The Effect of Phenytoin on Sodium Conductances in Rat Hippocampal CA1 Pyramidal Neurons

Z. ZENG1, Elisa L. HILL-YARDIN3, D.WILLIAMS3, T. O’BRIEN1,2 Andris SERELIS 1, C.R. FRENCH1,2
1. The Department of Medicine, the University of Melbourne, Melbourne, Victoria, Australia
2. Neurology Department, the Royal Melbourne Hospital, Melbourne, Victoria, Australia
3. The Department of Physiology, the University of Melbourne, Melbourne, Victoria, Australia

Author for Correspondence - CR French, Department of Medicine, University of Melbourne, Royal Pde, Parkville, Victoria, 3050 frenchc@unimelb.edu.au

Abbreviation
PHT: Phenytoin
AED: Anti-Epileptic Drug
DMSO: Dimethyl sulfoxide
HEPES: 2-[4-(2-hydroxyethyl)piperazine-1-yl]ethanesulfonic acid
PIPES: piperazine-N,N'-bis(2-ethanesulfonic acid)
TEA: Tetraethylammonium
4-AP: 4-Aminopyridine
EGTA: Ethylene glycol tetraacetic acid
IV: current-voltage
GV: conductance-voltage

Abstract
The anti-epileptic drug phenytoin (PHT) is thought to reduce the excitability of neural tissue by stabilising sodium channels (NaV) in inactivated states (Rogawski and Loscher 2004). It has been suggested the fast inactivated state (“IF”) is the main target (Kuo and Bean, 1994), though slow inactivation (“IS”) has also been implicated (Quandt, 1988). Other studies on local anaesthetics with similar effects on sodium channels have implicated the NaV voltage sensor interactions (Muroi and Chanda, 2009). Here, we re-
examine the effect of PHT in both equilibrium and dynamic transitions between fast and slower forms of inactivation in rat hippocampal CA1 pyramidal neurons. The effects of PHT on fast, slow and another identified “intermediate” inactivation processes were observed. The effect of enzymatic removal of fast inactivation was also studied as well as effects on the residual persistent sodium current (INaP). A computational model based on a gating charge interaction was derived that reproduced a range of PHT effects on NaV equilibrium and state transitions. No effect of PHT on I_F was observed, rather phenytoin appeared to facilitate the occupancy of other closed states, either through enhancement of slow inactivation or through formation of analogous drug-bound states. The overall significance of these observations is that our data are inconsistent with the commonly held view that the archetypal Nav channel inhibitor phenytoin stabilises fast inactivation states, and we demonstrate that conventional slow inactivation “I_s” as well as a more recently identified “intermediate” duration inactivation process I_l are the primary functional targets of phenytoin. Additionally, we show that the traditional explanatory frameworks based on the “modulated receptor hypothesis” (Hille 1976) can be substituted by simple, physiologically plausible interactions with voltage sensors. Additionally, I_{ap} was not preferentially inhibited compared with peak I_{Na} at short latencies (50 ms) by PHT.

New and Noteworthy

Phenytoin is an effective anti-epileptic drug. It has commonly been assumed that phenytoin has a specific affinity for the fast inactivated state of the sodium channel. The current study found no effect on fast inactivation, but that forms of slow inactivation were enhanced by phenytoin. Additionally, a theoretical study is presented showing the effects of phenytoin can be explained through an interaction with the activated voltage sensor.

Introduction

Phenytoin (PHT) is an anti-epileptic drug (AED) commonly used for a range of epileptic seizures (Browne and Holmes 2001). It is thought to achieve its therapeutic effect mainly through modulating the availability of voltage-gated sodium channels (NaV, Lipicky et al 1972, Mclean and Macdonald 1983) which are responsible for the initiation of action potentials in CNS neurons. Depolarization of the neuronal membrane results in a rapid phase of “activation” with channels entering open, conductive states. This activation phase
is followed by a reduction in sodium current amplitude termed “fast inactivation” (I_F), related to motion of the D3-D4 cytoplasmic linker (Bezanilla 2008). PHT selectively inhibits high frequency firing (Macdonald and McLean 1986) and also preferentially blocks sustained repetitive firing (Lampl et al 1998, Ragsdale and Avoli 1998). These effects have been considered to be a result of differential binding affinity of the drug to the different kinetic states of the channel, with highest affinity for inactivated states (the “modulated receptor hypothesis”, Hille 1977). However, although the fast inactivated state is generally considered the major target (Kuo and Bean 1994), PHT reduced sodium current amplitude in neuroblastoma cells after enzymatic removal of I_F (Quandt 1988). The relatively slow onset and offset of PHT action compared to the kinetic properties of I_F has been ascribed to slow binding of the drug to this state (Kuo and Bean 1994, Rogawski and Loscher 2004) rather than effects on slow inactivation as such.

Additionally, several studies report a stronger inhibition of the persistent sodium current (I_NaP) compared to the transient component by PHT. This would be expected to reduce repetitive firing (French et al 1990), augmenting the seizure suppressive action of PHT (Colombo et al 2013, Chao et al 1995).

In the present study we have examined the effects of PHT on equilibrium (steady state inactivation distributions), rates of transitions between closed and closed inactivated states for I_F and I_S, as well as an inactivation process with time constants intermediate to these processes, termed “I_I”. Additionally, the effect of PHT on I_Na after fast inactivation removal with papaine was examined. PHT did not significantly affect either transition or equilibrium properties of I_F but did affect slower forms of inactivation. I_NaP was not preferentially inhibited by PHT compared with the transient sodium current, I_NaT.

**Methods**

All procedures were carried out in accordance with protocols approved by University of Melbourne Animal Ethics Committee.

**Solutions and Drugs**

Stock aliquots of PHT (50 mM) in DMSO were stored at -20C and thawed at the time of experiment. Apart from the dose-reponse experiments, a concentration of 50 uM/l phenytoin was used, within the serum therapeutic range (40-80uM/l, Kozer et al, 2002).
Brain slices were prepared in a high sucrose bicarbonate-buffered “cutting solution” containing (in mM): NaCl 87, KCl 2.5, NaHCO₃ 25, NaH₂PO₄ 1.25, CaCl₂ 0.5, MgCl₂ 7, D-glucose 25, Sucrose 75 bubbled with 95% O₂ and 5% CO₂. Brain slices were maintained in bicarbonate buffered saline containing (mM): NaCl 125, KCl 2.5, NaHCO₃ 25, NaH₂PO₄ 1.25, CaCl₂ 2, MgCl₂ 1, D-glucose 25, bubbled with 95% O₂ and 5% CO₂ at room temperature. Recordings from dissociated neurons were performed in either HEPES buffered saline containing (mM): NaCl 125, KCl 2.5, CaCl₂ 2, MgCl₂ 1, D-glucose 25, HEPES 10, pH adjusted to 7.4 with NaOH; or low sodium buffered saline containing (mM): NaCl 35, KCl 2.5, CaCl₂ 2, MgCl₂ 1, D-glucose 10, 4-AP 4, Tris HCl 55, TEA 40, CdCl₂ 0.1, pH adjusted to 7.4 with Tris Base at 22 oC. Protease type XIV (Sigma-Aldrich) was dissolved in PIPES buffered saline containing (mM): NaCl 115, KCl 5, PIPES 20, CaCl₂ 1, MgCl₂ 4, D-glucose 25 and the pH was adjusted to 7.0 with NaOH. Recording electrodes were filled with intracellular CsF-based patch pipette solution containing (mM) CsF 85, NaCl 35, CaCl₂ 1, MgCl₂ 1, EGTA 10, HEPES 10, TEA 20, pH adjusted to 7.2 with CsOH), and osmolality of 280-300 mosmol l⁻¹.

**Dissociated Neuron Preparation**

2-6 week old Wistar rats (male and female) were deeply anaesthetized with pentobarbital sodium (Lethabarb) and decapitated with rapid brain removal. Coronal brain slices 400 µm thick were sectioned with a vibratome (Microm) at 4°C in cutting solution saturated with 95% O₂ and 5% CO₂. Hippocampal slices were subsectioned, then equilibrated in bicarbonate buffered saline at room temperature for 30 min. Small segments (~1mm²) of the CA1 region were treated with protease type XIV (1 mg/ml) in PIPES buffered saline which was constantly perfused with 95% O₂ and 5% CO₂ for another 30 min at 32°C in a temperature-controlled chamber. Treated slices were washed with HEPES buffered saline or, where appropriate, with low sodium buffered saline before being triturated to isolate individual neurons in the recording chamber.

**Electrophysiological Recording**

Pyramidal neurons were identified visually. Patch pipettes were made from borosilicate glass tubing (1.5mm outer diameter, 0.86mm inner diameter, Harvard Apparatus, USA) with a Sutter P-1000 electrode puller. Neurons were approached with patch pipettes under visual control with positive pressure. Upon contact the cell membrane, the positive
pressure was cancelled for seal formation. Access to the cell interior was obtained by applying further suction. The resistance of the recording pipette was between 1 to 2 MΩ using whole-cell voltage clamp configurations. Current and voltage were recorded using a Molecular Devices Axopatch 200B or AM systems Model 2400 patch clamp amplifier with approximately 90% predictive and compensatory series resistance compensation. Capacitive transients were minimized by maintaining the bath solution at a low level, and were further reduced by the capacitive compensation circuit of the amplifier. Signals were digitized at between 100-200 kHz with Axon 1320 (Molecular Devices, Sunnyvale, Ca) or National Instruments (NI USB-6259) devices, and low pass filtered at 10 kHz with a 4 pole Bessel filter. The digitizer was connected to a personal computer for stimulus generation and data acquisition with pClamp 10 (Molecular Devices) or Strathclyde WCP version 4.1.7 programs (University of Strathclyde, Scotland). A holding potential of -100 mV was routinely used unless otherwise noted. Potassium channels were blocked by external TEA (20 mM) and Cs ions in the patch electrode and calcium channels were blocked by Cd²⁺ (0.5 mM) externally. Leakage and capacitive currents were subtracted with a P/4 protocol. Experiments were performed at 22 °C with routine verification of chamber temperature. Control and drug containing solutions were applied via a custom made gravity flow manifold system, permitting solution changes within approximately 2 seconds, using a 100 um diameter outflow tube positioned approximately 500 um from the recorded cell. A liquid junction potential of ~ +10 mV was calculated for the pipette and bath solutions (Barry et al 1994), but potential measurements have not been adjusted for this offset.

Analysis and Statistics

Data analysis was performed with Molecular Devices Clampfit 10, Graphpad Prism and Microsoft Excel software packages. Statistical errors are presented as standard error of the mean (SEM) unless otherwise stated. Rates of onset and offset of inactivation were measured using exponential functions. Non-linear least-squares parameter estimations were performed with the Marquadt-Levenberg algorithm as implemented in Clampfit and Prism, and a quadratic minimization algorithm in the Microsoft Excel Solver package. Comparisons between single and double exponential data fits were analysed for significance using F statistics as implemented in Prism and Clampfit. Student’s t-tests were used for comparison of the means with a p value of < 0.05.
Steady-state inactivation ($h_{in}f$) curves were parameterised by a Boltzmann function

$$h_{in}f = \frac{1}{1 + e^{(V-V')/k}}$$

where $V$ is the command potential (mV), $V'$ is the half-inactivation potential (or half-activation potential) and $k$ the slope factor (mV). A similar equation was used for steady-state activation ($m_{in}f$).

Computational Modelling

Modelling of the NaV kinetics were carried out with JSim (Butterworth et al 2014) and Matlab (The MathWorks, Inc.) by generating Markov state models of the sodium channel which included slow inactivated states.

Results

Phenytoin Does Not Affect $I_{Na}$ Activation

The effect of phenytoin on activation of $I_{Na}$ was tested with step depolarisations before and after application of 50 uM phenytoin. Representative current traces with depolarisations from -80 mV to +50 mV with 10 mV increments are shown in Fig 1a and the peak normalized currents were fitted with a Boltzmann distribution. Activation occurred at -60 mV, with maximal conductance at +10 mV. The half activation potential ($V_{0.5}$) was $-32.1 \pm 3.3$ mV with a slope factor of 7.6 mV. Phenytoin (50 µM) had no significant effect on $V_{0.5}$ or slope. Time to peak, reversal potential and residual persistent current ($I_{NaP}$) were also unaffected by 50 µM PHT (Fig 1 b,c). The effect on $I_{NaP}$ is described in greater detail below. Application of PHT led to reduction of amplitude of $I_{Na}$ in a voltage-dependent manner as previously described (Matsuki et al, 1984; Willow et al, 1985; Ragsdale et al, 1991). At a holding potential of -100 mV, $I_{Na}$ was typically decremented by 20% after application of PHT (50 µM, Figure 2) and this inhibition was enhanced at more positive holding potentials. The time course of fast inactivation, as measured by the decrement of the macroscopic current, was a biexponential process as described previously (Sah et al, 1988). Neither the time constants or relative amplitudes of fast inactivation were affected by 50 µM PHT (Fig 2).

Dose-response of PHT on $I_{Na}$

Concentration-dependence of PHT on $I_{Na}$ inhibition was tested with 50 ms long depolarisations to -20 mV from a holding potential of -80 mV activated every 2 s as
control and drug containing solutions were applied to the neuron at concentrations from 1-200 µM. Peak $I_{Na}$ amplitude was normalised to the control solution amplitude then plotted against the log PHT concentration. An IC$_{50}$ of $72.6 \pm 22.5$ µM was calculated (Fig 3).

**Effect of PHT on Steady-state Inactivation with Conditioning Prepulses of Different Durations**

The equilibrium distribution of channels between closed and closed inactivated states is conventionally measured by the steady-state inactivation ($h_{inf}$) relation (Hodgkin and Huxley 1952). A minimum prepulse duration of approximately 3-5 time constants is needed for the channels to reach equilibrium, however longer pulses may start to engage other slower processes. Therefore a prepulse duration of 50 ms was used (Fig 4C) for the $I_F$ $h_{inf}$ curve based on the kinetics measured by Kuo and Bean (1994) as well as our own recordings to isolate the fast inactivation process. Additionally, as a component of inactivation with time constants of seconds had been observed previously (Kuo and Bean 1994) corresponding to previously described “slow inactivation” or “Is” (Rudy 1978), $h_{inf}$ curves were generated with a prepulse duration of 10 s (Fig 4E). We also used 500 ms duration prepulses (Fig 4D) because components of inactivation with time constants of the order of $10^2$ ms have been seen in neuronal preparations (Fliedervish et al 1996, Colbert et al 1997) including in this laboratory (French et al, 2015). Interestingly, simply increasing the duration of the prepulses from 50 to 500 ms (Figs 4D) resulted in an approximately 10 mV negative shift of the midpoint of the $h_{inf}$ curve, suggesting inactivation recruitment over this time period.

The $I_F$ $h_{inf}$ relation had a half inactivation ($V_{0.5}$) value of -69.8 ± 2.2 mV and a slope of 8.6 ± 0.4. PHT (50 µM) had no significant effect on these parameters (Fig 4c). The 500 ms and 10s V0.5 were shifted to negative potentials (-80.2 ± 4.1 mV and -84.8 ± 3.6 mV; 500 ms and 10 s protocols, respectively) compared to the $I_F$ relation in control solution. Application of 50 µM PHT caused a negative shift of the 500 ms and 10 s relations by 7 and 8 mV respectively, in marked distinction to the fast inactivation $h_{inf}$ relation.

**Effect of PHT on Fast Inactivation Transition Rates**

Rates of transition into the fast inactivated state were unaffected as measured by the rate of decay of $I_{Na}$ activated by step depolarisation to -20 mV (Fig 2D). Double pulse
experiments were then used to further explore the rates of transition to and from fast inactivated states before and after PHT application. Entry into fast inactivation was assessed by measuring the decrement of $I_{Na}$ to a series of depolarising prepulses incremented from 0 to 50 ms to -20 mV prior to a test depolarisation to -20 mV from a holding potential of -100 mV (Fig 5A). Recovery from $I_F$ was measured at a recovery potential of -100 mV after 20 ms depolarisations to -20 mV at 0-50 ms intervals (Fig 5b). Time constants of onset of inactivation were 1.4 ± 0.4 ms and 2.0 ± 0.3 ms before and after PHT ($n = 5, p = 0.1$). Recovery from fast inactivation at -100 mV was 10.7 ± 1.4 ms in control and 13.0 ± 2.9 ms in PHT (50 µM; $p=0.2, n=9$). Thus, as with the $I_F$ equilibrium relation, there was no effect of PHT on the dynamics of transition into and out of $I_F$ states.

**Effect of PHT on Slower Inactivated States**

As there was no significant effect of PHT on onset and offset of $I_F$ and the corresponding $h_{\text{inf}}$ curve (Fig 4C), effects of PHT on previously identified slow inactivation processes ($I_I$ and $I_S$) were investigated. This was particularly relevant as the equilibrium states (as determined by the $h_{\text{inf}}$ curves for these processes) were shifted to the left after exposure to 50 µM PHT (Fig 4D, E), which would conventionally imply an affinity for those states (see Hille 1977, Bean et al, 1983).

To test the effect of PHT application on the onset of slower forms of inactivation, neurons were depolarised to -20 mV from a holding potential of -100 mV for 10 s. During the depolarisation, cells were intermittently repolarised for 15 ms at -100 mV to remove fast inactivation and a 5 ms test pulse performed to measure slow inactivation onset. Four initial 250 ms intervals to capture “intermediate” inactivation components and subsequent 1 s intervals were used. The decrement of the $I_{NaT}$ of the test pulse is shown in the insert of Figure 6A and was best described by a double exponential function. The slow and intermediate inactivation time constants in control solutions were 4878.7 ± 230.4 ms and 111.8 ± 11.4 ms ($n=6$) respectively, the faster component being ~ 45% of the total decrement. The application of PHT (50 uM) reversibly decreased the $I_S$ time constant to 1519.6 ± 84.4 ms ($p<0.001, n=6$). In contrast, the intermediate inactivation time constants were not affected (123.1 ± 8.9 ms; $p=0.1, n=6$). The relative amplitude of $I_I$ to $I_S$ time constants were not altered by PHT (~ 45 %, $p=0.2$) and no additional time constant was seen.
The effect of phenytoin on the offset of slower forms of inactivation was then measured. The protocol was initiated with a 10 s pre-pulse to -20 mV from a holding potential of -100 mV. A series of 5 ms test pulses were sampled 265 ms apart for the first four pulses (to sample the faster component), and then sampled at 1 second intervals. The subsequent current amplitudes were normalised to the response at time zero and plotted against recovery time (Fig 6B). Again, the recovery was characterised by a sum of two exponentials. Both time constants were significantly increased; the slower component increasing from 2031.5 ± 114.6 ms to 3321.8 ± 463.0 ms (n=6, p=0.01) and the intermediate component increased from 198.1 ± 22.6 ms to 538.2 ± 188.0 ms in the presence of 50 µM PHT, which was reversed during washout. The intermediate component increase in amplitude did not reach statistical significance in these experiments (p = 0.08). However, in separate experiments with a larger number of sampling points over a shorter interval (700 ms) the time constant of offset of II was increased in the presence of 50 µM PHT. The relative amplitudes of I and I S were not significantly altered (~ 65 to 72 %, p = 0.2).

Effect of PHT on closed state transition at sub-threshold potentials

As noted above, macroscopic INa activates at approximately -60 mV. At hyperpolarised potentials, channel opening probability is low, but interchange between closed and closed-inactivated states occurs, as implied by the steady-state inactivation relation and as embodied in the Hodgkin-Huxley equations and Markov-state equivalent models. This can be termed “closed state transition inactivation” (CSI), as opposed to “open-state inactivation” (OSI) where channels have passed through the open state before inactivating (Goldman 1996, Bahring and Covarrubias 2011).

Initially, transitions between inactivated and closed states were observed by changing the membrane potential from -80 mV to -100 mV over an interval of 700 ms. 5 ms test pulses to -20 mV assessed channel availability. Figure 7A shows the protocol and a sample current trace (insert). The increase in amplitude of INa before and after application of 50 µM PHT was measured, normalised and plotted against the transition interval. Two components of current recovery were evident in both the control and drug affected conditions. A fast component corresponding to I F was present that was not significantly affected by the drug (p = 0.1, n = 4). The slower component corresponding to the previously identified “intermediate” duration process was slowed in the presence of drug
and changed from \(109.7 \pm 44.0\) ms to \(843.5 \pm 229.8\) ms (\(p = 0.04, n = 4\)). The relative amplitude of the intermediate component increased from \(19.7 \pm 5.3\) % to \(65.8 \pm 10.8\) % but did not quite reach significance (\(p=0.06, n=4\)).

The transition from closed to closed-inactivated states was investigated similarly, using voltage steps from a holding potential of -100 mV to -80 mV prior to a test pulse to -20 mV. The responses and fitted exponential curves in control and 50 µM PHT are shown in Figure 7B. \(I_f\) time constants were again not significantly affected by PHT, \(26.5 \pm 6.8\) ms and \(26.8 \pm 6.9\) ms in control and PHT respectively. Notably, \(I_i\) time constants of \(237.9 \pm 100.7\) ms in control and \(217.3 \pm 55.0\) ms in PHT were not significantly changed (\(p = 0.8, n=5\)), and there was no change in relative amplitudes. Nonetheless, PHT application decreased channel availability from about 40 % to 25 % at the end of 700 ms transition time.

### Relationship of Use-Dependent Block by PHT to Slow Inactivation Processes

Phenytoin and other sodium channel modulators enhance “use dependence”, whereby currents evoked by trains of depolarisations under voltage clamp or action potentials in voltage recording mode are attenuated by drug application. Although commonly associated with drug action, a similar intrinsic pattern of attenuation is seen in control conditions. We therefore sought to discover if intrinsic use-dependence could be related to the forms of inactivation identified here and if PHT affects these processes.

For this experiment, a train of 10 ms depolarisations to -20 mV at 25 Hz over one second from a holding of -100 mV was employed. A sample protocol is shown in the insert of Figure 8B. \(I_{Na}\) amplitude decreased over time, consistent with the accumulation of inactivated channels with repeated depolarisations. These responses were plotted against time and were best described by a double exponential timecourse, with components similar to the previously identified fast and intermediate inactivation time constants, plausibly relating these observations to the previously identified inactivation processes.

Using a 50 ms inter-pulse interval protocol, fast and intermediate inactivation time constants were \(40.2 \pm 4.1\) ms and \(542.2 \pm 146.3\) ms; \(52.3 \pm 9.0\) ms and \(560.1 \pm 126.5\) ms before and after 50 µM PHT. The relative amplitude of the fast inactivation component was significantly decreased in the presence of PHT (63.3 \(\pm 5.9\) % and 44.0 \(\pm 5.4\) %, before and after PHT; \(p=0.01, n=4\)). The total inactivated fraction of \(I_{Na}\) was \(\sim 18\%\) in control and \(\sim 30\%\) in PHT after the 1 second duration protocol. When the same pulse protocol was
performed at 40 Hz, the two time constants were again seen, but with smaller amplitudes; 21 and 354 ms respectively. Similarly, time constants were not affected by PHT, but the proportion of the slower component and total inactivated fraction increased to 55% in the presence of PHT. Although this experiment showed two time constants of inactivation similar to those reported above, as repolarisation occurs between pulses, it would not be expected that the time constants correspond exactly with the more specific protocols used in the previous experiments.

Effect of Removal of Fast Inactivation by Papaine

The use of protease or papain to remove macroscopic fast inactivation of $I_{Na}$ has been used in several previous studies (Armstrong et al., 1973; Oxford G.S., 1981; Zilberter and Motin, 1991), however, this approach has been less frequently used in central neurons. We found that addition of papaine (0.75mg/ml) to the patch pipette solution essentially removed fast inactivation over a duration of about 15 min (Fig 9a). PHT (50 µM) reversibly decreased $I_{Na}$ amplitude by a similar proportion to that seen with untreated cells (~20%, Fig 9a). When depolarisation was prolonged over several seconds, the currents in control solution attenuated with two time constants, similar to the slow and intermediate processes identified above (Fig 9b). The reversal potential of $I_{Na}$ remained constant during these depolarisations (data not shown).

Using a five second depolarisation protocol, the two inactivation time constants (intermediate and slow) were $331.1 \pm 85.8$ ms and $1689.2 \pm 404.8$ ms (in control conditions) and $206.5 \pm 52.4$ ms and $839.4 \pm 212.8$ ms in 50 µM PHT, findings consistent with an acceleration of entry into inactivated states (p=0.02 for intermediate inactivation; p=0.02 for slow inactivation; n=6 in each group). No additional time constants of decay corresponding to a possible binding rate of phenytoin were observed, and these effects were reversible with washout.

We also tested the effect of papaine on $h_{inf}$ curves (Figures 10A and B). Papaine largely removed the transition to inactivation with a 50 ms conditioning pulse presumably due to the abolition of $I_F$. However, the conventional sigmoid steady-state inactivation relation was restored with the long duration (500 ms) conditioning pulse, most likely due to the preservation of $I_{II}$ with papaine.

Effect of Phenytoin on Persistent Sodium Current ($I_{NaP}$)
PHT has been reported to inhibit the persistent sodium current $I_{Nap}$ to a greater extent than the transient component $I_{Na(t)}$ (Chao et al, 1995; Colombo et al, 2013). The effect of PHT (50 µM) on the fractional amplitude change of $I_{Nap}$ compared to $I_{Na(t)}$ was measured at 48 ms, about 24 times the time constant of fast inactivation at -20 mV. $I_{Nap}$ constituted 2.1 ± 0.4 % and 1.8 ± 0.5 % of the peak current before and after 50 µM PHT respectively, a non-significant variation ($p=0.6, n=6$).

**Computer Modelling**

As the results above could be consistently explained by effects on slower forms of inactivation, a model for the sodium conductance incorporating a slow inactivation state was developed. The aim of this model was to explore possible mechanisms of action, rather than to generate a detailed reproduction of experimental data. We had observed in separate studies that two of the most commonly described effects of phenytoin and other sodium channel modulators (SCMs) under voltage clamp conditions, the negative shift in the $h_{inf}$ curve and slowing of recovery from inactivation, were reproduced by small changes in the voltage parameter of the forward rate constant of inactivation ($\alpha_h$) in the Hodgkin Huxley equations. Additionally, a recent atomic-resolution model of Nav-phenytoin interactions revealed a potential binding site in the voltage sensor region in the activated state (Boiteaux et al 2014). A Markov-state model with 2 closed, one open as well as single fast and intermediate duration inactivation states was constructed (Fig 11a) with voltage sensitive rate constants derived from the HH equations (see appendix 1). By simply changing the voltage parameter of the forward rate constant of intermediate inactivation ($\alpha_{hI}$) from 100 to 110 mV, the leftward shift in the 500 ms $h_{inf}$ curve (Fig 11e) as well as significant slowing of recovery were reproduced (Fig 11c,d). The notable voltage sensitivity of the phenytoin effect is also reproduced (Fig 11b). Additionally a proportionally smaller change (25%) in the rate of onset of inactivation was seen, similar to the experimental data for the intermediate form of inactivation. The longer IS component was not included in this model for simplicity. Of note, no assumptions are made about relative binding affinities to different states as proposed by the modulated receptor hypothesis and its derivatives.

Although the experimental data and simulation was consistent with modulation of slow inactivation, possibly through effects on voltage sensors, we sought to compare a conventional modulated receptor model with slow binding of phenytoin with the
experiments with papaine-induced removal of IF. A modified Markov Nav model with $I_t$ and $I_s$ incorporated, with $I_f$ removed (Fig 11g), was developed to model the papaine experiments with long depolarisations. A modulated receptor modification of the model was constructed by adding two parallel drug bound slow inactivated states ($I_t^*$ and $I_s^*$) as illustrated in Fig 11g with rate constants derived from previous estimates (Kuo and Bean 1994). With long duration depolarisations, little change in the time constants of intermediate and slow inactivation occurred (230 ms and 1250 ms respectively), however a large separate 2100 ms additional time constant of decay occurred, corresponding to the slow phenytoin binding. This result is discordant with the experimental finding of an acceleration of the components of slow inactivation, with no additional time constant.

**Discussion**

Phenytoin primarily reduces the amplitude and rate of recovery of sodium currents in neurons (Schwarz and Vogel, 1977, Matsuki et al, 1984). Like local anaesthetics (LAs) and antiarrhythmic drugs, the phenytoin inhibition of $I_{Na}$ is strongly dependent on holding potential. A cogent explanation of the effect of sodium channel modulators such as LAs and antiarrhythmic drugs was developed by Hille (1977) and Hondeghem and Katzung (1977). It was proposed that these drugs have a higher binding affinity for inactivated states of the channel than other states. This theory explains the leftward shift of the steady-state inactivation relation and slowing of recovery from inactivation observed with local anaesthetics (Courtney et al 1975, Chen et al. 1975). This framework has been extended to PHT, with the relatively slow on and off rates of the drug ascribed to slow binding kinetics (Kuo and Bean, 1994). Further, it has been generally interpreted that the fast inactivated state has the higher affinity for drug binding (for example Kuo and Bean 1994, Errington et al 2008, Karoly et al 2010, Brodie and Sills 2011). Starmer et al (1983) proposed the “gated receptor hypothesis”: that the motion of the activation and inactivation gates results in exposure of binding sites (rather than inactivated states) as the targets of SCMs, explaining the voltage sensitivity of block and other SCM effects. However Khodorov et al (1976), Matsuki et al (1984) and Quandt (1988) have suggested that slower forms of inactivation are involved which would explain the effects of local anaesthetics and PHT. Nevertheless Karoly et al (2010), while noting the difficulty in
experimentally differentiating effects of SCMs on slow versus fast inactivation processes,

have suggested that fast inactivation is likely to be the main target of these drugs.

Although the present study cannot definitively identify the mechanism of action of

phenytoin, we have attempted to place the drug effects in the context of “normal” channel

inactivation behaviour throughout these experiments, including “intermediate” timescale

processes reported by several groups.

Phenytoin may influence $I_F$ by slow binding kinetics as suggested by Kuo and Bean (1994)

and the current experiments have not completely excluded this possibility. Nonetheless,

the qualitative amplitude of effect of PHT remains despite fast inactivation removal, so it

is unlikely that $I_F$ is the only and/or main target. It is also unexpected that the steady-state

inactivation relation for fast inactivation is remains constant with PHT – as has similarly

been reported by Matsuki et al (1984) and Kuo and Bean (1994).

A second plausible mechanism is that PHT affects slow inactivation processes, possibly

including innate “use-dependence”. A consistent observation in this study has been the

presence and drug modification of two components of slow inactivation, a more

“conventional” slow form with time constants of the order $10^3$ ms and a form that has

been termed “intermediate” with time constants of the order $10^2$ ms (French et al, 2015).

Both ranges of time constants have been observed previously in CA1 neurons (eg Migliore

and Fleidervish 1996, faster component; Kuo and Bean 1994, slower component) but have

generally been treated as a single process. The shift in the 500 ms and 10s, but not 50 ms,

$h_{inf}$ curves with phenytoin would conventionally suggest a higher affinity for the slower

states. Matsuki et al (1984) and Meves and Vogel (1977) found significant negative shifts

(>10 mV) with 60 s and 5 s prepulses respectively, the latter protocol being most similar

to the current study. Interestingly, Vandenplas et al (2013) did not see a significant shift in

the steady-state inactivation inactivation curve of the slow inactivation process in

neuroblastoma cells with phenytoin, but they used a rather long (500 ms) repolarisation

before the test pulse which would likely reduce or abolish such an effect. A notable

observation in our study was that the $I_S$ time constant of onset was reduced to 1.6s by 50

uM phenytoin, almost exactly the value cited by Kuo and Bean (1994) as the time constant

of onset of PHT effect, and interpreted as being incompatible with $I_S$ involvement.

Additionally, a recent molecular dynamical study of PHT interaction with Nav channels

reported a time constant of interaction with identified potential binding sites of less than

one millisecond (Boiteux et al 2014), i.e. much faster than that assumed by Kuo and Bean
(1994b) to explain the slow onset of PHT. A similarly short binding rate can be calculated from an additional study by Martin and Corry (2014), although these rates are estimates from rather short simulations periods in both studies. Alternatively, a phenylalanine residue, F1764 in the DIVS6 region is commonly considered the binding site for LAs and AEDs (Ragsdale et al, 1996) and mutation of this residue has been found to alter slow inactivation (Bai et al, 2003). It is therefore possible that binding of PHT to this site might affect slow inactivation to account for at least some of the experimental observations.

As the steady-state inactivation curve is the equilibrium distribution of closed and inactivated states resulting from rates of entry and exit, it was of interest to observe what effect phenytoin had on the onset and recovery rates into and out of inactivated states. A simple “increased affinity” for inactivated states would suggest an increased rate into and slowed exit rate from inactivation. This pattern was seen with conventional slow (~ order $10^3$ms) inactivation (Fig 5). Intriguingly, a different pattern was seen with the intermediate inactivation component – the rate of entry into this state was not greatly affected, but recovery was slowed considerably, as discussed below.

**Effect of Removal of Fast Inactivation with Papaine**

The inhibitory effect of PHT was preserved after removal of I_F with cytoplasmic papaine. Prolonged depolarisations for several seconds revealed two components of current decrement, similar to the time constants of onset of I_I and I_S identified with double pulse protocols. A striking acceleration of both components of inactivation with PHT was evident and reversible. If PHT were slowly binding to Nav channels without affecting slow inactivation as suggested by Kuo and Bean (1994b), it might be expected that the original time constants of inactivation would be preserved, with a third component corresponding to the PHT binding rate; however this was not seen. Rather, the modulated receptor simulation produced an extra time constant corresponding to slow binding (see Fig 11f, g), discordant with the experimental observations. These observations were again consistent with PHT enhancing or catalysing entry of open channels into slow inactivated states.

**Possible Role of Voltage Sensors as Phenytoin Binding Targets**

Channel voltage sensors have been implicated in LA and antiarrythmic drug effects. Local anaesthetics have been found to reduce gating charge (Keynes and Rojas 1974, Hanck et al
1994) and stabilise the domain III voltage sensor in an activated state (Arcisio-Miranda et al. 2010). A possible interaction of phenytoin with voltage sensors in Nav channels has also been considered by Kuo and Bean (1994). Additionally, slow inactivation of sodium channels is associated with a slow component of gating current immobilisation (Bezanilla et al., 1982) and Silva and Goldstein (2013) found that voltage sensor motion is intrinsically associated with the development of slow inactivation in sodium channels.

While there is no effect on the steady-state activation curve or rate of fast inactivation in this and previous studies with phenytoin, we hypothesise that drug interactions with gating charge affect slower inactivation processes that are initiated by outward motion of the charged S4 domains with depolarisation. These processes would produce “charge immobilisation” and subsequent and possibly related slow inactivation. Entry into slower inactivated states is related to outward voltage sensor movement with depolarisation, and the steady-state inactivation relation can be shifted by charge mutations in the S4 region (Muroi and Chanda 2009). Additionally, we could reproduce the salient effects of PHT by adjusting a voltage sensitivity parameter in a simple model of slow inactivation.

Specifically, using a Markov model based on the Hodgkin Huxley equations with an additional slow inactivated state, a modest 10 mV change in the voltage term of one of the inactivation rate constants ($\alpha_{Ih}$) produced both a hyperpolarising shift in the 500 ms steady-state inactivation curve as well as marked slowing of time constants of recovery (105 to 237 ms at -100 mV), with a much smaller prolongation of entry into slow inactivation (138 to 185 ms at -70 mV). These data are similar to our experimental observations for I\textsubscript{i} (see Fig 11 and appendix for equations). Another line of evidence supporting a voltage sensor role in phenytoin action are the molecular dynamical simulations of Boiteux et al (2014) that have shown a potential binding regions for phenytoin, in the voltage sensor region. Notably, the Boiteux model used voltage sensors in the activated state, i.e. in the conformation expected with depolarisation. Interactions of phenytoin with the four voltage sensors could produce the approximately 10 kcal energy change needed for this magnitude of voltage sensitivity alteration. It should be noted that this model does not have a strong dependence on the HH model, but only uses the equations for voltage sensitivity to provide a simple link between voltage sensor state and the experimentally observed equilibrium between closed and closed slow-inactivated states.
In summary we propose that phenytoin exerts at least some of its effects by interactions with S4 gating charges. Depolarisation with outward movement of the S4 voltage sensors in Nav likely exposes binding residues that interact with phenytoin (Boiteux et al, 2014), enhancing charge immobilisation and hence slow inactivation, and slowing return from these states. This is might be considered a form of the “gated receptor hypothesis”, with the addition that the actual binding target is the voltage sensor, and that this then enhances entry into slow inactivation. This is reminiscent of a “foot in the door” mechanism, in which exposure of the voltage sensors by outward motion of S4 are “latched” by binding to PHT. In support of this mechanism for the intermediate inactivation component, it was observed that the rate of entry into the intermediate inactivation state with depolarisation was minimally accelerated by PHT, compared with repolarisation which was quite slowed. This observation is compatible with “trapping” of voltages sensors once exposed. Both enhancement of transition into and slowing of recovery from I₈ was observed, also explainable by this mechanism. The final effect may occur via stabilisation of the selectivity filter P loop structures that is thought to underlie slow inactivation (Ulbrich 2005), also reported by Boiteaux et al (2014). It would therefore be of interest to examine the effects of phenytoin after mutagenesis of candidate S4 charge residues, as well as track S4 and P loop motion in the presence of drug using fluorometric techniques (Loots and Isacoff, 1998, Chanda and Bezanilla, 2002). A potentially analogous situation has been reported for a potassium channel agonist, NH29, which is thought to produce therapeutic effects by direct stabilisation of activated voltage sensors (Peretz et al, 2010).

**Lack of Phenytoin effect on Persistent Sodium Current (INaP)**

Several studies have reported that the persistent sodium current INaP is preferentially affected by phenytoin (Chao and Alzheimer 1995, Colombo et al 2013, Lampl 1998, Segal 1997). In the current study this was not found, reflecting probably both semantic and methodological issues. If the persistent sodium current is considered as the small residual current, persisting at long durations compared to the time constants of fast inactivation, then it did not appear preferentially affected by phenytoin using the 50 ms depolarising protocol in this study. However, we did not measure it during extended depolarisations where it may be reduced by the slow inactivation processes noted above, and enhanced by phenytoin. Nonetheless, even in this case, the amplitude of INaT at the point of
measurement should also be evaluated in order to evaluate whether $I_{\text{NaP}}$ is a more specific
target of phenytoin as concluded by Chao and Alzheimer (1995). Our conclusions are
similar to those Colombo et al (2013), who note that inactivation seems to be the process
affected by phenytoin in the suppression of $I_{\text{NaP}}$ with long duration depolarisations.

**Conclusion**

The mechanism of the inhibitory action of phenytoin on sodium channels has been
controversial and several issues remain to be resolved, including the exact mechanism of
interaction with the channel, the nature of the binding site and whether there is more
than one physiologically active site. The present study did not find any clear inhibition of
fast inactivation, a commonly assumed functional target for phenytoin, but rather the
observed effects could be interpreted as the modulation of slow inactivation mechanisms,
including a form that we have termed “intermediate”. This interpretation was extended
by showing that both experimental data and simulation studies were compatible with
such modulation of slow inactivation. These data are also compatible with the proposal
that the Nav voltage sensors may be targets for sodium channel modulators.

**Appendix 1**

Equations used for Markov state equations used in Fig 11A

\[
\alpha_m = 0.0978\frac{(V(t)+42.13)}{(1-\exp(-(V(t)+42.13)/6.49))}
\]

\[
\beta_m = 3.98\exp(-(V(t)+64.178)/18.43)
\]

\[
\alpha_{hf} = 0.08\exp(-(V(t)+92.3)/23.6)
\]

\[
\beta_{hf} = 2.54/(1+\exp(-(V(t)+7.5)/13.4))
\]

\[
\alpha_{hI} = 0.04\exp(-(V(t)+\alpha_{hI_V})/11); \alpha_{hI_V} = 100 \text{ or } 110 \text{ as described in text.}
\]

\[
\beta_{hI} = 0.0044/(1+\exp(-(V(t)+83.7)/13.4))
\]
References


Fig 1

A

B

C

Normalized Current

V_{\text{command}} (\text{mV})

Fraction of Channel Available

V_{\text{command}} (\text{mV})
Fig 2
Dose-response

Fig 3
Fig 4

A

B

C

D

E

Conditioning pulse potentials (mV)
Normalized current

Conditioning pulse potentials (mV)
Normalized current

Conditioning pulse potentials (mV)
Normalized current

Conditioning pulse potentials (mV)
Normalized current

Conditioning pulse potentials (mV)
Normalized current
Fig 5

A

Normalized current vs. Pre-pulse duration (ms)

- **CON**
- **50 µM PHT**

B

Normalized current vs. Interpulse interval (ms)
Fig 6

A

Test pulse duration (ms)

Normalized current

B

Interpulse interval (ms)

Normalized current

- CON
- 50 µM PHT
- WO
Fig 8

A

B

Normalized current

Duration of repetitive stimuli (ms)

-20mV
-100mV
$\Delta t = 25$ ms

1 s

0.5 nA

100 ms

CON

50 µM PHT

WO
Fig 9

A

-20 mV
-100 mV

20 ms

5 nA

5 ms

CON
50 µM PHT
WO

B

-20 mV
-100 mV

2 s

2 nA

500 ms
Conditioning pulse potentials (mV)

Normalized current

-120 -100 -80 -60 -40

0.0

0.2

0.4

0.6

0.8

1.0

Without papain

With papain

Fig 10
Figure 1. Current-potential (I-V) and Conductance-potential (G-V) relation before and after 50 μM PHT.
A, I-V voltage-clamp protocol is shown at the left panel, cells were held at -80 mV, a series of increments of 10 mV to 50 mV for 50 ms in duration. Representative current trances were shown in the right panel elicited by the I-V protocol. B, I-V relation plotted as a graph with control (blue circle) and 50 μM PHT (red triangle), n=7. C, G-V relation derived from the I-V in B and fitted with Boltzmann’s function. Half-activation potential ($V_{0.5}$) were found to be -32.1±3.3 mV and -32.6±2.7 mV in control and PHT respectively. Slope factor (k) were 7.6±1.6 mV and 9.5±1.8 mV in control and PHT respectively. No statistical significance were found in $V_{0.5}$ (p=0.7) and k (p=0.1) using paired T-test.

Figure 2. Changes in $I_{NaT}$ amplitude and macroscopic fast inactivation ($I_F$) with 50 μM PHT.
Representative $I_{NaT}$ generated by depolarization to -20 mV from holding potentials at -120 mV (A), -100 mV (B) and -80 mV (C) before (solid line) and after (dashed line) 50 μM PHT exerted effects. The percentage reduction in $I_{NaT}$ amplitude were 14.1±6.0 % (n=5), 23.8±3.4 % (n=6) and 38.7±9.5 % (n=6) at -120 mV, -100 mV and -80 mV respectively. D, A closer look at the macroscopic $I_F$ revealed double exponential components for both before (solid line) and after (dashed line) PHT effect (holding potential is -100 mV). There is no significant changes in both time constant components (paired t-test, p=0.4 for the slow component, and p=0.3 for the fast component) when 50 μM PHT was applied (n=6).
Figure 3. Dose-response curve of PHT in CA1 hippocampal pyramidal neurons. Fraction maximal response is plotted against the log concentration of PHT (n=6). A representative $I_{\text{NaT}}$ in control solution as well as in different concentration of PHT is shown in the insert together with the protocol. The holding potential was -80 mV. IC$_{50}$ was found to be 72.6±22.5 µM.

Figure 4. Steady-state inactivation of sodium current with 50 µM PHT. Data points with SEM’s are fitted to Boltzmann distributions. A, Steady-state inactivation protocol representative current traces shown. B, In control solution increasing conditioning pulse duration (50 ms, 500 ms and 10 s) produced hyperpolarising shifts (see text for values). Effect of 50 µM PHT on 50 ms (C), 500 ms (D) and 10 s (E) prepulse durations are shown. No significant change in $V_{0.5}$ was found with 50 ms prepulse ($p=0.97$, $n=6$), but were found with 500 ms (7 mV, $p<0.001$, $n=3$~6) and 10 s (8 mV, $p<0.001$, $n=3$) conditioning pulse durations.
Figure. 5 The effect of PHT on $I_F$ onset and recovery with double pulse protocol. Control data blue circle and drug treated red triangle. Recovery curves were well described by single exponential function. A, $I_F$ onset curve generated by the double pulse onset protocol shown (n=5)- a depolarizing prepulse to -20 mV for a variable interval (0-50 ms) was followed by a brief 10 ms repolarization to -100, followed by a test pulse to -20 mV as shown in th insert. Time constants of $I_{NaT}$ decrement of 1.4±0.4 ms and 2.0±0.3 ms for control and 50 µM PHT respectively were not significantly different (p=0.1). B, Recovery from $I_F$ with double pulse offset protocol shown – an initial 20 ms depolarization from -100 to -20 mV was followed by a 0-50 ms recovery period at -100 mV, with a subsequent 20 ms test pulse as illustrated in the insert (n=9). Time constants of recovery of $I_{NaT}$ are 10.7±1.4 ms and 13.0±2.9 ms in control and PHT respectively with no significant difference (p=0.2).
Figure 6. Effect of PHT on slow components of inactivation. Control blue circle, drug red triangle and washout black rectangle. All current responses were best described by a double exponential function. A, Onset of slow inactivation observed from 0 to 10s using test depolarisations with durations that initially increased in 4 250 ms increments and then in 9 1 s increments. PHT decrease the available current from about 25% in control to about 15% after 10s. Two components of inactivation termed “I1” and “I5” and 111.8±11.4 (45% total) and 4878.7±230.4 ms respectively were found. 50 µM PHT significantly reduced the slower time constant 1519.6±84.4 ms (p<0.001, n=6) but not I1 time constant (p=0.10, n=6). B, Modified double pulse protocols were used for the recovery of I5. PHT decreased about 10% channel availability after 9s. Again, two components were constantly seen. Only I5 time constants were affected significantly (p=0.01, n=8), but not I1 time constants (p=0.08, n=8). Paired t-test throughout. Both protocols and representative current traces are shown in the insert of the corresponding curves.

Figure 7. The effect of phenytoin on closed-state transition. Control blue circle, drug treated red triangle. All cells showed two time constants. A, -80 mV to -100 mV produces a transition from closed-inactivated to closed state. The time constants of recovery of I_{NaT} had components related to I_F and I_I (n=4). I_I time constant was significantly slowed by PHT (p=0.04, n=4), but not the I_F time constant. B, -100 mV to -80 mV produces a transition from closed to closed-inactivated state. The time constants of decrement of I_{NaT} were also found to contain I_F and I_I (n=5), but neither were significantly altered by PHT. Both protocols and representative current traces are shown in the insert of the corresponding curves.
Figure 8. Use-dependent block enhanced by phenytoin
Repeated stimulation pulse with the interpulse interval of 25 ms were shown before and after 50 µM PHT. A, Left panel: repeated stimulation protocol. Right panel: representative current trace. B, Normalised current peak vs stimulation duration. Control is blue circle, drug treated is red triangle and washout is black rectangle. All cells were better fitted with double exponential function. Only the amplitude of $I_F$ component was found to significantly decreased ($p<0.0001$, n=4). This suggests that $I_I$ components were significantly increased while the time constants were unaltered.

Figure 9. Enzymatic Removal of $I_F$ leaves phenytoin sensitivity intact.
0.75 mg/ml of papain was added to the intracellular solution remove $I_F$. Representative current trace of a single depolarisation to -20 mV from a holding potential of -100 mV is shown with different depolarisation durations, 20 ms (A), 2 s (B). Reversibly reduction of amplitude of $I_{Na}$ with 50 uM PHT is evident in the absence of $I_F$. The longer duration depolarization used in B revealed biexponential attenuation of $I_{Na}$ similar to the intermediate and slow components of inactivation seen in the normal preparation.

Figure 10. A) The steady-state inactivation curve shows little decrement with 50 ms conditioning pulses after papaine treatment (red squares) compared with control, (black circle, n=2~9). B) The steady-state inactivation curve with 500 ms pulses after papaine treatment showed significant decrement with depolarisation, consistent with the retention of the intermediate inactivation component evident in the long depolaristaion data (Fig 9b)
Fig 11. Simulations related to experimental findings - A) State diagram for sodium channel with fast and intermediate inactivation; slow inactivation state left out for clarity. The voltage parameter ("Vparhl") for the backward rate constant for alphah_i was changed from 100 mV (standard value) to 110 mV to simulate possible PHT interaction with gating charge resulting in an increased energy barrier to departure from the inactivated state. B) Effect of Vparhl parameter change on I_{Na} amplitude evoked by depolarisations from -100 and -80mV respectively- there is an approximate 25% reduction in I_{Na} amplitude at -80 compared with control, and a much smaller effect with the same depolarisation from -100 mV, ie very similar to the classical effect of phenytoin as shown in Fig 2A-C. C) recovery from inactivation at –100 mV with Vparhl = 100 mV and D) Vparhl = 110 mV showing slowing of recovery similar to PHT experimental findings (see Fig 7A). The fast recovery component was unaffected, but the slow component increased from 105 to 227 ms (182%); the rate of onset of the slow component was much less affected, decreasing from 185 to 138 ms (~25% reduction), again showing a similar pattern to experimental results. E) Steady-state inactivation curves from model—black diamonds -I_f, fast inactivation; black squares—I_i with Vparhl = 100 mV (standard model) and triangles showing shift to left of curve with Vparhl changed to 110 mV, as seen experimentally. G) Modified version of state diagram in A) with I_i and I_S, without fast inactivation to model PHT interaction sodium channels in the papaine preparation. Preferential interaction with inactivated states according to the modulated receptor hypothesis is simulated—PHT is assumed to interact with equal affinity for intermediate and slow inactivated states, with rate constants for 48uM PHT derived from Kuo and Bean 1994b to compare with experimental data shown in Fig 9—with this model, (panel F) a third time constant with large
Fig 11 (cont) amplitude (2.1 seconds, relative amplitude 37%) is introduced to the decay phase with the initial intermediate and slow time constants preserved (1.25 seconds and 230 ms, relative amplitudes 45% and 18%), as opposed to the experimental observation of an acceleration of both components without the addition of a third time constant. A similar pattern was observed when interaction of PHT with slow inactivation ($I_{S}$) alone was modelled.