Mechanism of Modification, by Lidocaine, of Fast and Slow Recovery from Inactivation of Voltage-Gated Na\(^+\) Channels

Vaibhavkumar S. Gawali, Peter Lukacs, Rene Cervenka, Xaver Koenig, Lena Rubi, Karlheinz Hilber, Walter Sandtner, and Hannes Todt

Center for Physiology and Pharmacology, Department of Neurophysiology and Neuropharmacology (V.S.G., P.L., R.C., X.K., L.R., K.H., H.T.) and Center for Physiology and Pharmacology (W.S.), Medical University of Vienna, Vienna, Austria

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ABSTRACT

The clinically important suppression of high-frequency discharges of excitable cells by local anesthetics (LA) is largely determined by drug-induced prolongation of the time course of repriming (recovery from inactivation) of voltage-gated Na\(^+\) channels. This prolongation may result from periodic drug-binding to a high-affinity binding site during the action potentials and subsequent slow dissociation from the site between action potentials ("dissociation hypothesis"). For many drugs it has been suggested that the fast inactivated state represents the high-affinity binding state. Alternatively, LAs may bind with high affinity to a native slow-inactivated state, thereby accelerating the development of this state during action potentials ("stabilization hypothesis"). In this case, slow recovery between action potentials occurs from enhanced native slow inactivation. To test these two hypotheses we produced serial cysteine mutations of domain IV segment 6 in rNa,1.4 that resulted in constructs with varying propensities to enter fast- and slow-inactivated states. We tested the effect of the LA lidocaine on the time course of recovery from short and long depolarizing prepulses, which, under drug-free conditions, recruited mainly fast- and slow-inactivated states, respectively. Among the tested constructs the mutation-induced changes in native slow recovery induced by long depolarizations were not correlated with the respective lidocaine-induced slow recovery after short depolarizations. On the other hand, for long depolarizations the mutation-induced alterations in native slow recovery were significantly correlated with the kinetics of lidocaine-induced slow recovery. These results favor the "dissociation hypothesis" for short depolarizations but the "stabilization hypothesis" for long depolarizations.

Introduction

Blockers of voltage-gated Na\(^+\) channels are used as local anesthetics, antiarrhythmics, antiepileptics, analgesics, and in the treatment of specific skeletal muscle diseases. Potential "noncanonical" roles of Na\(^+\) channels in nonexcitable cells may open new drug targets for Na\(^+\) channel blockers, such as cancer treatment (Black and Waxman, 2013). Recently, there has been an increasing interest in developing blockers with high specificity for certain Na\(^+\) channel isoforms. For example, targeting the neuronal isoforms Nav1.7 and Nav1.8 may lead to the development of potent analgesic drugs with few side effects (Leffler et al., 2007; Dib-Hajj et al., 2009; Waxman, 2013; Lampert et al., 2014). Furthermore, targeting the late sodium current in the heart may be used in the treatment of arrhythmias, ischemic heart disease, and heart failure (Coppini et al., 2013; Antzelevitch et al., 2014). Functional specificity of Na\(^+\) channel blockers is thought to arise from their use-dependent effect, which is responsible for the suppression of high-frequency discharges in excitable cells, which underlies their analgesic, anticonvulsive, and antiarrhythmic efficacy (Macdonald and Kelly, 1993; Pisanii et al., 1995; Weirich and Antoni, 1998; Dupere et al., 1999).

The use-dependent effect of Na\(^+\) channel blockers arises from high-affinity binding to specific states that are periodically populated during the firing of action potentials. Thus, local anesthetic drugs bind preferentially to activated (Hille, 1977; Yeh and Tanguy, 1985; Wang et al., 1987; McDonald et al., 1989; Vedantham and Cannon, 1999) and/or inactivated states (Hille, 1977; Cahalan, 1978; Yeh, 1978; Bean et al., 1983; Bennett et al., 1995; Balser et al., 1996a). As a result, the restoration of excitability between action potentials is prolonged. This latter effect can result from two processes: Drug-associated slowing of recovery of excitability could result from: 1) a slow rate of dissociation of the drug, which slows recovery from inactivation, ("dissociation-hypothesis"); or 2) an increased stability of an intrinsic, slow-inactivated state, on the basis of an increased rate of entry into the slow-inactivated state in the presence of drug ("stabilization hypothesis"); Fig. 1). These are two fundamentally different mechanisms that have qualitatively similar effects on excitability. With regard to the dissociation hypothesis, drugs are thought to bind with high affinity to a specific state-dependent conformation of the...
A three-affinity modulated receptor model in which NI, IF, IM, and IS represent noninactivated states (i.e., pooled open and closed states), fast, intermediate, and slow-inactivated states, respectively. $\alpha_F$ and $\alpha_M$ are the voltage-dependent forward rates into IF and IM; $\beta_F$ and $\beta_M$ are the respective voltage-dependent backward rates ($\alpha_F$, $\alpha_M$, $\beta_F$, $\beta_M$ refer to the respective rates among lidocaine-bound states). NI-L, IF-L, and IM-L are the lidocaine-bound NI, IF, and IM states. $k_{off,NI}$, $k_{off,IF}$, and $k_{off,IM}$ are the off-rates for lidocaine binding to NI, IF, and IM. During short depolarizations channels move from NI to IF, during repolarization the transit is from IF to NI (recovery from IF). During long depolarizations channels move from NI to IF and then further to IM. Since both $\alpha_M$ and $\beta_M$ are smaller than $\alpha_F$ and $\beta_F$, both development of and recovery from IF require a longer time course than the respective transitions into and out of IF. The “dissociation hypothesis” assumes high affinity binding of lidocaine to IF such that during short depolarizations channels mainly enter the IF-L state. Upon repolarization channels recover slowly via the small $k_{off,F}$ rate (drug-dissociation), which determines slow dissociation from the high affinity state (IF-L) and via $\beta_F$. The “stabilization hypothesis” assumes high affinity binding of lidocaine to IM (small value for $k_{off,IM}$). Thus, with lidocaine, even during short depolarizations, channels move from IF-L (low affinity) to IM-L (high affinity). This occurs because, owing to the high affinity of lidocaine to IF-L, the value for $k_{off,IM}$ is small (relative to $k_{off,IF}$ and $k_{off,NI}$). Consequently, from detailed balances, $\alpha_F$ has to be large, resulting in an acceleration of development of IM-L. Hence, with the “stabilization hypothesis” recovery from short depolarizations is prolonged by lidocaine, because channels are moving slowly from IM-L to NI (Chen et al., 2000). The states IM-M and IM-MUT are not considered in this scheme but would be located between IF and IM.

**Materials and Methods**

**Mutagenesis and Electrophysiology**

Mutagenesis and electrophysiology were performed as reported (Zarrabi et al., 2010; Lukacs et al., 2014). Mutagenesis of rat muscle NaV1.4 (rNaV1.4-A) vector consisting of the rNaV1.4 coding sequence flanked by Xenopus globin 5’ and 3’ untranslated regions was provided as a gift by R. Moorman (University of Virginia, Charlottesville, VA). This was used as the template for inserting oligonucleotide-directed point mutations by either four-primer polymerase chain reaction and subsequent subcloning into the template using directional ligations or the QuikChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA). Oligonucleotide primers containing a mutation were designed with a change in a silent restriction site to allow rapid identification of the mutation. Incorporation of the mutation into the template was then confirmed by DNA sequencing.
prepulses of varying durations in wild-type Nav1.4. Shown are test pulse currents recorded with electrophysiologic protocols similar to the protocol shown in Fig. 2. Channels were driven into inactivation by conditioning prepulses to $-20$ mV for the indicated durations. Test-pulse currents were normalized to the respective maximum value attained at full recovery and plotted as a function of the recovery interval. The time courses of recovery were assessed for rNav1.4 channels under drug-free conditions (A) and during superfusion with 500 mM lidocaine (B, solid symbols). For analysis the data points were fit with exponential functions (eqs.1–3; connecting lines). In (B) some fitted lines are reproduced from (A) for comparison. See Table 1 for the parameter estimates. Prolonging the duration of the conditioning prepulse induces a slow phase of recovery. This effect is enhanced by lidocaine.

Transfection Procedure

tsa201 cells were grown in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum and 20 IU/ml each of penicillin and streptomycin (Life Technologies, Carlsbad, CA). Cells were maintained at 37°C in a humid atmosphere containing 5% CO₂. A mixture of plasmids coding 1.5 μg of rNav1.4 α subunit, 0.2 μg of voltage-gated sodium channel (VGSC) β1 subunit, and 0.02 μg of eGFP were transiently transfected into tsa201 cells in 35-mm dish (Nunc, Roskilde, Denmark) using TurboFect Transfection Reagent (Thermo Fisher Scientific Baltics UAB, Vilnius, Lithuania) according to the manufacturer’s instructions. Sixteen hours later, cells were dissociated from the dish surface by treatment with a 0.25% trypsin solution (Life Technologies) for approximately 2 minutes, pelleted, resuspended in growth medium, and allowed to settle to the bottom of the recording chamber. Prior to recording, the growth medium was removed and changed to bath solution.

**TABLE 1**

<table>
<thead>
<tr>
<th>Prepulse Duration</th>
<th>$r_1$</th>
<th>$r_2$</th>
<th>$r_3$</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT (Control)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ms</td>
<td>0.57 ± 0.03</td>
<td></td>
<td></td>
<td>0.59 ± 0.01</td>
<td></td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>50 ms</td>
<td>0.71 ± 0.03*</td>
<td>100</td>
<td></td>
<td>0.72 ± 0.01</td>
<td>0.05 ± 0.007</td>
<td></td>
<td>8</td>
</tr>
<tr>
<td>10 s</td>
<td>0.71</td>
<td>76.53 ± 9.63</td>
<td>1755</td>
<td>0.12 ± 0.04</td>
<td>0.75 ± 0.03</td>
<td>0.11 ± 0.03</td>
<td>4</td>
</tr>
<tr>
<td>20 s</td>
<td>0.71</td>
<td>98.39 ± 6.45</td>
<td>3044 ± 993</td>
<td>0.07 ± 0.02</td>
<td>0.79 ± 0.02</td>
<td>0.14 ± 0.02</td>
<td>5</td>
</tr>
<tr>
<td>WT (Lidocaine)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10 ms</td>
<td>0.81 ± 0.05**</td>
<td>185.4 ± 10.83</td>
<td></td>
<td>0.38 ± 0.01</td>
<td>0.42 ± 0.006</td>
<td></td>
<td>4</td>
</tr>
<tr>
<td>50 ms</td>
<td>2.34 ± 0.36***</td>
<td>176.8 ± 10.6</td>
<td></td>
<td>0.30 ± 0.01***</td>
<td>0.69 ± 0.01</td>
<td></td>
<td>7</td>
</tr>
<tr>
<td>10 s</td>
<td>2.34</td>
<td>154.8 ± 6.46***</td>
<td>3044</td>
<td>0.08 ± 0.01</td>
<td>0.80 ± 0.01***</td>
<td>0.09 ± 0.01*</td>
<td>8</td>
</tr>
</tbody>
</table>

*P < 0.05, **P < 0.01, ***P < 0.001.
time constants, which should be independent of the prepulse potential. Lidocaine hydrochloride was obtained from Sigma-Aldrich. Unless stated otherwise lidocaine was applied at a concentration of 500 μM.

**Data Evaluation.** Nonlinear least squares fit of the normalized time course of recovery was performed using following equations:

\[ y = A_1 \left(1 - \exp\left(-\frac{t}{t_1}\right)\right) + C \]  

\[ y = A_1 \left(1 - \exp\left(-\frac{t}{t_1}\right)\right) + A_2 \left(1 - \exp\left(-\frac{t}{t_2}\right)\right) + C \]  

\[ y = A_1 \left(1 - \exp\left(-\frac{t}{t_1}\right)\right) + A_2 \left(1 - \exp\left(-\frac{t}{t_2}\right)\right) + A_3 \left(1 - \exp\left(-\frac{t}{t_3}\right)\right) + C \]  

where \( t_1, t_2, \) and \( t_3 \) are the time constants of distinct components of recovery; \( A_1, A_2, \) and \( A_3 \) are the respective amplitudes of these components; and \( C \) is the final level of recovery. The order (i.e., first order, eq. 1; second order, eq. 2) of exponential fit was chosen after an increase in the order of the fit produced no significantly better fit as reported by the "extra sum-of-squares F test". Graphic representation and data analysis was performed using GraphPad Prism, version 5.00 for Windows (GraphPad Software, LaJolla, CA).

Statistics-data are expressed as means ± S.E.M. Statistical comparisons were made using the two-tailed Student’s t tests. \( P < 0.05 \) was considered significant.

**Results**

**Protocol of Testing Time Course of Recovery from Inactivation.** To examine the time course of recovery in wild-type rNaV1.4 channels, a conditioning prepulse to -20 mV was introduced from a holding potential of -140 mV. Thereafter, the potential was returned to -140 mV for varying intervals followed by a 5-millisecond test pulse to -20 mV, which opened the fraction of channels that had recovered during the hyperpolarized interval after the conditioning prepulse. The inward currents elicited by the test pulse were normalized to the respective maximum value attained at full recovery and plotted as a function of the recovery interval. Figure 2 shows the original traces of test pulse currents obtained after the indicated intervals at -140 mV following a 10-second inactivating prepulse to -20 mV. Shown are currents from wild-type NaV1.4 channels and from channels carrying a mutation in the DIV-S6 segment at site 1585, which has been shown...
previously to destabilize slow inactivation (Hayward et al., 1997; Takahashi and Cannon, 2001). Clearly, the time course of recovery is substantially slower in wild-type Nav1.4 channels than in the mutation M1585C.

**Long Prepulse Durations Induce up to Three Inactivated States.** The normalized time courses of recovery of wild-type NaV1.4 channels following conditioning prepulses of varying durations to −20 mV are shown in Fig. 3; the estimated fitting parameters are presented in Table 1. The time course of recovery following a 10-millisecond prepulse could be fitted by a single exponential equation (eq. 1) with a time constant of 0.57 ± 0.03 milliseconds, indicating that all inactivated channels are recovering from a single inactivated state that we refer to as fast-inactivation (IF; Balser et al., 1996b; Kambouris et al., 1998), defined by time constants of recovery on the order of several milliseconds. If the duration of the conditioning prepulse was prolonged to 50 milliseconds, the time course of recovery could be fitted by the double exponential eq. 2, yielding time constants of 0.71 ± 0.03 milliseconds and 100 milliseconds and amplitudes of 0.72 ± 0.01 and 0.05 ± 0.007, respectively. This indicates that this conditioning prepulse recruited a minimum of two inactivated states. The faster phase of recovery most probably corresponds to recovery from IF as observed following a 10-millisecond prepulse. Because of the low amplitude of the slower phase the time constant was constrained to 100 milliseconds. We operationally refer to this phase as recovery from intermediate fast inactivation (IFM). Increasing the prepulse duration to 5 seconds gave rise to a time course of recovery that could be fitted by three exponentials (eq. 3) (Cummins and Sigworth, 1996; Nuss et al., 1996; Hayward et al., 1997; Kambouris et al., 1998). The time constants of the three phases were 0.71 milliseconds, 76.53 ± 9.63 milliseconds, and 1755 milliseconds, the respective amplitudes were 0.12 ± 0.04, 0.75 ± 0.03, 0.11 ± 0.03. Most probably the first phase corresponds to recovery from I_F (Kambouris et al., 1998), although we cannot exclude a contribution by recovery from IFM. The second phase may reflect recovery from a state previously termed “intermediate inactivation” (IM), characterized by time constants on the order of several hundred milliseconds (Kambouris et al., 1998). The third phase is characterized by time constants on the order of several seconds and has been referred to as slow inactivation (IS) (Kambouris et al., 1998). Increasing the prepulse duration to 20 seconds increased the fraction of channels recovering from IM and IS, as indicated by the effect on the amplitudes A2 and A3.

**Effect of Lidocaine on Time Course of Recovery in Wild-Type NaV1.4 Channels.** During superfusion with 500 μM lidocaine the time course of recovery from a 50-millisecond prepulse (further on referred to as “short depolarization”) had two exponential phases with time constants of 2.34 ± 0.36 milliseconds and 176.8 ± 10.6 milliseconds and amplitudes of 0.30 ± 0.01 and 0.69 ± 0.01, respectively (Fig. 3B). Thus, lidocaine substantially increased the time constant of the first rapid phase of recovery and the amplitude of the second phase, whose time constant is similar to recovery from the native condition in a given construct.

**Fig. 6.** Modulation of amplitudes of two phases of recovery from a short depolarization (50-millisecond) by mutations in DIV-S6 and by lidocaine. Shown are the amplitudes of the two phases of recovery as derived from fitting eq. 2 to the data of Fig. 4. The voltage of the conditioning prepulse was either −50 mV (A, C) or −20 mV (B, D) in most constructs lidocaine increased the amplitude of the second phase of recovery (A3). *P < 0.05; **P < 0.01; ***P < 0.001 compared with the respective value for wild-type channels. +P < 0.05; ++P < 0.01; +++P < 0.001 for the effect of lidocaine compared with drug-free condition in a given construct.
I₉ state (Table 1). This could indicate that lidocaine accelerates the entry into the I₉ state (Kambouris et al., 1998). Alternatively, lidocaine could bind only to the I₉ state during the conditioning prepulses, but, upon repolarization, dissociate from this state with a time constant similar to the time constant of recovery from the native I₉ state (Fig. 1). With longer prepulse durations (10 seconds) lidocaine increased both the time constant and the amplitude of recovery from I₉.

**Mutations in DIV-S6 Modulate Fast Inactivation.** As reported previously, all tested mutations of the DIV-S6 segment gave rise to robust inward currents with the exception of N1584C (Sunami et al., 2004). The time course of recovery from fast inactivation was examined by a prepulse-test pulse protocol as described before, whereby the duration of the prepulse was set to 50 milliseconds, which has previously been shown to be sufficient to cause NaV1.4 channels to populate mainly fast-inactivated states (Wang et al., 2003). Figure 4, A and B, show the time courses of recovery of the tested constructs for conditioning pulse voltages of −20 mV and −50 mV, respectively. Clearly, mutations in DIV-S6 produced alterations in the time course of recovery. In some mutations the time course of recovery was monoexponential, whereas in others a second phase of low amplitude was observed (inset of Fig. 4, A and B). For example in the mutation F1579C this second slow phase had amplitude of 0.04 ± 0.007. In Figs. 5 and 6 the time constants and amplitudes of the time course of recovery are presented. The time constants of recovery from I₉ (τ₁) were substantially modulated by the mutations. Thus, in wild-type τ₁ was 0.71 ± 0.03 milliseconds, in F1579C τ₁ was decreased to 0.38 ± 0.02 milliseconds (P < 0.001), and in M1585C τ₁ was increased to 1.31 ± 0.04 milliseconds (P < 0.001).

In general, with conditioning voltages of −20 mV τ₁ values were significantly different compared with wild type for all constructs, with the exception of S1578C and V1582C (Fig. 5). With conditioning voltages of −50 mV the observed changes were similar to those observed with −20 mV, with the exception of higher amplitude of noninactivating currents. As shown in Fig. 6 the amplitudes of the first phase of recovery were substantially modified by mutations in DIV-S6. This most probably reflects mutation-induced shifts of the half-point of steady state inactivation, which is highly modulated by mutations in DIV-S6 (Yarov-Yarovoy et al., 2002; Wang et al., 2003).

**Effect of Lidocaine on Recovery from Short Depolarizations.** Superfusion with 500 μM lidocaine resulted in significantly increased in the time constant of recovery from I₉ (τ₁) in all constructs with the exception of F1579C and Y1586C (Fig. 5). The prolongation of τ₁ was greatest in the mutations I1575C and M1585C (see below for detailed analysis)

The most dramatic effect of lidocaine was to increase the amplitude of the second phase of recovery (A₂) in all constructs with the exception of F1579C. This increase in A₂ occurred at the expense of decrease in A₁ (Fig. 6) or by a decrease in the amplitude of the noninactivating fraction (Fig. 4). The time constant of this late phase (τ₂, Fig. 7) showed some variation between constructs but, in general, was on the order of 100–200 milliseconds, as reported previously (Vedantham and Cannon, 1999; Kondratiev and Tomaselli, 2003).

**Mutations in DIV-S6 Modulate Slower Forms of Inactivation.** In some mutations the time course of recovery from inactivation induced by 10-second depolarizing prepulses (further on referred to as “long depolarization”) was substantially altered (Fig. 8). Figure 9 shows the effect of serial S6 mutagenesis on the time constants of recovery from I₉. Clearly, most cysteine replacements of DIV-S6 residues modulated the time constant of recovery from I₉, with the greatest reduction produced by M1585C and the greatest increase generated by Y1586C.

According to the classic Hodgkin-Huxley paradigm, changing the voltage of the prepulse should alter the time constant of entry into inactivation and, thus, the distribution of channels among different channel states at the end of the prepulse, but not the time constants of recovery from these states [e.g., see Takahashi and Cannon (1999)]. If the serial cysteine replacements in DIV-S6 per se altered the time course of recovery from I₉, then we would expect a strong correlation between the τ₂ values measured at inactivating voltages of −50 mV and −20 mV. As shown in Fig. 10 there is indeed a strong positive correlation between the time constants measured at these two voltages. This supports the conclusion that the differences in τ₂ values are the result of the mutation itself rather than a result of protocol-dependent variabilities. These data also demonstrate that the introduced mutations produce variations of time constants of recovery from I₉ that are sufficiently large to give rise to a significant correlation (see Fig. 12)

**Effect of Lidocaine on Time Constants of Recovery from I₉.** As shown in Fig. 9 lidocaine tended to increase the time constants of recovery from I₉ in most constructs with the exception of I1575C, F1579C, and Y1586C, which have
previously been shown to per se reduce binding of local anesthetics (Ragsdale et al., 1994).

**Lidocaine Reduces Amplitude of Recovery from IS during Long Depolarizations.** As shown in Fig. 3, recovery from long depolarizations occurred mostly from the $I_M$ state. There is also a small component of even slower recovery, with time constants on the order of several seconds, which most probably results from recovery from a very slow-inactivated state, $I_S$ (Kambouris et al., 1998). The values of the time constants of this phase are not reported, because the small amplitude of this phase precluded their precise estimation. As shown in Fig. 11, lidocaine tended to increase the amplitude of recovery from $I_M$ ($A_2$), mainly at the expense of a reduction in the amplitude of recovery from $I_S$ ($A_2$, arrows in Fig. 8) and the noninactivating component. This could result from the binding of lidocaine to and stabilization of $I_M$, thereby enhancing the rate of entry into $I_M$ ($a_M$ in Fig. 1) and reducing the rate of entry into $I_S$ during the conditioning pulse (via small $k_{off,M}$, Fig. 1). Alternatively, lidocaine could bind to $I_F$, thereby reducing the time constant of entry into $I_M$ and $I_S$ (via a small $k_{off,F}$; Fig. 1). Upon repolarization lidocaine would dissociate from $I_F$ with a time constant similar to recovery from the native $I_M$.

**Lidocaine-Induced Slow Recovery following Short Depolarizations Does Not Reflect Recovery from Endogenous $I_M$.** As mentioned above, the most dramatic effect of lidocaine when tested with short depolarizations was to increase the amplitude of a slower phase of recovery (Fig. 7) with a time constant similar to that of recovery from the native $I_M$ (Fig. 9). As shown in Fig. 9 the cysteine replacements of amino acids in DIV-S6 produced alterations in the time course of recovery from $I_M$. If the lidocaine-induced slower phase of recovery from short depolarizations represented recovery from lidocaine-induced $I_M$ (i.e., $I_M$-L in Fig. 1) rather than slow dissociation from $I_F$ ($I_F$-L in Fig. 1), then we would expect a strong correlation between the time constant of this lidocaine-induced $I_M$ state and the mutation-specific value of recovery from the native $I_M$. As shown in Fig. 12 the kinetics of the lidocaine-induced slower phase of recovery from short depolarizations did not correlate with the kinetics of either the...
anism different from recovery from I_M, perhaps by slow recovery from short depolarizations (Fig. 7) occurs by a mechanism distinct from lidocaine modification of slow recovery (IM).

Fig. 9. Lidocaine tends to increase the time constant $\tau_2$ for the intermediate phase of recovery, whereas several of the mutations decrease $\tau_2$ with respect to the wild-type construct under the same conditions. The time constants of recovery from intermediate inactivation (IM) were derived by fitting of eq. 3 to the time courses of recovery shown in Fig. 8. $*P < 0.05; **P < 0.01; ***P < 0.001$ compared with the respective value for wild-type channels ($n = 5–8$). $+=P < 0.05; ==P < 0.01; +++P < 0.001$ for the effect of lidocaine compared with drug-free condition in a given construct. Prepulse voltage was $-20$ mV (A) and $-50$ mV (B).

native I_M state (Fig. 12A) or the lidocaine-modified I_M state (Fig. 12B). This suggests that the lidocaine-induced slow recovery from short depolarizations (Fig. 7) occurs by a mechanism different from recovery from I_M, perhaps by slow dissociation of lidocaine from $I_F$ ($k_{off,F}$ in Fig. 1).

Lidocaine-Induced Slow Recovery following Long Depolarizations Reflects Recovery from Endogenous I_M. Conversely, one could ask whether the modulation of the slow phase of recovery by lidocaine with long depolarizations (Fig. 9) represents slow dissociation from fast inactivation rather than recovery from the stabilized native I_M state. In the latter case we would expect a correlation within the tested constructs between the time constant of recovery from I_M under control conditions and the time constant of recovery during lidocaine exposure. As shown in Fig. 12C this is indeed the case. Hence, lidocaine-induced slowing of recovery from fast inactivation appears to be mechanistically distinct from lidocaine modification of slow recovery (IM).

The Mutations M1585C and I1576C Induce a Modified I_M State. Mutations at site 1585 destabilize slow inactivation (Hayward et al., 1997). Indeed, the time constant of recovery from I_M was substantially shorter in M1585C compared with wild-type channels ($32.78 \pm 4.27$ milliseconds versus $98.39 \pm 6.45$ milliseconds, respectively $P < 0.0001$; Fig. 9, Table 2). We wondered whether this modified I_M state, which we refer to as modified intermediate inactivation (IM_MUT), represented an additional new I_M state, or a modification of the native I_M. Therefore, we explored whether prolongation of the conditioning prepulse could drive M1585C channels into the native I_M. As shown in Fig. 13A prolonging the duration of the conditioning prepulse from 10 to 30 seconds resulted in a substantial additional slowing of the time course of recovery of M1585C channels, reflected by an increase of the dominant time constant ($\tau_2$) to a value similar to the time constant of recovery from I_M in wild-type channels ($73.61 \pm 14.11$ milliseconds; Table 1). This suggests that prolonging the duration of the prepulse caused M1585C channels to move from IM_MUT to I_M. Exposure of M1585C channels to lidocaine had an effect similar to prolongation of the prepulse duration, suggesting that lidocaine accelerated the transition of IM_MUT to I_M during the conditioning prepulse (Fig. 13A, Table 2).

Figure 13B shows the response of lidocaine-modified M1585C to conditioning prepulses of the following durations: 10 milliseconds, 50 milliseconds, and 10 seconds. With lidocaine, recovery from inactivation after a 10-millisecond prepulse ($-20$ mV) occurred by two distinct phases (filled triangles in Fig. 13B, Table 1). However, when the prepulse duration was extended to 50 milliseconds, the clear separation of these two phases was lost (filled circles in Fig. 13B). We suspected that this was the result of the emergence of a third phase of recovery that “bridged” the fast and slow phases observed with the 10-millisecond prepulse. This third phase had a time constant $22.9 \pm 8.18$ milliseconds, i.e., a value similar to the time constant of recovery from the native IM_MUT state (see Table 2, $\tau_2$ M1585C control, 10-second versus M1585C lidocaine 50 milliseconds). A similar trend toward the lidocaine-induced emergence of a third phase of recovery with short depolarizations was observed in the mutation I1576C (Fig. 13, C and D, arrow). Interestingly, as with M1585C, this mutation also shortened the time constant of recovery from I_M with long depolarizations under drug-free conditions (Fig. 9). These results suggest that with I1576C and
M1585C lidocaine appears to stabilize IM-MUT during 50-millisecond depolarizing pulses.

The assumption of an increased entry into IM-MUT upon lidocaine exposure also explains the substantial drug-induced increase in \( \tau_1 \) with I1576C and M1585C shown in Fig. 5. Most probably, with these constructs, \( \tau_1 \) reports a mixture of time constants of recovery from fast inactivation and from IM-MUT. Hence, the enhanced recovery from IM-MUT during lidocaine exposure may artifactually produce a substantial increase in \( \tau_1 \). Lidocaine exposure also explains the substantial drug-induced increase in the time constant of fast phase during lidocaine exposure (Fig. 12D, \( \tau_1 \)-Lido). If the lidocaine-modified IF or substantially slow recovery from native IF, whereas prolonged recovery from inactivation of unmodified IF is a result of binding to IFM, i.e., a native state with recovery kinetics slightly slower than IF. Thus, IFM may be a state similar to IM-MUT in M1585C and I1576C.

**The Mutation W1531G Accelerates Recovery from Lidocaine Block.** As mentioned above, the lidocaine-induced delay in recovery can arise from drug-dissociation or from drug-induced stabilization of native slow recovery. These two processes are difficult to distinguish because, with lidocaine treatment, the drug-induced slow phase of recovery from short depolarizations (Fig. 7) has a time constant similar to that for recovery from unmodified IM (Fig. 9). Such separation would be possible if a mutation would either accelerate recovery from lidocaine-modified IF or substantially slow recovery from native IM. We recently reported that mutation of a tryptophan in the outer vestibule of DIV produced a rapid access and egress pathway for external QX-222, a membrane impermeant derivative of lidocaine (Lukacs et al., 2014). As shown in Fig. 14 this mutation, W1531G, substantially shortened the time constant of recovery from lidocaine-modified IF. This phase most probably reflects rapid egress of lidocaine from its binding site. However, with W1531G, lidocaine modified the time course of recovery from 10-second prepulses in a way similar to wild-type Na\(_{1.4}\) channels, i.e., lidocaine substantially reduced the amplitude of recovery from IM \((A_2)\) in Fig. 14 consistent with binding to and stabilizing IM. These data support the idea that the lidocaine-induced increase in \( \tau_1 \) (Fig. 5) is a result of binding to IFM, i.e., a native state with recovery kinetics slightly slower than IF. Thus, IFM may be a state similar to IM-MUT in M1585C and I1576C.
produced by long depolarizations reflects recovery from drug-modified $I_M$.

**Discussion**

**Electrophysiological Assessment of Inactivation.** In the present study we examined the effect of lidocaine on the time course of recovery from inactivation from both short and long depolarization. Such protocols allow for judgment of the limiting number of inactivated states populated during a given conditioning prepulse but provide only limited information on the voltage dependency of inactivation. We did not apply classic availability protocols for different inactivated states

<table>
<thead>
<tr>
<th>Prepulse Duration</th>
<th>$\tau_1$</th>
<th>$\tau_2$</th>
<th>$\tau_3$</th>
<th>$A_1$</th>
<th>$A_2$</th>
<th>$A_3$</th>
<th>$n$</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>M1585C (Control)</strong></td>
<td></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10 ms</td>
<td>1.15 $\pm$ 0.08</td>
<td>100</td>
<td>1 $\pm$ 0.04</td>
<td>0.84 $\pm$ 0.01</td>
<td>0.04 $\pm$ 0.008</td>
<td>0.15 $\pm$ 0.02</td>
<td>5</td>
</tr>
<tr>
<td>50 ms</td>
<td>1.13 $\pm$ 0.04</td>
<td>1035 $\pm$ 439.3</td>
<td>0.12 $\pm$ 0.03</td>
<td>0.57 $\pm$ 0.02</td>
<td>0.15 $\pm$ 0.02</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>10 s</td>
<td>1.13</td>
<td>73.61 $\pm$ 14.11</td>
<td>1119 $\pm$ 299</td>
<td>0.04 $\pm$ 0.02</td>
<td>0.61 $\pm$ 0.05</td>
<td>0.33 $\pm$ 0.05</td>
<td>8</td>
</tr>
<tr>
<td><strong>M1585C (Lidocaine)</strong></td>
<td></td>
<td></td>
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</tr>
<tr>
<td>10 ms</td>
<td>1.9 $\pm$ 0.02**</td>
<td>290 $\pm$ 34.3</td>
<td>0.49 $\pm$ 0.02</td>
<td>0.5 $\pm$ 0.01</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>50 ms</td>
<td>1.9</td>
<td>22.9 $\pm$ 8.18</td>
<td>290</td>
<td>0.09 $\pm$ 0.03</td>
<td>0.65 $\pm$ 0.02</td>
<td>7</td>
<td></td>
</tr>
<tr>
<td>10 s</td>
<td>1.9</td>
<td>141.8 $\pm$ 18.81***</td>
<td>1010 $\pm$ 353.8</td>
<td>0.04 $\pm$ 0.02</td>
<td>0.69 $\pm$ 0.07**</td>
<td>0.24 $\pm$ 0.07</td>
<td>5</td>
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<tr>
<td><strong>I1576C (Lidocaine)</strong></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>50 ms</td>
<td>1.9</td>
<td>78.95 $\pm$ 10.32</td>
<td>290 $\pm$ 34.3</td>
<td>0.16 $\pm$ 0.01</td>
<td>0.51 $\pm$ 0.04</td>
<td>0.31 $\pm$ 0.04</td>
<td>7</td>
</tr>
</tbody>
</table>

**P** $< 0.01$, ****P $< 0.001$. 

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Fig. 12. Lidocaine modifications of recovery from short and from long depolarizations are distinct processes. Correlation analysis is performed to explore the effects of lidocaine on the time constants of recovery. The mutations I1575C, F1579C, and Y1586C are excluded from this analysis because these mutations abolished the effect of lidocaine on the time constants of recovery from long depolarizations at $-20$ mV, consistent with earlier reports implicating these sites in lidocaine binding (Fig. 9) (Ragsdale et al., 1994). M1585C is also excluded because of mutation-induced complete destabilization of $I_M$. (A) The time constant of the second phase of recovery from inactivation produced by 50-millisecond pulses to $-20$ mV during lidocaine exposure (Fig. 7A) is plotted as a function of the time constant of the second phase of recovery from 10-second prepulses to $-20$ mV under drug-free conditions (open bars in Fig. 9A). The line is the best-fit linear regression of the form $y = 0.27x + 125.9$ ($R^2 = 0.04$, $P = 0.63$). (B) The time constant of the second phase of recovery from inactivation produced by 10-second prepulses to $-20$ mV during lidocaine exposure (Fig. 7A) is plotted as a function of the time constant of the second phase of recovery from 10-second prepulses to $-20$ mV during lidocaine exposure (solid bars in Fig. 9A). The line is the best-fit linear regression of the form $y = 0.54x + 113.7$ ($R^2 = 0.61$, $P < 0.001$). (C) The time constant of the second phase of recovery from inactivation produced by 10-second prepulses to $-20$ mV during lidocaine exposure (Fig. 9A, solid bars) is plotted as a function of the time constant of the second phase of recovery from 10-second prepulses to $-20$ mV during drug-free conditions (Fig. 9A, open bars). The line is the best-fit linear regression of the form $y = 0.54x + 113.7$ ($R^2 = 0.61$, $P = 0.02$). This analysis suggests the presence of a link between the mechanism of generation of $I_M$ and the lidocaine-induced modification of the second phase of recovery from long depolarizations. (D) Lidocaine-induced changes in the time constant of the first rapid phase of recovery from short depolarizations (i.e., the difference among the values denoted by the solid and open bars in Fig. 5A) are plotted as a function of the amplitude of the second phase of recovery from short depolarizations (inset of Fig. 4B). The line is the best-fit linear regression of the form $y = 0.29x^2 - 0.022$ ($R^2 = 0.81$, $P = 0.0022$). Constructs with red labels were excluded from the analysis. This analysis suggests that the lidocaine-induced increase in the time constant of the first rapid phase of recovery from short depolarizations results from a modification of the second slow phase of recovery observed under drug-free conditions (inset of Fig. 4B, i.e., recovery from $I_{M2}$).
In most constructs we observed five major effects of lidocaine: inactivation. The DIV-S6 segment as an important player in the control of these results confirm previous reports implementing (et al., 1994; Hayward et al., 1997; Bai et al., 2003). In general these results confirm previous reports implementing the DIV-S6 segment as an important player in the control of inactivation.

**Effect of Lidocaine on Recovery from Inactivation.** In most constructs we observed five major effects of lidocaine:

1. An increase in the time constant of the first phase of recovery from short depolarizations (Figs. 4, 5, 13).
2. The emergence of a second, slow phase of recovery from short depolarizations (Figs. 4, 6, 7, 11, 13, 14).
3. An increase in the time constant of recovery from $I_M$ following long depolarizations (Fig. 9).
4. An increase in the amplitude of recovery from $I_M$ following long depolarizations (Fig. 11).
5. A decrease in the amplitude of recovery from $I_S$ following long depolarizations (Fig. 11).

Recovery from short depolarizations in lidocaine-exposed channels occurs by two phases, a fast phase commonly interpreted as recovery of drug-unbound channels (Ragsdale et al., 1994) and an additional slow phase representing recovery of drug-bound channels (Vedantham and Cannon, 1999). We find that the first rapid phase is also substantially slowed by lidocaine in most tested constructs (Fig. 5), as has been reported previously by numerous studies but not systematically investigated. Crumb and Clarkson (1990) suggested that this effect is caused by open-channel unblock during the test pulse. The most dramatic slowing of this first phase was observed in I1576C and M1585C. Notably, for both mutations, the intermediate time constant of recovery was also substantially shortened (Fig. 9).

This probably indicates destabilization of $I_M$ by the mutations $I_{M,MUT}$ (Hayward et al. (1997)). As shown in Fig. 13B lidocaine-modified M1585C channels recovered from a 10-millisecond depolarizing prepulse with two distinct phases, as observed in most other constructs. However, if the prepulse duration was increased to 50 milliseconds, a third phase of recovery appeared to emerge. This phase had a time constant of $22.9 \pm 8.18$ milliseconds and thus “bridged” the two distinct phases of recovery observed with a 10-millisecond duration.
identical to IM-MUT, which was observed in the mutations with long depolarizations (Fig. 9). Hence, according to the IFM, which is populated during short depolarizations and changes in the amplitude of IFM, i.e., the slow phase of recovery, is significantly correlated with the mutation-induced observed in most constructs (Fig. 5). As shown in Fig. 12D this of the fast phase of recovery from short depolarizations, as was similar to the time constant of recovery from IM observed with a time constant of 50 milliseconds (control, lidocaine). The parameters of the fits are:

- A1 = 0.99 ± 0.03, A2 = 0.10 ± 0.02; for 50 milliseconds (lidocaine, 500 μM, n = 5).
- r1 = 1.42 ± 0.12 milliseconds, r2 = 40.42 ± 22.91 milliseconds, A1 = 0.49 ± 0.05, A2 = 0.52 ± 0.06; for 10 seconds (control, n = 4).
- 1 = 1.42 ± 0.12 milliseconds, r2 = 166.5 ± 14.54 milliseconds, A1 = 0.01 ± 0.01, A2 = 0.55 ± 0.00; for 10 seconds (lidocaine, 1 mM, n = 3).
- r1 = 2.13 ± 0.55 milliseconds, r2 = 140.2 ± 11.02 milliseconds, A1 = 0.01 ± 0.01, A2 = 0.15 ± 0.02; for 10 seconds (lidocaine, 1 mM, n = 3).

The most dramatic effect of lidocaine on recovery from short depolarizations is the emergence of a slower phase of recovery during short depolarizations lidocaine. A similar mechanism may appear to occur mainly from the lidocaine-modified IM state. It has to be noted that in these constructs, with short depolarizations, lidocaine stabilizes IM-MUT (Fig. 9). A similar mechanism may account for the lidocaine-induced increase in the time constant of the fast phase of recovery from short depolarizations, as observed in most constructs (Fig. 5). As shown in Fig. 12D this increase significantly correlates with the mutation-induced changes in the amplitude of IP, i.e., the slow phase of recovery from short depolarizations under drug-free conditions (insets of Fig. 4, A and B). Hence, lidocaine may bind to IP, which is populated during short depolarizations and recovers with a time course slightly slower than that for IP. Further studies will be needed to elucidate whether IP is identical to IM-MUT, which was observed in the mutations I1576C and M1585C.

The most dramatic effect of lidocaine on recovery from short depolarizations was the emergence of a slower phase of recovery with a time constant of ~170 milliseconds. This time constant was similar to the time constant of recovery from IM observed with long depolarizations (Fig. 9). Hence, according to the “stabilization hypothesis,” during short depolarizations lidocaine may accelerate entry into the native IM state. Alternatively, according to the “dissociation hypothesis,” lidocaine may bind to IP during the short depolarization and, upon repolarization, dissociate with a time constant that, by coincidence, has a value similar to the time constant of recovery from native IM. As shown in Fig. 9 the time constants of recovery from IP were substantially modulated by serial cysteine mutagenesis of DIV-S6 residues. Figure 10 demonstrates that the mutation-induced alterations of the time constants of recovery from IP are independent of the prepulse voltage, as would be expected from a Markovian kinetic scheme, and that the changes are sufficiently large to be explored by linear correlation analysis. Figure 12, A and B, demonstrates that there is no correlation between the mutation-induced alterations of the time constant of recovery from IM (unmodified and drug-modified) and the time constant of the lidocaine-induced slow phase of recovery from short depolarization. Hence, this latter phase of recovery appears to be mechanistically different from recovery from IM and probably does not result from drug-induced acceleration of entry into IM (Fig. 1). Most probably this phase represents drug dissociation from the IP state (koff in Fig. 1), as proposed previously (Hille, 1977; Ragsdale et al., 1994). This notion is supported by the effects of lidocaine on the mutation W1531G (Fig. 14). W1531G is located in the P-loop of DIV and is part of a highly conserved “ring of tryptophanes” forming a portion of the outer vestibule (Lipkind and Fozzard, 2000; Durell and Guy, 2001; Tikhonov and Zhorov, 2011). W1531G opens an external access/egress pathway for local anesthetics (Lukacs et al., 2014). The lidocaine-induced slow phase of recovery from short depolarizations was substantially accelerated in W1531G compared with wild-type channels, whereas lidocaine-modified recovery from inactivation produced by long depolarizations occurred at a much slower rate. Thus, upon recovery from short depolarizations lidocaine most probably dissociates from fast-inactivated channels. This dissociation is accelerated by the external egress pathway created by W1531G. Although the high-affinity state for lidocaine with short depolarizations appears to be the fast-inactivated state, it has to be noted that there is increasing experimental evidence for local anesthetic drug-binding along the activation pathway that precedes entry into inactivation (Vedantham and Cannon, 1999; Muroi and Chanda, 2009).

On the other hand, after long depolarizations recovery appears to occur mainly from the lidocaine-modified IM state. Lidocaine significantly increased both the time constant and the amplitude of recovery from IM in most examined mutations (Fig. 9 and Fig. 11, A and B). The modification of recovery from IM by lidocaine has been reported previously (Kambouris et al., 1998). The authors noted that “lidocaine enhanced occupancy of a nonconducting state with intermediate recovery kinetics similar (but not identical) to IM.” In this study lidocaine consistently increased the time constant of IM with prepulses to ~20 mV (Fig. 9). With prepulses to ~50 mV the effect was less dramatic, mainly because of the lower amplitude of recovery at this voltage, which reduces the accuracy of estimation of the time constants by nonlinear curve fitting. The lidocaine-induced increase in the time constant of recovery from IM may reflect slow drug dissociation from that state at hyperpolarized voltages.

Figure 12C shows that there is a significant correlation between the mutation-induced changes in the time constant of recovery from IM and the lidocaine-modified time constant of IM, suggesting a link between recovery from the native IM state and lidocaine-induced slowing of recovery.

The significant reduction of the amplitude of recovery from IM by lidocaine (Fig. 11, C and D) most probably results from the drug-induced stabilization of IM, which prevents channels from being reversibly inactivated by the long depolarizing prepulse (Fig. 13, B–D). This effect may have generated artfactually a dramatic increase in the time constant of the first phase if this phase was fitted with a single exponential equation, as performed in Fig. 5. A similar phenomenon occurred with I1576C (Fig. 13, C and D). Thus, it appears that in these constructs, with short depolarizations, lidocaine stabilizes IM-MUT (Fig. 9). A similar mechanism may account for the lidocaine-induced increase in the time constant of the fast phase of recovery from short depolarizations, as observed in most constructs (Fig. 5). As shown in Fig. 12D this increase significantly correlates with the mutation-induced changes in the amplitude of IP, i.e., the slow phase of recovery from short depolarizations under drug-free conditions (insets of Fig. 4, A and B). Hence, lidocaine may bind to IP, which is populated during short depolarizations and recovers with a time course slightly slower than that for IP. Further studies will be needed to elucidate whether IP is identical to IM-MUT, which was observed in the mutations I1576C and M1585C.

Figure 14. Modification of the time course of recovery in W1531G. The mutation W1531G creates a pathway for rapid external drug access and egress (Lukacs et al., 2014), thereby allowing for assessment of gating-independent drug dissociation. Here, we make use of this mutation to examine the contribution of drug-dissociation to the drug-induced prolongation of channel repriming. Shown here are the time courses of recovery from prepulse durations of the indicated durations (prepulse voltage = ~20 mV). Open and filled symbols denote data recorded under drug-free conditions and during application of 1 mM lidocaine, respectively. Connecting broken lines are fits of eqs. 2 and 3 to the data points of recovery from 50-millisecond and 10-second prepulses to ~20 mV, respectively. Compared with wild-type, recovery from 50-millisecond pulses in lidocaine-exposed channels is substantially accelerated in W1531G. However, the time courses for lidocaine-modified recovery from 10-second depolarizations are similar for the two different constructs. For comparison, fitted lines to the indicated wild-type data are reproduced from Fig. 3.
from entering into other connected states (Kambouris et al., 1998). This effect also explains the substantial acceleration of recovery from long depolarizations in W1531G (Fig. 14). With this mutation lidocaine reduced the fraction of channels that recovered from I3 with long depolarizations from 37% to 15% (Fig. 14). A similar phenomenon has been observed in Na1.7 channels (Sheets et al., 2011) and may allow for development of a new generation of analgesic drugs.

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Authorship Contributions

Participated in research design: Gawali, Lukacs, Todt.
Conducted experiments: Gawali, Lukacs.
Performed data analysis: Gawali, Lukacs, Cervenka, Koenig, Rubi, Sandtner, Todt.
Wrote or contributed to the writing of the manuscript: Gawali, Cervenka, Koenig, Hilber, Sandtner, Todt.

References


Address correspondence to: Dr. Hannes Todt, Center for Physiology and Pharmacology, Department of Neurophysiology and Neuropharmacology, Medical University of Vienna, 1090 Vienna, Austria. E-mail: hannes.todt@meduniwien.ac.at