Blockade of Cardiac Sodium Channels by Amitriptyline and Diphenylhydantoin

Evidence for Two Use-Dependent Binding Sites

Michael J. Barber, C. Frank Starmer, and Augustus O. Grant

Cardiac toxicity is a frequent manifestation in amitriptyline overdose and is felt to be due, in part, to sodium channel blockade by the drug. Another agent with sodium channel blocking properties, diphenylhydantoin, has been used clinically to reverse cardiac conduction abnormalities induced by amitriptyline. This reversal of toxicity is believed to occur secondary to competition for the sodium channel binding site. We evaluated individually and in combination the effects of amitriptyline (0.4 μM) and diphenylhydantoin (10–80 μM) on the sodium current in isolated rabbit atrial and ventricular myocytes at 17°C. Using the whole-cell variant of the patch-clamp technique, we found that both amitriptyline and diphenylhydantoin reduced the sodium current in a use-dependent fashion. The time constant of recovery (τr) from block by amitriptyline at −130 mV was very slow (13.6±3.2 seconds), whereas τr during diphenylhydantoin exposure was fast (0.71±0.21 seconds, p<0.0001 compared with amitriptyline). During exposure of cells to a mixture of the two drugs, τr was found to be 6.6±1.8 seconds, but no evidence of direct competition between amitriptyline and diphenylhydantoin was seen. Attempts to fit the recovery data of the mixture to two exponentials resulted in no significant improvement in the fit when compared with that using a single exponential. Use of the sodium channel blocking agent lidocaine (similar kinetics to diphenylhydantoin) in competition with amitriptyline resulted in findings consistent with direct competition of these two drugs for a single binding site. These observations prompted us to evaluate the possibility that diphenylhydantoin was not acting at (and therefore not competing for) the same channel binding site as amitriptyline. Experiments altering pHr and pHc revealed dramatic differences between amitriptyline and diphenylhydantoin. When pHr was increased from 7.4 to 8.0, τr was reduced approximately threefold (from 13.6±3.2 to 4.2±0.1 seconds, p<0.0001) during exposure to amitriptyline, but no effect was seen on τr after exposure to diphenylhydantoin. Conversely, when pHc was increased from 7.3 to 8.0, τr after amitriptyline was unaffected, but τr after diphenylhydantoin markedly increased (from 0.71±0.21 to 2.60±1.30 seconds, p<0.001). Additionally, diphenylhydantoin block demonstrated profound voltage dependence across the range of −130 to −90 mV, whereas amitriptyline block appeared less voltage sensitive. Single-channel studies using patch-clamp techniques in isolated ventricular myocytes supported these data. Superfusion of cells with diphenylhydantoin did not change mean channel open time but increased the probability of failure of the channel to open. When diphenylhydantoin was placed in the micropipette, no effect on sodium channel kinetics could be demonstrated. Our experiments show that amitriptyline blocks sodium channels via a drug-receptor complex site sensitive to changes in pHr, whereas diphenylhydantoin blocks from a pHr-insensitive site that is susceptible to changes in pHc. The decrease in amitriptyline-induced τr by diphenylhydantoin may result from allosteric modulation and suggests the presence of a separate (intracellular) binding site for diphenylhydantoin action. (Circulation Research 1991;69:677–696)

From the Departments of Medicine and Computer Science, Duke University Medical Center, Durham, N.C., and the Department of Medicine, Division of Cardiology, University of Virginia Health Sciences Center, Charlottesville, Va.

Supported in part by grants HL-32994 and HL-32708 from the National Institutes of Health, National Research Service Award ST32 HL-07101 from the National Heart, Lung, and Blood Institute, a Grant-in-Aid and an Established Investigatorship from the American Heart Association (A.O.G.), a Grant-in-Aid (VA-90-G25) from the American Heart Association (Virginia Affiliate), and contract 44 14 808 from the Office of Naval Research.

Address for correspondence: Dr. Augustus O. Grant, Box 3504, Duke University Medical Center, Durham, NC 27710.

Received December 20, 1990; accepted May 6, 1991.
Amitriptyline, a frequently prescribed tricyclic antidepressant, is often associated with drug overdose.\(^1\)\(^2\) Cardiac toxicity in the form of impaired conduction,\(^3\)\(^4\) decreased contractility, and life-threatening arrhythmias\(^5\)-\(^7\) is seen. The mechanism(s) by which amitriptyline produces its toxic effects is unknown, but some of these actions may be due to its sodium channel blocking properties.\(^1\)\(^8\)\(^9\) Hagerman and Hanashiro\(^1\) and Boehnert and Lovejoy\(^8\) demonstrated improvement in cardiac conduction as measured by narrowing or normalization of PR interval and QRS duration abnormalities when patients who had taken an overdose of amitriptyline were treated with diphenhydantoin. Some authors\(^10\)\(^11\) even recommend administration of diphenhydantoin prophylactically in tricyclic antidepressant overdose. Callaham\(^12\) and Davis,\(^13\) on the other hand, could demonstrate no beneficial effect of diphenhydantoin after amitriptyline overdose. Experimental data in whole animals have been equally confusing when amitriptyline toxicity is treated with diphenhydantoin or other type IB agents.\(^14\)-\(^16\)

Recent theoretical and experimental studies\(^17\)-\(^22\) suggest that under certain circumstances the combination of two sodium channel blocking agents with markedly different binding kinetics may produce smaller levels of sodium channel block than that produced by the agent with the slow kinetics alone. A drug with rapid association and dissociation kinetics could theoretically compete for sodium channel binding sites and displace a drug with slower binding kinetics. This could lead to a reversal of cardiotoxic manifestations that result from sodium channel blockade. We recently have demonstrated such a phenomenon between the opiate analgesic propoxyphene and lidocaine.\(^22\)

Prior voltage-clamp experiments have shown that diphenhydantoin has rapid binding and unbinding kinetics to the cardiac sodium channel\(^23\)-\(^25\) and as such would in principle displace amitriptyline from its binding site. We set out to determine whether such a competitive blockade of the cardiac sodium channel could be demonstrated in vitro. First, we analyzed the kinetics of development of and recovery from block of the sodium current recorded from isolated rabbit atrial myocytes under voltage-clamp conditions during exposure to amitriptyline. Then, we confirmed that diphenhydantoin had rapid blocking and unblocking kinetics under our study conditions. Although we could demonstrate modulation of amitriptyline by diphenhydantoin, we could not demonstrate a true reversal of amitriptyline-induced blockade of the cardiac sodium channel by diphenhydantoin. This suggested that the two drugs may be acting at different sites.

We evaluated the effects of internal and external pH changes on the kinetics of recovery from block by amitriptyline (pK 9.4) and diphenhydantoin (pK 8.3). The kinetics of recovery from block by amitriptyline were slowed by a reduction of external pH only. In contrast, the kinetics of recovery from diphenhydantoin-induced block were accelerated by a decrease in internal pH. These data suggest that diphenhydantoin blocks the cardiac sodium channel from a site (intracellular?) insensitive to external protons. We were able to show that lidocaine, a drug with fast binding and unbinding kinetics to a site sensitive to external protons, may produce partial reversal of amitriptyline-induced sodium channel blockade.

**Materials and Methods**

**Cell Preparation**

We performed the whole-cell voltage-clamp experiments on myocytes isolated from the atria of adult rabbits. The methods of cell isolation and culture have been described in detail in previous publications from our laboratory.\(^26\) Briefly, the heart was isolated and perfused for 5 minutes with Ca\(^{2+}\)-free Krebs-Henseleit (K-H) solution (see below for solution composition). Sterile technique was used, and all solutions were maintained at 37°C. After 5 minutes, the K-H Ca\(^{2+}\)-free perfusate was changed to K-H solution containing 180 units/ml collagenase (Worthington Biochemical Corp., Freehold, N.J.) and 0.1 mg/ml hyaluronidase (Sigma Chemical Co., St. Louis, Mo.), and the hearts were perfused for 25–45 minutes. Enzyme activity was terminated with K-H solution and 10% fetal bovine serum. Ventricular tissue was separated from the atria. The atria were minced into small segments, transferred to K-H medium containing elastase (0.5 mg/ml, Sigma), and gently agitated. On completion of the dissociation procedure, single myocytes were separated from tissue chunks by filtration through a 200-μm nylon mesh. The isolated myocytes were washed twice with K-H solution and resuspended in a medium consisting of F-12 Ham/Dulbecco’s modified Eagle’s Medium/antibiotics. Cells were then plated onto 18×18-mm laminin-coated coverslips and stored in a CO\(_2\) incubator (Queue Systems, Inc., Parkersburg, W.Va.) at 37°C. Under these conditions, rodlike atrial cells assume a spherical shape after 24–48 hours in culture. Atrial cells were used after they had assumed a spherical configuration, because theoretical analysis has indicated that the spherical shape is most favorable for obtaining a homogeneous potential under voltage-clamp conditions.\(^25\)\(^26\) Ventricular cells were used 2–4 hours after isolation.

**Solutions**

The K-H solution used in the cell isolation procedure had the following composition (mM): NaCl 118.2, CaCl\(_2\) 2.7, KCl 4.7, MgSO\(_4\)·7H\(_2\)O 1.2, NaHCO\(_3\) 25, NaH\(_2\)PO\(_4\) 2, and glucose 10. Calcium-free K-H solution had no added calcium. K-H solution was gassed with a 95% O\(_2\)-5% CO\(_2\) mixture.

For whole-cell recording, the standard intracellular solution had the following composition (mM): CsF 120, MgCl\(_2\) 5, K\(_2\)ATP 5, KH\(_2\)PO\(_4\) 1, EGTA 5, glucose 5, and HEPES 10. The pH of the internal solution was adjusted to 7.3 or 8.0 on the day of the
experiment using CsOH (1 M). The internal solution was filtered with a 0.3-μm filter at time of use. The external solution contained the following composition (mM): NaCl 75, MgCl₂ 5.0, CaCl₂ 1.5, glucose 5, and HEPES 10. The pH of the external solution was adjusted to the desired value (7.4 or 8.0) on the day of the experiment with NaOH (1 M).

For cell-attached patch recordings, the standard micropipette solution had the following composition (mM): NaCl 182, MgCl₂ 5.0, CaCl₂ 0.2, CsCl 1.0, and HEPES 5. The pH was adjusted to 7.4 with NaOH. The isolated myocytes were superfused in these experiments with a high external potassium solution of the following composition (mM): KCl 70, potassium aspartate 80, NaCl 5, MgCl₂ 3, K₂EGTA 0.05, HEPES 5, NaH₂PO₄ 1.2, and glucose 10. The pH was adjusted to 7.4 with KOH. The high external potassium solution was used to depolarize the cells to −0 mV and to enable us to express membrane potentials as absolute voltage.

In whole-cell experiments, the cells were perfused with the external solution alone (control) or with external solution containing 0.4 μM amitriptyline (Sigma), 10–160 μM diphenylhydantoin (Sigma), or a mixture of 0.4 μM amitriptyline and 10–80 μM diphenylhydantoin dissolved in the superfuse. Additionally, in a series of experiments, lidocaine (80 μM) was added and superfused in combination with amitriptyline (0.4 μM). On the day of each experiment, fresh stock solutions of amitriptyline (0.4 mM), diphenylhydantoin (1 mM), and/or lidocaine (80 mM) were made up by dissolving the drug in the external solution. Diphenylhydantoin goes into solution poorly and dissolves only at high pH (>11.0). This required the stock solution to be very alkaline. Stock solutions then were added to buffered external solution in appropriate amounts to give the desired final concentration of drug(s). Solutions containing diphenylhydantoin were rechecked to ensure that no significant alteration in pH occurred.

**Whole-Cell Recording Techniques**

Micropipettes were pulled from 1.5-mm o.d. borosilicate glass (N-51A, Drummond Scientific Co., Broomall, Pa.) using a horizontal puller (model P 80/PC Flaming Brown, Sutter Instrument Co., Novato, Calif.). Micropipettes were coated with Sylgard 184 (Dow Corning, Midland, Mich.) to lower microelectrode capacitance and then fire-polished on a microforge (model MF-83, Narishige, Tokyo). For whole-cell current recordings, 400–1,000-kΩ microelectrodes were used. Each microelectrode was coupled to the input of the patch-clamp amplifier (model EPC 7, List Electronics, Darmstadt, FRG, or model 3900, Dagan Corp., Minneapolis, Minn.) by an Ag/AgCl wire coated with Teflon up to its tip (In Vivo Metric Systems, Healdsburg, Calif.). A similar wire was used to make a bath reference, which was inserted in an agar bridge (3% agar dissolved in internal solution). With this bridge, voltage offset was typically 1–5 mV. These were nulled before obtaining recordings.

Whole-cell currents were filtered at a corner frequency of 5 kHz with an eight-pole Bessel filter (model 902, LPF Frequency Devices, Haverhill, Mass.) and digitized using a Compaq 386/20 microcomputer at 40 kHz. Data were stored on magnetic tape and analyzed off-line (see data analysis).

**Whole-Cell Experimental Protocols**

On the day of the study, an 18×18-mm coverslip containing the atrial cells was fixed to the base of the recording chamber, which sat on a thermostatically controlled Peltier device (TS-2 thermal microscope stage, Sensortek, Clifton, N.J.) on the stage of an inverted microscope (Nikon Diaphot, Nikon Instruments, Garden City, N.Y.). The temperature was set at 17°C, and the bath was perfused at 1 ml/min with cooled external solution. The inflow to the bath was controlled by a series of solenoid valves, which permitted the rapid switch of perfusion solutions. To record in the whole-cell configuration, a gigohm seal was obtained as outlined by Hamill et al.28 The capacitance of the microelectrode and amplifier input were nulled. The membrane patch then was ruptured by a brief pulse of suction, and the cell capacitance and series resistance were nulled. Finally, series resistance compensation was applied to a level just below that which produced ringing (usually 70–90%). The holding potential was fixed at −130 mV and a current–voltage curve was obtained as shown in Figure 1. Voltage error, peak current, and series resistance were determined using readings from the patch-clamp amplifier and from the equation

\[ \Delta V = I_p (1-a) R \]

where \( \Delta V \) is voltage error, \( I_p \) is peak current, \( a \) is percent compensation, and \( R \) is series resistance. If \( \Delta V \) was >3 mV, the experiment was abandoned.

Before obtaining other information, the current–voltage relation was determined using 50-msec pulses of increasing amplitude applied at 1,500-msec intervals. The pulse was incremented in 5-mV steps from a potential of −120 to +70 mV. We proceeded with the experimental protocol if the currents of the negative limb of the current–voltage curve showed a gradual increase with progressively larger depolarizations (onset to peak sodium currents spanning 30–40 mV, see Figure 1).

A steady-state inactivation curve was determined using a 1-second prepulse to potentials from −170 to −50 mV, followed immediately by a 10-msec test pulse to −30 mV. The prepulse potential was incremented by 5 mV in each subsequent test. A representative steady-state inactivation curve is shown in Figure 1C with the data plotted in Figure 1D.

In the first series of experiments, trains of 40 pulses (pulse duration, 50 msec; holding potential, −130 mV; step, to −30 mV) with a frequency of 5 Hz were applied from the holding potential to determine the amount of steady-state block in the absence or presence of drug(s) (see below). In these studies, current from the first pulse and last pulse of the train was...
measured, and steady-state block was expressed as a percent of the first pulse current. To determine recovery from steady-state block, pulse trains were followed by a variable interval of recovery (2-50,000 msec) to the holding potential, and a subsequent 20-msec test pulse to $-30$ mV was applied to assess available sodium current. Measurements were made in the absence of drugs (control) as well as in mixtures containing amitriptyline alone (0.4 μM), diphenylhydantoin alone (10-80 μM), amitriptyline (0.4 μM) plus diphenylhydantoin (20-80 μM), and amitriptyline (0.4 μM) plus lidocaine (80 μM). During control conditions and in the presence of diphenylhydantoin or lidocaine alone there was a 15-second rest interval between each train. During any protocol in which amitriptyline was used as a component of the superfusate, a 50-second rest interval was used between each test pulse and the succeeding train or prepulse.

The development of frequency-dependent block was determined by the application of trains of 50-msec pulses to $-30$ mV from the holding potential of $-130$ mV with stimulus frequencies ranging from 0.1 to 5 Hz. There were 40 pulses in trains with stimulus frequencies of 5, 4, and 2 Hz, 30 pulses in trains with stimulus frequencies of 1 and 0.5 Hz, and 20 pulses in trains with stimulus frequencies of less than 0.5 Hz. This was done because steady-state block was achieved with a smaller number of pulses at slower stimulus frequencies.

We analyzed the dependence of development of steady-state block on membrane potential in the absence of drug and in the presence of amitriptyline or diphenylhydantoin. By using trains of forty 50-msec pulses stepped to $-30$ mV at a frequency of 5 Hz, the holding potential was varied from $-130$ to $-90$ mV in 10-mV increments during each successive pulse train series. In control or in the presence of diphenylhydantoin, a rest interval of 15 seconds was

**FIGURE 1.** Typical characteristics of sodium current in rabbit atrial myocytes. Panel A: Currents recorded under voltage-clamp conditions during test potentials from $-75$ mV to $-35$ mV. Currents were obtained from a holding potential of $-130$ mV. Panel B: The current–voltage curve for all points. The continuous line represents the fit of the data to a Hodgkin-Huxley $m^n h$ model, where $m$ is the activation variable and $h$ is the inactivation variable. Panel C: Recordings depicting characteristics of sodium currents during the inactivation protocol. From a holding potential of $-130$ mV, 1 second prepulses to various potentials were followed by a 20-msec test pulse to $-30$ mV. There is no crossover of the currents, because their size changes with holding prepotential and the peak of each current occurs at approximately the same time. Panel D: Steady-state inactivation curve for all points. The continuous line depicts the predicted ideal fit to a relation of the form $h=1/[\exp(V-V_h)/s]$ (where $h$ is the inactivation variable, $V$ is membrane potential, $V_h$ is the potential for half-maximal inactivation, and $s$ is the slope factor) for the data. For this experiment, $V_h=94.5$ mV and $s=6.27$. 
used between trains, whereas in the presence of amitriptyline, the rest interval was 50 seconds.

The rate of development of block also was determined with a two-pulse protocol.26 We evaluated channel availability using conditioning pulses of increasing duration (holding potential, −130 mV; prepulse potential, −30 mV; prepulse duration, 2–10,000 msec), followed with a return to the holding potential for 500 msec and a subsequent test pulse of 20 msec to −30 mV.

The rate of recovery from block in the absence or presence of drug also was determined with a two-pulse protocol. Using conditioning pulses of 2 seconds (maximal drug uptake without evidence of slow inactivation of the sodium channel as shown by the uptake protocol) to −30 mV, a variable period of recovery to the holding potential was used. The recovery interval ranged from 100 to 40,000 msec and was followed by a 20-msec test pulse to −30 mV to evaluate sodium current availability.

Single-Channel Recording Techniques

The results of the whole-cell voltage-clamp experiments suggested that diphenylhydantoin may produce block from an internal membrane site. We did a series of single-channel experiments to determine the nature of diphenylhydantoin block and whether it was producing block from a drug pool immediately accessible to the internal or external membrane surface. We performed these experiments in cell-attached patches of ventricular myocytes, because this configuration afforded the most stable single-channel kinetics.29–32

Experiments were performed in the absence of diphenylhydantoin, with drug-free microelectrodes attached to cells exposed to drug in the superfusate, or with drug-containing microelectrodes attached to cells that had not been exposed to drug.

Single-Channel Experimental Protocols

Our protocol consisted of the application of a 1-second conditioning pulse to −20 mV, a recovery interval of 500 msec, and a 40-msec test pulse to −20 mV. The 500-msec recovery period permitted the recovery of drug-free channels from inactivation (see “Results”). A rest period of 5 seconds separated each test pulse and the subsequent conditioning pulse. This allowed recovery of the channels from block by the drug. Whole-cell voltage-clamp experiments with diphenylhydantoin suggested rapid recovery from block at a holding potential of −120 mV. We performed preliminary experiments with the holding potential set at −120 and −90 mV. Block of single channels could be demonstrated with the holding potential equal to −90 mV but was not well detected at −120 mV. The remaining single-channel experiments were performed at −90 mV.

We obtained an ensemble of 50 conditioning and test pulses and accepted the data only if two such ensembles were obtained under a given condition. Further, in all experiments we waited a minimum of 5 minutes (7 minutes with the experiments with diphenylhydantoin-containing microelectrodes) after gigohm seal formation before data were acquired. This circumvented the period of rapid spontaneous changes in single-channel kinetics during which latency to first opening decreased and mean open time increased. An experiment required a stable seal for at least 15 minutes.

Data Analysis

As noted above, filtered currents from whole cells were digitized and subsequently transferred to a SUN 4/280 microcomputer (SUN Microsystems, Inc., Mountainview, Calif.), where the peak values of individual current tracings were determined by using custom software developed in our laboratory and written in C programming language. These peaks were plotted, and exponentials were fitted to the data describing the recovery from block and the development of block and inactivation using the Marquardt routine or Gauss–Newton method.33 From the residual error an F statistic was calculated and considered a good fit at a significance level <0.05.

Single-channel currents were filtered at a corner frequency of 2.5 kHz and digitized at 25 kHz. To reduce the size of the files, we digitized only the first 40 msec of the conditioning pulse. At −20 mV, the averaged currents relaxed to zero in ≤20 msec.34 On the rare occasions in which bursts were observed in either the conditioning or the test pulse, we excluded that pulse and its associated conditioning or test pulse from the analysis.

The single-channel currents also were analyzed on a SUN 4/280 microcomputer using methods outlined by Grant et al.34 Briefly, residual capacitive and leakage currents were reduced by subtracting the average of null sweeps from each sweep. We used an automatic event detection algorithm with the threshold at 0.5. The performance of the algorithm was checked routinely by direct comparison of the original and idealized records.

We compared single-channel closing kinetics by using mean open times. Particularly in the test pulses in the experiments using diphenylhydantoin, the number of events was sometimes too small to construct a histogram. The apparent probability of failure of a channel to open was obtained by taking the Nth root of the number of null sweeps, where N is the apparent number of functioning channels in the patch. N was estimated from the maximum number of overlapping events at a strongly depolarized potential (−30 mV). Because the holding potential was usually set at −90 mV, the estimate of N was conservative. The conservative estimate does not change the major conclusions of the paper.

In reporting results, all data are expressed as mean ± SD. The statistical significance of the differences was determined using the appropriate Student’s t test for paired or unpaired data. When multiple group comparisons were made, a one-way or two-way analysis of variance was performed. When an analysis of variance demonstrated a significance
between some data group(s), a t test or Scheffé’s test was used to determine significance between individual means. Significance was considered to be present at \( p < 0.05 \). Statistical techniques were taken from Gilbert\(^3\) and Snedecor and Cochran.\(^3\)

**Results**

**Characteristics of the Cultured Rabbit Atrial Myocytes**

For reasons reported earlier,\(^2,6,27\) experiments in this study were performed on spherical cells only. For all of the experiments (total \( n = 141 \) cells), the cell capacitance averaged \( 26 \pm 1 \) pF, which was in excellent agreement with our previous report.\(^6\) We were able to compensate \( \geq 70\% \) of the series resistance in all experiments. At all test potentials used in this study, the capacitive current was clearly separated from the sodium current. The threshold potential of our cell population was \(-66 \pm 5 \) mV with peak sodium current of the current–voltage relation occurring at \(-35 \pm 4 \) mV. All steady-state inactivation curves were fitted with a Boltzmann function and demonstrated a half maximal inactivation value of \(-90 \pm 5 \) mV. Because of the absence of sodium ions in the pipette solution, no current reversal was seen. There was no crossover of the current tracing during steady-state inactivation determination or threshold phenomena on the negative limb of the current–voltage curve (see Figure 1). These characteristics suggest that adequate voltage-clamp conditions existed during our experiments despite the comparatively high concentration of sodium in the external solution.\(^26\)

**Description of Kinetics of Sodium Current Block by Amitriptyline**

As illustrated in Figure 2, amitriptyline blocked the sodium current in a use-dependent manner. In the absence of drug (panel A), sodium current declined by \( 11\% \) during trains of 40 pulses with a stimulation frequency of 5 Hz. During exposure to \( 0.4 \) \( \mu \)M amitriptyline (panel B), sodium current declined progressively until reaching a steady-state block of \( 74\% \). The steady-state block was achieved after 21 pulses in the 40-pulse train (panel C). Using multiple frequencies of stimulation from 0.1 to 5 Hz, we observed a progressive increase in the amount of steady-state block achieved. Summary of the level of steady-state block as a function of stimulus frequency seen in 15 cells is shown in panel D. Amitriptyline block during pulse train stimulation was not dependent on the holding potential (panel E).

The progressive decline in sodium current during pulse train stimulation resulted from enhanced binding of amitriptyline to a sodium channel receptor during the depolarizing pulses. This enhanced binding may be due to differential affinity of amitriptyline with various states of the channel (i.e., open, inactivated, and resting) and/or differential access to "guarded" binding sites of fixed affinity.\(^27,28\) Between depolarizing pulses, some block dissipates because of drug dissociation from the channel binding site. The rate of drug dissociation (recovery from block) under given experimental conditions is a constant. Therefore, if the stimulus frequency is greater than five times the recovery time constant, block accumulates until steady-state is reached; that is, the amount of block gained per pulse is equal to the relief of block. As shown in Figure 2D, accumulation of block, even at slow stimulus frequencies, is significant with amitriptyline and suggests slow dissociation of drug from the sodium channel at \( 17^\circ\)C.

We examined the kinetics of recovery from steady-state block using trains of pulses (40 pulses; duration, 50 msec; test, to \(-30 \) mV) in the absence (Figure 3A) and presence (Figure 3B) of \( 0.4 \) \( \mu \)M amitriptyline. After each pulse train, a variable recovery period (100–50,000 msec) was used before application of a recovery test pulse. Currents from the test pulse were compared with the peak (first pulse) sodium current at 10–15 recovery intervals after the pulse train, and these data were fitted to a single exponential equation to determine the time constant of recovery (\( \tau_r \)) (Figure 3C). In the absence of drug (control), only a small amount (<10%) of steady-state block developed, and the current recovered rapidly with a mean \( \tau_r \) of \( 30 \pm 8 \) msec in our cell population (Table 1). This value agreed with previously published \( \tau_r \) in myocytes under similar voltage-clamp conditions.\(^2,23,39\) During exposure to amitriptyline, significant steady-state block developed (63±10%), and \( \tau_r \) was increased to \( 13,600 \pm 3,200 \) msec, demonstrating slowed kinetics of recovery from steady-state block.

Hille\(^40\) has suggested that for tertiary amines, the neutral species may access its binding site when the channel is open or closed, whereas the charged species may access the binding site only when the channel is open. The charged and uncharged species are thought to have different dissociation rates from the channel. To evaluate the role played by the charged versus neutral species of amitriptyline, the rate of recovery from block in the absence (Figure 3D) and presence (Figure 3E) of amitriptyline was evaluated in the same cell using a two-pulse protocol. Because charged species access open sodium channels, pulse train data reveal kinetics predominantly of the charged species, whereas holding the cell at \(-30 \) mV (prepulse) inactivates sodium channels and allows access to channel binding sites predominantly by the neutral form of the drug.

By using a fixed conditioning prepulse (duration, 2,000 msec) followed, after a variable period (100–50,000 msec), by a test pulse of 20 msec to \(-30 \) mV, sodium current recovery was measured. In the absence of drug, no significant decrease in peak sodium current was seen between consecutive conditioning pulses (no slow inactivation).\(^44\) Perfusion with external solution containing amitriptyline showed development of significant block. The data are shown graphically in Figure 3F and demonstrate a \( \tau_r \) similar to that seen with pulse train stimulation. Table 1 compares results from the pulse train and two-pulse protocols. In six experiments, the \( \tau_r \) value using the
two-pulse protocol was 45±10 msec in the absence of drug (p=NS for τ, in absence of drug with pulse train stimulation). When the cells were perfused with amitriptyline, significant block developed (83±8%). This value was statistically different from that seen during pulse train stimulation protocol (83±8% versus 63±10%, p<0.001) in spite of equal total time of depolarization in both protocols. The smaller amount of block with pulse train stimulation may result from partial recovery from block between pulses. Using the two-pulse protocol, the τ, for amitriptyline was 14,800±4,100 msec. This value was not significantly different from the value of τ, in the absence of drug.

The kinetics of decline of the sodium current during pulse train stimulation is the result of a combination of uptake of the drug at the binding site during the pulse and dissociation of drug from the binding site between pulses. Recovery data evaluate dissociation from the binding site. We also examined drug uptake in our model.

By using a two-pulse protocol to evaluate uptake kinetics, prepulses of varying duration (2–10,000 msec) were followed 500 msec later by a 20-msec test pulse to −30 mV to evaluate sodium current. A representative experiment is shown in Figure 4. In the absence of drug (panel A), no change was seen in sodium current measured during the test pulses with prepulse durations of ≤2,000 msec. With prepulses of >3,000 msec, the current seen during the test pulse began to decline slightly, secondary to slow inactivation of the sodium channel.41 In the presence of amitriptyline (panel B), progressively longer prepulses caused a decline in the sodium current. The results of a single study are shown in panel C. The decline in sodium current in the presence of amitriptyline was significantly faster than in the absence of drug.
tyline could be fitted with a single exponential with an uptake time constant of 1,410 msec in this experiment. The mean value for all experiments using amitriptyline (n=6) was 1,350±60 msec.

The τ, measured for amitriptyline was considered to be quite long, on the same order of magnitude as the τ, reported by Whitcomb et al.² for propoxyphene. As these authors demonstrated, the long τ, during exposure to a drug was consistent with the large amount of cumulative block observed even at slow stimulus rates. Whereas the τ, of amitriptyline is shorter than that of propoxyphene (13.6 versus 24.6 seconds), the τ, of the type IB agents (i.e., lidocaine and diphenylhydantoin) is shorter, <1 second. If the slow binding agent and the agent with more rapid kinetics shared a common binding site, they would “compete” for available binding sites during each depolarizing pulse. During these pulses, the drug with the more rapid kinetics might compete more effectively for the binding site and

TABLE 1. Steady-State Block and Time Constants of Recovery for Pulse Train and Two-Pulse Protocols With Amitriptyline and With Diphenylhydantoin

<table>
<thead>
<tr>
<th></th>
<th>Pulse trains</th>
<th>Two-pulse protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Drug</td>
</tr>
<tr>
<td>Amitriptyline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSB (%)</td>
<td>6±3</td>
<td>63±10*</td>
</tr>
<tr>
<td>τ, (msec)</td>
<td>30±8</td>
<td>13,600±3,200*</td>
</tr>
<tr>
<td>n</td>
<td>15</td>
<td>27</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6</td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SSB (%)</td>
<td>9±3</td>
<td>14±2</td>
</tr>
<tr>
<td>τ, (msec)</td>
<td>35±8</td>
<td>710±210*</td>
</tr>
<tr>
<td>n</td>
<td>10</td>
<td>22</td>
</tr>
</tbody>
</table>

Values are mean±SD. Control, absence of drug; Drug, in the presence of amitriptyline or diphenylhydantoin; SSB, steady-state block expressed as a percent of first pulse current; τ, time constant of recovery; n, number of cells.

*p<0.0001 and †p<0.001 vs. corresponding control value.
therefore displace the slower drug. If this occurred, at appropriate rates of stimulation the amount of sodium channel blockade in the presence of two channel-blocking agents could actually be less than the amount seen in the presence of the single agent with slow kinetics. Theoretically, a drug such as diphenylhydantoin or lidocaine might lessen the amount of sodium current block seen with amitriptyline when also present in the perfusate.

**Description of Kinetics of Sodium Current Block by Diphenylhydantoin**

Diphenylhydantoin (10–160 μM) also acted as a sodium channel blocking agent, demonstrating use- and frequency-dependent block but to a lesser extent than amitriptyline. In the absence of drug (Figure 5A), little steady-state block developed, and with the addition of 20 μM diphenylhydantoin (Figure 5B), block increased only slightly. Steady-state block was reached in 13 pulses (Figure 5C). By using several frequencies of stimulation, trains of forty pulses (50 msec; test, to −30 mV) were applied to obtain steady-state block (Figure 5D). The level of steady-state block for 20 μM diphenylhydantoin increased as the stimulus frequency increased, although much less total block (even at 160 μM diphenylhydantoin) of the sodium current was seen than in the presence of amitriptyline. Diphenylhydantoin, in contrast to amitriptyline, showed a steep dependence of steady-state block on the holding potential (Figure 5E).

We examined the kinetics of recovery from steady-state block during pulse train and two-pulse protocols in the absence (Figure 6A) and presence (Figure 6B) of 20 μM diphenylhydantoin. In the absence of drug, very little block developed (<10%), and the $r_t$ measured at steady-state during pulse trains was 40 msec. With the addition of diphenylhydantoin, the $r_t$ increased to 810 msec in this cell. Determination of $r_t$ is shown in Figure 6C. As shown in Table 1, the $r_t$ for a population of 10 cells in the absence of drug was
35±8 msec. In these same cells as well as in an additional 12 cells, the \( \tau \) in the presence of diphenylhydantoin increased to 710±210 msec. This value was in agreement with data from other voltage-clamp studies.\(^{23,24} \) The \( \tau \) increased significantly compared with the \( \tau \) in the absence of drug but was \( \sim 20 \) times less than the \( \tau \) in the presence of amitriptyline seen with pulse train stimulation. Using a two-pulse protocol (Table 1), the \( \tau \) in the absence of drug was 50±10 msec, whereas in the same cells subsequently exposed to diphenylhydantoin, the \( \tau \) was 1,100±500 msec. As seen with amitriptyline, a single pulse of 2,000 msec resulted in a greater decrease in the sodium current than was seen in the pulse train protocols (31±6% versus 14±8%, \( p<0.01 \)).

By using the two-pulse protocol as above, the uptake time constant of diphenylhydantoin was measured. Figures 6D and 6E show results in a single cell. With progressively longer prepulses, there was a decline in sodium current in the presence of diphenylhydantoin (Figure 6E), whereas in the absence of drug (Figure 6D), no decrease in sodium current was seen until the prepulse duration increased to \( >2 \) seconds. The results of this study are plotted in Figure 6F. The uptake time constant in six cells for diphenylhydantoin was 1,150±380 msec.

Because of the differences in \( \tau \) between amitriptyline and diphenylhydantoin (13,600±3,200 versus 710±210 msec, \( p<0.0001 \)), we predicted that competition between the two agents might occur. A series of experiments was designed to test this hypothesis.

**Description of Kinetics of Sodium Current Block With a Combination of Amitriptyline and Diphenylhydantoin**

We examined whether addition of diphenylhydantoin altered the amount of steady-state block from that seen in the presence of amitriptyline alone. By using various stimulus frequencies, trains of 40 pulses (50 msec; holding potential \(-130\) mV; test, to \(-30\) mV) were applied as above. As with the single drug perfusion experiments, the level of steady-state block in the presence of both drugs increased as the stimulus frequency increased. This is demonstrated for a single experiment in Figure 7A. In the presence of the two sodium channel blocking agents, a higher level of steady-state block was seen at every stimulus frequency.\(^{25} \)
frequency when compared with the level of steady-state block seen with amitriptyline alone. Unlike Whitcomb et al., who described a “crossover” of the propoxyphene and propoxyphene–lidocaine curves at slower stimulus rates (<1 Hz), we were unable to demonstrate less block at any stimulus frequency in the presence of a combination of amitriptyline and diphenylhydantoin than with amitriptyline alone in 10 cells.

We evaluated whether τ₁ was modified in the presence of amitriptyline and diphenylhydantoin perfusion. Results from a single experiment are shown in Figure 7B. During exposure to a combination of amitriptyline and diphenylhydantoin, the sodium current recovered faster (τᵱ, 4,800 msec) than during exposure to amitriptyline alone (τᵱ, 12,400 msec) but did not recover as rapidly during control conditions or in the presence of diphenylhydantoin alone. The τᵱ for 23 experiments in which cells were exposed to a combination of amitriptyline and diphenylhydantoin was 6,600±1,800 msec.

Surprisingly, single exponential fits of the sodium current recovery curve in the presence of the drug combination provided as good a fit to the data as a
double exponential. These results differed from the results seen with competition between other drugs. Our data suggested that competition for a single channel binding site might not be occurring. There appeared to be significant modification of the recovery from sodium channel blockade in the presence of the drug combination without obvious evidence for displacement of the slow agent by the drug with the faster kinetics.

We considered several possibilities to determine why the competition between amitriptyline and diphenylhydantoin did not seem the same as that seen by Whitcomb et al. with propoxyphene and lidocaine. Amitriptyline has characteristics similar to propoxyphene and other sodium channel blocking agents, but it was possible that in some way it differed in its effect at the level of the blocking site. To evaluate this, we performed experiments examining whether amitriptyline could be displaced from the receptor site by other agents (i.e., lidocaine). The results of one of these experiments are shown in Figure 7C. In the presence of amitriptyline, decreasing amounts of block of the sodium current were seen as the stimulus frequency decreased. With the addition of lidocaine to the preparation, larger amounts of total block were seen at faster stimulus frequencies, but as the stimulation rate approached 1 Hz, the two curves merged and actually crossed in a manner similar to that described by Whitcomb et al. for propoxyphene and lidocaine. Although these differences in the percent of block were small, the crossover of the amitriptyline and the amitriptyline–lidocaine curves occurred consistently in all eight cells studied. This crossover reflected less block in the presence of two sodium channel blocking agents than was seen in the presence of the single agent (amitriptyline) alone.

From this information, it appeared that amitriptyline was acting in a manner similar to propoxyphene and could be competitively displaced from its receptor site by lidocaine. We considered that lidocaine could be competing more effectively than diphenylhydantoin for the sites occupied by amitriptyline within the sodium channel. The concept of competition requires consideration of two processes—binding and unbinding of drugs at the level of the channel receptor site. Examining the \( \tau \), (unbinding) of the drugs lidocaine and diphenylhydantoin would not explain the results found when these drugs were combined with amitriptyline. The \( \tau \) of diphenylhydantoin as found in this and other studies is ~500–800 msec, whereas the reported \( \tau \) for lidocaine under similar experimental conditions is 1–2 seconds. If lidocaine were to compete more effectively than diphenylhydantoin for the sites within the channel, one would predict that the unbinding of lidocaine would be faster, not slower, than the unbinding of diphenylhydantoin. Additionally, the “ratio” of the \( \tau \) of the slow agents to the \( \tau \) of the fast agents would appear to favor diphenylhydantoin

---

**Figure 7.** Graphs showing block and recovery with combinations of drugs. Panel A: Steady-state block in a single atrial myocyte in the presence of 0.4 \( \mu \)M amitriptyline alone (■) and during superfusion with 0.4 \( \mu \)M amitriptyline in combination with 20 \( \mu \)M diphenylhydantoin (+) as a function of stimulus frequencies ranging from 0.1 to 5 Hz. Note that for all frequencies examined, no evidence of crossover of the curves is seen. The continuous lines connect the data points. Panel B: Results from another cell showing recovery from block in the presence of 0.4 \( \mu \)M amitriptyline (■) and 0.4 \( \mu \)M amitriptyline and 20 \( \mu \)M diphenylhydantoin (+). \( \tau \), Time constant of recovery. The continuous lines represent the single exponential fit of the data. Panel C: Data from atrial myocyte in the presence of 0.4 \( \mu \)M amitriptyline (■) and 0.4 \( \mu \)M amitriptyline plus 80 \( \mu \)M lidocaine (●). The amount of steady-state block seen with the mixture is greater than that seen with amitriptyline alone at higher stimulus frequencies. For stimulus frequencies <1 Hz, the block observed at steady-state was less in the presence of the mixture than with amitriptyline alone. Though these differences were small, this phenomenon was reproduced in all eight cells with the crossover point occurring at 0.7±0.1 Hz (mean±SD).
as the more effective competitive agent when compared with lidocaine.

**Effects of Changes in Internal and External pH on Kinetics of the Sodium Current**

Failure of diphenylhydantoin to reverse amitriptyline block was consistent with the presence of two separate binding sites capable of modulating sodium channel kinetics. There are reports in the literature that describe modulation of sodium channel function that appears to be the result of more than a single binding site within the sodium channel.17,18,20,45

As a test of this possibility, we measured the $\tau_r$ and uptake rates as internal and external pH were varied (Table 2). Under control conditions, the nominal internal pH was 7.3 and external pH was 7.4. The $\tau_r$ values in the absence of drug, presence of amitriptyline, and presence of diphenylhydantoin were 30, 13,600, and 710 msec, respectively. In separate experiments, the $\tau_r$ was evaluated in the absence and presence of drug under conditions of high external pH (8.0). In the absence of drug ($n=6$), the $\tau_r$ though not significantly different (42±10 msec) from control conditions (30±8 msec), tended to be longer. Similar results under somewhat different voltage-clamp conditions have been seen when altering external pH.46

In cells perfused with pH 8.0 solution during amitriptyline exposure, the $\tau_r$ was decreased from ~14,000 to 4,200±100 msec ($p<0.001$). Because amitriptyline is a weak base (pK 9.4), increasing the pH of the perfusing solution containing amitriptyline should increase the amount of neutral drug and make the drug more effective in accessing and leaving the binding site, thus lowering the $\tau_r$. In cells perfused with diphenylhydantoin (pK 8.3) in external solution with pH 7.4, the $\tau_r$ was 710±210 msec. When the pH of the external perfusate was increased to 8.0, the $\tau_r$ ($n=10$ cells) was 700±125 msec. This was not different from the value seen under conditions of normal pH. Additionally, changes in external pH from 7.3 to 8.0 did not alter the uptake time constants measured with two-pulse protocols of amitriptyline (1,351±61 versus 1,221±202 msec, $p=NS$) or diphenylhydantoin (1,152±380 versus 1,010±161 msec, $p=NS$).

Similar experiments were performed keeping the pH of the external perfusate constant at 7.4 but now altering the pH of the pipette solution from 7.3 to 8.0. In the absence of drug ($n=6$), the $\tau_r$ was not altered ($\tau_r$ 33±11 msec) from that seen when the pH of the solution in the pipette was 7.3. In cells perfused under these same pH conditions with amitriptyline ($n=6$), the $\tau_r$ was not significantly changed from the $\tau_r$ at internal pH 7.3 (13,300±1,700 versus 13,600±3,200 msec, $p=NS$). On the other hand, in experiments in which the internal pH was increased and cells were superfused with diphenylhydantoin ($n=17$), the $\tau_r$ was increased to 2,600±1,300 msec ($p<0.001$ compared with the $\tau_r$ of diphenylhydantoin at internal pH 7.3). Because diphenylhydantoin is an anion, a larger fraction of receptor bound drug should be charged at the higher pH. Assuming that charged drug dissociates more slowly from the receptor, the results should be internally consistent with the opposite effect of external pH on amitriptyline recovery. The uptake time constants measured with two-pulse protocols in the presence of diphenylhydantoin with the internal solution at pH 8.0 decreased from 1,152±380 to 650±90 msec ($p<0.001$); the uptake time constant for amitriptyline was unchanged from control conditions (1,351±61 versus 1,290±71 msec, $p=NS$). These data suggest that sodium channel blockade by diphenylhydantoin was modulated by changes in the internal pH only, whereas that by amitriptyline was modulated from a site sensitive to changes in the external pH.

**Results of Single-Channel Studies**

We hoped to demonstrate block of single sodium channels during diphenylhydantoin exposure by comparing the current during the conditioning and the test pulses. The recovery interval between the condition and the test had to be sufficiently long to permit recovery from inactivation but not to allow substantial recovery of diphenylhydantoin-induced block. Figure 8 shows single sodium channel current in the absence of drug with a recovery interval of 500 msec.
recovery interval in this run was 250 msec. In comparing the averaged current in the conditioning pulses in panels A and B, there was significant rundown of current over the 15-minute period. The number of null sweeps was increased, and the averaged current for the test pulse was decreased. This suggests that significant inactivation occurs after a recovery interval of only 250 msec. Data from five experiments with a recovery interval of 500 msec are summarized in Table 3. The 500-msec recovery period was sufficient to permit recovery from inactivation.

Figure 9 illustrates current in conditioning and test pulses in an experiment in which the cells had been exposed to 80 \( \mu \)M diphenylhydantoin and the micro-electrode was drug free. The averaged current in the test pulse was less than that during the conditioning pulse. The decrease in current in the test pulse resulted primarily from an increase in the fraction of null sweeps. In the sequences 2, 3, 4, 5, and 10, openings in the conditioning pulse were followed by nulls in the test pulse. In this experiment, the mean open time was 0.73 msec during the conditioning pulse and 0.64 msec during the test pulse. The results of five experiments are summarized in Table 3. The integral of the current decreased by 46% during the test pulse and the apparent probability of a channel to fail to open increased significantly from 0.52±0.19 during the conditioning pulse to 0.70±0.11 during the test pulse. The mean open time was not significantly decreased between the conditioning and the test pulses.

Similar experiments in the absence of drug \((n=4)\) performed at a holding potential of -120 mV demonstrated that mean open time of the sodium channel was not different between conditioning (0.65±0.11 msec) and test (0.67±0.10 msec) pulses. These values also were not different from those at -90 mV. The probability that a channel would fail to open in both conditioning and test pulses was identical (0.21±0.10). When diphenylhydantoin was added to the superfusate \((n=4)\), no difference in mean channel open time was seen between conditioning (0.70±0.18 msec) and test (0.68±0.13 msec) pulses. Additionally, the probability of the channel to fail to open was not different between conditioning (0.19±0.08) and test (0.21±0.11) pulses. These experiments show that diphenylhydantoin can produce block of single sodium channels when a membrane patch is exposed to drug-free solution and that this block is voltage dependent. It is assumed that during the prior incubation in diphenylhydantoin-containing solution the drug was able to diffuse across the cell membrane and produce block from an intracellular or intramembrane site.

We performed six experiments in which the micro-electrode contained 80 \( \mu \)M diphenylhydantoin and the cells were not exposed to drug in the solution. Although there was a trend for the drug to decrease the current during the test pulse and to increase the
TABLE 3. Effects of Diphenylhydantoin on Single Sodium Channel Current

<table>
<thead>
<tr>
<th></th>
<th>&lt;1&gt; (msec)</th>
<th>(p_t)</th>
<th>(\int I dt) (pA · msec)</th>
<th>(\int V dt/\int V_{t0} dt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_c)</td>
<td>0.71±0.11</td>
<td>0.51±0.11</td>
<td>115±36</td>
<td>1.02</td>
</tr>
<tr>
<td>(V_i)</td>
<td>0.71±0.10</td>
<td>0.52±0.14</td>
<td>118±40</td>
<td></td>
</tr>
<tr>
<td>Diphenylhydantoin</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bath (n=5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_c)</td>
<td>0.66±0.06</td>
<td>0.52±0.19</td>
<td>139±77</td>
<td></td>
</tr>
<tr>
<td>(V_i)</td>
<td>0.62±0.05</td>
<td>0.70±0.11*</td>
<td>75±35*</td>
<td></td>
</tr>
<tr>
<td>Pipette (n=6)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(V_c)</td>
<td>0.56±0.10</td>
<td>0.73±0.16</td>
<td>74±40</td>
<td></td>
</tr>
<tr>
<td>(V_i)</td>
<td>0.50±0.07</td>
<td>0.77±0.12</td>
<td>50±18</td>
<td></td>
</tr>
</tbody>
</table>

Values are Mean±SD. <1>, Mean open time; \(p_t\), probability channel will fail to open; \(\int I dt\), integral of single-channel current; \(V_c\), conditioning pulse; \(V_i\), test pulse.

*\(p<0.05\) compared with corresponding value for \(V_c\).

probability of the channel to fail to open, the changes were not statistically significant.

**Discussion**

**Major Findings**

Based on previous studies demonstrating the competitive relief of block, our studies have examined the individual and combined blocking actions of amitriptyline and diphenylhydantoin on the sodium current in cultured rabbit atrial myocytes. Our major findings are as follows: 1) Both amitriptyline and diphenylhydantoin demonstrate use- and frequency-dependent blockade of the sodium channel. 2) Amitriptyline demonstrates a longer \(\tau_i\) (intracellular pH 7.3, extracellular pH 7.4) than diphenylhydantoin under similar conditions. 3) Uptake time constants of the two drugs are similar. 4) Increased external pH significantly decreased the \(\tau_i\) of amitriptyline (pK 9.4) without affecting the \(\tau_i\) of diphenylhydantoin (pK 8.3), whereas increased internal pH increased the \(\tau_i\) for diphenylhydantoin without affecting the \(\tau_i\) of amitriptyline. 5) Lidocaine attenuated the amitriptyline-induced block at low rates of stimulation. Unlike previous studies\(^{22,43}\) using similar agents, the combined application of amitriptyline and diphenylhydantoin did not appear to reverse the blockade. This difference in our results might have been caused by the location of the binding site of the diphenylhydantoin anion and the cationic form of tricyclic antidepressants or local anesthetics.

**Effects of Amitriptyline on the Sodium Current**

Little in vitro data exist concerning the effects of amitriptyline as a sodium channel blocking agent. More data exist on the tricyclic antidepressant imipramine, which has similar structure to amitriptyline. Voltage-clamp studies in *Myxicola* giant axon\(^{47}\) found imipramine to reversibly suppress sodium conductance at low concentrations while not affecting the resting membrane potential. Imipramine did shift the conductance-voltage curve in the hyperpolarizing direction in a concentration-dependent fashion but did not change the time to peak current or the time to half-maximal steady state.
In cardiac tissue, most of the effects of tricyclic antidepressants have been described using information obtained from $V_{\text{max}}$ data in Purkinje fibers. Rawling and Fozzard showed that imipramine reduced the rate of rise of $V_{\text{max}}$ and markedly slowed conduction velocity in canine Purkinje fibers. Similar studies by Brennan, Weld and Bigger, and Muir et al in Purkinje fibers and by Weld and Bigger in ventricular muscle corroborated the effects of tricyclic antidepressants on cardiac tissue. Sasyniuk and Jhamandas observed that, although amitriptyline (1.8 $\mu$M) did not affect maximum diastolic potential of the cells, it significantly depressed action potential amplitude and duration as well as $V_{\text{max}}$. This concentration of amitriptyline was two to four times the therapeutic level of the drug but was well within the range of concentrations obtained in patients with overdose. These studies all implied that the tricyclic antidepressants, including amitriptyline, acted as sodium channel blocking agents.

Ogata and Narahashi demonstrated that at very negative holding potentials (-140 mV) imipramine caused little resting block but that shifting the holding potential to less negative values increased the amount of resting block seen. During repetitive stimulations, significant use-dependent block was seen. There was also voltage dependence, but no shift in the current-voltage relation was seen when compared with the drug-free state. Steady-state inactivation was negatively shifted -18 mV. The recovery from drug-induced block was relatively slow (1.8±0.3 seconds).

The results of our study with amitriptyline support the concept of potent sodium channel blockade by this group of drugs in cardiac tissue with some important differences. Although both amitriptyline and imipramine have use- and frequency-dependent blocking properties on the sodium current, amitriptyline appears to be more potent. At concentrations of 0.4 $\mu$M, amitriptyline caused marked reduction of sodium current (78% block at 5 Hz; 60-70% block at 2 Hz, see Figure 2), whereas under similar voltage-clamp conditions, 3-Hz stimulation in the presence of 3 $\mu$M imipramine resulted in -50% block (Figure 6 from Reference 39). Neither drug appeared to significantly block resting sodium channels at hyperpolarized potentials (holding potential, -130 to -140 mV).

Imipramine and amitriptyline seemed to demonstrate relatively different properties of dissociation from the blocking site under voltage-clamp conditions. Compared with lidocaine with a $\tau$, of 1-2 seconds or diphenylhydantoin with a $\tau$, of 0.7 second, the $\tau$, of imipramine (1.8 seconds) was similar, whereas the $\tau$, of amitriptyline was very slow (13.6 seconds).

Drugs with slow dissociation kinetics from cardiac tissue may demonstrate greater degrees of cardiotoxicity than drugs with more rapid $\tau$. Slowed time constants of recovery of sodium current and decreased conduction velocity of cardiac tissues by tricyclic antidepressants most probably contribute to the clinical findings associated with toxicity including increased atrioventricular conduction time, decreased intraventricular conduction, and increased propensity for arrhythmias.

Effects of Diphenylhydantoin on the Sodium Current

Interest in examining whether diphenylhydantoin and amitriptyline were interacting at the same site on the level of the sodium channel was stimulated by the clinical controversy describing reversal of tricyclic antidepressant-induced cardiac conduction abnormalities with diphenylhydantoin. We recently reported normalization of cardiac conduction abnormalities in a case of propoxyphene overdose when lidocaine, an agent with significantly different recovery kinetics from those of propoxyphene, was administered. Review of the literature yielded support of the concept that amitriptyline had slow recovery kinetics and diphenylhydantoin had fast recovery kinetics at the level of the sodium channel and that, under appropriate conditions, competitive relief of block from amitriptyline by diphenylhydantoin might be demonstrable.

The effects of diphenylhydantoin on the sodium current have been examined in studies using several models. In neuroblastoma cells under voltage-clamp conditions, Willow et al found use-dependent block of voltage-activated sodium currents with a rapid onset of block (steady-state block reached in seven to 10 pulses) at concentrations of 30 $\mu$M diphenylhydantoin. Depolarization of the holding potential increased the amount of resting block as well as the amount of block seen at steady state. Half time of recovery from block was 1.36 seconds at a holding potential of -90 mV.

Sanchez-Chapula and Josephson evaluated the effects of diphenylhydantoin in rat ventricular cells during voltage-clamp experiments. They found the blocking effect of diphenylhydantoin on the sodium current to be both voltage and use dependent. At a concentration of 20 $\mu$M diphenylhydantoin and a holding potential of -90 mV, sodium current was reduced 45% during steady-state stimulation. Using a two-pulse protocol, the $\tau$, of six cells was 1.367±126 msec. When the holding potential was reduced to -80 mV, $\tau$, increased to 1.514 msec, and when the cells were hyperpolarized to -105 mV, $\tau$, decreased to 718 msec. Their $\tau$, for cells in the absence of drug was 70-80 msec. Xu et al using rat ventricular myocytes and 40 $\mu$M diphenylhydantoin, reported low levels of steady-state block (31±4%) with 950-msec pulses. They also demonstrated a rapid $\tau$, (520±140 msec) when cells were held at -140 mV.

Our results agree with those previously published. Under voltage-clamp conditions we demonstrated low levels of steady-state block (14±2%) when the cells were held at -130 mV and stimulated with 50-msec pulses. Our preliminary experiments ($n=4$) had shown that longer pulse durations resulted in greater amounts of steady-state block at the same stimulus frequency. Our $\tau$, for diphenylhydantoin correlated well with those seen in neuroblastoma.
cells and in rat ventricular myocytes. Onset of block in our preparation was rapid and reached steady state in 10–15 pulses. The amount of steady-state block was steeply voltage dependent.

Because of clinical information using diphenylhydantoin to treat amitriptyline overdose and experimental demonstration of competitive relief of block with other drugs, we felt that investigation of reversal of amitriptyline effects by diphenylhydantoin was reasonable.

**Effects of Combinations of Sodium Channel Blocking Agents on Sodium Channel Kinetics**

Hille and Schwarz proposed a model of local anesthetic action based on a common receptor site located within the sodium channel. This receptor site could bind charged and neutral drugs. This common receptor site has gained widespread support in the literature through the years.

Rimmel et al. examined the concept of the common receptor site by looking at the interaction of two drugs—procaine and benzocaine—at the level of the sodium channel in the frog node of Ranvier. Using kinetic analysis, these authors concluded that procaine (pK 8.9, cationic form at pH 7.0) appeared to have limited access to the receptor site because of the charged nature of the molecule over a wide range of pHs, whereas benzocaine (pK 6.5, uncharged over a wide range of pH) essentially had unlimited access to the receptor site at all pHs tested. When the kinetics of the channel were analyzed in a mixture of procaine and benzocaine with equieffective concentrations of the two drugs, the membrane responded as it did in benzocaine alone. The authors postulated that benzocaine and procaine acted at a common receptor site and that the rate of sodium channel block appeared to be limited by access to the receptor site. Benzocaine, the neutral drug, had easy entry into the channel and, hence, access to the binding site, whereas entry into the channel by procaine, the charged drug, was limited. In three experiments, the authors saw an increase in V_	ext{max} during exposure to benzocaine and procaine compared with procaine alone. The “overall” binding kinetics of the mixture were similar to those seen in the presence of benzocaine alone. Mathematical modeling (Figure 9 of Reference 18) of this reversal could not be fitted using two separate and independent binding sites for the drugs. Modeling for one binding site resulted in close agreement with their experimental results. Modeling by Starmer supported the concept of a single binding site for the drugs but stressed the importance of varying frequency of stimulation as a means to demonstrate apparent reversal of blockade.

Evidence exists for the presence of more than a single receptor site that may affect the sodium channel. Mrose and Ritchie observed that competition for blockade of the sodium channel existed between the drugs benzocaine and lidocaine. From their data, they concluded that there appeared to be a second site of action for benzocaine in addition to the sodium channel receptor site classically affected by lidocaine. Previously, Shrivastav et al. had demonstrated that volatile compounds such as alcohols or general anesthetics produced effects on the inactivation curve of the sodium channel similar in magnitude, but by a different mechanism, to effects produced by benzocaine. This led to the postulate that, although a common receptor may reside within the sodium channel itself and predominate in the modulation of sodium channel function, at least one other site of drug action appeared to be involved in the control of sodium channel kinetics and function. Alpert et al. have postulated the presence of a second local anesthetic binding site present outside or near the outside of cardiac sodium channels.

The concept of competitive reversal in the presence of two sodium channel blocking agents in cardiac tissue has been examined by several authors. Whitcomb et al. described that a drug with rapid association (binding) and dissociation (unbinding) kinetics may, in some circumstances, displace a drug with slower binding/unbinding kinetics such that effects produced by the slower drug may be altered or “reversed” with the addition of an agent with faster kinetics. We further showed that the degree of steady-state sodium channel blockade by a drug with a slow unbinding rate could be reduced by adding a drug with faster recovery kinetics. In these studies, the “slow” drug, propoxyphene, exhibited a τ of ~23 seconds, whereas the “fast” competitor, lidocaine, exhibited a τ of 1–2 seconds. In our present studies, we did not observe a reduction in the steady-state blockade when amitriptyline (τ, 13.6 seconds) competed with diphenylhydantoin (τ, 0.7 seconds). Was this evidence of two separate binding sites where a competitive effect would not occur, or was this compatible with a single binding site?

To explore this question, we looked at the dynamics of drug mixture. In the presence of both amitriptyline and diphenylhydantoin, there was no evidence of competitive displacement of amitriptyline at any stimulus frequency examined (no crossover as seen with propoxyphene and lidocaine or when lidocaine competed with amitriptyline in the present study). Though the τ of amitriptyline and diphenylhydantoin varied by a factor >10, we were unable to reproduce results similar to those obtained with propoxyphene and lidocaine or amitriptyline and lidocaine. We observed that the binding rate for amitriptyline was slightly larger than that of diphenylhydantoin. Increasing the concentration of diphenylhydantoin by 30 to reverse this relation also was ineffective in producing a crossover. However, competition with 80 μM lidocaine did produce this phenomenon. These data suggested to us that, whereas amitriptyline and lidocaine were binding to a common site, diphenylhydantoin was binding to a separate site. This stimulated us to explore alternative experimental approaches to distinguish whether a common binding
some were significantly modified. Because of this observation, they suggested that lidocaine-associated channels may conduct with a decreased open time. Their experiments were performed with lidocaine present in both the microelectrode and the bath.

The fact that we observed block in the experiments without diphenhydantoin in the microelectrode is consistent with block occurring from an intracellular and/or intramembrane site. The fact that we did not observe significant block when drug was present only in the microelectrode may be a concentration-dependent phenomenon. With drug in the microelectrode only, the cell acts as a large sink for drug and dilution of the drug concentration may occur. Resolution of this uncertainty would not be made any easier by patch excision, because the entire bath would then act as a sink for the drug.

The results of the whole-cell voltage-clamp experiments, including those with variations of intracellular and extracellular pH, and the single-channel recordings can best be integrated by observing that diphenhydantoin is able to cross the cell membrane and bind to some (internal?) site that is not accessible to external protons and that amitriptyline binds to a more superficial site that is accessible to external protons. We and others21 have reported that a model of binding to separate sites predicts enhanced block during exposure to the combination of drugs with fast and slow kinetics.

**Acknowledgments**

We would like to acknowledge the excellent technical support of Ms. Anne Stone and thank Ms. Pat Dean for preparing the manuscript.

**References**


38. Starmer CF, Grant AO, Strauss HC: Mechanism of use-dependent block of sodium channels in excitable membranes by local anesthetics. Biophys J 1984;46:15–27


46. Wendt D, Starmer F, Grant A: Recovery kinetics of sodium current are accelerated by decrease in external pH (abstract). Biophys J 1990;57:300a


52. Sasyiniuk BI, Jhamandas V: Mechanism of reversal of toxic effects of amitriptyline on cardiac Purkinje fibers by sodium bicarbonate. J Pharmacol Exp Ther 1984;231:387–394


**KEY WORDS** - lidocaine • amitriptyline • sodium channel • diphenylhydantoin
Blockade of cardiac sodium channels by amitriptyline and diphenylhydantoin. Evidence for two use-dependent binding sites.

M J Barber, C F Starmer and A O Grant

Circ Res. 1991;69:677-696
doi: 10.1161/01.RES.69.3.677

Circulation Research is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1991 American Heart Association, Inc. All rights reserved.
Print ISSN: 0009-7330. Online ISSN: 1524-4571

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://circres.ahajournals.org/content/69/3/677

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Circulation Research can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Circulation Research is online at:
http://circres.ahajournals.org//subscriptions/