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Frequency and Voltage-Dependent Inhibition of Type IIA Na^+ Channels, Expressed in a Mammalian Cell Line, by Local Anesthetic, Antiarrhythmic, and Anticonvulsant Drugs

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SUMMARY

This study examined the actions of phenytoin, carbamazepine, lidocaine, and verapamil on rat brain type IIA Na^+ channels functionally expressed in mammalian cells, using the whole-cell voltage-clamp recording technique. The drugs blocked Na^+ currents in both a tonic and use-dependent manner. Tonic block was more pronounced at depolarized holding potentials and reduced at hyperpolarized membrane potentials, reflecting an overall negative shift in the relationship between membrane potential and steady state inactivation. Dose-response relationships with phenytoin supported the hypothesis that the voltage dependence of tonic block resulted from the higher affinity of the drugs for inactivated than for resting channels. At −62 mV, approximately 50% of the Na^+ channels were blocked by phenytoin at 13 μM, compared with therapeutic brain levels of 4–8 μM. The use-dependent component of block developed progressively during a 2-Hz train of 40-msec-long stimulus pulses from −85 mV to 0 mV. At 2 Hz, verapamil was the most potent use-dependent blocker, lidocaine and phenytoin had intermediate potencies, and carbamazepine was least effective. The use-dependent block resulted from drug binding to open and inactivated channels during the depolarizing pulses and the slow repriming of drug-bound channels during the interpulse intervals. Verapamil, lidocaine, and phenytoin all bound preferentially to open channels, but this open channel block was most striking for verapamil. Use-dependent block was less pronounced at hyperpolarized membrane potentials, due to more rapid repriming of drug-bound channels. The results indicate that type IIA Na^+ channels expressed in a mammalian cell line retain the complex pharmacological properties characteristic of native Na^+ channels. These channels are likely to be an important site of the anticonvulsant action of phenytoin and carbamazepine. Lidocaine and verapamil, drugs with well characterized effects on peripheral Na^+ and Ca^{2+} channels, are also effective blockers of these brain Na^+ channels.

Voltage-gated Na^+ channels are large glycoproteins that form voltage-dependent, Na^+-selective pores through the membranes of excitable cells (1). At hyperpolarized membrane potentials, most Na^+ channels are in a closed resting state. The channels open in response to depolarization, resulting in inward Na^+ flux, and then rapidly convert to a nonconducting inactivated state. Repolarization of the membrane removes inactivation, converting the channels back to the resting state. The depolarization-activated ion flux through Na^+ channels plays a central role in the regenerative electrical properties of neurons and muscle cells.

The main functional component of the channel is the 260-kDa α subunit (2–5). Na^+ channels in rat brain also contain two smaller β subunits of 36 and 33 kDa, whereas Na^+ channels of rat skeletal and cardiac muscle contain a single β subunit of 38 kDa. Na^+ currents in neurons, cardiac cells, and skeletal muscle cells display different electrophysiological and pharmacological properties, and Na^+ channel subtypes with distinct primary structures are expressed in these tissues (6, 7). Four α subunit cDNAs (types I, II, IIA, and III), with >85% sequence identity, have been isolated from rat brain cDNA libraries (8, 9). Types II and IIA differ by only six amino acids. The α subunit alone forms a functional channel when expressed in *Xenopus* oocytes (8, 9), whereas the role of the β subunits is not known.

An increasing body of evidence indicates that a number of clinically important drugs exert their therapeutic effects principally by blocking Na^+ channels. For example, the anticonvulsants phenytoin and carbamazepine, which are effective against grand mal and partial seizures, are potent blockers of neuronal Na^+ channels at therapeutically relevant concentrations (10, 11). The local anesthetic lidocaine suppresses cardiac arrhythmias by blocking cardiac Na^+ channels (12) and is also an effective, although less potent, blocker of neuronal Na^+ channels (13). Verapamil, another effective antiarrhythmic, is believed to exert its effect principally by blocking cardiac Ca^{2+}

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ABBREVIATIONS: CHO, Chinese hamster ovary; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid.

channels (12), but it also blocks cardiac Na^+ channels (14). Na^+ channel block by verapamil is not well characterized but may be an important mechanism by which verapamil affects the excitability of cardiac cells. Verapamil and lidocaine also suppress epileptiform activity in brain neurons (15–18), perhaps by blocking brain Na^+ channels.

In voltage-clamp experiments, the blocking action of local anesthetics, anticonvulsants, and antiarrhythmics is greater at more depolarized holding potentials, and an additional "use-dependent" component of block accumulates during a train of stimulus pulses (12, 13, 19). The modulated receptor hypothesis (12, 13, 19) proposes that these characteristics reflect the higher affinity of local anesthetics, anticonvulsants, and antiarrhythmics for open and inactivated channels than for resting channels. Thus, use-dependent block during a train of pulses reflects the accumulation of drug binding to open and inactivated channels during each depolarizing pulse, whereas the dependence of block on holding potential reflects the predominance of inactivated channels at depolarized holding potentials and resting channels at hyperpolarized potentials. Neurons and cardiac cells in seizure and arrhythmic foci are typically more depolarized than normal cells and fire rapid bursts of action potentials. Thus, the use- and voltage-dependence of anticonvulsant and antiarrhythmic block may explain the ability of these drugs to selectively suppress the abnormal cell electrical activity that is responsible for seizures and arrhythmias, while not appreciably altering normal cell activity (10, 12).

The functional role and pharmacological significance of the different Na^+ channel subtypes is not clear. The type I and II channels are heterogeneously distributed in the rat brain (20, 21) and are localized to specific regions of individual neurons (22). They are not expressed at high levels in peripheral neurons. Determining the pharmacological properties of Na^+ channels expressed from single α subunit cDNAs and characterizing the relative effects of local anesthetics, anticonvulsants, and antiarrhythmics on the Na^+ channels encoded by different α subunit subtypes will be important steps in understanding the clinical effects of these drugs. Na^+ currents expressed in oocytes injected with RNA from α subunit cDNAs inactivate abnormally slowly, compared with Na^+ currents in mammalian neurons (9). Thus, for Na^+ channels, results obtained from oocytes must be interpreted with caution. We have recently developed a mammalian cell line, called CNaIIA-1, that has been stably transfected with cDNA encoding the type IIA subtype of the Na^+ channel α subunit and expresses high levels of functional type IIA Na^+ channels. These channels have electrophysiological characteristics that are similar to those seen in mammalian neurons (23, 24). In this study, we have used whole-cell voltage-clamp recording to characterize the effects of the anticonvulsants phenytoin and carbamazepine and the antiarrhythmics lidocaine and verapamil on the rat brain Na^+ channels expressed in CNaIIA-1 cells. Our results provide a more detailed characterization of the effects of these drugs on brain sodium channels than has been possible with previous physiological preparations.

Materials and Methods

Cell culture. All experiments were performed using the cell line CNaIIA-1, which was derived from a CHO cell line (CHO-K1; American Type Cultures) transfected with the vector ZEM2580 containing a cDNA encoding the rat brain type IIA Na^+ channel. The rat IIA sequence used contains the natural leucine at position 860, conferring normal voltage-dependent properties (25). The vector places the Na^+ channel cDNA under the control of the mouse metallothionein promoter. The vector also contains a neomycin resistance gene, which confers resistance to the antibiotic G418. A more detailed description of cloning of the type IIA Na^+ channel and its transfection into CHO cells is given elsewhere (9, 23–25).

CNaIIA-1 cells were cultured in 35-mm plastic Petri dishes in RPMI medium (GIBCO) with 10% fetal calf serum and streptomycin (2 mg/ml) and penicillin (4 mg/ml) to inhibit microbial growth. G418 was also included to select for transfectants. The cells were grown either directly on the bottom of the Petri dishes or on pieces of glass coverslips in the dishes.

Electrophysiology. Electrophysiological recording was performed either with the whole Petri dishes or with pieces of glass coverslips transferred to a 200-μl recording chamber. The culture medium was replaced by an extracellular recording solution containing (in mM) NaCl, 130; KCl, 5.0; $CaCl_2$, 1.5; $MgCl_2$, 1.0; glucose, 5.0; and HEPES, 5.0; pH 7.4 with NaOH. All recordings were at room temperature (~20–22°).

Na^+ currents were recorded using the whole-cell configuration of the patch-clamp recording technique (26). Recording pipettes were pulled from hematocrit microtubes and back-filled with an intracellular solution containing (in mM) CsF, 90; Cs-EGTA, 10; CsCl, 60; NaF, 10; and HEPES, 10; pH 7.4 with CsOH. The pipettes had input resistances of ~1–2 MΩ. The cells were clamped using a conventional patch-clamp amplifier (List L/M EPC-7). Data acquisition and analysis were performed with a personal computer, using commercially available software (Basic-Fastlab, Indec Systems). The data were filtered at 10 kHz, digitized, and stored on the computer hard disk. Theoretical curves were fit to the data using a least squares algorithm (27). Series resistance and capacitive transients arising from voltage steps were compensated using the internal clamp circuitry. In some cases, the remaining capacitive currents and leak currents were subtracted by the $P/4$ subtraction procedure (28). The settling time of the clamp before series resistance compensation was <100 μsec. The series resistance in the whole-cell configuration was approximately 3 mΩ; 70–75% of the series resistance was compensated. We estimate that, for typical Na^+ currents of 2–5 nA, the voltage drop across the series resistance was <4 mV.

Drug application. Stock solutions (20 mM) of phenytoin, carbamazepine, lidocaine, and verapamil were prepared in dimethyl sulfoxide and then diluted into the recording solution to the desired concentrations for experiments. Control recordings showed that 1.0% dimethyl sulfoxide, the highest concentration used in any experiment, had no detectable effects on the Na^+ currents in CNaIIA-1 cells. For the dose-response curves and some other experiments, the drugs were applied by superfusion; however, it was difficult to maintain gigaseals on CNaIIA-1 cells with this technique. Thus, for most experiments drugs were applied by adding the appropriate volume of stock solution directly to the bath and then gently mixing the drug into the bath for about 5 min. There were no obvious differences in the drug effects using these two methods, and we were able to routinely record from cells for >1 hr by directly adding the drugs to the bath.

Results

Effects of anticonvulsants and antiarrhythmics on Na^+ currents. Depolarization of CNaIIA-1 cells from −85 to 0 mV evoked transient inward currents that inactivated within a few milliseconds, were blocked by tetrodotoxin, and reversed around the Na^+ equilibrium potential. Thus, the currents were due to activation of voltage-gated Na^+ channels. Untransfected CHO cells display little inward current under the same recording conditions (23, 24), indicating that the Na^+ channels expressed in CNaIIA-1 cells were encoded by the transfected cDNA, rather than being native to the cell line.

Fifty micromolar phenytoin, carbamazepine, lidocaine, and verapamil all rapidly reduced the amplitude of the Na^+ currents evoked by infrequently applied stimulus pulses (i.e., ≤0.05 Hz),

without having any obvious effects on their time course (Fig. 1, *curves A* and *B*). Verapamil was the most potent blocker, typically reducing Na^+ currents by 60–80% (Table 1). Phenytoin and carbamazepine were somewhat less effective, reducing Na^+ currents by 20–40%, whereas lidocaine was the least potent tonic blocker, reducing the currents by ~10%.

In addition to this tonic block, the antagonists also inhibited Na^+ currents in a use-dependent manner. Thus, the amplitude of the Na^+ currents became progressively smaller during a 2-Hz train of depolarizing pulses in the presence of the blockers (Fig. 1, *curve C*). As with tonic block, there was considerable variability in the potency of the different drugs for use-dependent block. Verapamil was the most effective, whereas lidocaine and phenytoin had intermediate potencies and carbamazepine was relatively ineffective (Table 1). Both tonic and use-dependent block displayed complex properties, which we characterized in more detail, as described below.

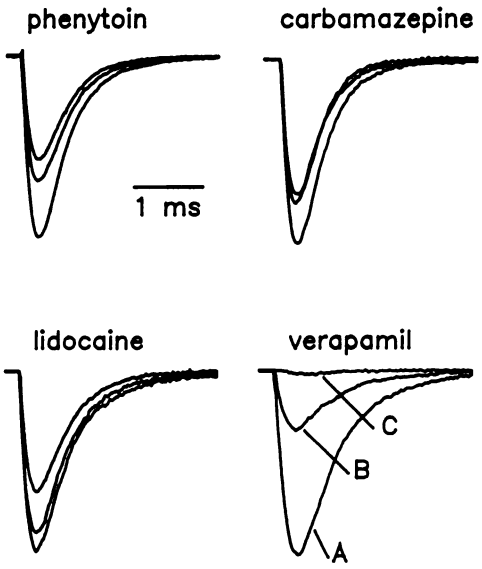

Fig. 1. Tonic and use-dependent block of Na^+ currents in CNaIIA-1 cells by phenytoin, carbamazepine, lidocaine, and verapamil. The cells were held at a membrane potential of −85 mV, and Na^+ currents were evoked by applying stimulus pulses to 0 mV. Each set of current traces is from a single cell and shows currents elicited by stimulus pulses in control conditions (*curve A*), about 5 min after the drugs were washed on (*curve B*), and by the 20th pulse in a train of repetitive, 40-msec-long pulses, at a frequency of 2 Hz (*curve C*). In this and subsequent figures, the drug concentrations were 50 μM, unless stated otherwise. Note that the current records were normalized so that the control currents for each cell are the same size. The amplitudes of the control currents actually ranged from about 3 to 6 nA.

Characterization of tonic block of Na^+ currents by local anesthetics, anticonvulsants, and antiarrhythmics. To examine whether tonic block reflected altered activation or permeability properties of Na^+ channels in CNaIIA-1 cells, we determined current-voltage relationships in control conditions (i.e., in normal bath solution) and then in the presence of the blockers (Fig. 2). From a prepulse potential of −100 mV, inward Na^+ currents were just detectable with depolarizations to approximately −50 mV. The peak Na^+ currents were maximal at around 0 mV, and the currents became outward at depolarizations more positive than about +65 mV, which corresponds to the Na^+ equilibrium potential with the internal and external recording solutions used in these experiments. In the presence of blockers, the currents were smaller at all test potentials, and there were no changes in the activation properties or reversal potential of the currents. The effects of the drugs were partially reversible during washout for as long as stable seals could be maintained. Fig. 2 shows a typical experiment with phenytoin. Similar results were obtained with carbamazepine, lidocaine, and verapamil.

One of the most striking features of tonic block was its strong dependence on the holding membrane potential of the cells. For example, 50 μM phenytoin reduced Na^+ currents by <10% in cells depolarized to 0 mV from a holding potential of −128 mV, but the currents were reduced by >90% when the holding potential was −66 mV (Fig. 3). Carbamazepine, lidocaine, and verapamil were also more potent blockers at more positive holding potentials. This potential-dependence is similar to previous findings (10–13, 19). According to the modulated receptor model, potential-dependence stems from the higher affinity of the drugs for inactivated than for resting channels, resulting in an overall negative shift in the relationship between membrane potential and steady state inactivation.

We investigated the effects of anticonvulsants and antiarrhythmics on steady state inactivation in more detail by determining complete steady state inactivation curves in the absence and presence of drugs, using two different experimental proto-

TABLE 1

Summary of drug effects on rat brain type IIA Na^+ channels expressed in cultured mammalian cells

	Tonic block[a]		Use-dependent block[b]	h_∞ shift (1-min prepulses)[c]	Channel repriming[d]	
	V_m = −85 mV	V_m = −128 mV			τ_1	τ_2
	%		%	mV	msec	
Phenytoin	28.1 ± 3.6 (*n* = 9)	13.9 ± 6.0 (*n* = 4)	19.9 ± 1.5 (*n* = 6)	−9.50 ± 0.40 (*n* = 4)	7.22 ± 0.60 (*n* = 3)	150.60 ± 39.34 (*n* = 3)
Carbamazepine	28.3 ± 4.5 (*n* = 6)	6.8 ± 3.4 (*n* = 3)	8.1 ± 1.8 (*n* = 5)	−6.13 ± 0.73 (*n* = 4)	7.60 ± 0.31 (*n* = 2)	52.91 ± 2.78 (*n* = 2)
Lidocaine	8.6 ± 3.2 (*n* = 7)	2.4 ± 1.4 (*n* = 3)	27.8 ± 2.4 (*n* = 5)	−7.35 ± 1.47 (*n* = 3)	11.63 ± 0.63 (*n* = 3)	667.00 ± 62.12 (*n* = 3)
Verapamil	72.9 ± 3.5 (*n* = 8)	32.4 ± 16.7 (*n* = 3)	79.1 ± 8.6 (*n* = 6)	−16.14 ± 1.91 (*n* = 3)	8.67 ± 0.97 (*n* = 2)	5234.50 ± 390.66 (*n* = 2)
Control			6.7 ± 1.0 (*n* = 17)		5.08 ± 0.25 (*n* = 11)	

[a] The experimental protocol is described in Fig. 1. Data are presented as mean ± standard error and show the percentage of inhibition of the peak currents in the presence of a 50 μM concentration of each drug.

[b] The protocol is described in Fig. 7. The data show the percentage of inhibition of the current evoked by the 20th pulse in a 2-Hz train, compared with the current evoked by the first pulse.

[c] The protocol is described in Fig. 5.

[d] The protocol is described in Fig. 11.

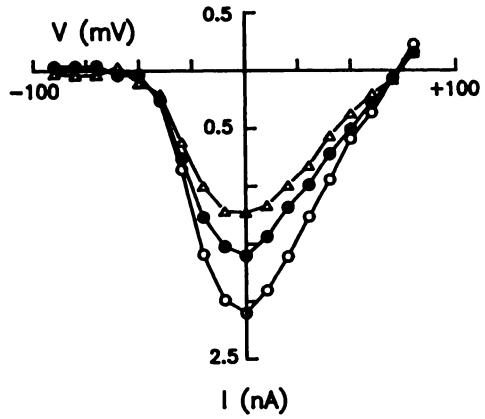

Fig. 2. Effects of phenytoin on the voltage dependence of Na^+ channel activation. The data are from a representative cell in control conditions (○), in the presence of phenytoin (△), and about 10 min after washing out the drug (●). The current-voltage relationships were determined by application of 100-msec prepulses to −95 mV, followed by test pulses to potentials ranging from −85 to +76 mV. The pulses were applied every 3 sec, and the cells were held at −76 mV between stimuli. The amplitudes of the peak currents were plotted as a function of test pulse potential.

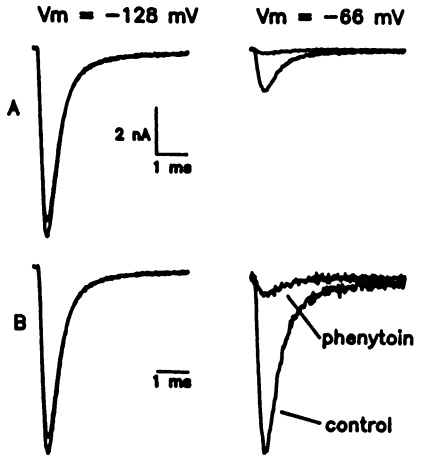

Fig. 3. Effects of holding potential on tonic block of Na^+ currents by phenytoin. The records are from a single representative cell. A, Na^+ currents were evoked by pulsing to 0 mV, from holding potentials of −128 mV (*left*) or −66 mV (*right*), without a prepulse in control conditions and then in the presence of phenytoin. B, The same traces were normalized so that the control currents at −128 and −66 mV are the same size.

cols. In the initial experiments, inactivation was assessed by the amplitude of the currents elicited by a test pulse to 0 mV after a 100-msec prepulse to various potentials. Fig. 4 shows a typical result for phenytoin. The smooth curve through the control data is according to the equation:

$$1/(1 - \exp((E - E_h)/k)) \quad (1)$$

where E = membrane potential, E_h = the prepulse potential where the current is half-maximal, and k = the slope factor. In the presence of drugs, there were relatively small (typically 1–5-mV) negative shifts in the midpoints of the inactivation curves determined in this way. In addition, the inactivation curves in the presence of the drugs had more shallow slopes than control curves, did not reach a plateau at very negative prepulse potentials, and were not well fit by Eq. 1. This probably indicated that, in the presence of the drugs, the 100-msec prepulses were not long enough to reach a steady state at a

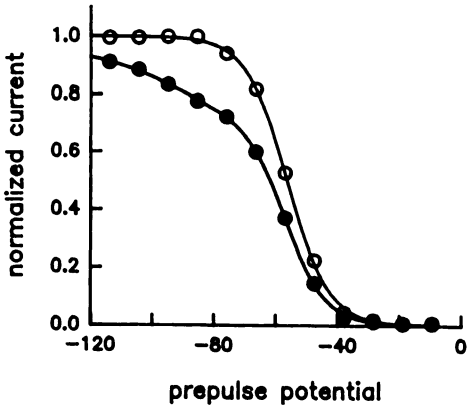

Fig. 4. Effect of phenytoin on the voltage dependence of Na^+ channel inactivation, determined using short prepulses. The data are from a representative cell in control conditions (○) and in the presence of 50 μM phenytoin (●). The fraction of available channels as a function of membrane potential was assessed by application of 100-msec prepulses to potentials from −120 to −10 mV, followed by test pulses to 0 mV. Pulses were applied every 3 sec, and the cell was held at a membrane potential of −76 mV between stimuli. The data were normalized with respect to the amplitude of the current evoked in control conditions after a prepulse to −120 mV. The *smooth curve* through the control data is according to Eq. 1, with $h_{1/2}$ = −56.23 mV and k = 6.73.

given prepulse potential (see below), perhaps due to slowed transitions of drug-bound channels. Thus, to obtain a true steady state measure of the voltage dependence of inactivation in the absence and presence of drugs, the cells were held at various potentials for 1 min, followed by a test pulse to 0 mV. Using this procedure, the shifts in half-inactivation for phenytoin, carbamazepine, lidocaine, and verapamil were approximately −10, −6, −7, and −16 mV respectively (Fig. 5; Table 1). In addition, the inactivation data in the presence of the drugs reached a plateau at negative holding potentials and were well fit by Eq. 1, suggesting that these results were obtained under steady state conditions. Interestingly, there was still significant block with phenytoin, carbamazepine, and verapamil that was constant between holding potentials of about −100 to −128 mV (Fig. 5; Table 1), suggesting that these drugs blocked some resting channels at a concentration of 50 μM.

To examine more directly the difference in affinity of the drugs for resting and inactivated channels, we determined dose-response relationships for phenytoin at holding potentials of −85 mV, where most channels were in the resting state in control conditions, and −62 mV, where more than half the channels were inactivated in control recordings. A typical result from a single cell is shown in Fig. 6. At −85 mV, block of the Na^+ current was just detectable at 10 μM, and even at 100 μM the current was blocked by <50%. The Hill coefficient of the best fit line through the data points was 0.92, and the approximate EC_{50} determined by extrapolation of the best fit line was 162 μM. In contrast, at −62 mV, 1 μM phenytoin reduced the currents by about 10%, and at 100 μM the currents were almost completely blocked. The EC_{50} for phenytoin at −62 mV was 10 μM, and the Hill slope was 0.72. Similar results were obtained in two other cells; the EC_{50} values for all cells were 12.6 ± 3 μM (mean ± standard error) at −62 mV and 195 ± 71 μM at −85 mV.

Use-dependent block with anticonvulsants and antiarrhythmics. Fig. 7 shows the amplitudes of the currents evoked by a train of 20 pulses from −85 to 0 mV, in control conditions and in the presence of 50 μM phenytoin, carbamazepine, lido-

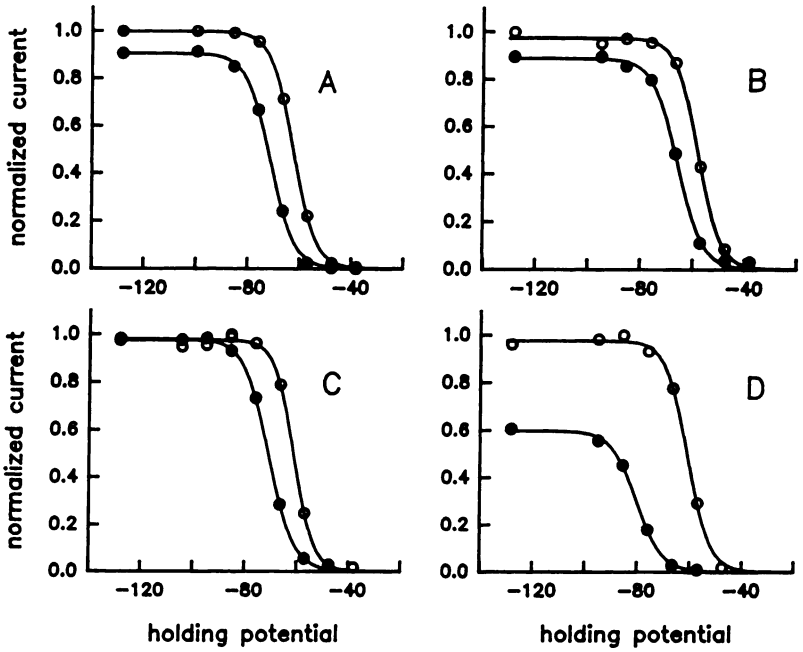

Fig. 5. Drug effects on the voltage dependence of steady state Na^+ channel inactivation. Each graph shows data from a single cell in control conditions (○) and after addition of one of the drugs at 50 μM (●), i.e., phenytoin (A), carbamazepine (B), lidocaine (C), or verapamil (D). The relationship between steady state inactivation and holding potential was determined by holding the cells for 1 min at membrane potentials ranging from −128 to −38 mV and then applying test pulses to 0 mV. The data were normalized with respect to the amplitude of the currents evoked from −128 mV in control conditions. The smooth curves are according to Eq. 1, with the following values for $h_{1/2}$ and k: A, control: $h_{1/2}$ = −62.50 mV, k = 4.32; phenytoin: $h_{1/2}$ = −71.30 mV, k = 4.62; B, control: $h_{1/2}$ = −57.89 mV, k = 4.23; carbamazepine: $h_{1/2}$ = −65.73, k = 4.78; C, control: $h_{1/2}$ = −61.04 mV, k = 3.77; lidocaine: $h_{1/2}$ = −70.76, k = 4.86; D, control: $h_{1/2}$ = −60.71, k = 4.24; verapamil: $h_{1/2}$ = −80.10 mV, k = 4.90.

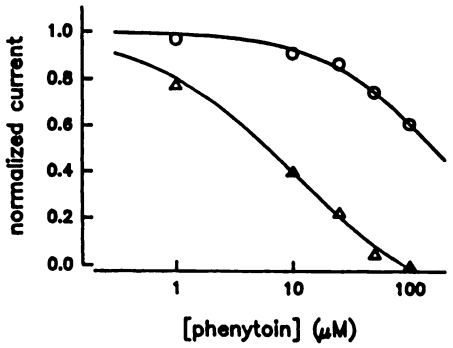

Fig. 6. Dose-response relationship for phenytoin at two different holding potentials. The data are from a representative cell. The drug concentration was varied from 1 to 100 μM by superfusion. Na^+ currents were evoked by pulsing to 0 mV from holding potentials of −85 mV (○) and −62 mV (△). The data were normalized with respect to the amplitudes of the currents evoked under control conditions at each holding potential. The smooth curves are according to the formula $1 - (1/(1 + (EC_{50}/[drug])^{\gamma}))$, with EC_{50} = 162 and 10 μM and n = 0.92 and 0.72 for holding potentials of −85 and −62 mV, respectively.

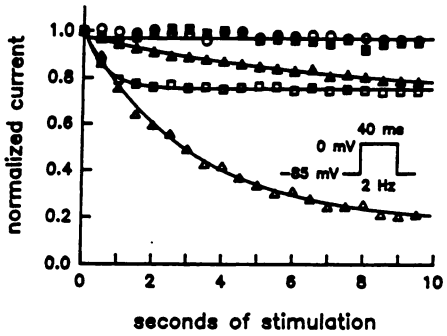

Fig. 7. Use-dependent block of Na^+ currents. The data points are from different cells in the presence of phenytoin (▲), carbamazepine (■), lidocaine (□), or verapamil (△). An example of typical control data is also shown (○). The cells were held at −85 mV, and Na^+ currents were elicited by trains of 20 stimulus pulses to 0 mV, at a frequency of 2 Hz. The duration of the pulses was 40 msec. For each experiment, the current amplitudes were normalized with respect to the current evoked by the first pulse in the train.

caine, and verapamil. The stimulus frequency was 2 Hz and the duration of each pulse was 40 msec. In the control recording, there was a small steady decline in the current amplitude. This use-dependent "droop" was usually ~5% by the 20th pulse in the train. In the presence of phenytoin, lidocaine, and verapamil, there was an additional decline in the currents, due to the accumulation of use-dependent block. The effect was clearly most pronounced with verapamil, where the currents typically declined to 10–30% of those in the first pulse by the end of the pulse train (Table 1). Lidocaine was the next most potent use-dependent blocker. With lidocaine, currents declined in the first few pulses to a steady state amplitude that was ~65–75% of the current evoked by the first pulse in the train. Use-dependent block developed more slowly with phenytoin, and by the 20th pulse in the train Na^+ currents were about 80% of the first pulse. Carbamazepine at a stimulus frequency of 2 Hz displayed virtually no use-dependent block.

The extent of use-dependent block increased with increasing frequency, and there were differences among the drugs in the stimulus frequency at which use-dependent block became clearly detectable (Fig. 8). From a holding potential of −85 mV, stimulus frequencies of about 4 Hz were necessary to clearly observe use-dependent block with 50 μM carbamazepine, whereas 50 μM phenytoin and lidocaine blocked Na^+ currents in a use-dependent manner with stimulus frequencies greater than or equal to about 1 and 0.5 Hz, respectively. The most striking effects were with 50 μM verapamil, where use-dependent block was present at frequencies as low as four pulses/min (0.067 Hz).

Rates of drug association. The accumulation of use-dependent block during a train of stimulus pulses results from the association of the drugs to open and inactivated channels during each depolarizing pulse and the relatively slow recovery of drug-bound channels during the interpulse intervals. To determine whether the large differences in use-dependent block seen with the different blockers reflected differences in either the association or recovery kinetics, we investigated the kinetics of use-dependent block in more detail.

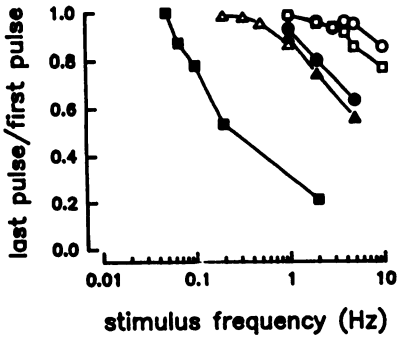

Fig. 8. Extent of use-dependent block at different stimulus frequencies. The data were obtained from five different cells in the presence of phenytoin (●), carbamazepine (□), lidocaine (△ and ▲, representing two different cells), or verapamil (■). Typical control data are also shown (○) (same cell as for carbamazepine). At each frequency, stimulus pulses were applied until the use-dependent block was approximately at steady state. The graph shows the ratio of the amplitudes of the currents evoked by the last pulse in the train divided by the currents evoked by the first pulse in the train, plotted as a function of stimulus frequency.

The rate of drug association was examined by applying a conditioning pulse to 0 mV for varying durations (from 1 to 100 msec), followed by a recovery prepulse to −85 mV for 30 msec and then a test pulse to 0 mV. The fraction of channels that bound drug during the conditioning pulse was determined by taking the ratio of peak Na^+ currents evoked by the test pulse and the conditioning pulse. The recovery interval was chosen to be short enough to "catch" most of the drug-bound channels before the drug dissociated but long enough for complete repriming of unbound channels (see below).

The results indicated that there were differences in the kinetics of association for the different drugs (Fig. 9). With 50 μM phenytoin, carbamazepine, and lidocaine, there was a small rapid component of block with conditioning pulse durations up to 2 msec (Fig. 9, *right*), and then the extent of block increased steadily as the pulse duration was increased up to 100 msec (Fig. 9, *left*). With 100 and 200 μM lidocaine and phenytoin, the overall extent of block was greater, and the initial rapid component was more pronounced. The rapid component of block corresponded to the brief period when most Na^+ channels were open (see the current traces above the panels in Fig. 9). These results indicate that the biphasic time course of association of these drugs resulted from rapid binding to open or partially activated channels, followed by slow binding to inactivated channels. Phenytoin, carbamazepine, and lidocaine bound significantly to both open and inactivated channels. However, because the channel open time was so brief, the use-dependent block shown in Fig. 7 was probably due primarily to drug binding to inactivated channels. In fact, use-dependent block was strongly dependent on pulse duration far beyond the time during which channels were open. When tested with trains of stimulus pulses of variable duration, with the interpulse interval maintained constant at 500 msec, no use-dependent block was seen with phenytoin or lidocaine during pulse trains of 4 msec or less (Fig. 10). Increased use-dependent block was observed for pulse durations of 20, 40, and 100 msec for these drugs, even though the channels were open only during the first 3 msec.

The time course of association for verapamil was clearly different from that for the other drugs. With 50 μM verapamil, the extent of block increased rapidly for pulse durations up to 2 msec and then increased much more gradually as the pulse duration was further increased up to 100 msec (Fig. 9). When the verapamil concentration was increased to 100 μM, the extent of the rapid block was approximately doubled, but the slower component seen at longer pulse durations was essentially unchanged. These results indicate that verapamil bound rapidly to open channels and much more slowly to inactivated channels. Consistent with this, in the presence of 50 μM verapamil dramatic use-dependent block was seen with stimulus pulses as short as 0.6 msec, and the extent of block increased substantially for stimulus pulses of 1 msec (Fig. 10). In contrast, the increment in use-dependent block observed when the pulse duration was increased from 1 msec to 20, 40, and 100 msec was modest, indicating a less important role for binding to inactivated channels in the use-dependent block by verapamil. (Fig. 10).

Rates of drug dissociation. The rate of channel recovery from inactivation was examined by application of a 100-msec conditioning pulse to 0 mV, followed by a recovery pulse to −85 mV for varying durations and then a test pulse to measure the extent of channel repriming. In control conditions, the rate of recovery was well fit by a single exponential function with a time constant of about 4–6 msec (Fig. 11; Table 1). Recovery was essentially complete within 30 msec. In the presence of 50 μM phenytoin, carbamazepine, lidocaine, or verapamil, recovery had two distinct components and was well fit by the sum of two exponentials. The time constant for the fast component was typically around 6–12 msec, whereas the time course of the slow component depended on which drug was present, i.e., about 50 msec for carbamazepine, 100–200 msec for phenytoin, 600–700 msec for lidocaine, and 5 sec for verapamil (Table 1). Presumably, the rapid component of recovery reflected the rapid recovery of channels that did not bind drug during the conditioning pulse, whereas the slow component of repriming reflected the relatively slow recovery of drug-bound channels. The large difference in the rate of the slow recovery for the different drugs was the main factor determining their potencies as use-dependent blockers.

It has previously been shown that recovery of both unbound and drug-bound Na^+ channels is faster at hyperpolarized membrane potentials (12). Consistent with this, we found that, for phenytoin, lidocaine, and verapamil, use-dependent block was less when stimulus pulses were applied from a holding potential of −128 mV, compared with a holding potential of −85 mV (Fig. 12). The dependence of recovery on membrane potential was also more directly examined with lidocaine, using the two-pulse protocol described in Fig. 10, with recovery prepulses to −85 mV and −128 mV (Fig. 13). In control conditions, the time constants of recovery at −85 mV and −128 mV were 5.5 and 1.0 msec, respectively. Thus, recovery from inactivation was about 5 times faster at −128 mV than at −85 mV. In the presence of 50 μM lidocaine, the fast component of recovery had time constants of 10.9 and 1.6 msec at −85 and −128 mV, whereas the slow component of recovery had time constants of 775 and 136 msec. Thus, lidocaine-bound channels also recovered more rapidly at −128 mV than at −85 mV.

Discussion

Inhibition of type IIA Na^+ channel α subunits by local anesthetic, antiarrhythmic, and anticonvulsant drugs, in a frequency- and voltage-dependent manner. A major finding of this study is that local anesthetic, antiarrhythmic, and anticonvulsant drugs block cloned rat brain type IIA Na^+

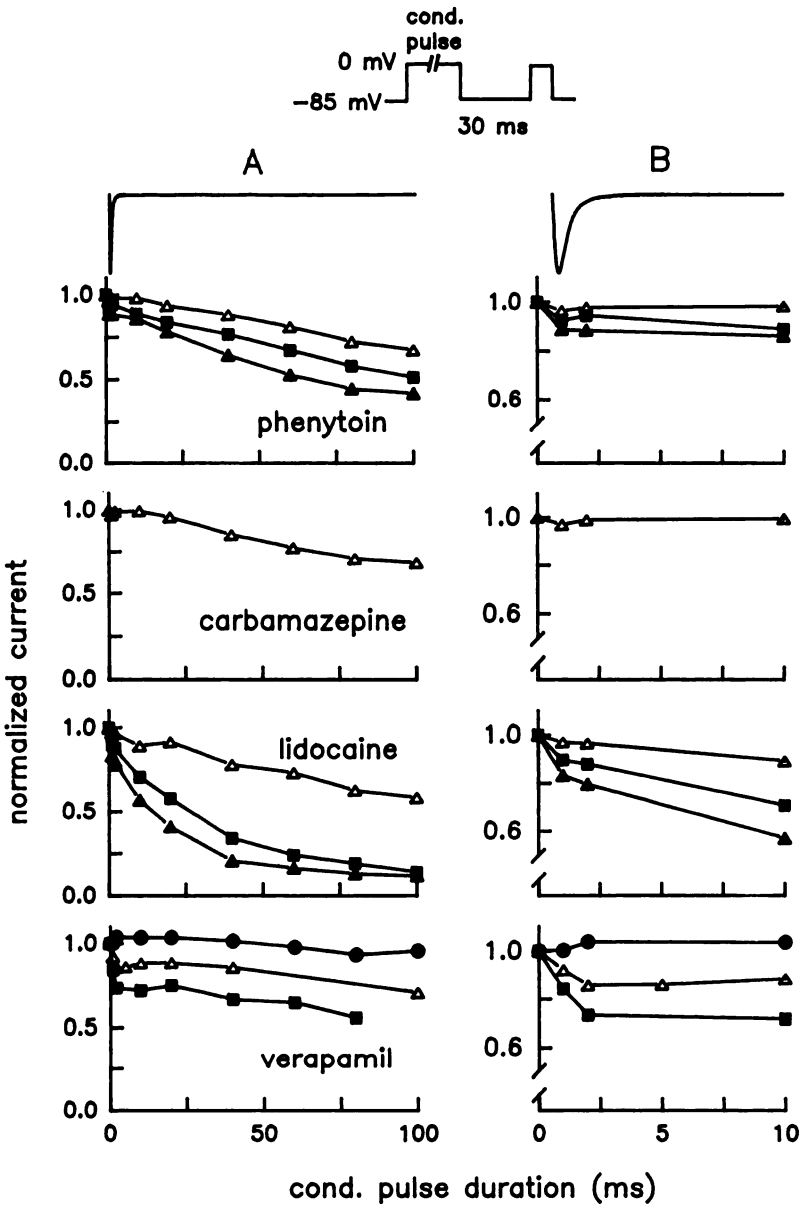

Fig. 9. Rate of development of drug block during depolarization. The data were obtained in the presence of phenytoin, carbamazepine, lidocaine, or verapamil, at concentrations of 50 (△), 100 (■), or 200 (▲) μM. The data for phenytoin, lidocaine, and verapamil were from two different cells for each drug (designated by *open* and *filled symbols*). The graph for verapamil also shows typical control data (●). A, Development of drug block was assessed by application of a conditioning pulse to 0 mV, of variable duration (1–100 msec), followed by a 30-msec recovery interval to −85 mV and then a test pulse to 0 mV. The amplitudes of the currents evoked by the test pulse were normalized with respect to the currents elicited by the conditioning pulse and are plotted as a function of conditioning pulse duration. B, The same data, showing conditioning pulses up to 10 msec on an expanded time scale. *Traces above each column*, typical Na^+ currents, during depolarizations to 0 mV, on the same time scales as the respective graphs.

channel α subunits, expressed in a heterologous mammalian cell line, in a manner that is dependent on holding potential and frequency of stimulation, as for native sodium channels. The results indicate that these drugs bind with higher affinity to open and inactivated channels than to resting channels. They are consistent with extensive previous studies of frequency- and voltage-dependent block of Na^+ channels in neurons and cardiac myocytes (10–13, 19). Assuming that CHO cells do not express endogenous Na^+ channel β subunits, the results show that the α subunit alone is responsible for the characteristic pharmacological interactions between Na^+ channels and local anesthetics, antiarrhythmics, and anticonvulsants. The preparation used in this study presents important experimental advantages, because it consists of a homogeneous population of a known rat brain Na^+ channel subtype expressed in a cell line that has low endogenous levels of Na^+, Ca^{2+}, and K^+ channels and is amenable to high resolution whole-cell voltage-clamp recording techniques. These favorable characteristics have allowed us to make a more complete study of the interactions of these drugs with brain sodium channels than was possible previously.

Inhibition of type IIA Na^+ channels as an anticonvulsant mechanism. The findings for phenytoin and carbamazepine are directly relevant to their pharmacological actions, because these drugs are thought to exert their anticonvulsant effects mainly by blocking brain Na^+ channels (10, 11). Typical therapeutic concentrations for these drugs in the cerebrospinal fluid are about 3–8 μM (10, 11). Although 10 μM phenytoin (approximately the therapeutic cerebrospinal fluid concentration) had little effect on Na^+ currents in CNaIIA-1 cells held at −85 mV, it strongly blocked currents when the holding potential was −62 mV. Thus, at clinically relevant concentrations, phenytoin blocked Na^+ currents mediated by type IIA α subunits at the relatively positive resting membrane potentials characteristic of neurons during seizure activity, but not at the more hyperpolarized resting membrane potentials typical of neurons during normal cell activity. These results are similar to those of previous drug binding, ion flux, and electrophysiological studies, in which phenytoin and carbamazepine were shown to bind to and inhibit Na^+ channels in mammalian neurons at therapeutically relevant concentrations (29–34).

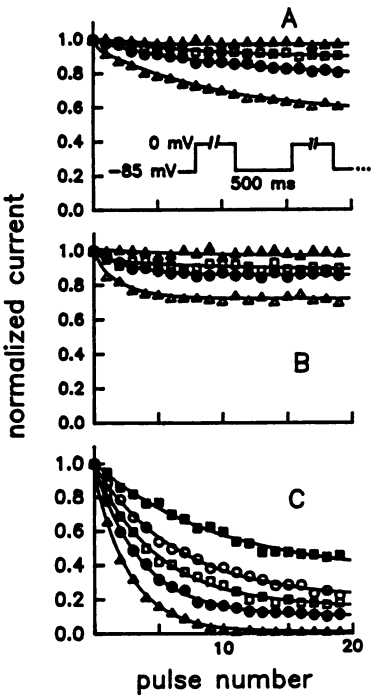

Fig. 10. Effect of pulse duration on use-dependent block. Each graph shows data from a single cell, with phenytoin (A), lidocaine (B), or verapamil (C). The cells were held at −85 mV, and Na^+ currents were evoked by a train of 20 stimulus pulses to 0 mV. The durations of the pulses were 4 (▲), 20 (□), 40 (●), and 100 (△) msec for A and B and 0.6 (■), 1 (○), 20 (□), 40 (●), and 100 (△) msec for C. The duration of the interpulse interval was held constant at 500 msec. The data were plotted as described in Fig. 7.

The voltage dependence of drug binding presumably explains, at least in part, the ability of phenytoin to block seizures without affecting normal brain function (10, 11, 32).

Neither phenytoin nor carbamazepine was a potent use-dependent blocker of type IIA Na^+ channels at a stimulus frequency of 2 Hz. However, use-dependent block with these drugs became more pronounced at higher stimulus frequencies. These use-dependent effects may be important for filtering the high frequency bursts of action potentials characteristic of epileptiform activity.

Type I and II/IIA Na^+ channels account for >85% of adult brain Na^+ channels in the rat but are not highly expressed in peripheral neurons or peripheral nerves (20). Type II/IIA Na^+ channels are preferentially localized in axons of central neurons (22). They may be responsible for initiation of the conducted action potential at the axon initial segment and conduction along the axon. Our results imply that both initiation and propagation of action potentials in central neurons would be inhibited by phenytoin and carbamazepine. Type I Na^+ channels are localized primarily in the cell bodies of major projection neurons in the brain (22). It will be of interest in the future to compare the actions of anticonvulsant drugs on type I and type IIA Na^+ channels expressed in a mammalian cell line.

Lidocaine and verapamil also have anticonvulsant properties (15–18); however, it is not clear whether either of these drugs would be clinically useful for treating seizures. In at least one case, lidocaine suppressed seizures in a human epileptic after more conventional anticonvulsants had proven ineffective (16). Thus, lidocaine may have some utility. However, verapamil, at concentrations sufficiently high to inhibit seizures, would have undesired side effects, due to its actions on peripheral Ca^{2+} channels. It, therefore, would probably not be a clinically useful anticonvulsant, even if it crossed the blood-brain barrier effectively.

Characterization of frequency- and voltage-dependent drug effects. The strong voltage dependence of block by the local anesthetics, anticonvulsants, and antiarrhythmics studied here reflected an overall negative shift in the relationship between steady state inactivation and membrane potential. The time constants for Na^+ channel inactivation in various cell types are typically a few milliseconds and, therefore, experimental protocols that assess steady state inactivation usually use relatively short (e.g., 100-msec) prepulses, followed by a test pulse to measure the ratio of resting and inactivated channels. However, in this study 100-msec prepulses were too

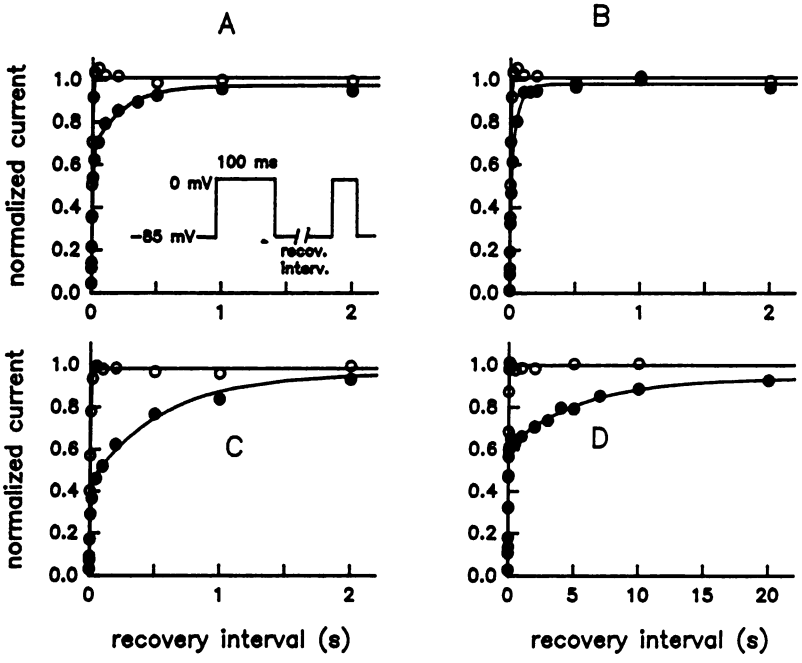

Fig. 11. Drug-induced slowing of Na^+ channel recovery. The graphs show data from four cells in control conditions (○) and after addition of 50 μM levels of the indicated drug (●), i.e., phenytoin (A), carbamazepine (B), lidocaine (C), or verapamil (D). The extent of channel repriming was determined by applying 100-msec conditioning pulses to 0 mV and then stepping to −85 mV for varying durations, followed by test pulses to 0 mV. The amplitudes of the currents evoked by the test pulses were normalized with respect to the currents elicited by the conditioning pulses and are plotted as a function of the recovery interval. The *smooth lines* through the control data are according to the equation $1 - \exp(-t/\tau)$, where t is recovery interval and τ = 5.20, 5.86, 6.13, and 4.72 msec for A, B, C, and D, respectively. The data in the presence of drugs were fit by $1 - (c_1 \exp(-t/\tau_1) + c_2 \exp(-t/\tau_2))$, with τ_1 = 7.17, 7.27, 11.03, and 7.69 msec and τ_2 = 228.6, 55.6, 559.8, and 5624.0 msec for A, B, C, and D, respectively. Note the different time scale for D.

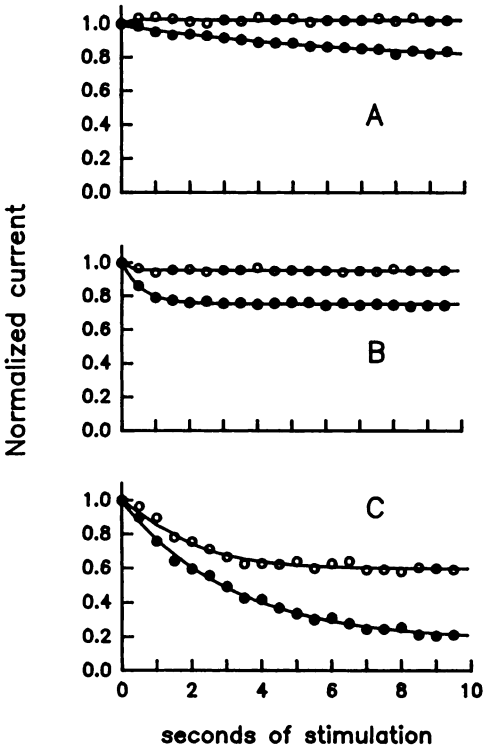

Fig. 12. Effect of holding potential on use-dependent block. Each graph shows data from a single cell, with phenytoin (A), lidocaine (B), or verapamil (C). A 2-Hz train of 40-msec-long pulses was applied from holding potentials of −128 (○) and −85 (●) mV. Current amplitudes were normalized with respect to the first pulse of the train.

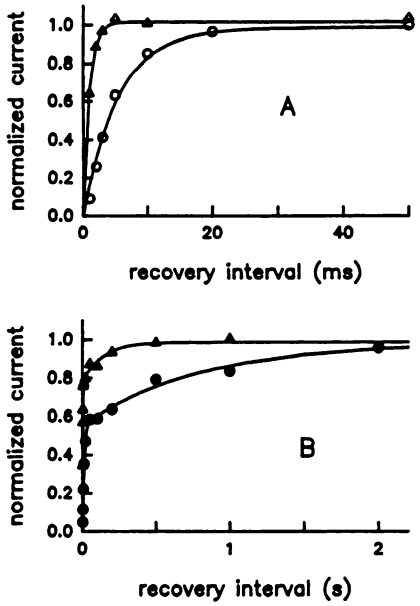

Fig. 13. Voltage dependence of Na^+ channel recovery. The rate of recovery from inactivation was assessed by the two-pulse protocol described in Fig. 10, but with the membrane potentials during the recovery intervals set to −85 mV (*circles*) and −128 mV (*triangles*), in control conditions (A) and in the presence of 50 μM lidocaine (B). The *smooth lines* were according to the equations given in Fig. 10, with the following time constants: A, τ = 5.5 and 1.0 msec for recovery pulses to −85 mV and −128 mV, respectively; B, τ_1 = 10.9 and 1.6 and τ_2 = 775 and 136 msec for −85 mV and −128 mV, respectively. Note the different time scales for A and B.

short to reach steady state conditions in the presence of the drugs, probably because of the relatively slow rate of drug dissociation during hyperpolarizing prepulses and slow association during depolarizing prepulses (see Figs. 9 and 11). Thus, a more accurate measure of steady state inactivation was obtained by varying the holding potential for 1 min before application of the test pulses. The dependence of steady state inactivation on prepulse duration could also be explained if the drugs stabilize Na^+ channels in "slow" inactivation states (30, 35, 36). These slow inactivation states have time constants of hundreds of milliseconds to many seconds and, therefore, would not be measured with short prepulses. Na^+ channels in CNaIIA-1 cells appear to have slow inactivation states, although they have not been studied in detail. The role, if any, of slow inactivation in the effects of anticonvulsants and local anesthetics in CNaIIA-1 cells is not clear and requires further investigation.

The drugs bound to both open and inactivated channels; however, association was faster to open channels. Similar fast association to open Na^+ channels has been observed for lidocaine in rabbit cardiac muscle (37) and toad sciatic nerve fibers (38). These results are consistent with the model proposed by Hille (19), in which local anesthetics bind rapidly to open channels by a hydrophilic route through the cytoplasm but bind more slowly to inactivated channels by a hydrophobic route through the plasma membrane.

The mechanism by which local anesthetic, antiarrhythmic, and anticonvulsant drugs slow channel repriming is not well understood. Slow repriming could reflect either slow drug dissociation from inactivated or resting channels or slow conversion of drug-bound channels from the inactivated to the resting states. Drug-bound Na^+ channels in CNaIIA-1 cells recovered more rapidly at hyperpolarized membrane potentials. Similar voltage dependence has been observed for lidocaine in cardiac cells (12, 37) and phenytoin in mouse neuroblastoma cells (30). Thus, in these preparations, there is a voltage-dependent, rate-limiting step for repriming of drug-bound channels. However, no voltage dependence of repriming was reported for lidocaine in toad sciatic nerve (38) or squid axon (39). This suggests that there may be different mechanisms of drug action for different types of Na^+ channels or that different recovery steps may be rate limiting.

Verapamil as a Na^+ channel blocker. Verapamil was a surprisingly potent blocker of rat brain Na^+ channels in CNaIIA-1 cells. The concentrations of verapamil used in this study were more than 10 times higher than those that are effective in blocking cardiac Ca^{2+} channels (40, 41). However, because both the tonic and use-dependent effects of verapamil were quite pronounced, it will be interesting to determine whether it also blocks cardiac Na^+ channels at concentrations closer to those at which it is an effective Ca^{2+} antagonist. An important implication of the results is that, when both Na^+ and Ca^{2+} channels are present, effects that are attributed strictly to verapamil block of Ca^{2+} channels may also be due, at least in part, to block of Na^+ channels.

It has previously been shown that, at concentrations ranging from 50 to 200 μM, verapamil and its methoxy derivative D600 block Na^+ currents in squid axons and cardiac muscle cells (14, 42) and inhibit veratridine-activated Na^+ flux in rat synaptosomes, chick cardiac cells, and mouse neuroblastoma cells (43, 44). However, the electrophysiological properties of this block were not well characterized in any of these studies. In this

study, we found that verapamil produced strong negative shifts in steady state inactivation and pronounced use-dependent block. These characteristics were similar to the effects of local anesthetics and anticonvulsants, suggesting that these drugs all have a similar mode of action. However, in several respects, the properties of verapamil block differed from those of the other drugs. For example, compared with phenytoin, carbamazepine, or lidocaine, verapamil bound more rapidly to open channels and more slowly to inactivated channels. Repriming of verapamil-bound channels was also quite slow, compared with the other drugs. In these respects, the characteristics of verapamil block of Na^+ channels were more similar to its effects on Ca^{2+} channels (40). This suggests that verapamil may have a similar mechanism of action for these two channel types and may act at analogous receptor sites on both.

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