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Lidocaine: A Foot in the Door of the Inner Vestibule Prevents Ultra-Slow Inactivation of a Voltage-Gated Sodium Channel

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ABSTRACT

After opening, Na^+ channels may enter several kinetically distinct inactivated states. Whereas fast inactivation occurs by occlusion of the inner channel pore by the fast inactivation gate, the mechanistic basis of slower inactivated states is much less clear. We have recently suggested that the inner pore of the voltage-gated Na^+ channel may be involved in the process of ultra-slow inactivation (I_{US}). The local anesthetic drug lidocaine is known to bind to the inner vestibule of the channel and to interact with slow inactivated states. We therefore sought to explore the effect of lidocaine binding on I_{US}. rNa_V 1.4 channels carrying the mutation K1237E in the selectivity filter were driven into I_{US} by long depolarizing pulses (−20 mV, 300 s). After repolarization to −120 mV, 53 ± 5% of the channels recovered with a very slow time constant (τ_{rec} = 171 ± 19 s), typical for recovery from I_{US}. After exposure to 300 μM lidocaine, the fraction of channels recovering from I_{US} was reduced to 13 ± 4% ($P < 0.01$, $n = 6$). An additional mutation in the binding site of lidocaine (K1237E + F1579A) substantially reduced the effect of lidocaine on I_{US}, indicating that lidocaine has to bind to the inner vestibule of the channel to modulate I_{US}. We propose that I_{US} involves a closure of the inner vestibule of the channel. Lidocaine may interfere with this pore motion by acting as a "foot in the door" in the inner vestibule.

Sodium channels are believed to have several distinct inactivated states. These states can be identified by their contribution to the time course of recovery from inactivation. When depolarized for up to 5 s, adult rat skeletal muscle (μ1; Na_V 1.4) Na^+ channels recover with three distinct components having time constants in the order of several milliseconds ("fast inactivation", I_F), several hundred milliseconds ("intermediate inactivation", I_M), and several thousand milliseconds ("slow inactivation", I_S; Kambouris et al., 1998). Furthermore, certain mutations in the selectivity filter region promote an ultra-slow inactivated state (I_{US}). Entry into and exit from I_{US} requires up to 20 min (Todt et al., 1999; Hilber et al., 2001, 2002).

Whereas the molecular basis of I_F is likely to be a "hinged lid" mechanism involving a cytoplasmic loop between the third and fourth domain [the III–IV linker, (West et al., 1992)], little is known about the mechanistic basis of the slower forms of inactivation.

It has been suggested that slower forms of inactivation occur by a closure of the extracellular mouth of the channel (Tomaselli et al., 1995; Balser et al., 1996; Townsend and Horn, 1997; Kambouris et al., 1998; Todt et al., 1999; Ong et al., 2000; Hilber et al., 2001, 2002; Vilin et al., 2001; Xiong et al., 2003).

Lysine 1237 is located in the outer vestibule of the rNa_V 1.4 channel and has a pivotal role in ionic selectivity (Favre et al., 1996), suggesting that this residue is part of the selectivity filter (Lipkind and Fozzard, 1994). The mutation K1237E favors entry into I_{US} supporting the notion that I_{US} is produced by a conformational change of the outer vestibule. On the other hand, we recently found that I_{US} is inhibited by closure of the intracellular inactivation gate indicating that the *intracellular* vestibule may participate in the molecular motion that underlies I_{US} (Hilber et al., 2002).

To explore the latter notion, we sought to explore the effect of lidocaine on I_{US}. Lidocaine blocks voltage-gated Na^+ channels by binding to the inner vestibule and by interaction with the inactivated conformation of the channel (Hille, 1977; Hondeghem and Katzung, 1977; Ragsdale et al., 1994). We

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W.S. and J.S. contributed equally to this work.

ABBREVIATIONS: I_{US}, ultra-slow inactivation; I_M, intermediate inactivation; I_S, slow inactivation; $I_{M,S}$, intermediate and slow inactivation.

examined the effect of lidocaine in the mutant K1237E because 100% of channels can be driven into I_{US} in this mutant, whereas in wild-type channels only ~20% of channels enter into I_{US} in response to long depolarizing pulses (Todt et al., 1999). We found that lidocaine dramatically increased the apparent time constant of entry into I_{US} whereas the time constant of recovery from I_{US} was unaffected. These data are consistent with a model in which I_{US} occurs by a closure of the inner vestibule. Lidocaine may enter the inner vestibule and inhibit the channel closure via a foot-in-the-door mechanism. Some of the data have been published in abstract form (Sandtner et al., 2004).

Materials and Methods

A detailed description of *Materials and Methods* is given in our previous work (Hilber et al., 2001).

Site-Directed Mutagenesis. Detailed methods for the mutagenesis have been published previously (Dudley et al., 1995; Sunami et al., 1997; Todt et al., 1999).

Electrophysiological Recordings. Stage V and VI *Xenopus laevis* oocytes were isolated from female frogs (Nasco, Ft. Atkinson, WI), washed with Ca^{2+}-free solution (90 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 1 mM $NaHPO_4$, and 5 mM HEPES titrated to pH 7.6 with 1 N NaOH), treated with 2 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) for 1.5 h, and their follicular cell layers were manually removed. As judged from photometric measurements, approximately 50 to 100 ng of cRNA was injected into each oocyte with a Drummond micro-injector (Drummond Scientific Co., Broomall, PA). Either native or mutant α subunit cRNA was mixed with rat brain β_1 cRNA at a molar ratio of 1:1. Oocytes were incubated at 17°C for 12 h to 3 days before examination.

Recordings were made in the two-electrode voltage clamp configuration using a TEC 10CD clamp (NPI Electronic GmbH, Tamm, Germany). For accurate adjustment of the experimental temperature (18 ± 0.5°C), an oocyte bath cooling system (HE 204; Dagan Corp., Minneapolis, MN) was used. Oocytes were placed in recording chambers in which the bath flow rate was about 100 ml/h, and the bath level was adjusted so that the total bath volume was less than 500 μl. Electrodes were filled with 3 M KCl and had resistances of less than 1 MΩ. Using pCLAMP6 software (Axon Instruments Inc., Foster City, CA), data were acquired at 71.4 kHz after low-pass filtration at 2 kHz (−3 db). Curve fitting was performed using ORIGIN 5.0 (OriginLab Corp., Northampton, MA). Recordings were made in a bathing solution that consisted of 90 mM NaCl, 2.5 mM KCl, 1 mM $BaCl_2$, 1 mM $MgCl_2$, and 5 mM HEPES titrated to pH 7.2 with 1 N NaOH. $BaCl_2$ was used as a replacement for $CaCl_2$ to minimize Ca^{2+}-activated Cl^- currents. Lidocaine was obtained from Sigma-Aldrich.

Data Evaluation. The normalized time courses of recovery from I_{US} of normalized peak inward currents were fit with the double exponential function:

$$y = A_1(1 - \exp(-t/\tau_1)) + A_2(1 - \exp(-t/\tau_2)) \quad (1)$$

where τ_1 and τ_2 are the time constants of distinct components of recovery, A_1 and A_2 are the respective amplitudes of these components.

The time course of entry into I_{US} was fitted with the monoexponential function:

$$y = y_0 + A(1 - \exp(-t/\tau)) \quad (2)$$

Data are expressed as means ± S.E.M. Statistical comparisons were made using one-way analysis of variance. A $P \leq 0.05$ was considered significant.

Results

Lidocaine Reduces Occupancy of I_{US}. When depolarized for several minutes, a small fraction of wild-type Na_V 1.4 channels enter into a long lived inactivated state, from which channels recover with a time constant of ~100 s. We refer to this state as I_{US} (Todt et al., 1999). To test, whether exposure

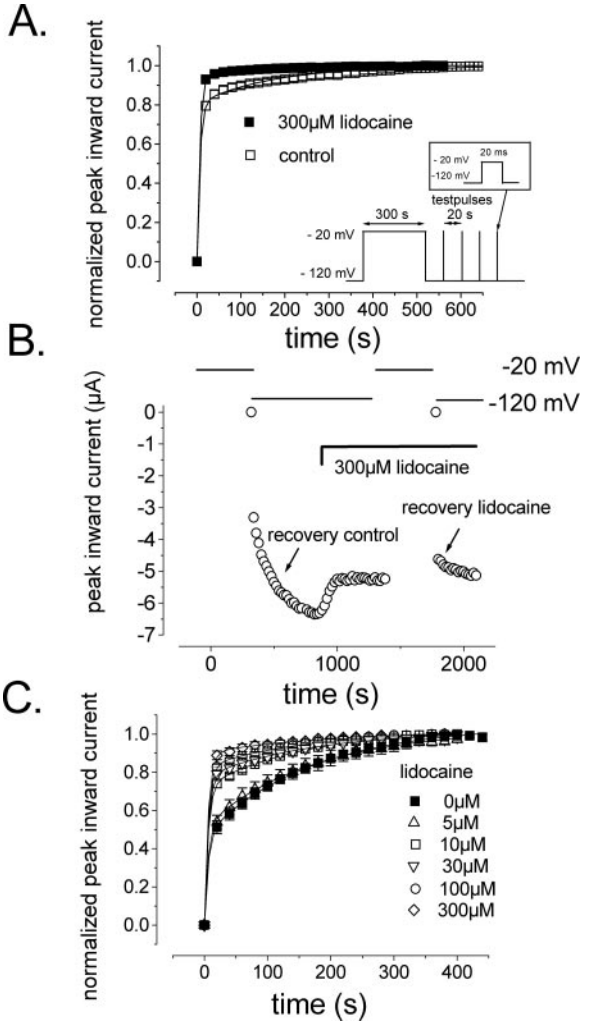


Fig. 1. A, effect of lidocaine on I_{US} in wild-type Na_V 1.4. From a holding potential of −120 mV, the channels were inactivated by a 300-s depolarizing step to −20 mV. After returning to −120 mV, recovery from inactivation was monitored by repetitive 20-ms test pulses to −20 mV at 20-s intervals (inset). Maximum inward currents elicited by the test pulses were normalized to the values attained after full recovery (mean values of five experiments). Data points were fitted with two exponentials (eq. 1). Fitting parameters were $\tau_1 = 6.8 \pm 0.3$ s, $\tau_2 = 170 \pm 5.8$ s, $A_1 = 0.80 \pm 0.01$, and $A_2 = 0.20 \pm 0.01$ under lidocaine-free conditions. Exposure to 300 μM lidocaine almost abolished the ultra-slow recovering component of recovery. For analysis, the time constants τ_1 and τ_2 were fixed to 6.8 and 170 s, and only the amplitudes were allowed to float, which resulted in the following estimates: $A_1 = 0.96 \pm 0.003$, $A_2 = 0.04 \pm 0.001$ ($P < 0.05$ when compared with control). B, recovery from I_{US} is modulated by lidocaine in K1237E. Data points are peak inward currents evoked by 20-ms step depolarizations to −20 mV from the indicated holding potential. During a drug-free control, recovery from a 300-s prepulse to −20 mV was monitored analogously to the protocol described in Fig. 1 (arrow: recovery control). Thereafter, the protocol was repeated during exposure to 300 μM lidocaine. C, summary of results of several experiments (as in B) using different concentrations of lidocaine. The time courses of recovery were normalized to the final level of recovery, and the data points were fitted with two exponentials (eq. 1). The parameters obtained from fitting are summarized in Table 2.

to lidocaine affects I_{US}, recovery from I_{US} was assessed in Na_V 1.4 channels during a drug-free control and after exposure to 300 μM lidocaine (Fig. 1A). The membranes were depolarized to −20 mV for 300 s. Thereafter, the potential was returned to −120 mV, and the time course of recovery from inactivation was monitored by 20-ms test pulses to −20 mV applied at 20-s intervals. Under control conditions, recovery had a biexponential time course with 80% of channels recovering with a time constant of 6.5 s, reflecting recovery from slow inactivation, which is the predominant state from which wild-type channels recover in response to long depolarizations (Kambouris et al., 1998; Todt et al., 1999). Twenty percent of channels recovered with a time constant of 170 s, reflecting recovery from I_{US}. During lidocaine exposure, the fraction of channels recovering from I_{US} was almost completely removed. The small fraction of channels recovering from I_{US} in wild-type channels even under lidocaine-free conditions precluded a systematic analysis of this drug effect in wild-type channels.

Previously, we reported that the mutation K1237E substantially increased the fraction of channels which enter into I_{US} in response to long depolarizations (Todt et al., 1999). Therefore, we examined the effect of lidocaine on I_{US} in the mutant K1237E. Figure 1B shows the effect of lidocaine in an oocyte-expressing K1237E channels. Recovery from I_{US} was examined as in Fig. 1A. Clearly, under control conditions most K1237E channels recovered with a very slow time course (Fig. 1B; arrow "recovery control"). After full recovery from I_{US}, 300 μM lidocaine were washed in, resulting in blockage of 17% of inward current. Because this block developed at −120 mV, it most likely reflects low-affinity binding of lidocaine to Na^+ channels in the rested state ("tonic block"). Following the establishment of steady-state tonic block, the time course of recovery from I_{US} was assessed again. Lidocaine substantially speeded recovery from I_{US} (Fig. 1B, arrow "recovery lidocaine"). In Fig. 1C, the normalized time courses of recovery from I_{US} during exposure to lidocaine concentrations of 5 to 300 μM are presented. Fitting two exponentials (eq. 1) to the time courses of recovery revealed that lidocaine had no effect on the time constant of recovery from I_{US} (τ_2) but decreased the fraction of channels recovering from I_{US} (A_2), suggesting that lidocaine may have increased the time constant of entry into I_{US} without affecting the time constant of exit from this state (Table 1).

Lidocaine Modulates I_{US} through a Binding Site in the Inner Vestibule. The residue Phe1579, which is located in the intracellular vestibule of the Na^+ channel, forms a part of the binding site of local anesthetic drugs. Accordingly, the mutation F1579A reduced the binding affinity of open and inactivated channels for lidocaine by 2- and 24.5-fold, respectively (Ragsdale et al., 1996). We wondered whether the effect of lidocaine on I_{US} was mediated by binding of lidocaine to this site. Hence a double mutant carrying the mutations K1237E and F1579A was constructed. To confirm that the double mutant K1237E + F1579A has a reduced binding affinity for lidocaine, we tested the effect of 300 μM lidocaine on currents evoked by repetitive 15-ms test pulses at varying frequencies. As shown in Fig. 2A, the single mutant K1237E was inhibited by lidocaine in a frequency-dependent manner. Such frequency-dependent block is due to the transient availability of higher affinity channel states during each stimulus pulse. The high sensitivity of K1237E channels to frequency-dependent block by lidocaine indicates a high affinity of open and/or inactivated channels for lidocaine. By contrast, the frequency-dependent block was almost abolished in the double mutant K1237E + F1579A (Fig. 2A). Thus, the additional mutation F1579A effectively abolished lidocaine binding to the high-affinity states. Next we tested the effect of lidocaine on I_{US}, which takes several minutes to develop. Recovery from I_{US} was assessed as in Fig. 1. Lidocaine (300 μM) had only a slight effect on I_{US} in the double mutant K1237E + F1579A (Fig. 2B) when compared with the single mutant K1237E (Fig. 1). This suggests that lidocaine has to bind to the cytoplasmic vestibule of the channel to inhibit entry into I_{US}.

Lidocaine Increases Occupancy of I_M in the Mutant K1237E. In wild-type Na_V 1.4 channels, one reported effect of lidocaine is to increase occupancy of I_M (Kambouris et al., 1998; Chen et al., 2000; Ong et al., 2000). Given the dramatic effect of lidocaine on I_{US} in K1237E, we wondered whether the lidocaine-induced occupancy of I_M is preserved in this mutant. To assess the development of binding of lidocaine to I_M, oocytes expressing K1237E channels were exposed to 300 μM lidocaine. From a holding potential of −120 mV, channels were depolarized to −20 mV for 10 ms (Fig. 3, A and B, inset). Thereafter, the membrane potential was returned to −120 mV and the time course of recovery from inactivation was assessed by 50 ms test pulses to −20 mV applied after variable recovery intervals (Fig. 3A). Channels recovered with two time constants τ_1 = 2.2 ± 0.25 ms and τ_2 = 747 ± 252.9 ms, reflecting the time constants of recovery from fast inactivation (I_F) and I_M, respectively (Chen et al., 2000). The fractions of channels recovering from I_F and from I_M were 0.74 ± 0.04 and 0.26 ± 0.02, respectively (A_1 and A_2 in eq. 1)

With longer prepulse durations, the fraction of channels recovering from I_M (A_2) increased substantially (0.76 ± 0.03 after a prepulse of 500 ms duration); however, prolonging the prepulse duration from 500 to 1000 ms did not result in any further substantial increase in the fraction of channels entering into the I_M state. These data suggest that lidocaine

TABLE 1

Parameters of biexponential fits (eq. 1) to the normalized time courses of recovery from ultra-slow inactivation in K1237E (Fig. 1C)

τ_1, τ_2, and A_1, A_2 are time constants (s) and amplitudes of recovery from slow inactivation and recovery from I_{US}, respectively.

	Lidocaine					
	0 μM (n = 6)	5 μM (n = 3)	10 μM (n = 3)	30 μM (n = 4)	100 μM (n = 5)	300 μM (n = 6)
τ_1	6.5 ± 1	7 ± 0.6	5.1 ± 0.9	5.6 ± 1.1	5.7 ± 1.1	5.2 ± 0.4
τ_2	171.2 ± 19.0	166.1 ± 24.3	165.9 ± 11.2	178.5 ± 19.2	168.1 ± 10.3	170.1 ± 19.1
A_1	0.47 ± 0.05	0.49 ± 0.02	0.63 ± 0.04*	0.76 ± 0.05**	0.80 ± 0.02**	0.87 ± 0.03**
A_2	0.53 ± 0.05	0.51 ± 0.02	0.36 ± 0.04*	0.24 ± 0.05**	0.20 ± 0,02**	0.13 ± 0.03**

* Statistically significant difference when compared with control ($P < 0.05$).
** Statistically significant difference when compared with control ($P < 0.01$).

increased the occupancy of I_M and the development of this process was completed within about 500 ms. Thus, similar to wild-type Na_V 1.4 channels (Chen et al., 2000), conditioning prepulses of 1000 ms duration result in steady-state occupancy of I_M by lidocaine in K1237E.

Next we assessed the concentration dependence of the lidocaine-induced occupancy of I_M. K1237E channels were depolarized for 1 s and recovery from inactivation was examined with a double pulse protocol. Under lidocaine-free conditions, full recovery of test pulse current was attained after ~10 s (Fig. 3B). Lidocaine did not change the time to full recovery but produced a concentration-dependent decrease in the fraction of channels recovering from I_F and a corresponding increase in the fraction of channels recovering from I_M (Fig. 3C, Table 2). According to Khodorov et al. (1976) and Bean et al. (1983), the strength of lidocaine binding to an inactivated state can be deduced from the relative fraction of channels recovering from that state. We quantified the interaction of lidocaine with I_M by calculating the fraction of channels unavailable to recover from I_M ($F_{non\text{-}IM}$) at a given concentration of lidocaine according to $F_{non\text{-}IM} = 1 - (A_{2\text{-}lido} - A_{2\text{-}control})/(A_{2\text{-}max} - A_{2\text{-}control})$, where $A_{2\text{-}lido}$ and $A_{2\text{-}control}$ are the fractions recovering from I_M during exposure to lidocaine and during drug-free control, respectively. $A_{2\text{-}max}$ is the maximal fraction recovering from I_M that was found during exposure to 1000 μM lidocaine. The apparent K_D for the reduction in $F_{non\text{-}IM}$ produced by lidocaine was 22.1 ± 2.6 μM (Fig. 3D). These data show that K1237E retains the state-dependent lidocaine sensitivity typical for wild-type μ1 channels (Kambouris et al., 1998).

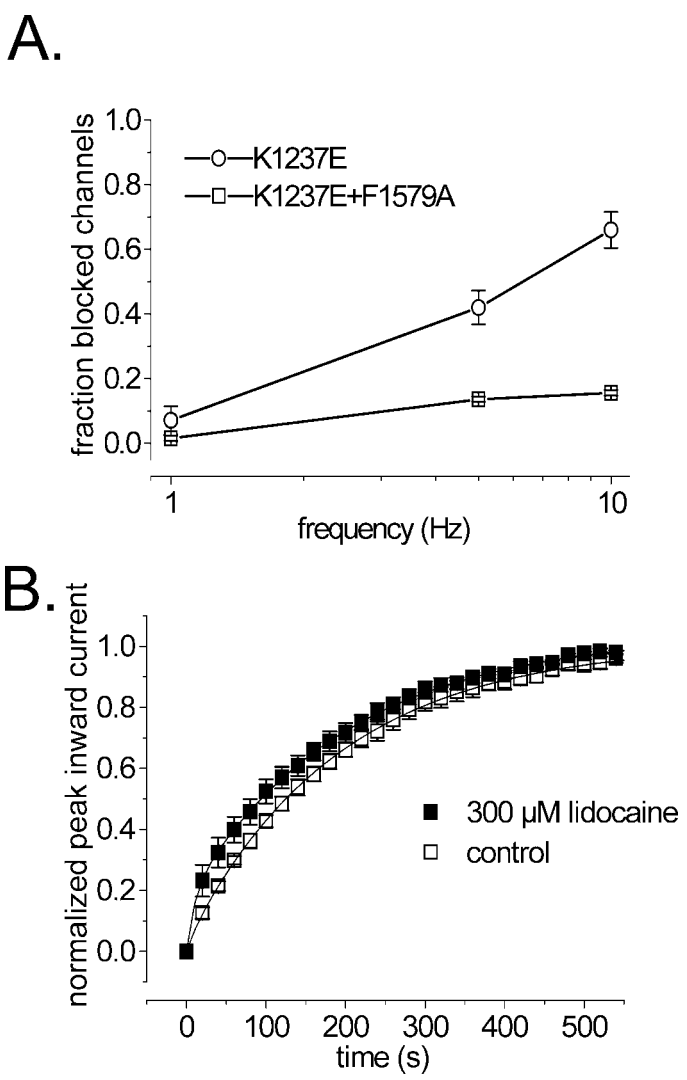


Fig. 2. Comparison of the effect of lidocaine on channel kinetics in the mutants K1237E and K1237E + F1579A. A, frequency-dependent block by 300 μM lidocaine. Inward currents were elicited by trains of 20 repetitive test pulses of 15 ms duration from the holding potential of −120 to −20 mV. The test pulses were applied at the indicated frequencies, and the fraction of blocked channels was calculated by the formula: fraction of blocked channels = (peak current during the 20th pulse)/(peak current during the 1st pulse), $n = 4$. B, effect of 300 μM lidocaine on recovery from I_{US} in K1237E + F1579A. The protocol is the same as shown in Fig. 1B, $n = 6$.

Lidocaine Does Not Alter the Distribution of Channels between I_M and I_S. As mentioned above, I_S in Na_v 1.4 channels is defined as an inactivated state with time constants of recovery on the order of several thousand milliseconds (Kambouris et al., 1998). To examine the effect of lidocaine on I_S wild-type Na_v 1.4 channels were depolarized for 25 s to −20 mV, and the time course of recovery was assayed as shown in Fig. 3C. Because lidocaine has a substantial effect on I_{US}, we chose to evaluate the effect of lidocaine on I_S in wild-type channels to minimize possible *interfering* effects of the drug on the neighboring I_{US} state. As shown in Fig. 4, the time course of recovery from a 25-s prepulse could be fit with a double exponential formula (eq. 1) yielding two time constants τ_1 = 932 ± 210 ms and τ_2 = 5620 ± 810 ms, reflecting the time constants of recovery from I_M and I_S, respectively. The fractions of channels recovering from I_M and from I_S were 0.36 ± 0.04 and 0.64 ± 0.04, respectively (A_1 and A_2 in eq. 1). Addition of 300 μM lidocaine to the bath had no significant effect on the time constants, and the relative amplitudes of I_M and I_S (τ_1 = 793 ± 206 ms and τ_2 = 5542 ± 781 ms, A_1 = 0.34 ± 0.06 and A_2 = 0.66 ± 0.06). These data show that the relative distribution of channels between I_M and I_S remained unchanged by lidocaine. If lidocaine had a higher affinity to either one of the two states, then this state would be stabilized relative to the state with the lower affinity, resulting in a change of the relative distribution of channels between the two states. Since this is not the case, we conclude that the affinity of lidocaine to I_M and I_S must be similar. Thus I_M and I_S represent high-affinity binding states for lidocaine.

A Model for Lidocaine Interaction with the Cytoplasmic Pore. In a previous study, we demonstrated that binding of the inactivation gate to the cytoplasmic vestibule of the voltage-gated Na^+ channel can protect the channels from entry into I_{US} (Hilber et al., 2002). We suggested that I_{US} may reflect a dynamic rearrangement involving the cytoplasmic vestibule of the channel. When the inactivation gate is bound to the cytoplasmic vestibule, it may stabilize the structure of the cytoplasmic vestibule and thereby prevent channels from entering into I_{US}. The data presented in this report indicate that lidocaine binds to a site in the cytoplasmic vestibule of the channel and interferes with the pore motion that underlies I_{US}.

Which mechanism might underlie the interaction of lidocaine with I_{US}? We propose a simple mechanism in which we assume that the lidocaine-bound states and the I_{US} state are mutually exclusive.

This hypothetical mechanism can be represented by

Scheme 1, where $I_{M,S}$ represents the high-affinity states for lidocaine binding (I_M and I_S), $I_{M,S\text{-lido}}$ represents the lidocaine-bound inactivated states, I_{US} is the ultra-slow inactivated state, and k represents the forward rate constant into the I_{US}. The transitions leading from the closed state to $I_{M,S}$ have been omitted because they are very rapid compared with the development of the I_{US} state. I_{US} is absorbing (Fig. 5A and see below), therefore the rate of exit from I_{US} can be assumed to be zero. This scheme predicts that the apparent time constant of entry into I_{US} should increase in a concentration-dependent manner. To test this prediction, we determined the time course of entry into I_{US} in the following manner: prepulses to −20 mV of variable duration were applied to K1237E channels from a holding potential of −120 mV, and the time course of recovery at −120 mV was monitored for each prepulse duration. The time course of recovery for each prepulse duration was then fitted with two exponentials that yielded time constants and amplitudes of I_S and I_{US}. To derive the apparent time constant of entry into I_{US} (τ_{entry}), the fraction of channels recovering from I_{US} following a given prepulse duration (A_2) was plotted as a function of the respective prepulse duration. These data were well fitted with a monoexponential function (eq. 2) allowing for estimation of τ_{entry}, which was 348 ± 29 s under control conditions and 649 ± 44 s during exposure to 10 μM lidocaine (Fig. 5A). Figure 5A also demonstrates that I_{US} is absorbing under drug-free conditions and during exposure to lidocaine. In Fig. 5B, the drug-induced change in τ_{entry} is plotted as a

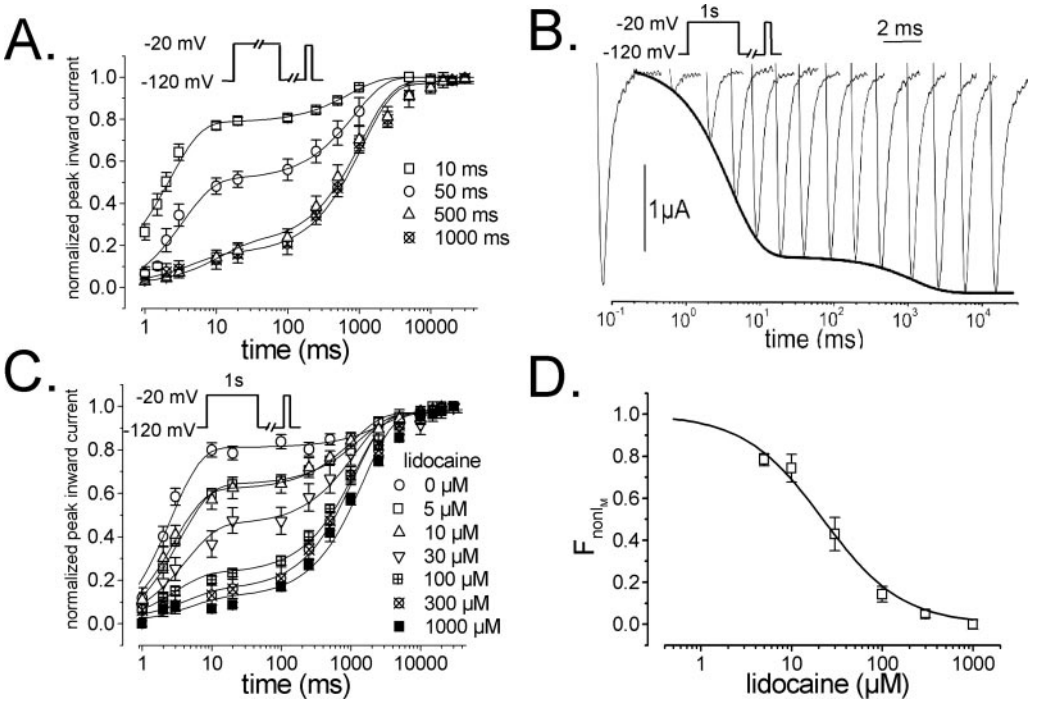


Fig. 3. Effect of lidocaine on the time course of recovery of K1237E from intermediate inactivation (I_M). A, development of intermediate inactivation during exposure to lidocaine. Oocytes were bathed in 300 μM lidocaine. From a holding potential of −120 mV channels were depolarized to −20 mV for 10 ms (n = 5), 50 ms (n = 3), 500 ms (n = 4), and 1000 ms (n = 6). The time course of recovery from inactivation was assessed by 50-ms test pulses to −20 mV applied at varying intervals following repolarization to −120 mV (inset). The inward currents elicited by the test pulses were normalized to the final current level following complete recovery. Data were fitted with two exponentials (eq. 1). B, recovery of inward currents following a conditioning 1-s prepulse to −20 mV compared with the current elicited by a test pulse before stepping to the conditioning potential. Data are from one oocyte expressing K1237E channels bathed in lidocaine-free solution. Recovery assumed a biexponential time course (connecting line) and was completed by ~10 s after the conditioning pulse. C, concentration-dependent increase in recovery from intermediate inactivation by lidocaine. Channels were depolarized for 1000 ms, and the time course of recovery was assessed by 50-ms test pulses to −20 mV applied at the indicated intervals following return to the holding potential. Inward currents elicited by the test pulses were normalized to the final level of recovery. The data points were fitted with two exponentials (eq. 1). The kinetic parameters derived from fitting two exponentials to the time courses of recovery (eq. 1) are summarized in Table 2. D, concentration-response curve of the interaction of lidocaine with the I_M state. $F_{non\text{-}IM}$ = the fraction of channels unavailable to recover from I_M (see text for details). The data were fitted with the single component Langmuir equation $y = K_D/([\text{lidocaine}] + K_D)$.

TABLE 2

Parameters of biexponential fits (eq. 1) to the normalized time courses of recovery from inactivation produced by a 1-s prepulse to −20 mV in K1237E (Fig. 3C)

τ_1, τ_2, and A_1, A_2 are time constants (ms) and amplitudes of recovery from fast inactivation and recovery from I_M, respectively.

	Lidocaine						
	0 μM (n = 6)	5 μM (n = 3)	10 μM (n = 3)	30 μM (n = 4)	100 μM (n = 5)	300 μM (n = 6)	1000 μM (n = 4)
τ_1	2.8 ± 0.30	3.4 ± 0.3	3.0 ± 0.3	2.9 ± 0.5	2.1 ± 0.5	1.8 ± 0.8	1.8 ± 1.1
τ_2	2443 ± 1269	1142 ± 176	1108 ± 184	1180 ± 174	1064 ± 101	1027 ± 100	1387 ± 154
A_1	0.83 ± 0.02	0.68 ± 0.02*	0.65 ± 0.04**	0.44 ± 0.05**	0.24 ± 0.02**	0.17 ± 0.02**	0.14 ± 0.02**
A_2	0.17 ± 0.02	0.32 ± 0.02*	0.35 ± 0.04**	0.56 ± 0.05**	0.76 ± 0.02**	0.83 ± 0.02**	0.86 ± 0.02**

* Statistically significant difference when compared with control ($P < 0.05$).
** Statistically significant difference when compared with control ($P < 0.01$).

function of the lidocaine concentration. Clearly, lidocaine produces a concentration-dependent increase in τ_{entry}.

If binding and unbinding of lidocaine is fast compared with the development of I_{US}, the above scheme also predicts that the apparent time constant of entry into I_{US} (τ_{entry}) will be increased by exactly the same factor by which the fraction of channels recovering from $I_{M,S}$ is increased by lidocaine: -fold change in the fraction of blocked channels ($I_{M,S\text{-}lido}$) = τ_{entry} (during lidocaine exposure)/τ_{entry} (under lidocaine free conditions).

This relationship allows the estimation of K_D for lidocaine binding to the inactivated high-affinity states ($I_{M,S}$). The K_D for lidocaine binding, derived by this method, was 12.8 ± 2.5 μM (Fig. 5B). This value is in good agreement with the K_D for occupancy of the I_M state by lidocaine (Fig. 3C), which strongly supports the validity of the proposed model. (As shown in Fig. 4, the K_D for lidocaine binding to I_S is similar to the K_D for lidocaine binding to I_M).

Lidocaine Has Similar Effects on I_{US} in K1237E and K1237S. The amino acid Lys1237 is a part of the selectivity filter of the Na^+ channel (Lipkind and Fozzard, 1994). Previously, Sunami et al. (1997) suggested that this residue electrostatically interacts with the binding of lidocaine. We wondered whether in the mutant K1237E an electrostatic interaction between lidocaine and Glu1237 plays a role in the modulation of I_{US} by lidocaine. Therefore, we examined the effect of lidocaine on I_{US} in a mutant in which the positively charged Lys1237 was replaced by the neutral amino acid serine. As shown in Fig. 6, the effect of 300 μM lidocaine on I_{US} in K1237S was similar to the effect of lidocaine on I_{US} in the mutant K1237E (Fig. 1C). The fact that the kinetic effect of lidocaine was not influenced by the charge of residue 1237 argues against the notion that the effect of lidocaine on I_{US} is the result of a direct interaction of lidocaine with the selectivity filter region.

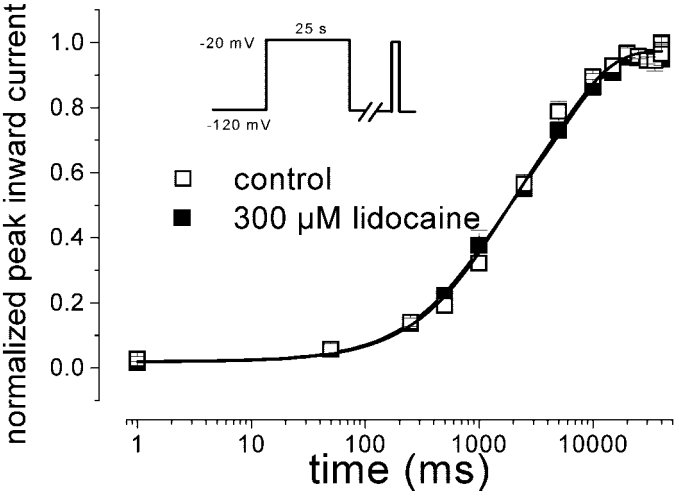
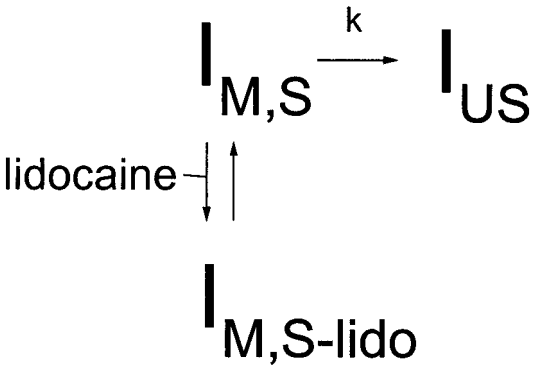


Fig. 4. Lidocaine does not alter the distribution of channels between I_M and I_S. Na_V 1.4 wild-type channels were depolarized for 25,000 ms to allow channels to enter both I_M and I_S states (inset). The time course of recovery was assessed by 50-ms test pulses to −20 mV applied at the indicated intervals following return to the holding potential. Inward currents elicited by the test pulses were normalized to the prepulse current. The data points were fitted with two exponentials (eq. 1), reflecting time constants and amplitudes of recovery from I_M and I_S. Fitting parameters are given in the text.



Scheme 1.

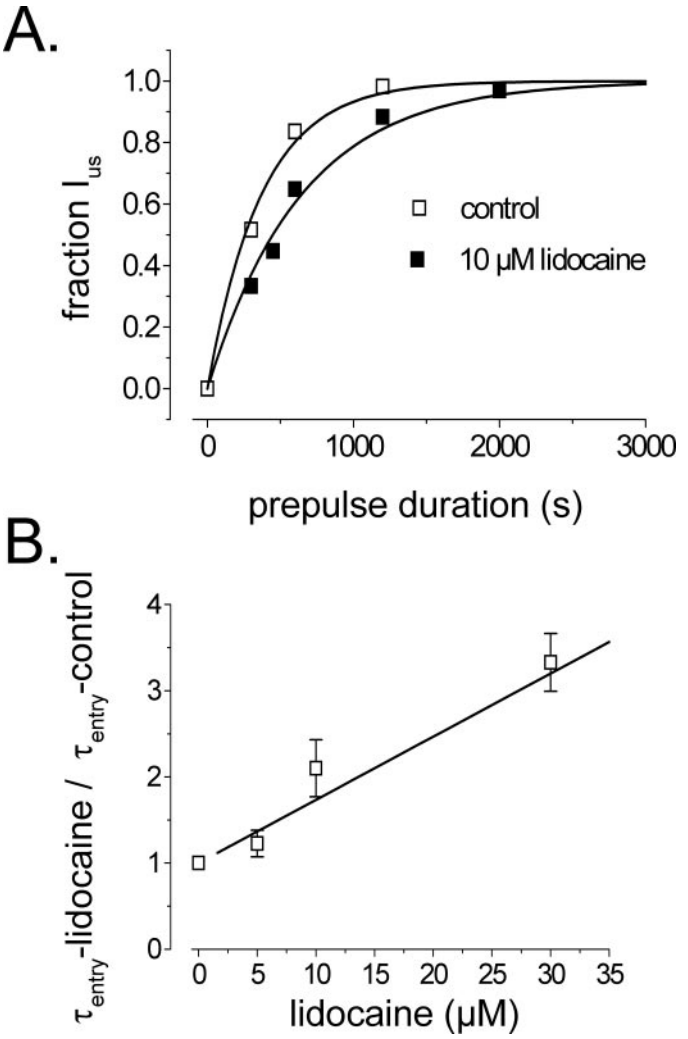


Fig. 5. Lidocaine prolongs the apparent time course of entry into I_{US}. A, effect of lidocaine on the time course of entry into I_{US} in an oocyte expressing K1237E channels. The time courses of recovery from inactivation produced by conditioning prepulses of the indicated durations were fitted with eq. 1, which yielded the amplitudes of the ultra-slow recovering components [A_2 in eq. 1 = fraction I_{US} (A_2)]. The resulting time course of entry into I_{US} was fitted with a monoexponential function (eq. 2, solid lines). B, concentration-dependent slowing of the apparent time constant of entry into I_{US} in K1237E. The solid line is the result of fitting the data points to the single component Langmuir equation: $y = 1 + ([\text{lidocaine}]/K_D)$. n = 3, 3, and 4 for 5, 10, and 30 μM lidocaine, respectively.

Discussion

The main finding of the present study is that lidocaine slows the development of I_{US} by interacting with the inner pore of the channel. Although the effect can be demonstrated in wild-type channels (Fig. 1A), most of the experiments were performed in channels carrying the mutation K1237E, which increases the number of channels entering I_{US} (Todt et al., 1999) and thus allows for a more accurate quantitative analysis.

Local anesthetic drugs like lidocaine have long been known to produce voltage- and frequency-dependent block of Na^+ channels (Weidmann, 1955). It has been proposed that local anesthetic drugs bind with high affinity to the fast inactivated conformational state, resulting in stabilization of this state. Upon repolarization the drug-bound fast inactivated state recovers more slowly than the drug-free fast inactivated state, giving rise to frequency-dependent block (Hille, 1977; Hondeghem and Katzung, 1977; Bean et al., 1983).

This view has recently been challenged by the observation that the time course of opening of the fast inactivation gate during repolarization is unchanged by lidocaine (Vedantham and Cannon, 1998). Alternatively, it has been suggested that local anesthetics bind to and stabilize slower inactivated states (I_M, I_S) (Fakler et al., 1990; Kambouris et al., 1998; Chen et al., 2000; Ong et al., 2000). In this case, recovery from inactivation is prolonged because lidocaine increases the number of channels entering these long lived inactivated states at depolarized potentials. The I_M state is characterized by time constants of recovery on the order of ~100 to 1000 ms (Kambouris et al., 1998). We show that binding of lidocaine to I_M is preserved in the mutation K1237E (Fig. 3). Furthermore, as shown in Fig. 4, channels residing in the I_S state seem to bind lidocaine with similar affinity as channels in the I_M state, which is consistent with data obtained in wild-type Na_V 1.4 channels (Ong et al., 2000). This result is not unexpected since lidocaine can only prolong the development of the I_{US} state if it is still bound to the channel during the time course of entry into this state. The I_{US} state takes several minutes to develop (Fig. 5A), thus most channels will reside in the I_S state during the entry phase into I_{US}.

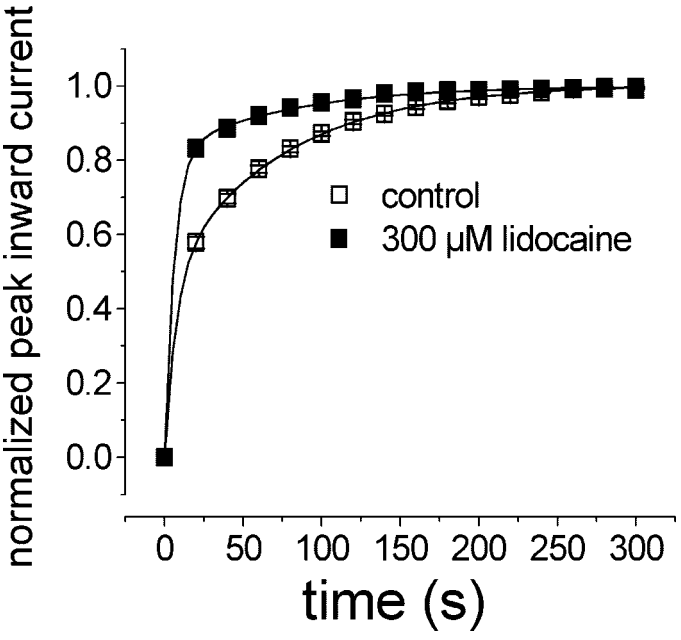


Fig. 6. Effect of 300 μM lidocaine on I_{US} in the mutant K1237S. The protocol was the same as shown in Fig. 1A (n = 4). Lines are the results of fitting of a biexponential function to the data points (eq. 1). Fitting parameter were τ_1 = 7.1 ± 0.4 s, τ_2 = 73.6 ± 1.5, A_1 = 0.48 ± 0.008, and A_2 = 0.52 ± 0.006 under lidocaine-free conditions and τ_1 = 6.0 ± 0.34 s, τ_2 = 74.5 ± 2.3, A_1 = 0.81 ± 0.006, and A_2 = 0.18 ± 0.006 during exposure to 300 μM lidocaine.

The interaction of lidocaine with the I_{US} state was mediated by binding of the drug to the residue Phe1579 in the inner vestibule of the channel as the double mutant K1237E + F1579A substantially reduced the modulation of I_{US} by lidocaine. Apart from binding to Phe1579, interactions of lidocaine with residues in S6 segments of domains I and III have also been reported (Wright et al., 1998; Yarov-Yarovoy et al., 2001, 2002). However, these interactions are substantially weaker than the interaction with Phe1579 (Ragsdale et al., 1996; Yarov-Yarovoy et al., 2002). Nevertheless, we cannot exclude that residues in other domains may play an additional role in the modulation of I_{US} by lidocaine.

Binding of lidocaine resulted in an increase in the apparent time constant of entry into I_{US} (Fig. 5), whereas the time course of recovery from I_{US} remained unchanged (Table 1). These results could be explained if the lidocaine-bound states and the I_{US} state were mutually exclusive. In this case, binding of lidocaine would reduce the number of channels available to enter into I_{US}, thereby prolonging entry into I_{US}, whereas recovery from I_{US} remains unchanged. Application of this model to our experimental data allowed for a correct prediction of the K_D for interaction of lidocaine with the I_M state, which serves as a successful consistency check of the model.

Our model assumes high-affinity binding of lidocaine to slow inactivated states. Alternatively, it may be hypothesized that lidocaine binds to and stabilizes the I_F state. In this case, dissociation of lidocaine would be the rate-limiting step during recovery from inactivation. The concentration-dependent increase in the amplitude of a component recovering with a time constant of ~1000 ms (Fig. 3C, Table 2) would then reflect dissociation of lidocaine from blocked fast inactivated channels. It may be argued that if fast inactivated channels were unable to enter the I_{US} state, then stabilizing channels in the fast inactivated state would reduce the number of channels in the I_{US} state by mass action. On the other hand, ultra-slow inactivation was produced by holding the channels at −20 mV for 300 s. At such a depolarized potential, all channels enter fast inactivation, even in the absence of lidocaine (see Fig. 1 in Todt et al., 1999). Nevertheless, 100% of K1237E channels were able to enter into ultra-slow inactivation if the duration of the conditioning prepulse was increased to 2000 s, irrespective of whether lidocaine was present or absent (Fig. 5A). Thus, lidocaine could not have increased the number of channels entering I_F at −20 mV, and entry into I_F could not have prevented channels from entry into I_{US} during long conditioning prepulses. As mentioned above, Vedantham and Cannon (1998) have shown that lidocaine-induced slowing of Na^+ channel repriming does not result from a slowing of recovery of the fast inactivation gate, which argues against a direct interaction between lidocaine binding and I_F. This notion is supported by a more recent study demonstrating that the action of lidocaine on gating charge cannot be interpreted as stabi-

lizing the Na^+ channel in an I_F state (Sheets and Hanck, 2003). Accordingly, a number of studies have postulated a linkage between I_S and use-dependent local anesthetic block (for review, see Ong et al., 2000). Therefore, we interpret our data by assuming that lidocaine binds with high affinity to slower inactivated states (I_M, I_S).

Which *molecular* mechanism may account for the failure of lidocaine-bound channels to enter into I_{US}? Slow inactivated states have been suggested to reflect a closure of the outer vestibule of the channel (Tomaselli et al., 1995; Balser et al., 1996; Kambouris et al., 1998; Todt et al., 1999; Ong et al., 2000; Hilber et al., 2001, 2002; Vilin et al., 2001; Xiong et al., 2003). Specifically, both the I_M state and the I_{US} state are sensitive to mutations in the outer vestibule of the channel (Balser et al., 1996; Todt et al., 1999; Hilber et al., 2001), and the I_{US} state can be modified by binding of a mutated μ-conotoxin GIIIA to the outer vestibule (Todt et al., 1999; Hilber et al., 2001). Recently, Ong et al. (2000) tested the state-dependent accessibility of an engineered cysteine in the outer vestibule to sulfhydryl-modifying agents. It was found that slow inactivation and lidocaine block inhibited side chain accessibility, suggesting that lidocaine block and slow inactivation share a common outer pore structural rearrangement. These data suggest that I_M and I_{US} may reflect conformational changes of the outer vestibule. Lidocaine may bind to the cytoplasmic side of the selectivity filter, thereby stabilizing the outer vestibule (as suggested in Ong et al., 2000), resulting in a reduction of the I_{US} state as observed in this study. However, as shown in Fig. 6, lidocaine exerted similar effects on I_{US} irrespective of the charge at position 1237 in the selectivity filter. This suggests that the modulation of I_{US} by lidocaine does not result from an interaction with the outer vestibule of the channel.

The notion that slow inactivation involves a closure of the outer vestibule has recently been challenged by the demonstration that accessibility of cysteines, engineered to some positions in the outer vestibule, to the charged sulfhydryl-modifying agent methanethiosulfonate ethyltrimethylammonium (MTS-ET) was preserved during slow inactivation (Struyk and Cannon, 2002); however, only residues external to the selectivity filter (DEKA motif, inner ring of charge) could be studied. Hence, if slow inactivation encompassed a closure of the outer vestibule, this closure most likely occurs at the level of the selectivity filter. In support of this notion, the mutation W402C, located close to the selectivity filter region, substantially reduced I_M (Balser et al., 1996).

Entry into I_{US} is inhibited by binding of a mutated μ-conotoxin GIIIA to the outer vestibule (Todt et al., 1999; Hilber et al., 2001). μ-Conotoxin GIIIA does not interact with the selectivity filter (Dudley et al., 2000), which is inconsistent with the idea that I_{US} solely reflects a localized constriction

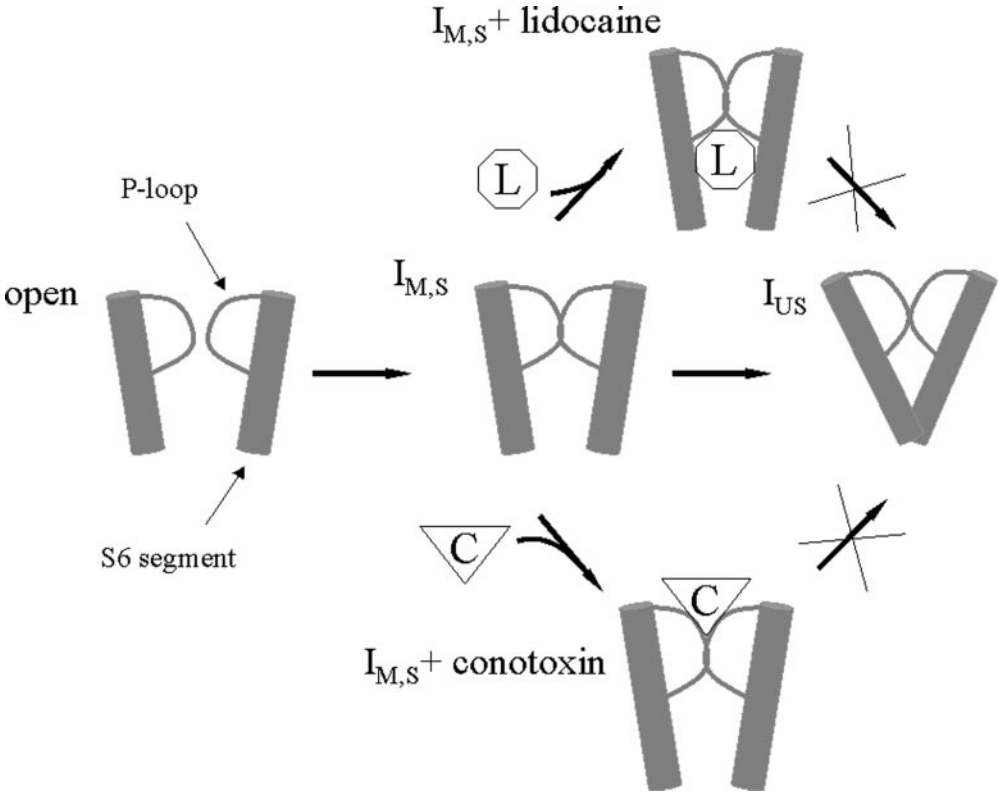


Fig. 7. Hypothetical molecular mechanism of modulation of I_{US} by lidocaine. Shown are P-loops and adjacent S6 segments of two domains. Only the open, I_M, I_S, and I_{US} states are considered. Upon depolarization, channels first open and then enter the I_M and I_S states, which may reflect a closure of the outer vestibule at the level of the selectivity filter ($I_{M,S}$). This closure reduces the accessibility of P-loop residues to sulfhydryl-modifying reagents, consistent with (Ong et al., 2000). As the depolarization is maintained, the outer vestibule widens consistent with the regained accessibility of some P-loop residues (Struyk and Cannon, 2002). This lateral motion of the outer vestibule causes the S6 segments to move toward the pore axis resulting in closure of the inner vestibule, consistent with the reduction of the volume of the inner pore in potassium channels during slow inactivation (Jiang et al., 2003a). This closure of the inner vestibule produces a longer lived inactivated state (I_{US}). Lidocaine (L) binds to the $I_{M,S}$ states and inhibits the closure of the inner vestibule by a foot-in-the-door mechanism. Binding of μ-conotoxin GIIIA (C) to the P-loops stabilizes the outer vestibule, thereby inhibiting the hypothetical lateral movement of the P-loops, which prevents S6 segments to close the inner vestibule, consistent with the inhibition of I_{US} by μ-conotoxin GIIIA (Todt et al., 1999; Hilber et al., 2001).

of the selectivity filter. If I_{US} encompassed a motion of the outer vestibule as suggested by the modulatory action of μ-conotoxin GIIIA, but the accessibility of the outer vestibule remained intact during prolonged depolarization as suggested by the data of Struyk and Cannon (2002) then it follows that the motion underlying I_{US} might not be a closure of the outer vestibule. Alternatively, the outer vestibule might become wider during $I_{US,}$ which is consistent with the demonstration that the outer vestibule is a very flexible structure (Benitah et al., 1997). But how can a widening of the outer vestibule produce a long-lived nonconducting state? Recent data from a crystallized voltage-gated K^+ channel suggest that gating involves a complex rearrangement of the channel protein. Opening of K^+ channels most likely results from a motion during which the S5 helices are pulled away from the pore axis, which causes the S6 helices to move apart resulting in opening of the pore (Jiang et al., 2003b). Hence motions of the S5 segments and, most likely, of the adjacent P-loops seem to be transmitted to the S6 segments.

From these findings, we speculate that P-loops exert a lateral motion during I_{US} associated with a widening of the outer pore, which is transmitted from the P-loops to the adjacent S6 segments, resulting in a closure of the inner vestibule of the channel (Fig. 7). Binding of lidocaine to the inner vestibule of the channel might interfere with this closure of the inner vestibule by a foot-in-the-door mechanism thereby preventing the channels from entry into I_{US}. Such a foot-in-the-door model would be consistent with the fact that lidocaine slowed entry into I_{US} but did not affect recovery from I_{US} and would explain why the lidocaine-bound inactivated states (I_M, I_S) and the I_{US} state are mutually exclusive. A similar model has previously been proposed for the interaction of tetraethylammonium with the fast inactivated state in Shaker K^+ channels (Choi et al., 1991).

The idea that I_{US} involves a rearrangement of the inner vestibule of the channel is supported by the following findings. First, an interaction of the inactivation particle with the inner vestibule modulates I_{US} in a mutant in which I_{US} is not absorbing (Hilber et al., 2002). Second, a number of mutations in S6 segments have been reported to affect slow inactivated states (Wang and Wang, 1997; Takahashi and Cannon, 1999; O'Reilly et al., 2001). Third, G protein-coupled receptor- and protein kinase-dependent reductions in Na^+ channel availability have recently been shown to be mediated by modulation of slow inactivation (Carr et al., 2003), consistent with a significant role of the internal parts of the Na^+ channel in slow inactivation. Fourth, in K_V 1.4 channels, slow inactivation is associated with a decrease in intracellular aqueous pore volume (Jiang et al., 2003a), again pointing toward a significant role of the inner vestibule in slow inactivation gating. If the proposed model holds true then slow inactivated states may represent a heterogeneous group of molecular rearrangements, some involving the outer pore (I_M) and others the inner vestibule (I_{US}). Given the importance of slow inactivation in a great number of disease states (Goldin, 2003), a detailed understanding of the mechanistic basis of slow inactivated states has significant implications with regard to designing pharmacologic modulators of slow inactivation.

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