Late Sodium Channel Openings Underlying Epileptiform Activity Are Preferentially Diminished by the Anticonvulsant Phenytoin

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Segal, Michael M. and Andrea F. Douglas. Late sodium channel openings underlying epileptiform activity are preferentially diminished by the anticonvulsant phenytoin. J. Neurophysiol. 77: 3021-3034, 1997. Late openings of sodium channels were observed in outside-out patch recordings from hippocampal neurons in culture. In previous studies of such neurons, a persistent sodium current appeared to underlie the ictal epileptiform activity. All the channel currents were blocked by tetrodotoxin. In addition to the transient openings of sodium channels making up the peak sodium current, there were two types of late channel openings: brief late and burst openings. These late channel openings occurred throughout voltage pulses that lasted 750 ms, producing a persistent sodium current. At −30 mV, this current was 0.4% of the peak current. The late channel openings occurred throughout the physiological range of trans-membrane voltages. The anticonvulsant phenytoin reduced the late channel openings more than the peak currents. The effect on the persistent current was greatest at more depolarized voltages, whereas the effect on peak currents was not substantially voltage dependent. In the presence of 60 μM phenytoin, peak sodium currents at −30 mV were 40–41% of control, as calculated using different methods of analysis. Late currents were 22–24% of control. Phenytoin primarily decreased the number of channel openings, with less effect on the duration of channel openings and no effect on open channel current. This set of findings is consistent with models in which phenytoin binds to the inactivated state of the channel. The preferential effect of phenytoin on the persistent sodium current suggests that an important pharmacological mechanism for a sodium channel anticonvulsant is to reduce late openings of sodium channels, rather than reducing all sodium channel openings. We hypothesize that pharmacological interventions that are most selective in reducing late openings of sodium channels, while leaving early channel openings relatively intact, will be those that produce an anticonvulsant effect while interfering minimally with normal function.

INTRODUCTION

The persistent sodium current is made up of rare “late” openings of sodium channels that continue to occur many milliseconds after the beginning of a depolarization of cellular voltage. In contrast, the peak sodium current is made up of the more numerous transient openings of the sodium channels. It appears that both the late and transient channel openings reflect different states of the same channel molecule, because both types of openings are produced by sodium channels encoded by the same brain (Moorman et al. 1990) or muscle (Ukomadu et al. 1992; Zhou et al. 1991) sodium channel mRNA expressed in cells.

The importance of this persistent sodium current in neurons has become increasingly apparent (Taylor 1993). Three lines of evidence, taken together, suggest that persistent sodium currents may be important in the treatment of convulsions and are a possible site of abnormalities producing convulsive seizures.

1) The sodium current is a well-characterized pharmacological target for controlling seizures. Most clinically useful anticonvulsants with known mechanisms of action reduce sodium currents (Rogawski and Porter 1990). Among the best studied of these drugs are phenytoin (Kuo and Bean 1994; Matsuki at al. 1984; Willow et al. 1985), carbamazepine (Willow et al. 1985), and lamotrigine (Lang et al. 1993).

2) The persistent sodium current may be a particularly important component of the sodium current involved in seizures because the hallmark of the “ictal” epileptiform activity is a persistent depolarization of the neuronal voltage lasting seconds to minutes (Kandel and Spencer 1961; Matsumoto and Ajmone Marsan 1964). Evidence from a simplified culture system displaying epileptiform activity suggests that a persistent sodium current may be an important component of this sustained depolarization (Segal 1994). In that simplified system, neurons with the ictal sustained depolarizations also have endogenous bursts of action potentials and depolarization even when synaptic transmission is blocked, which is evidence for a nontransmitter persistent inward current.

3) Persistent sodium currents underlie the hyperexcitability diseases in the other excitatory tissues in which cells display pathological persistent depolarizations. Some variants of the muscle diseases hyperkalemic periodic paralysis (Cannon et al. 1991) and paramyotonia congenita (Chahine et al 1994) and the cardiac long QT syndrome (Bennett et al. 1995) are caused by mutations, leading to an increase in late openings of sodium channels.

This study uses single channel recordings to study persistent sodium currents in neurons in the culture system displaying epileptiform activity (Segal 1994; Segal and Furspan 1990) and also explores the actions of the anticonvulsant phenytoin on these currents.

Most of the studies of anticonvulsant actions on sodium currents to date have examined the effects of the drugs on the peak sodium current (Kuo and Bean 1994; Lang et al. 1993; Matsuki at al. 1984; Willow et al. 1985), which is easier to study because it is two orders of magnitude larger than the persistent sodium current. However, if a persistent sodium current is crucial in maintaining ictal depolarization, it may be the most relevant portion of the sodium current to study.
A previous study of phenytoin actions on late openings of sodium channels used neuroblastoma cells in which fast inactivation had been removed proteolytically (Quandt 1988). This study found a reduction in channel open times and number of channel openings, but the relevance to intact channels was unclear because of the proteolytic digestion of the channel.

We report here that hippocampal neurons in culture have late openings of sodium channels. The anticonvulsant phenytoin has a preferential effect on these late openings, with a lesser action on early channel openings making up the peak current. These findings have been reported in preliminary form (Segal et al. 1995).

METHODS

Culture methods

Cultures were prepared according to the microculture method of Segal and Furshpan (1990), with minor modifications (Segal 1994). Briefly, drops of collagen were sprayed onto a dried film of agarose to define microislands. Glial cells and then neurons adhered to form microcultures on these islands. The cultures were grown in medium containing synaptic antagonists (11 mM Mg2+, and 1 mM kynurenate), conditions that led to epileptiform activity in mass cultures (Furshpan and Potter 1989) and microcultures (Segal and Furshpan 1990) when the cultures were returned to solutions with standard magnesium (1 mM) and no kynurenate.

Recording methods

Recordings were made 14–30 days after plating. Neurons in microcultures or mass cultures that were >17 μm diam were chosen for recording. Such cells are highly likely to release a glutamate-like transmitter (Segal 1991). Cultures were continually perfused during recording and drugs were added via perfusion with delay times of ~1 min. The temperature was maintained at 27.5°C in the central well of the dish using a heating coil.

Outside-out patch recordings were made using standard techniques (Hamill et al. 1981). The signals were recorded using an Axopatch 200 or 200A amplifier and an IBM-type computer running pClamp 6.0 software (Axon Instruments). RMS current was 0.25–0.40 pA using pipettes that were coated with Syldgard (Dow-Corning) such that the visible component of the Syldgard was ~200 μm from the tip. Pipettes were fire polished to 5–10 MΩ. Holding potential was ~100 mV.

Records were filtered at 5 kHz using the Axopatch amplifier. For 100-ms pulses, runs of 33 pulses were sampled at 20 kHz and recorded every 1 s. For 750-ms pulses, runs of 56 pulses were sampled at 15.4 kHz (limited by the maximum of 12,800 points in the 832-ms recording) recorded every 2 s.

Solutions

The extracellular perfusion solution was based on Ca2+/Mg2+-free Hanks’ balanced salt solution, to which was added (in mM) 25 glucose, 5 N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid (HEPES), 1.5 Ca2+, and 1.0 Mg2+. The pipette solution contained (in mM) 60 CsCl, 60 CsF, 5 MgCl2, 10 Cs ethylene glycol-bis(β-aminoethyl ether)-N,N’,N’’,N’’’-tetraacetic acid (has Cs+ 20), and 10 Cs HEPES (has Cs+ 4), pH 7.4.

Drugs

Phenytoin was dissolved to make 10–60-mM stock solutions in a solvent vehicle consisting of water (5%), propylene glycol (40%), and ethanol (10%). The stock solutions were diluted 1:1000 in perfusion solution minutes before perfusion so as to minimize loss of drug by adsorption. Solvent vehicle was added to control solutions, resulting in the same solvent concentration in all drug and control solutions. Similar channel openings were seen with or without the vehicle.

Data analysis

Peak currents were assessed using blank traces for subtracting capacitive currents. Because there were usually no records without any transient channel openings, blanks were obtained using one of the following two procedures: 1) tetrodotoxin blanks were recorded directly using the drug (1 μM) to block all channel currents or 2) calculated blanks were constructed for each run of 56 pulses. Channel-free segments were combined using the segmented average feature of the Fetchan program of pClamp. Such segmented averages are not accurate in the first ~20 ms of the pulse due to the presence of transient channel openings in virtually every record. Accordingly, the opening-free end part of the pulse was copied, inverted, and used to form a symmetric beginning of the pulse, with appropriate correction for leak current. The calculated blanks appeared the same as tetrodotoxin blanks, but calculated blanks were used preferentially because they could be computed for each run of 56 pulses, thus minimizing the effects of drift of leakage current during the experiment. The blank construction procedure was automated using a program written in the programming language C, for which the source code is available on request from the authors, and also has been provided to Axon Instruments. Calculated blanks then were used in the pClamp software to generate blank-subtracted records.

Late currents were assessed using two methods: 1) In the ensemble current method, used for 100-ms pulses, blank segmented averages were made from each run of 33 pulses and subtracted from each trace in the run to leave single channel events with no leakage current. This process was time consuming but was highly accurate and resulted in removal even of noise from segments of the data in which there are no channel openings (e.g., Fig. 6). It was not accurate to use blank traces from different runs of 33 pulses because changes in leak current were of similar magnitude to the current due to late openings of sodium channels. 2) In the idealized trace method, used for 750-ms pulses, the current from 100 to 750 ms was analyzed using pClamp to form an idealized record of channel openings. A threshold of half the open current channel was used for detection, and the idealized record events were counted and measured using the pSTAT program of pClamp to calculate late current during the 100–750 ms period. The number of channel events was counted, and the time distribution was obtained as an average using pClamp or analyzed from a dwell time histogram using the simplex least square method in pClamp (using two exponentials) to get τ values.

Records were filtered a second time for display, at 1–3 kHz, using the Gaussian filter in pClamp or a Gaussian filter macro run in Sigmaplot for Windows 1.02, implementing the algorithm detailed by Colquhoun and Sigworth (1983). Baselines for drug applications were taken as the average of the values before the drug and after washout of the drug so as to control for the slow decreases in sodium currents which were a factor more for peak currents than for late currents (e.g., Fig. 7).

For all statistical test, two-tailed t-tests were used to calculate P values.

RESULTS

Late sodium channel openings

Outside-out patches from all neurons studied (n = 17) displayed both early and late openings of channels displaying
inward current. Early openings were taken as those openings occurring in the first few milliseconds, comprising the peak current, and late openings were taken as those after 50 ms (for 100-ms pulses) or after 100 ms (for 750-ms pulses). There were no patches with only a single channel. Potassium channel currents in these patches were blocked by the cesium (144 mM) in the patch pipette. No outward currents were seen in these recordings, using depolarizations to a range of voltages from −60 to +30 mV.

The late channel openings were present across a wide range of voltages typical of electrically active neurons (Fig. 1; selected records show channel activity at each voltage). Near resting potential (approximately equal to −62 mV for these neurons) (Segal 1994), there were brief, punctuated channel openings, often occurring in bursts (Fig. 1). At more depolarized potentials, the channel openings were longer and more ‘‘box-like’’, not interrupted by frequent closings.

The most common late channel openings were the brief late openings (e.g., Fig. 5, Control, top trace), which were seen in all patches in most or all of the depolarizing pulses. There were also prominent burst openings lasting tens or hundreds of milliseconds (e.g., Fig. 5, Control, 6th trace), as noted in previous studies, including cell-attached patch recordings from neocortical neurons (Alzheimer et al. 1993). These open-channel bursts were rare: in 16 of the 17 patches, the bursts occurred in <1% of depolarizing pulses, and in many of these 16 patches, the bursts were seen far more rarely or not at all. Some of this variability may be due to differences between different neurons, but
some of the variability appeared to be from the tendency of channels to go into long-lasting states that were predisposed to bursting. The sequence of bursts shown in Fig. 5 was the longest encountered. It demonstrates that these bursts often recur in consecutive pulses despite the channel being closed in the 1.25-s period at the holding potential between subsequent pulses. There was no absolute demarcation between the duration of brief late openings and the long burst openings, because events of intermediate duration often were seen. The rarity of the burst openings makes quantitative studies of the bursts difficult to do, because typically only several bursts of >100 ms would be seen in the ~1,000 pulses obtained during 30 min of recording. In addition, statistical analysis of the bursts was made more difficult by the tendency of bursts to occur in clusters and thus not represent statistically independent events.

Three lines of evidence demonstrate that these channel openings are due to sodium channels.

1) As expected for sodium channels, the single channel currents at different voltages extrapolate to a positive reversal potential (Fig. 2) with a chord channel conductance of 14.3 pS, in the expected range for mammalian sodium channels (Hille 1992). Because sodium was present in the extracellular solution absent in the intracellular solution, there was no actual reversal potential. The amplitudes for single cellular solutions but absent in the intracellular solution, there 14.3 pS, in the expected range for mammalian sodium chan-

2) As expected for sodium channels, there was a negative slope region to the peak current-voltage curve (Fig. 3), and the current did not pass near the origin as would be expected from a non-selective cation channel.

3) As expected for sodium channels, all channel currents were blocked reversibly by perfusion with tetrodotoxin (1 μM; Fig. 4; n = 9). In a similar set of experiments in which tetrodotoxin was included in the pipette during cell-attached patch experiments, no inward currents were seen (n = 12) (S. Yilma and M. Segal, unpublished results).

Phenytoin reduces late channel openings more than early openings

Phenytoin (10–60 μM) reduced both late and peak sodium currents in all patches (n = 17). Traces from one trial with 60 μM phenytoin are shown in Fig. 5, using a series of consecutive current traces to show the channel activity. The records are stacked to show many current traces, although the stacking obscures part of the peak currents in all but the final record in each panel.

In phenytoin, both brief-late and burst openings occurred but less frequently than in control solution. In all cases, phenytoin reduced late openings more than early openings. Two methods were used to quantitate these late current re-

results: ensemble currents and idealized traces. Ensemble current analysis is more accurate for estimating the total current because no simplifying assumptions are made about the shape of the channel current. Idealized traces are more versatile for analysis because one can quantitate single channel properties. Because the analysis is more automated (see METHODS), longer records can be processed, giving results that are more significant statistically.

The single channel studies were limited by the lifetime of the outside-out patches (0.5–2 h) and the small number of late channel openings occurring with each depolarization. These constraints limit the number of channel openings that can be studied from each individual patch. For this reason, long samples were obtained at a single voltage and a single drug concentration in most experiments so as to sample enough depolarizing pulses to get quantitatively reliable information. In other experiments, a total of 112 depolarizations at each of several voltages or drug concentrations were used so as to develop a profile of the pharmacology of the channels, albeit one with more variability than found with the longer records obtained at only one voltage and phenytoin concentration.

Analysis of the phenytoin effect using ensemble currents

Single channel events were studied in most detail at a holding potential of −30 mV and a phenytoin concentration of 60 μM. Analysis using the ensemble current method in other systems typically consists of a straightforward process of adding individual patch current traces such as those in Fig. 5. However, in the case of late openings of sodium channels, additional caution is needed. The late current is <1% of the peak current, similar in magnitude to typical changes in leakage current that occur between runs of voltage pulses. To add single channel events to get an ensemble current, it is best to control for baseline changes by subtracting all nonchannel segments of the record using a blank trace constructed from each run of traces (see METHODS). This process is time-consuming, but it removes all leak current, which changes significantly over time. Because this technique uses a mathematical subtraction of traces that includes the noise in the recording, the segments of the records free of channel openings can be entirely free of noise and baseline drift (e.g., the opening-free segments of Fig. 6).

Ensemble currents were made for three phenytoin trials, those employing 100-ms pulses. With a holding potential of −100 mV and a test potential of −30 mV, late sodium currents (50−100 ms) averaged 0.48 ± 0.27% of peak current (mean ± SE; n = 3).

Both ensemble peak current and late current were reduced in phenytoin (60 μM). The drug reduced peak currents to 41.1% of control. Late currents (50–100 ms) were reduced to 24.3% of control. To display the relative effect of the drug on peak versus late currents, the phenytoin ensemble current was scaled up so that its peak value was the same as control (Fig. 6, inset). Late current in phenytoin was significantly lower than the current that would be expected from the null hypothesis that phenytoin would reduce peak and late currents equally (current = 59 ± 8% of null hypothesis prediction, n = 3; P < 0.05).
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Analysis of the phenytoin effect using idealized traces

Idealized traces were used to analyze late sodium channel openings in seven patches in which all trials used 750-ms pulses to a holding potential of −30 mV and a phenytoin concentration of 60 μM. The idealized traces were constructed as described in METHODS. The peak currents were measured after subtraction of calculated blank pulses as described in METHODS. The use of 750-ms pulses for these experiments allowed recording of enough late channel openings in each run of 56 pulses to quantify the time course of the peak and late currents reliably over the ~1-h period that patches were held (Fig. 7). Peak currents declined to approximately one-half by 1 h, whereas late currents remained more stable. There was more point to point variation for late currents than peak currents, chiefly because of the rare occurrences of late bursts (e.g., the unusual number of burst recurrences in Fig. 5, left, are reflected in the outlying high value for late current at 35 min in Fig. 7).

The late currents averaged 0.39 ± 0.10% of peak current (n = 7). The drug effects appear to be completely reversible, though there was a slow decline in peak current that was present before and after the drug exposures (Fig. 7, top). Maximal drug effects were seen after the 2-min wash-in period (Fig. 7). In the seven phenytoin trials analyzed in this way, the drug reduced peak current to 39.9% of control. Late current (100–750 ms) was reduced to 22.1% of control. Late current in phenytoin was significantly lower than the current that would be expected from the null hypothesis that phenytoin would reduce peak and late currents equally (current = 55 ± 5% of null hypothesis prediction, n = 7; P < 0.001). The effect of phenytoin was therefore similar using both ensemble current and idealized trace methods. In both cases, phenytoin demonstrates a preferential effect of the drug on the late openings of the channels at −30 mV and 60 μM drug concentration.

The main effect of phenytoin was to reduce the number of channel openings, rather than the duration of individual openings. The number of late channel openings in phenytoin
was reduced to 28% of control \( (n = 4, P < 0.001) \). In contrast, the duration of openings was reduced less: when assessed by the average duration of openings, the openings in phenytoin were 79% of control duration \( (n = 4, P < 0.05) \). When three of these four experiments with enough values to fit time constants were assessed using a simplex least square exponential fit of channel open times in pClamp, the fast \( \tau \) \((\sim 0.3 \text{ ms}) \) of the late openings was the same as control \( (4\% \text{ increase, n.s.}) \) and the slow \( \tau \) \((\sim 2 \text{ ms}) \) was decreased to 73% of control \( (P < 0.005) \). A pair of histograms from this analysis is shown in Fig. 8. These findings indicate that the major effect of the drug was to decrease the number of late channel openings with both fast and slow \( \tau \) time courses, but there may in addition have been a small amount of additional reduction in the duration or numbers of the slow \( \tau \) events.

Burst openings occurred both in the absence (Fig. 5, Control) and presence of the drug (Fig. 5, Phenytoin). The bursts were less frequent in phenytoin, but it is difficult to determine whether such a reduction in frequency is significantly different from the reduction of brief late openings because of the small number of the bursts \( (\text{typically } \leq 1\% \text{ of traces or } 0–2 \text{ bursts per drug condition}) \) and the lack of statistical independence of successive bursts in those traces that have clusters of successive bursts \( (\text{e.g., Fig. 5}) \). In contrast, the numerous brief late events were reduced by phenytoin to a degree similar to the effect on the total late events, as determined by mathematically excluding burst openings \( >5 \text{ ms} \) from the analysis \( (\text{cf. concentration dependence studies below}) \) and by the similarity of results in patches in which the bursts were rare or absent.

Concentration dependence of the phenytoin effect

The experiments described above used 60 \( \mu \text{M} \) phenytoin, which is above the therapeutic range of free phenytoin concentrations of 4–8 \( \mu \text{M} \) \( (\text{McLean and Macdonald 1983}) \). The 60-\( \mu \text{M} \) dose was high enough to obtain results that were robust to variations in channel opening such as those produced by channels bursting.

The effect of phenytoin on peak and late sodium currents also was tested in concentrations from 10 to 60 \( \mu \text{M} \). The experiments were done as multiple exposures of a single patch to different concentrations to control for possible variations due to different states or types of sodium channels in a patch. A dose-response curve was obtained from the trials \( (\text{all with a test voltage of } -30 \text{ mV; } n = 4; \text{Fig. 9}) \). At each concentration, phenytoin reduced both the peak and persistent currents.

The persistent current was reduced 15% at the lowest concentration \( (10 \mu \text{M}; \text{near the therapeutic drug concentration of } 4–8 \mu \text{M}) \). The variability for the persistent current records at 10 \( \mu \text{M} \) phenytoin was particularly high due to bursts that occurred during two of the four trials at this concentration. Because a single burst typically added \( \sim 10\% \) to the current recorded during a set of 112 pulses at a particular drug concentration, single bursts can skew significantly the analysis at low drug concentrations where the actual drug effects are small. Accordingly, the effect of phenytoin on the brief late openings was analyzed in isolation from the late bursts \( (\text{by discarding all openings lasting } >5 \text{ ms}) \). For the brief late channel openings alone, the effect of 10 \( \mu \text{M} \) phenytoin was a 22% reduction in current; this may be a more plausible value for the reduction of persistent current because the reduction of peak current was 15% and reductions of persistent current were greater than reductions of peak current at all other concentrations tested.

Voltage dependence of the phenytoin effect

The effect of phenytoin on peak and persistent sodium currents also was tested at different voltages from -60 to 0 mV. These tests were done as multiple voltage pulses for each patch \( (n = 3; \text{all at } 60 \mu \text{M} \text{ phenytoin}; \text{Fig. 10}) \) so as to control for possible variations due to different states or types of sodium channels in a patch. The reduction of peak currents by phenytoin was similar at all voltages tested \( (\bullet) \), while the effect on the late currents was greater at more depolarized voltages \( (\blacksquare) \).

Discussion

Late sodium channel openings

This study adds to the growing body of evidence for the presence of persistent sodium currents in neurons \( (\text{Alonso and Llínás 1989; Alzheimer et al. 1993; French and Gage 1985; French et al. 1990; Masukawa et al. 1991; Stafstrom et al. 1985; reviewed in Taylor 1993}) \). Such persistent currents appear to account for the pacemaker potentials associated with repetitive firing \( (\text{Hotson et al. 1979; Stafstrom et al. 1982, 1984}) \) and appear to underlie the ictal activity in a
culture system with epileptiform activity (Segal 1994 and these studies).

No evidence was found in these single channel recordings for the presence of ‘cation channels’ currents, as have been described in neurons with activation by voltage (Alzheimer 1994) neurotransmitters (Guérineau et al. 1995; Haj-Dahmane and Andrade 1996) or calcium (Partridge and Swandulla 1988). Such currents have reversal potentials ranging from −60 to −9 mV (Alzheimer 1994; Guérineau et al. 1995). However, additional studies would be required before reaching firm conclusions as to the absence of cation channels in the system. Some posited cofactors for cation currents, such as cyclic-nucleotides, calcium and neurotransmitters, were reduced or absent in the outside-out patch recordings, so one cannot rule out the presence of cation channels from these experiments that were designed to examine sodium channels. It is also possible that the posited cation channels were blocked by the cesium used in these experiments, though described currents have been permeable to cesium (Alzheimer 1994; Guérineau et al. 1995). It is also possible that a small population of tetrodotoxin (TTX)-sensitive cation channels is buried among a more numerous population of sodium channels, though described current appear to be TTX insensitive (Alzheimer 1994). Experiments designed specifically to test for cation channels are needed to resolve these questions. However, this study was aimed to identify the currents underlying endogenous bursts of action potentials and depolarization associated with ictal activity, which are TTX-sensitive events that occur in the absence of neurotransmitter action or calcium entry (Segal 1994), so those endogenous bursts likely are to be driven largely or completely by a persistent sodium current.

The sodium channel recordings shown here were obtained using outside-out “off-cell” patch recording. Outside-out patches offer an important advantage in studying the action of drugs, because it is possible to perfuse the drugs on and off the patch rapidly. Although it is possible to perfuse drugs from the inside of a patch pipette, this would be difficult to do for a drug as hydrophobic and as likely to stick to surfaces as phenytoin. However, there is concern about whether off-cell patches accurately reflect physiological channel kinetics. Some investigators find that under the less disrupting cell-attached patch-clamp conditions, the sodium channel openings are more brief than those seen in off-cell recordings from cardiac (Kirsch and Brown 1989) or neuronal channels (Aldrich and Stevens 1987). However, some have found little difference between cell-attached patch and off-cell patch recordings in neurons (Kirsch and Brown 1989), and ‘box-like’ channel openings that look very similar to those found here (e.g., Fig. 5) have been recorded using cell-attached patch recording (Alzheimer et al. 1993). In studies in progress in this culture system, we also have used cell-attached patch recordings and found instances in which

FIG. 4. Tetrodotoxin (TTX; 1 μM) blocked all channel currents in outside-out patches. No channel currents were seen in TTX traces, but channel currents were present in almost all control traces before and after TTX. Cesium (144 mM) in the patch pipette blocked any potassium channels.
only punctuated sodium channel openings were found as well as instances at the same voltage in which the common type of openings was box-like (M. Segal, S. Yilma, and A. Douglas, unpublished data). This indicates that the outside-out channel recordings shown here are representative of at least some physiological circumstances present in neurons. The nature of these conditions governing channel open properties is being investigated.

**Late sodium channel openings and epileptiform activity**

The demonstration of late sodium channel openings in these neurons is relevant to our understanding of the currents that generate epileptiform activity. In this culture system, some neurons display “interictal” epileptiform activity [with wedge-shaped Paroxysmal Depolarizing Shifts (PDSs)], whereas others display “ictal” epileptiform activ-
Phenytoin

Phenytoin reduces late openings of sodium channels. The ictal neurons generally had endogenous (nonsynaptic) bursts of action potentials and depolarization, whereas interictal neurons lacked such endogenous bursts (Segal 1994). Because the endogenous bursts of action potentials and depolarization underlying the ictal epileptiform activity were TTX sensitive and occurred in the absence of calcium or neurotransmitter action, it was hypothesized that these endogenous events were driven by a persistent sodium current (Segal 1994), but this remained to be tested by voltage-clamp studies as done here.

The presence of late sodium channel openings at the resting potential ($\approx -62$ mV) (Segal 1994) and at the voltage needed to sustain plateau depolarizations ($\approx -30$ mV) (Segal 1994) suggests that these late channel openings can be physiologically significant in producing epileptiform activity. Because...
application of the sodium channel blocking TTX abolishes the ramp depolarization that occurs at resting potential and leads to initiation of the ictal-associated endogenous bursts in neurons (Segal 1994), it is likely that these late sodium channel openings at the resting potential play an important role in initiating ictal epileptiform activity. Because the plateau depolarizations appear to underlie the continuation of ictal epileptiform activity in these neurons (Segal 1994), it is likely that the late sodium channel openings seen at approximately equal to $-30 \text{ mV}$ contribute to the current underlying the continuation of the ictal activity in this system.

Therefore it is likely that examination of late openings of sodium channels provides a direct measurement of the currents crucial for initiating and continuing epileptiform activity in this simplified system. In the future, this hypothesis needs to be tested in more intact systems, though such questions are difficult to study in more complicated networks. However, the ability to search for drugs that are very selective for blocking late openings of sodium channels will provide a test of an important implication of the persistent sodium channel hypothesis, and may be used to screen for new anti-epileptic drugs.

Late sodium channel openings were present in all neurons from which outside-out patches were made. The brief openings were invariably present, but the presence of long burst openings was highly variable in different neurons. Late sodium channel openings also were present in all cell-attached patch and inside-out patch recordings in which early sodium channel recordings were present (M. Segal, S. Yilma, and A. Douglas, unpublished results). It was not attempted at this stage to compare epileptiform activity in a series of neurons followed by sampling of channels displayed by the same neurons, because of the low yield of obtaining prolonged outside-out patch recordings from neurons. Therefore there is no direct information about the relationship between variability in late sodium channel
openings and epileptiform activity. Because all excitatory neurons appear to have late openings of sodium channels, it is clear that the difference between “ictal” and “interictal” neurons in this system is not explained by a complete absence of all types of late sodium channel openings in the interictal neurons. However, a variety of less-stark differences between ictal and interictal neurons could be present: ictal neurons could have more of all types of sodium channel openings, all late openings, or a particular type of late opening such as the channel bursts. Alternatively, ictal neurons could have less countervailing potassium current than the interictal neurons.

Phenytoin effects on sodium currents

The outside-out patch sodium channel studies reported here demonstrate a preferential effect of phenytoin on late openings of sodium channels. This is in accord with the known use-dependent action of phenytoin (Kuo and Bean 1994; Lang et al. 1993; Matsuki et al. 1984; Willow et al. 1985). However, the use-dependence studies were done by analyzing peak sodium currents so the nature of effects on late sodium channel openings was unclear (Taylor 1993). The direct examination of persistent sodium currents allows a direct test of the effect of an anticonvulsant on a current that appears to be important in initiating and continuing epileptiform activity.
FIG. 8. Histograms of channel open time in vehicle control and phenytoin. More test depolarizations were included for the phenytoin condition than control (448 vs. 168; all from the same patch) to give comparable numbers of channel openings (2,119 vs. 2,572). Exponential curves were fit using the simplex least square algorithm in pClamp software using 2 exponentials.

In addition to the findings reported here and in our preliminary communication (Segal et al. 1995), one other study of macroscopic persistent currents has demonstrated that phenytoin reduces persistent sodium currents at low drug concentrations (Chao and Alzheimer 1995). This study, using whole cell recording, found inhibition of the persistent current by phenytoin with an EC$_{50}$ of 34 μM, lower than the 70–260 μM EC$_{50}$ values for peak currents reported in the literature (reviewed in Chao and Alzheimer 1995). The suppression of persistent currents to ~23% with 60 μM phenytoin reported in this paper falls directly on the macroscopic whole-neuron dose response curve in the Chao and Alzheimer (1995) study, providing confirmation for both the single channel and the whole neuron methods for analyzing late sodium currents.

The 15–22% decrease in late sodium current at 10 μM phenytoin appears to represent a larger effect of the drug at low doses than the ~5% decrease in late current obtained by Chao and Alzheimer (1995) at that concentration. The results could differ for a variety of reasons, such as the pulse parameters or the interpulse intervals (1.25 s here). Also, single channel methods may be inherently more accurate because one can correct for changes in leak current down to the level of single channel openings as was done here.
PHENYTOIN REDUCES LATE OPENINGS OF SODIUM CHANNELS

Because 8 μM phenytoin abolishes the plateau depolarizations in those neurons that are associated with ictal activity (Segal 1994), the effect of phenytoin on the persistent sodium current appears to be sufficient to account for the antiepileptic action of the drug in this system.

The single channel studies demonstrate that the primary effect of phenytoin is to reduce the number of sodium channel openings with a much smaller effect on reducing the duration of the openings. This provides direct evidence supporting the kinetic model suggested from analysis of transient sodium currents by Kuo and Bean (1994) proposing that phenytoin acts primarily by stabilizing inactivated states of the sodium channel, which would lead to fewer channel openings. Because the voltage dependence of the phenytoin effect on the persistent sodium current was substantial, and no voltage dependence of drug effect on peak currents was found (Fig. 10), this suggests that one of the steps involved in phenytoin’s effect on the late channel openings is voltage dependent.

Implications for development of anticonvulsant drugs

These results suggest that a persistent sodium current is important in initiating and continuing ictal epileptiform activity in this system. The action of the anticonvulsant phenytoin is to reduce this persistent current underlying seizures more effectively than it reduces the peak current underlying normal action potentials.

This preferential effect on late channel openings suggests that the important pharmacological action for a sodium channel anticonvulsant is to reduce late openings of sodium channels rather than reduce all sodium channel openings. It is reasonable to hypothesize that pharmacological interventions that are most selective in reducing late openings of sodium channels while leaving early channel openings relatively intact will be those that produce an anticonvulsant effect with minimal interference with normal function.

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