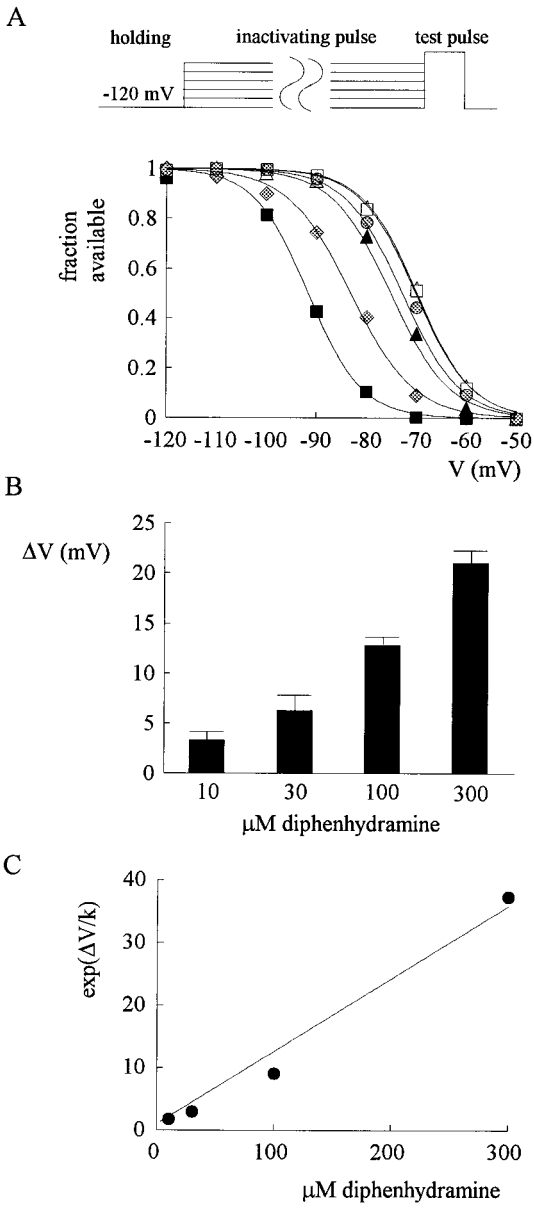


Fig. 2. Shift of the inactivation curve by diphenhydramine. A, the cell was held at −120 mV and stepped every 15 s to the inactivating pulse (−120 to −50 mV) for 9 s. The channels that remain available after each inactivating pulse were assessed by the peak currents during the following short test pulse to 0 mV for 6 ms. The pulse protocol was repeated every 15 s. The fraction available is defined as the normalized peak current (relative to the current evoked with an inactivating pulse at −120 mV) and is plotted against the voltage of the inactivating pulse. Two sets of control data were obtained before and after the experiments in different concentrations of diphenhydramine to demonstrate the absence of significant voltage drift during this long experiment. The lines are fits to each set of data of a Boltzmann function: fraction available = $1/\{1 + \exp[(V - V_h)/k]\}$, with V_h values of −70.2, −70.2, −72.9, −75.2, −83.2, and −91.6 mV and k values of 5.5, 5.6, 5.8, 5.5, 6.3, and 5.4 for control (before diphenhydramine), control (after diphenhydramine), and 10, 30, 100, and 300 μM diphenhydramine, respectively. B, the shift of the inactivation curve (ΔV) is determined in each cell by the difference between V_h in control and in various concentrations of diphenhydramine and is 3.3 ± 0.7, 6.2 ± 1.6, 12.8 ± 0.7, and 21.0 ± 1.2 mV (n = 3–5) for 10, 30, 100, and 300 μM diphenhydramine, respectively. C, $\Delta V/k$ values are calculated for each drug concentration in each cell. The mean values of $\Delta V/k$ in each drug concentration (n = 3–5) are then used to calculate $\exp(\Delta V/k)$, which is plotted against diphenhydramine concentration. The line is a fit to the data of the form: $\exp(\Delta V/k) = [1 + (D/8.6)]$, where D denotes diphenhydramine concentration (in μM).

ΔV, which could be related to K_I by equating $\exp(\Delta V/k)$ with $1 + (D/K_I)$ if one assumes that K_R is very large (Bean et al., 1983; Bean, 1984; D is the concentration of diphenhydramine). Figure 2, A and B, shows that with 10 to 300 μM diphenhydramine added, the inactivation curves indeed are shifted leftward with unchanged slope. Figure 2C shows the mean $\exp(\Delta V/k)$ values in various concentrations of diphenhydramine and a fit with the foregoing equation yielding a K_I value of 8.6 μM. This is consistent with the result from Fig. 1 that the K_I for diphenhydramine is probably around 10 μM.

Slow Binding Rate of Diphenhydramine onto the Inactivated Na^+ Channel. Except for K_I and K_R, we also explored the kinetics of diphenhydramine action on Na^+ channels. Figure 3A shows that after a few milliseconds at a recovery gap potential, the majority of normal inactivated channels recover, whereas most diphenhydramine-bound channels do not. Because diphenhydramine-bound inactivated channels recover much slower than "normal" inactivated channels, one may assess the binding rate of diphenhydramine onto inactivated Na^+ channels by another voltage protocol, in which the prepulse is gradually lengthened while the −120 mV gap is fixed at 5 ms (Fig. 3B). The decrease of Na^+ currents elicited during the test pulse subsequent to the −120-mV gap now mostly reflects the increase in drug-bound inactivated channels, with a little contamination from the concomitant increase in normal inactivated channels that have not recovered during the 5-ms gap. The contamination is corrected by taking the difference between the Na^+ currents in control and in the presence of diphenhydramine (Fig. 3C). Figure 3D shows that the macroscopic binding rates increase linearly with drug concentration, supporting the presumption in Fig. 1B that diphenhydramine interacts with Na^+ channels via a one-to-one binding (simple bimolecular) reaction. The lin-

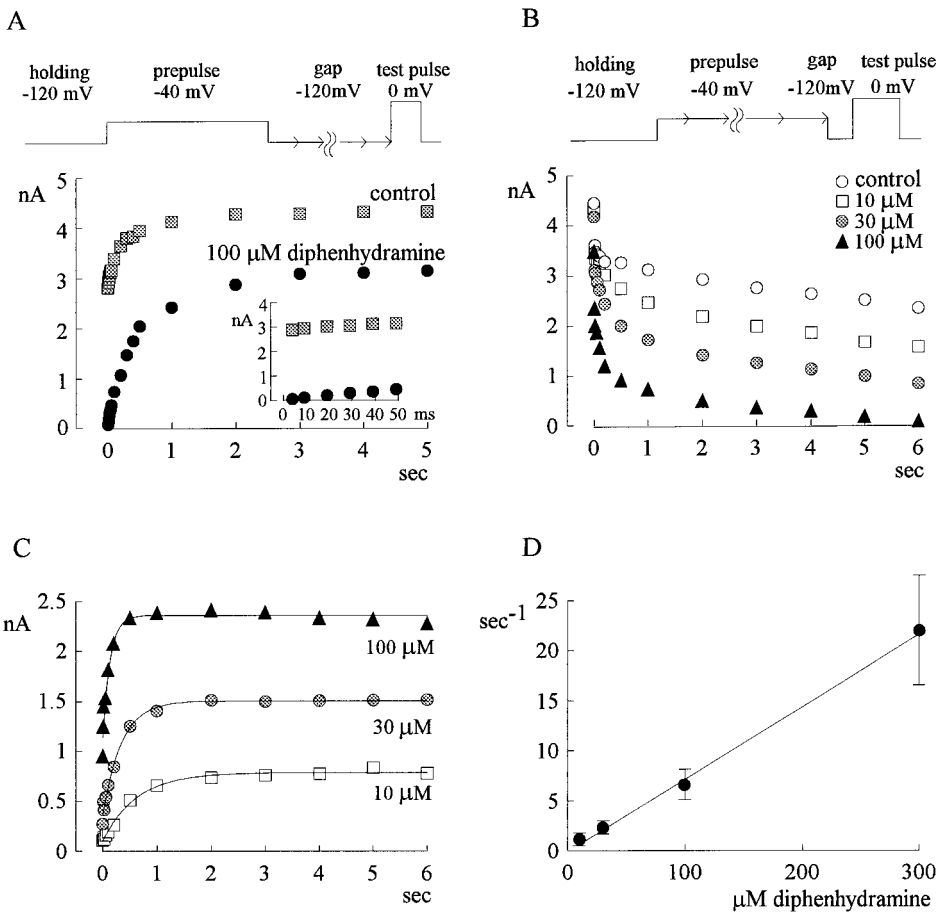


Fig. 3. Unbinding and binding rate of diphenhydramine. A, in control or in the continuous presence of 100 μM diphenhydramine, the cell was held at −120 mV and then prepulsed to −40 mV for 9 s. The cell was then stepped back to a recovery gap potential at −120 mV for variable length before being stepped again to a short test pulse at 0 mV for 6 ms to assess the available current. The pulse protocol was repeated every 15 s. The time courses of recovery are obtained by plotting the peak current at the test pulse against the length of the recovery gap potential. With very long (~5 s) recovery gap potential, the current in 100 μM diphenhydramine recovers to its steady-state block level at −120 mV, and thus is still smaller than the current at that time point in control. Inset, first 500 ms data are redrawn with a smaller horizontal scale to demonstrate that after short (e.g., 5–10 ms) recovery period, currents in control have largely recovered, but most currents in diphenhydramine have not. B, in control and 10, 30, or 100 μM diphenhydramine, the cell was held at −120 mV and prepulsed to −40 mV for variable length. Immediately after the prepulse, there is a recovery gap potential at −120 mV for 5 ms to recover normal (not diphenhydramine-bound) inactivated Na^+ channels, and then the available current is assessed by a short test pulse to 0 mV for 6 ms. The pulse protocol is repeated every 15 s. The peak Na^+ current at the test pulse is plotted against the duration of the prepulse to demonstrate the decay of the peak currents as the prepulse lengthens. C, differences between the currents in diphenhydramine and the current in control in B are plotted against the duration of the prepulse. The lines are monoexponential fits of the form: current (nA) = 2.46 − 1.35∗exp(−t/0.14), where t denotes the length of prepulse in seconds, the horizontal axis; current = 1.51 − 1.14∗exp(−t/0.35), and current = 0.79 − 0.69∗exp(−t/0.62) for 100, 30, and 10 μM diphenhydramine, respectively. D, inverses of the time constants in C (n = 3–6) are plotted against the concentration of diphenhydramine. The line is a linear regression fit to the mean values. The intercept and slope are 0.43 s^{-1} and 72,000 M^{-1} s^{-1}, respectively.

ear regression fit to the data yields a binding rate constant of ~72,000 M^{-1} s^{-1}.

Similar Effect of Tripelennamine and Diclofenac to that of Diphenhydramine. There are striking similarities between diphenhydramine and anticonvulsants phenytoin, carbamazepine, and lamotrigine in their actions on neuronal Na^+ channels (Kuo and Bean, 1994a,b; Kuo et al., 1997; Kuo and Lu, 1997). Because these anticonvulsants have been found to bind to the same binding site in neuronal Na^+ channels (Kuo, 1998), they probably share a common structural motif interacting with the channel. Examination of the chemical formulas reveals that each of the anticonvulsants contains two phenyl groups, and such a diphenyl structure is the only structural motif shared by these drugs. In this regard, it is interesting to note that diphenhydramine also contains the same diphenyl structure. We thus extend our observation to other drugs containing the diphenyl structure to further investigate the importance of such a motif. Figures 4 and 5 show that many other drugs containing the diphenyl structure also inhibit Na^+ currents by selective binding to the inactivated channels. For example, 100 μM tripelennamine (another H1 histamine receptor antagonist) and diclofenac (a cyclooxygenase inhibitor and anti-inflammatory drug, which consists of only two phenyl groups connected to an N atom) both inhibit Na^+ current, and the inhibitory effect is remarkably greater with more positive holding potentials (Fig. 4A). Moreover, similar to the case of diphenhydramine (Fig. 2A), the inactivation curve of Na^+ channel is significantly shifted toward more negative potentials by tripelennamine and diclofenac (Fig. 4B).

Structural Determinants underlying Selective Binding to Inactivated Na^+ Channels. Similar experiments were repeated with other drugs, whose chemical formulas are summarized in Fig. 5A. These compounds could be roughly divided into three groups according to their effects on Na^+ channels. Group I drugs (phenytoin, carbamazepine, lamotrigine, and diclofenac) strongly inhibit Na^+ currents when the holding potential is −70 mV, yet the inhibition becomes unremarkable when the holding potential is hyperpolarized to −120 mV, implying significant binding of these drugs (in the concentration of 100 μM) to the inactivated but not to the resting channels. Group II drugs (diphenhydramine,

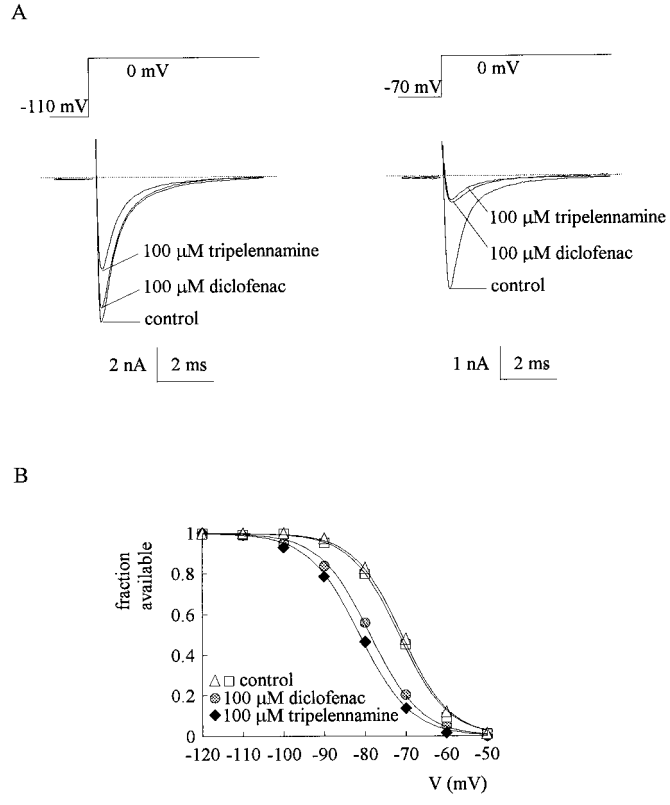


Fig. 4. Inhibition of Na^+ currents by tripelennamine and diclofenac. A, Na^+ currents were elicited by the same pulse protocol as that in Fig. 1. When the holding potential was −110 mV, 100 μM tripelennamine inhibited ~35% of the current, whereas the inhibition produced by 100 μM diclofenac was less. The inhibitory effects of both drugs were stronger with more depolarized holding potentials. B, tripelennamine and diclofenac (both in a concentration of 100 μM) also shifted the inactivation curve of Na^+ channels to more hyperpolarized potentials. The inactivation curves are obtained by the same protocols as those in Fig. 2. Two sets of control data were obtained before and after the experiments in tripelennamine and diclofenac. The lines are fits with a Boltzmann function $1/\{1 + \exp[(V - V_h)/k]\}$, with V_h values of −71.6, −71.0, −81.3, and −78.8 mV and k values of 6.2, 5.9, 6.0, and 6.3 for control (before drugs), control (after drugs), tripelennamine, and diclofenac, respectively.

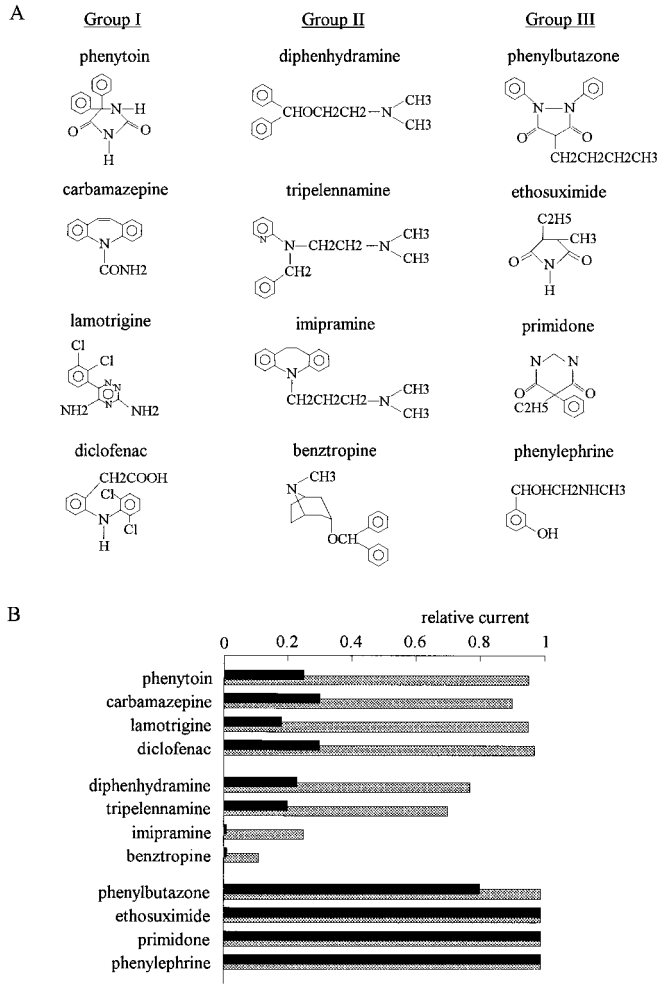


Fig. 5. Summary of the inhibitory effect of different drugs on Na^+ currents. A, chemical structure of the drugs. The chemical formulas are arranged into three columns, which correspond to drug groups I, II, and III (see text). B, the relative current in the presence of a drug is defined as that in Fig. 1 and is plotted for each drug. Na^+ currents were elicited from a holding potential of either −120 mV (gray columns) or −70 mV (black columns). All drugs were given at a concentration of 100 μM. The S.D. values are in general smaller than 10 to 20% of the mean (n = 3–6) and are omitted for clearer presentation.

tripelennamine, imipramine, and benztropine) have some inhibitory effect on the Na^+ current when the holding potential is −120 mV, but the effect is much more pronounced with a holding potential of −70 mV. Thus, group II drugs seem to have higher affinity to the inactivated channels than group I drugs (and may even have some binding to the resting channels). Group III drugs (phenylbutazone, ethosuximide, primidone, phenylephrine) have little effect on the Na^+ current regardless of whether the holding potential is −70 or −120 mV, implying little binding of these drugs to either inactivated or resting channels.

Similar Effect of Lidocaine to Group I Drugs. The grouping of drug effect in Fig. 5 is unrelated to the usual clinical categorization of the drugs. For example, diclofenac and phenylbutazone are both anti-inflammatory agents, but their effects on Na^+ channels are very different. Also, the anticonvulsants phenytoin, carbamazepine, and lamotrigine show significant inhibition of Na^+ currents when the holding potential is −70 mV, whereas the anticonvulsants primidone and ethosuximide show no such effect. At this point, it may be noteworthy that ethosuximide is different from phenytoin and carbamazepine in the therapeutic spectrum against seizures and has been proposed to act via inhibition of T-type Ca^{2+} current rather than Na^+ current (Coulter et al., 1989, 1990). A closer examination of the chemical formulas of ethosuximide and phenytoin reveals that they both consist of a very similar five-member ring (the "ureide structure"). The only difference is that phenytoin has two phenyl groups attached to the ureide structure, whereas ethosuximide has none. Further examination of the chemical formulas of the other drugs yields similarly interesting results, suggesting that the grouping of drug effect in Fig. 5 could be correlated with the diphenyl groups and some other structural features of the drugs. Group I drugs contain two phenyl groups separated by one or two C—C or C—N bonds but not any long linear (tertiary amine) side chain. Group II drugs have two phenyl groups as well as a long tertiary amine chain (totally five or six C or N atoms in a line with the amine N atom mostly charged at pH 7.4). Group III drugs have either one or no phenyl group (except phenylbutazone; see Discussion) and no tertiary amine chain. The stronger binding of group II drugs to Na^+ channels suggests that in addition to the two phenyl groups, a charged amine group contributes to drug binding. To further characterize the effect associated with the amine chain and the benzene ring, we examine the effect of lidocaine, a prototypical local anesthetic containing one tertiary amine chain and one phenyl group (Fig. 6). Very similar to the group I drugs in Fig. 5, 100 μM lidocaine has little effect on the Na^+ current elicited from a holding potential of −120 mV yet significantly inhibits Na^+ currents elicited from more positive holding potentials ($K_I \sim 25$ μM; data not shown). This finding implies that the amine group and the two phenyl groups are three important molecular determinants for drug binding onto the inactivated Na^+ channels. If a drug has two or more of the three determinants (and these determinants are arranged into favorable configurations, see *Discussion* and Fig. 7), the drug may selectively bind to the inactivated channels with "appropriate" binding and unbinding kinetics and thus produces the pharmacologically important use-dependent blocking effect on Na^+ current.

Discussion

Molecular Mechanisms Underlying the Local Anesthetic Effect of Diphenhydramine. In this study, we demonstrate that diphenhydramine binds to the inactivated Na^+ channels with a dissociation constant of ~10 μM, whereas the affinity between diphenhydramine and the resting channels is at least 30 times lower. Moreover, the binding rate of diphenhydramine is ~72,000 M^{-1} s^{-1}, yet the unbinding rate from the inactivated Na^+ channels is very slow (Fig. 3A). These characters are qualitatively similar to previous observations for the anticonvulsants phenytoin, carbamazepine, and lamotrigine (Kuo and Bean, 1994a,b; Kuo et al., 1997; Kuo and Lu, 1997) and quantitatively readily substantiate voltage- or use-dependent block of neuronal Na^+ currents. We therefore conclude that the local anesthetic effect of diphenhydramine is ascribable to selective binding to the in-

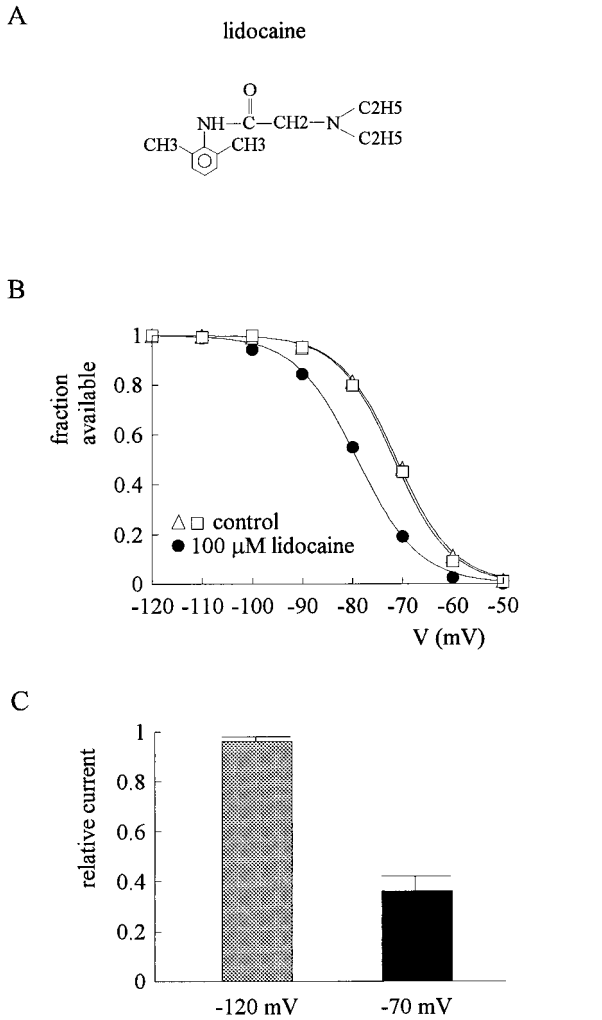


Fig. 6. Inhibition of Na^+ currents by lidocaine. A, the chemical formula of lidocaine. B, lidocaine also shifts the inactivation curve of Na^+ channels to more hyperpolarized potentials. Two sets of control data were obtained before and after the data for 100 μM lidocaine. The lines are fits with a Boltzmann function $1/\{1 + \exp[(V - V_h)/k]\}$, with V_h values of −71.2, −71.5, and −78.8 mV and k values of 5.8, 5.7, and 6.3 for control (before lidocaine), control (after lidocaine), and lidocaine, respectively. C, the relative current in the presence of 100 μM lidocaine is defined as that in Fig. 1B and is 0.96 ± 0.02 and 0.36 ± 0.06 ($n = 5$) for Na^+ currents elicited from holding potentials of −120 mV (gray column) and −70 mV (black column), respectively.

activated Na^+ channels with "appropriate" kinetics, the same molecular mechanisms as those underlying the action of lidocaine and most other classic local anesthetics.

Correlation between Inhibition of Na^+ Current and Clinical Effect of Drugs. Although at a concentration of 100 μM, diphenhydramine and many other compounds have significant effects on neuronal Na^+ channels (Fig. 5B), the inhibition of Na^+ current is not necessarily related to the usual clinical category or clinical effect of these drugs. An important consideration here is the drug concentration that can be achieved in clinical conditions. For example, the therapeutic plasma concentrations of the antidepressant imipramine and the antihistamine diphenhydramine are in the order of 10^{-7} M (0.1–1 μM; Carruthers et al., 1978; Amsterdam et al., 1980; Blyton et al., 1986; Benet et al., 1996). Because there is as much as 80 to 90% plasma protein binding of these drugs, the free drug concentrations in the cerebrospinal fluid are probably in the order of 10^{-8} to 10^{-7} M (10~100 nM). This is close to the half-inhibitory concentrations of imipramine on specific ligand binding to the 5-hydroxytryptamine$_2$ (5-HT$_2$) and α_1-adrenergic receptors (~472 and ~58 nM, respectively; U'Prichard et al., 1978; Enna and Kendall, 1981) and of common antihistamines (e.g., terfenadine and chlorpheniramine) on specific ligand binding to H1 receptors (70–700 nM; Wiech and Martin, 1982) but is one to two orders of magnitude smaller than the lowest effective concentrations inhibiting Na^+ currents (1–10 μM). Thus, the major clinical effect of systemic application of diphenhydramine or other H1 receptor antagonists is most likely ascribable to its action on the H1 receptors, whereas inhibition of Na^+ currents probably plays no role. We have seen that selective binding to the inactivated Na^+ channels with a K_I value in the micromolar range is a more general property shared by many compounds rather than a unique character of some classic local anesthetics or anticonvulsants. All such compounds theoretically could be anticonvulsants or local anesthetics if only they could reach a concentration of a few micromolar units around the Na^+ channel under therapeutic conditions. Thus, diphenhydramine is as good as lidocaine as a local anesthetic when injected locally but is by no means a useful anticonvulsant in systemic use. This is not due to a weaker effect on Na^+ channels of diphenhydramine than of phenytoin (because the two drugs have K_I values in a similar micromolar range). Instead, this is because phenytoin can reach 4 to 8 μM in the cerebrospinal fluid under most clinical conditions (Sherwin et al., 1973; Richens, 1979), but the therapeutic concentration of diphenhydramine in systemic use is much lower.

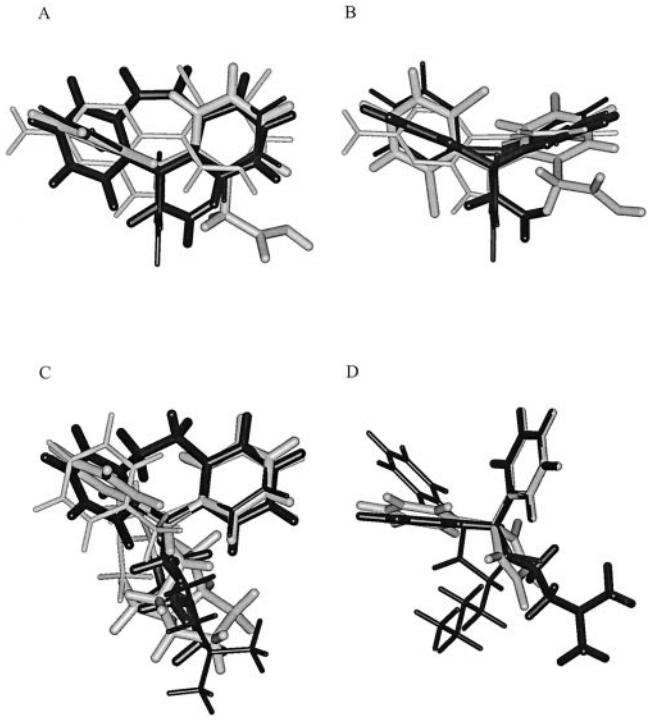


Fig. 7. The structure of different drugs given by molecular modeling. A, the minimum energy conformation of predominant conformers of group I drugs phenytoin (thin black line), carbamazepine (thick black line), lamotrigine (thin gray line), and diclofenac (thick gray line). Except for lamotrigine (which has no pivotal atom), the C1 atoms and the pivotal atom of each compound are superimposed, respectively. Lamotrigine was then placed by eye. It is evident that the two benzene rings take up a very similar space, demonstrating the very similar stem bond angle and center–center distance among group I drugs (Table 1). Although the torsion angles are different in some compounds, these angles may be flexible and thus are less important parameters in a comparison of the configuration of diphenyl groups in different drugs (see text). Note that other than the diphenyl groups, there is no common motif shared by all these drugs from a structural point of view. B, the structure of drugs in A is viewed from a different angle (perpendicular to the view angle in A). A and B together demonstrate the three-dimensional proximity of the diphenyl groups in group I drugs. C, the minimum energy conformation of predominant conformers of group II drugs diphenhydramine (thick gray line), tripelennamine (thin gray line), imipramine (thick black line), and benztropine (thin black line). Like in A, the C1 atoms and pivotal atoms from each compound are superimposed, respectively. (For tripelennamine, which has two pivotal atoms, only one C1 and one pivotal atom are arbitrarily chosen to line up with the other drugs.) It is evident that the diphenyl groups in these drugs are located in a very similar space. Also, it is interesting to note that the tertiary amine groups are clustered into roughly the same space as the diphenyl groups lined up with one another. The diphenyl groups of imipramine are plotted in a similar orientation as those of carbamazepine in A, so one may use the two compounds as references for a comparison of the other drugs in A and C. D, the minimum energy conformation of predominant conformers of group I drug phenytoin (thick gray line), group II drug diphenhydramine (thick black line), and group III drug phenylbutazone (thin line). The conformation of the first two drugs are taken from A and C, respectively. When the first benzene rings are superimposed onto one another, the other benzene rings in phenytoin and diphenhydramine have very similar spatial orientations, but that in phenylbutazone takes a different (i.e., more "vertical") position. Despite repeated trials with different placements of the drugs, it is impossible to orient the second benzene rings of phenylbutazone and phenytoin to the same space.

Structural Determinants of Drug-Channel Interactions. We noted in Fig. 5 that the drug effect on Na^+ currents is correlated with drug structure and that two phenyl and one tertiary amine groups probably are the three major molecular determinants interacting with Na^+ channels. The tertiary amine in group II drugs (pK_a = 8.7–10.0 for the protonated drugs) is mostly charged in physiological pH. Its interaction with the channel thus may involve electrostatic forces. On the other hand, the binding between the uncharged phenyl groups in drugs and its counterparts in the inactivated Na^+ channel probably involves hydrophobic or induced-dipole forces. Because effective hydrophobic or dipole bonds require close proximity (i.e., exact fit) between the binding counterparts, the two phenyl groups in all group I and group II drugs should conceivably have very similar spatial placements if they do bind to the same binding site like phenytoin, carbamazepine, and lamotrigine (Kuo, 1998). We therefore explored the configurations of the diphenyl groups in these drugs by computer-based molecular modeling (see Materials and Methods). The configuration of the two

phenyl groups can be grossly defined by the "stem bond angle" (the angle between the two bonds "holding" the two benzene rings), the distance between the two benzene rings, and the "torsion angle" (rotation of the benzene ring with the stem bond being the rotating axis). Table 1 shows that the two benzene rings in all group I and II drugs indeed have a very similar stem bond angle (~110 degrees, except for lamotrigine, which does not have this angle) and center–center distance (~5 Å), whereas the torsion angles are more variable. However, the torsion angle of a benzene ring itself tends to be a less-fixed parameter unless the pivotal atom (the atom to which the benzene ring is directly connected) is also in a ring structure. For example, the torsion angle is relatively fixed in carbamazepine where the pivotal N atom is in a seven-member ring. On the other hand, diclofenac has more than five similarly low-energy conformers with torsion angles distributed over a range of 50 degrees, implying that rotation of a benzene ring around its stem bond is not necessarily associated with major free energy changes. The flexibility of torsion angle thus makes this angle a less significant parameter in comparing the configuration of diphenyl groups in different drugs. However, this flexibility could play an important role in envisioning the affinity between a drug and the inactivated Na^+ channel (see below).

The structures of the minimum energy conformers of groups I and II drugs are plotted in Fig. 7, A to C, demonstrating that the diphenyl groups in these drugs indeed have very similar spatial orientations (except for some differences in torsion angles). On the other hand, Fig. 7D demonstrates the different configuration of the diphenyl groups in phenylbutazone, a diphenyl compound that has only a minimal effect on the inactivated Na^+ channels (Fig. 5). The stem bond angle of phenylbutazone (−18 degrees; Table 1) is quite different from those in group I and II drugs (~110 degrees). Moreover, although in phenylbutazone the center–center distance of the two phenyl groups is also ~5 Å, the difference between the C1–C1 distance and the C4–C4 distance is only ~3.3 Å. This is quite different from the very consistent ~5-Å difference in groups I and II drugs, indicating a different spatial relationship between the two benzene rings in phenylbutazone. It is evident in Fig. 7D that the benzene rings in phenylbutazone are positioned more parallel to each other than those in phenytoin and diphenhydramine. These structural data not only are consistent with the notion that the diphenyl structural motif plays an essential role in selective binding to the inactivated Na^+ channels but also demonstrate that the two phenyl groups must be arranged into "appropriate" configurations to have such an effect.

Implications for Organization of Drug-Binding Sites and Gating Conformational Changes in Neuronal Na^+ Channels. We mentioned that effective hydrophobic bonds require close proximity between the binding counterparts. A planar benzene ring thus has a very strong tendency to form bond with the other planar benzene ring (Zimmerman and Feldman, 1989). If the two phenyl groups in phenytoin and other drugs serve as the major binding ligands, then the key structure of the drug-binding site in the inactivated Na^+ channel probably also involves two phenyl groups, most likely the side chain groups of two aromatic amino acids of the channel protein. It should be noted that there is potential flexibility of the torsion angle of these aromatic binding ligands (both in the drugs and in the channel), and additional flexibility could also be contributed by the peptide chain. These flexibilities may be part of the reason why so many compounds could bind to the inactivated Na^+ channels but in general only with mediocre rather than very high affinity (the K_I values are mostly in the micromolar or even tens of micromolar range, rather than in the submicromolar or nanomolar range). Other than torsion angles, there are more fixed parameters of the "appropriate" configurations of the diphenyl structural motif in groups I and II drugs (e.g., stem bond angle, ~110 degrees; center–center distance, ~5 Å), and the two aromatic side chain groups in the drug-binding site in inactivated Na^+ channels presumably should be arranged

TABLE 1

Configuration of the two benzene rings in different drugs

The tertiary structures of the drugs were built through computer-based molecular modeling (see *Materials and Methods*). The minimum energy conformation of each drug is reported.

Agent	Stem Bond Angle[a]	Distance[c]	Distance	Distance	Torsion Angle[d]
	degree	*Å, center–center*	*Å, C1–C1*	*Å, C4–C4*	*degree*
Phenytoin	111.0	4.84	2.52	7.12	−57.4, −56.5
Carbamazepine	118.5	4.93	2.35	7.47	−63.1, 62.4
Lamotrigine	N.A.[b]	4.38	1.50	7.10	−59.9, 122.1
Diclofenac	123.3	5.00	2.54	7.46	−29.2, −55.5
Diphenhydramine	109.2	4.79	2.48	7.04	−72.0, −64.6
Tripelennamine	114.1	5.87	3.53	8.25	43.3, −61.3
Imipramine	119.4	4.92	2.49	7.39	−46.8, 67.2
Benztropine	109.6	4.77	2.48	7.07	−66.1, −65.7
Phenylbutazone	−18.4	4.79	3.10	6.40	−40.4, −39.7

[a] In each drug molecule, the carbon or nitrogen atom to which the two benzene rings directly connect is designated as the pivotal atom. The C—C or N—C bond connecting the pivotal atom and the benzene ring is designated as the stem bond. The angle made by the two stem bonds at the pivotal atom is the stem bond angle. In tripelennamine and phenylbutazone, the two benzene rings are connected to two neighboring atoms rather than to one atom. In such cases, the two neighboring atoms are still designated as pivotal atoms. The bonds connecting the benzene rings to these atoms are the stem bonds, and the stem bond angle is the angle formed by the two stem bonds with a view line through the two neighboring pivotal atoms.

[b] In lamotrigine, the two benzene rings are connected to each other directly, and thus no stem bond or stem bond angle is defined.

[c] The distance between the two benzene rings are measured from the center of one ring to the center of the other (center–center). The distance between the two C1 atoms (C1–C1) or the two C4 atoms in the rings (C4–C4) is also measured. C1 is the carbon atom forming the stem bond with the pivotal atom; in lamotrigine, it is defined as the atom directly connected to the other benzene ring. C4 is the atom farthest from C1 in the ring.

[d] The torsion angle defines rotation of the benzene ring. The stem bond is the rotating axis, and the plane formed by the two stem bonds is the reference plane (when there is only one pivotal atom). Positive angle means clockwise rotation if one views the rotation from the pivotal atom. When there are two pivotal atoms (tripelennamine and phenylbutazone), the definition remains the same except that the reference plane is now defined by the stem bond serving the rotating axis and the bond between the two pivotal atoms. For lamotrigine, there is a hypothetical reference plane that contains both rings by neglecting ring rotation first. Each ring then rotates with the bond connecting the two rings being the rotating axis. Positive angle means clockwise rotation if one views the rotation from the other ring.

into template conformations of these appropriate configurations. Because this drug receptor exists only in the inactivated, not in the resting, channels, it seems plausible that some aromatic side chain groups of Na^+ channel protein are realigned during the gating process. An exploration of the key ligands in drugs and their configurations thus may also provide important conformational information about the channel protein.

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