Amino acids located in the outer vestibule of the voltage-gated Na\textsuperscript{+} channel determine the permeation properties of the channel. Recently, residues lining the outer pore have also been implicated in channel gating. The domain (D) IV P-loop residue alanine 1529 forms a part of the putative selectivity filter of the adult rat skeletal muscle (\(\mu 1\)) Na\textsuperscript{+} channel. Here we report that replacement of alanine 1529 by aspartic acid enhances entry to an ultra-slow inactivated state. Ultra-slow inactivation is characterized by recovery time constants on the order of several hundred ms (fast inactivation), several hundred ms ("intermediate inactivation"), and several thousand ms ("slow inactivation") (1, 2). When the channels are inactivated for even longer periods, a component of recovery can be identified with a time constant in the range of 30–100 s (3–6). We refer to this inactivated state, from which channels recover with time constants in the order of ~100 s, as "ultra-slow inactivation" (7).

Prolonged inactivation may be of substantial significance in a broad variety of physiological and pathological settings. In neurons, accumulation of Na\textsuperscript{+} channel prolonged inactivation may influence activity-dependent neuronal excitability, especially under pathological conditions of intense discharge such as epilepsy (8). In skeletal muscle, differences in Na\textsuperscript{+} channel prolonged inactivation may underlie differences in fast and slow twitch muscle excitability (9). Several genetic skeletal muscle diseases are a result of defects in inactivation (10). In the heart, myocardial infarction is associated with a delay in recovery from inactivation of Na\textsuperscript{+} currents (11, 12). This effect may produce inhomogeneities of cardiac impulse conduction, setting the stage for reentrant arrhythmias and predisposing to sudden cardiac death. Understanding the mechanisms of prolonged inactivation also could define new targets for drug development and therapeutic strategies against neurologic, neuromuscular, and cardiac disorders.

The molecular basis of fast inactivation is thought to be a "ball and chain" mechanism involving a cytoplasmic loop between the third and fourth domain (the III-IV linker) (13–15), but little is known about the mechanism of the slower forms of inactivation.

We have shown previously that the mutations \(\mu 1\)-DIV-A1529D\textsuperscript{\textsuperscript{1237E}} and \(\mu 1\)-DIV-A1529D favor entry to the ultra-slow inactivated state (6, 16). Residues Lys\textsuperscript{1237} in DIII and Ala\textsuperscript{1529} in DIV are predicted to line the outer channel pore and, together with residues Asp\textsuperscript{1009} in DI and Glu\textsuperscript{775} in DII, are presumed to form a part of the selectivity filter of the channel (17–22).

Recent work from other laboratories suggests that the voltage sensors in DIII and DIV play a unique role in coupling fast inactivation to voltage-dependent activation (23, 24). Also, DIV voltage sensors have been shown to play a role in slow inactivation (25). In the present study we explore in detail the pro-

---

2 The abbreviations used are: D, domain; CTX, conotoxin; HPLC, high pressure liquid chromatography; BTX, batrachotoxin.
properties of ultra-slow inactivation in the mutant DIV-A1529D. We find that ultra-slow inactivation in DIV-A1529D has a U-shaped voltage dependence. It can be induced more efficiently by depolarizing voltage trains, suggesting that ultra-slow inactivation in DIV-A1529D occurs from intermediate closed states that are occupied on the way to the open state. This suggests that the P-loop in DIV may be involved in the activation mechanism of the channel.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis of the µ1—**The oligonucleotide-directed point mutation A1529D was introduced using four primer polymerase chain reaction. An oligonucleotide containing the mutation was designed with a change in a silent restriction site to allow rapid identification of the mutant. A vector consisting of the µ1 coding sequence flanked by Xenopus gilbertin 5'- and 3'-untranslated regions was provided as a gift by R. Moorman. This was used as the template for mutagenesis, and polymerase chain reaction fragments were isolated and subcloned into this template using directional ligations. Incorporation of the mutation was confirmed by DNA sequencing of the entire polymerized regions. The vector was linearized by SauI digestion and transcribed with SP6 DNA-dependent RNA polymerase using reagents from the mCAP RNA capping kit (Stratagene, La Jolla, CA). The rat brain

**RESULTS**

**Recovery from Ultra-slow Inactivation in µ1 and DIV-A1529D—**DIV-A1529D shows a slowly recovering component of inactivation. Fig. 1A shows the growth of inward currents through DIV-A1529D channels with subsequent pulses at 20-s intervals. From a holding potential of −120 mV, the channels were first inactivated by a 300-s depolarizing prepulse to −50 mV. Recovery from inactivation after returning to −120 mV was monitored by repetitive test pulses to −10 mV. The test pulse duration was 30 ms. About 50% of the current recovered within 20 s, whereas the remaining fraction took several minutes to complete recovery.

DIV-A1529D shows a larger component of a slowly recovering current in comparison with µ1. Fig. 1B summarizes the time course of recovery from inactivation, produced by a 300-s depolarizing prepulse to −50 mV in native µ1 and in constructs with the mutation DIV-A1529D (n = 6 for each data point). To ensure complete recovery from fast inactivation between test pulses, the test pulse duration was decreased to 20 ms. The data were normalized to the final current level after full recovery. Clearly, wild type µ1 currents recovered completely within ~50 s, whereas the time course of recovery of DIV-A1529D channels was substantially slower. The data points were well fitted with two exponentials (Eq. 1) reflecting two channel populations recovering from distinct inactivated states, which we refer to as slow and ultra-slow inactivation (6). Recovery from slow inactivation had a mean time constant of 7.9 ± 1.3 and 7.1 ± 1.4 s in µ1 and DIV-A1529D, respectively.

The corresponding amplitudes were 0.58 ± 0.04 in µ1 and 0.35 ± 0.02 in DIV-A1529D (p < 0.01). Time constants of recovery from ultra-slow inactivation were 78.4 ± 19.8 s in µ1 and 110.9 ± 11.5 s in DIV-A1529D. The amplitudes were 0.22 ± 0.02 in µ1 and 0.62 ± 0.04 in DIV-A1529D (p < 0.01). Thus, significantly more channels recovered from ultra-slow inactivation in DIV-A1529D than in µ1.

**Development of Ultra-slow Inactivation in DIV-A1529D—**To determine the time course of development of ultra-slow inactivation in DIV-A1529D, prepulses to −50 mV of variable duration were applied from a holding potential of −120 mV, and the time course of recovery at −120 mV was monitored for each prepulse duration by subsequent 30-ms test pulses to −10 mV at 20-s intervals. The time course of recovery for each prepulse duration was then fitted with two exponentials that yielded time constants and amplitudes of slow and ultra-slow inactivation (Equation 1). In Fig. 1C the amplitude of the ultra-slow exponential component of recovery, reflecting the fraction of channels recovering from ultra-slow inactivation (A2 in Equation $1 - F_{\text{inactivating}}$), is plotted as a function of the respective durations of the inactivating prepulse. The connecting line is the result of a single exponential fit (Equation 3) to the data points. The time constant of development of ultra-slow inactivation was 81 ± 17.9 s. Thus, both entry to and exit from ultra-slow inactivation in DIV-A1529D channels had time constants similar to those previously found in DIII-K1237E channels (6).

Coexpression of DIV-A1529D with the Rat Brain β2 Subunit—Coexpression of the β2 subunit with the α subunit of the µ1 Na+ channel speeds current decay and accelerates recovery from fast and slow inactivation (6, 27–30). We wanted to ex-
Fig. 1. Ultra-slow inactivation in DIV-A1529D. A, growth of inward current during recovery from ultra-slow inactivation in DIV-A1529D channels. From a holding potential of −120 mV, the channels were inactivated by a 300-s depolarizing step to −50 mV. Thereafter, the potential was returned to −120 mV, and recovery from inactivation was monitored by repetitive 30-ms test pulses to −10 mV at 20-s intervals. B, comparison of the time courses of recovery from inactivation, produced by a 300-s depolarizing prepulse to −50 mV in native μ1 (filled circles) and in DIV-A1529D (filled squares; n = 6 for each data point). The time course of recovery was monitored by successive 20-ms test pulses to −10 mV, applied at 20-s intervals. Peak inward currents were normalized to the final current level attained after full recovery. Recovery was substantially slower in DIV-A1529D channels than in wild type μ1. The time course of recovery was best fit with double exponential functions (Equation 1; connecting lines). C, the time course of development of ultra-slow inactivation in DIV-A1529D. The membrane potential was depolarized from −120 to −50 mV for variable durations, and the time course of recovery at −120 mV was monitored for each prepulse duration as described for B. The time course of recovery from ultra-slow inactivation for each prepulse duration was then fitted with two exponentials (Equation 1). The amplitude of the ultra-slow exponential component of recovery (Aρ, in Equation 1 = F_{inactivating}) is plotted as a function of the respective duration of the inactivating prepulse. The connecting line is the result of a single exponential fit (Equation 3) to the data points. The time constant of development of ultra-slow inactivation was 81 ± 17.9 s. D, growth of inward current during recovery from ultra-slow inactivation in DIV-A1529D, coexpressed with the rat brain β1 subunit. From a holding potential of −120 mV, the channels were inactivated by a 1200-s depolarizing step to −50 mV. Thereafter, the potential was returned to −120 mV, and recovery from inactivation was monitored by repetitive 30-ms test pulses to −10 mV at 20-s intervals. E, peak inward currents of the experiment shown in D were normalized to the final current level attained after full recovery. The time course of recovery was best fit with a double exponential function (Equation 1; connecting line). The time constant of the slower component representing recovery from ultra-slow inactivation was 130 s, which is similar to the mean value for DIV-A1529D α only channels (110.9 ± 11.5 s; see text). F, comparison of the amplitude of ultra-slow inactivation in DIV-A1529D + β1 channels following inactivating prepulses to −50 mV. Prepulse duration was 300 s (n = 5) and 1200 s (n = 4). Prolonging prepulse duration from 300 to 1200 s significantly increased the amplitude of ultra-slow inactivation to a value of −0.6. This amplitude was similar to the amplitude of ultra-slow inactivation in DIV-A1529D α alone channels, following prepulses of 300-s duration. Thus, coexpression with the β1 subunit slowed development of ultra-slow inactivation.

probe whether the β1 subunit affected recovery from ultra-slow inactivation in addition to well known modulating effects of β1 on fast and slow inactivation. We found that, compared with DIV-A1529D α alone, longer prepulse durations were required to drive DIV-A1529D + β1 channels into ultra-slow inactivation. Fig. 1 (D and E) shows the time course of recovery of DIV-A1529D + β1 channels from ultra-slow inactivation produced by a 1200-s inactivating prepulse to −50 mV. Note that the prepulse duration was four times as long as in the experiment with DIV-A1529D α alone, shown in Fig. 1A. Inward currents during recovery of DIV-A1529D + β1 were slowly increasing, suggesting that the channels recovered from ultra-slow inactivation. Comparison of the current traces in Fig. 1 (A and D) demonstrates that coexpression with β1 substantially accelerated current decay. However, the time course of recovery from ultra-slow inactivation of DIV-A1529D + β1 was similar to that of DIV-A1529D α alone (Fig. 1, B and E). Fig. 1F shows that increasing the prepulse duration from 300 to 1200 s significantly increased the fraction of DIV-A1529D + β1 channels recovering from ultra-slow inactivation. In contrast, entry into ultra-slow inactivation at −50 mV in DIV-A1529D α alone channels was completed after −300 s (Fig. 1C). Thus, coexpression of DIV-A1529D α with the β1 subunit did not abolish ultra-slow inactivation but delayed entry into the ultra-slow inactivated state.

Voltage Dependence of Ultra-slow Inactivation—The fraction of channels recovering from ultra-slow inactivation was strongly voltage-dependent. To examine the voltage dependence of ultra-slow inactivation in DIV-A1529D, the oocytes were depolarized for 300 s from −120 mV to prepulse voltages in the range of −90 mV to +30 mV. After each prepulse the potential was returned to −120 mV, and recovery was monitored by 20-ms test pulses to −10 mV at 20 s intervals (n = 4–12). The time course of recovery for each prepulse potential was then fit with a double exponential function (Equation 1) to estimate the fraction of channels recovering from ultra-slow inactivation (A2 = F_{inactivating}).

To facilitate comparison with standard availability curves, the fraction of channels not recovering from ultra-slow inactivation was plotted as a function of prepulse voltage in Fig. 2. The voltage dependence of ultra-slow inactivation was U-shaped. At
The membrane potential was depolarized from −120 mV to the indicated prepulse voltages for 300 s, and the time course of recovery at −120 mV was monitored for each prepulse potential as described for Fig. 1B (n = 4–12). The time course of recovery from ultra-slow inactivation for each prepulse potential was then fit with a double exponential function (Equation 1) to estimate the fraction of channels recovering from ultra-slow inactivation (1 − A∞). Channel availability defined as the fraction of channels not recovering from ultra-slow inactivation (1 − A∞ = Fnoninactivating) is plotted as a function of prepulse voltage. Inset, Current-voltage relationship in DIV-A1529D, determined by 20-ms test pulses to the indicated potentials, from a holding potential of −120 mV (open squares). Open circles, integrated inward currents, reflecting charge entry, versus voltage.

Fig. 2. Voltage dependence of ultra-slow inactivation in DIV-A1529D and DIV-A1529D + β1. The membrane potential was depolarized from −120 mV to the indicated prepulse voltages for 300 s, and the time course of recovery at −120 mV was monitored for each prepulse potential as described for Fig. 1B (n = 4–12). The time course of recovery from ultra-slow inactivation for each prepulse potential was then fit with a double exponential function (Equation 1) to estimate the fraction of channels recovering from ultra-slow inactivation (1 − A∞). Channel availability defined as the fraction of channels not recovering from ultra-slow inactivation (1 − A∞ = Fnoninactivating) is plotted as a function of prepulse voltage. Inset, Current-voltage relationship in DIV-A1529D, determined by 20-ms test pulses to the indicated potentials, from a holding potential of −120 mV (open squares). Open circles, integrated inward currents, reflecting charge entry, versus voltage.

The following protocol was designed to obtain an estimate of the voltage dependence of inactivated states that were elicited by conditioning prepulses short enough to avoid entry of channels into ultra-slow inactivation. 1-s conditioning prepulses to various voltages were applied, and the available, noninactivated fraction of channels was gauged by a subsequent test pulse (Fig. 3). Both in wild type μ1 and in DIV-A1529D channel availability after 1-s conditioning prepulses decreased monotonically with depolarization, asymptotically approaching zero at potentials positive to −40 mV. The absence of U-shaped voltage dependence of inactivation produced by a 1-s prepulse suggested that the ultra-slow inactivated state in DIV-A1529D was unlikely to be connected to those inactivated states that were produced by 1-s conditioning prepulses.

Reciprocal from a 1-s Conditioning Prepulse.—Prepulses of 1-s duration have been shown to recruit a minimum of three inactivated states in μ1 (1). Although the experiment shown in Fig. 3 suggested a monotonic voltage dependence of other than ultra-slow inactivated states, it failed to provide information regarding the relative partitioning among faster inactivated states at a given prepulse potential. Thus, we sought to explore whether conditioning prepulses of 1-s duration would be able to recruit a minimum number of three distinct states of inactivation in DIV-A1529D and, if so, whether any one of these states had a nonmonotonic voltage dependence similar to ultra-slow inactivation. Therefore, we examined the time course of recovery of DIV-A1529D channels after a 1-s conditioning prepulse to −50 mV (i.e. Fnoninactivating was at minimum) and to −10 mV (where Fnoninactivating at maximum). As shown in Fig. 4 the time course of recovery in DIV-A1529D was adequately fitted with three exponential decay functions (Equation 2), suggesting the presence of at least three inactivation processes (1). The amplitudes of recovery from inactivation, reflecting the fraction of channels recovering from anyone distinct state were similar for both prepulse potentials (see legend to Fig. 4). This result indicates that within the range of examined prepulse voltages, the mutation DIV-A1529D did not affect the voltage dependence of inactivated states produced by brief prepulse durations. Thus, it is unlikely that brief prepulses could produce inactivated states showing U-shaped voltage dependence.

Ultra-slow Inactivation in DIV-A1529D Is Favorably by Pulse Trains.—The fact that inactivation produced by 1-s prepulses did not exhibit U-shaped voltage dependence argues against a serial Markovian kinetic scheme, where ultra-slow inactivation...
FIG. 3. Voltage dependence of steady-state availability probed by 1-s conditioning prepulses. From a holding potential of −120 mV the membrane potential was changed to the level displayed on the abscissa. Subsequently, the membrane potential was stepped to −20 mV for 20 ms to assay the available peak inward currents. The available peak inward currents were normalized to 1 by the current measured at the holding potential of −120 mV. Both in wild type μ1 and in DIV-A1529D channel availability decreased monotonically with depolarization, reaching a minimum at potentials positive to −40 mV. The solid lines represent fits to a Boltzmann function $I_{\text{avail}} = I_{\text{max}} \frac{1}{1 + \exp ((V - V_{1/2})/k)}$ where $V_{1/2}$ is the midpoint of the curve and $k$ is the slope factor.

is reached via faster inactivated states. The voltage range where ultra-slow inactivation reached a local maximum was around −60 to −50 mV. This is the voltage range over which Na⁺ channels are activated, i.e. the channels pass through a number of closed states to reach a final open state. Therefore, we reasoned that ultra-slow inactivation could be connected to the kinetic processes of activation and/or deactivation. To test this we examined whether ultra-slow inactivation could be produced by repetitive brief depolarizations that enhance the probability of channels to undergo transitions between closed and open states. Fig. 5A shows the effect of 3333 repetitive step depolarizations to −20 mV, applied from a holding potential of −120 mV. Each depolarization had a duration of 2 ms, followed by an interpulse interval of 28 ms, at −120 mV (33.3 Hz). The entire duration of the pulse train was 100 s. Each data point in Fig. 5A indicates the inward current elicited by one out of 50 applied pulses. Clearly, the pulse train was associated with a slowly developing decline in peak inward current, reflecting cumulative channel inactivation. After 100 s the 33.3 Hz train was stopped, and recovery from cumulative inactivation was monitored by repetitive 20-ms test pulses to −20 mV, applied at 20-s intervals from a holding potential of −120 mV. The time course of recovery was very slow, and the initial current level (before the 33.3 Hz train) was not reached until >200 s had elapsed at −120 mV. Fitting a double exponential function to the normalized time course of recovery after the train (Fig. 5B, line) revealed that ∼36% of the channels recovered from ultra-slow inactivation ($\tau = 85$ s).

Table I presents a quantitative comparison between the fraction of channels recovering from ultra-slow inactivation, produced by a single 15-s prepulse to −50 mV, by a 33.3-Hz pulse train, and by a 20-Hz pulse train ($n = 6$ for each protocol). During the trains 2-ms step depolarizations to −20 mV were applied for a total duration of 100 s. The cumulative amount of time the channels spent at depolarized potentials was 6.6 and 4 s during the 33.3- and 20-Hz trains, respectively. After each train the time course of recovery was examined as described for the train in Fig. 5. The data in Table I demonstrate that the fraction of channels recovering from ultra-slow inactivation ($A_3$) was significantly greater after each train than after the single prepulse, even though channels spent substantially less time at depolarized potentials during each train than during the constant voltage. Previously we showed that during a single 300-s depolarization to −20 mV only ∼10% of channels entered the ultra-slow inactivated state, whereas following a 300-s depolarization to −60 mV, ∼70% of channels became ultra-slow inactivated. During the depolarizing trains channels were either at the holding potential of −120 mV or at the depolarized potential of −20 mV. Neither of these potentials should recruit a substantial amount of ultra-slow inactivation. The fact that multiple repeated depolarizations drove substan-
ability to be recruited at approximately −60 mV. Holding channels at −60 mV may accumulate those closed states from which a kinetic pathway leads into ultra-slow inactivation, explaining why entry to ultra-slow inactivation is maximal at this voltage. Similarly, cycling channels between closed and open states during pulse trains will also accumulate channels in specific closed states that could be connected to the ultra-slow inactivated state.

**Molecular Mechanism of Ultra-slow Inactivation in DIV-A1529D**—Previously, we have reported that the mutation DIII-K1237E in µ1 enhanced the probability of entry to ultra-slow inactivation (6). We hypothesized that ultra-slow inactivation may result from a conformational change of the outer channel vestibule. This hypothesis was supported by the observation that binding of the mutated µ-conotoxin GIIIA R13Q to the outer channel pore dramatically reduced the likelihood of DIII-K1237E channels entering ultra-slow inactivation. One explanation for this effect was that binding of µ-CTX R13Q to the outer channel vestibule protected channels from entry to ultra-slow inactivation by physical hindrance of the underlying conformational change of the outer channel vestibule. Assessment of channel gating kinetics in the toxin-bound state was possible because µ-CTX R13Q only partially occludes the outer vestibule, resulting in a residual current of about 25−30% of that in control (35).

We tested whether µ-CTX R13Q reduced the likelihood of DIV-A1529D channels to enter the ultra-slow inactivated state, as has been demonstrated with DIII-K1237E channels. Fig. 6A shows the time course of recovery of DIV-A1529D channels from a 300-s prepulse to −50 mV. Recovery at −120 mV was monitored by repetitive 20-ms test pulses at 20-s intervals. The currents were assessed during control and during superfusion with 27 µM µ-CTX R13Q. Binding of µ-CTX R13Q substantially reduced the amount of ultra-slow recovery from inactivation.

The time constants of recovery from ultra-slow inactivation (τ₂) were 120.4 ± 4.0 and 94.1 ± 11.2 s (p < 0.05; n = 6 for each measurement) during control and during superfusion with µ-CTX R13Q, respectively. µ-CTX R13Q also reduced the amplitudes of recovery from ultra-slow inactivation (A₂; control: 0.76 ± 0.02; µ-CTX R13Q: 0.47 ± 0.09; p < 0.05). Thus, µ-CTX R13Q reduced both the time constant and the amplitude of ultra-slow inactivation.

These results suggest that binding of µ-CTX R13Q protects a fraction of channels from entry to ultra-slow inactivation in DIV-A1529D as well as in DIII-K1237E. However, µ-CTX R13Q has been shown to shift voltage-dependent channel gating, perhaps by an electrostatic effect on the voltage sensors (35). To test whether the reduction of ultra-slow inactivation by µ-CTX R13Q may have resulted from a voltage shift of ultra-slow inactivation, we investigated the effect of µ-CTX R13Q on the voltage dependence of ultra-slow inactivation. As demonstrated in Fig. 6B, µ-CTX R13Q reduced ultra-slow inactivation over the voltage range where ultra-slow inactivation was maximal, i.e. between −60 and −40 mV, without shifting its voltage dependence. Thus, the toxin-mediated reduction in the probability of entry to ultra-slow inactivation did not result from a mere shift of the voltage dependence of ultra-slow inactivation.

These results support the notion that ultra-slow inactivation is produced by a conformational change in the outer vestibule of the channel. µ-CTX R13Q may act as a “splint in the vestibule,” thereby preventing channels from entry to the ultra-slow inactivated state.

**DISCUSSION**

The major findings of the present study are: (i) the mutation DIV-A1529D in µ1 increased the propensity of Na⁺ channels to enter an ultra-slow inactivated state, (ii) the voltage depend-
ence of the ultra-slow inactivated state was U-shaped, (iii) entry to the ultra-slow inactivated state was promoted by repeated brief depolarizing pulses, and (iv) entry to the ultra-slow inactivated state was reduced by blocking the outer channel vestibule with a mutant \( \mu \)-conotoxin GIHA.

**Kinetic Effects of Mutations in the Selectivity Filter Region**—Recently, we reported that replacement of the lysine at site 1237 in \( \mu \) by serine and by glutamic acid dramatically increased the likelihood of entry to an ultra-slow inactivated state (6). However, mutating residue K1237 not only altered channel gating but also caused a substantial loss of ionic selectivity (22, 36). This dual effect of amino acid replacements at site 1237 raised the question of whether ultra-slow inactivation was produced more inactivation than a single depolarizing pulse of the same duration, despite the fact that the net time charge had moved (43).

Recently, it was shown that U-shaped voltage dependence in delayed rectifier K⁺ channels and in N-type calcium channels arises from purely voltage-dependent mechanisms (41–43). It was suggested that U-shaped inactivation occurs preferentially from partially activated closed states, \( i.e. \) nonconducting states that have some or all voltage sensors in the activated position. This model was supported by the observation that a train of depolarizing pulses cycling channels through closed and open states produced more inactivation than a single depolarizing pulse of the same duration, despite the fact that the net time during which channels were depolarized was longer with a single conditioning prepulse (41). The observation that accumulation of intermediate closed states during repetitive pulses produced more inactivation than single pulses of the same duration suggests that inactivation occurred mainly from intermediate closed states (41, 42). Additional strong support for preferential closed state inactivation as the basis for U-shaped voltage dependence of inactivation came from the observation in voltage-gated Ca²⁺ channels that the inactivation rate was fastest at a voltage where only one-third of the total gating charge had moved (43).

“Cumulative inactivation” (44), \( i.e. \) inactivation that builds up during repetitive brief depolarizations, has been reported in

---

**Fig. 6. Recovery from ultra-slow inactivation in DIV-A1529D is modulated by a mutant \( \mu \)-conotoxin known to bind at the outer vestibule.** A, recovery from ultra-slow inactivation was examined during a toxin-free control (solid squares) and during superfusion with 27 \( \mu \)M \( \mu \)-CTX R13Q (open squares). Ultra-slow inactivation was produced by a 300-s inactivating prepulse to \(-50\) mV. Recovery was assessed as described in the legend of Fig. 1B. Connecting lines represent double exponential fits (Equation 1). The parameters are given in the text. Clearly, superfusion with \( \mu \)-CTX R13Q substantially speeded recovery from ultra-slow inactivation. B, \( \mu \)-CTX R13Q (27 \( \mu \)M, open squares) substantially raised the nadir of U-shaped voltage dependence of ultra-slow inactivation in DIV-A1529D (control, solid squares). The voltage dependence of ultra-slow inactivation was determined as described for Fig. 3.
a subset of K+ channels (44–52). Aldrich (53) suggested that during pulse trains, where the pulse duration is sufficiently short to avoid inactivation within a pulse, channels inactivate predominantly from closed states. Upon repolarization, after each pulse, recovery from inactivation is sufficiently slow that little recovery occurs during the short interpulse interval.

In the present study, we found that DIV-A1529D channels could be driven into ultra-slow inactivation by repetitive brief depolarizations. Furthermore, a steady depolarization to −50 mV of the same duration as the applied pulse train produced substantially less ultra-slow inactivation despite the fact that channels spent substantially more time at depolarized potentials. These results are consistent with the idea that ultra-slow inactivation is reached preferentially via closed states. Hence, DIV-A1529D channels are most likely to undergo ultra-slow inactivation at the voltage range of −60 to −50 mV because this voltage range has the highest probability to accumulate intermediate closed states, which may provide a pathway for entry to the ultra-slow inactivated state.

\(\mu\)-CTX R13Q Interferes with Ultra-slow Inactivation in DIV-A1529D—Na\(^+\) channel block by \(\mu\)-CTX R13Q is incomplete, leaving residual single channel current, which allows the examination of channel gating in the blocked state (6, 35, 54, 55).

Recently, we presented evidence that \(\mu\)-CTX R13Q is capable of destabilizing the ultra-slow inactivated state by a mechanism unrelated to simple electrostatic interaction with the gating process (6), suggesting that \(\mu\)-CTX R13Q interacted with the outer channel vestibule in a way that prevented ultra-slow inactivation. We proposed that ultra-slow inactivation most likely reflected a rearrangement of the outer pore, similar to C-type inactivation in voltage-gated K\(^+\) channels and that binding of \(\mu\)-CTX R13Q to the outer pore stabilized the structure of the outer vestibule, thereby protecting channels from ultra-slow inactivation. In the present study ultra-slow inactivation in DIV-A1529D was substantially reduced when \(\mu\)-CTX R13Q was bound to the outer vestibule. Furthermore, as shown in Fig. 6B, superfusion with \(\mu\)-CTX R13Q did not result in a shift of the voltage dependence of ultra-slow inactivation, but the U-shaped dependence on prepulse voltage appeared blunted relative to the unblocked state. This is consistent with the hypothesis that the reduction of ultra-slow inactivation by \(\mu\)-CTX R13Q did not result from electrostatic interaction with the voltage-sensing channel structures, but most likely resulted from an impediment of entry to the ultra-slow inactivated state, similar to the interaction of \(\mu\)-CTX R13Q with DIII-K1237E (6). The molecular mechanism of the protection from ultra-slow inactivation by \(\mu\)-CTX R13Q remains to be elucidated. In theory, a part of \(\mu\)-CTX R13Q protruding into the outer pore could act as a splint in the vestibule, preventing a pore collapse as the molecular event underlying ultra-slow inactivation. Alternatively, the toxin might, by virtue of multiple interactions with the outer surface of the channel (56, 57), act as a molecular scaffold, thereby stabilizing the structure of the outer vestibule.

The Outer Vestibule of the Na\(^+\) Channel May Be Involved in Activation Gating—For DIV-A1529D the dynamic rearrangement of the pore, which most likely forms the basis of ultra-slow inactivation is preferentially reached through intermediate closed states, on the way to the open state of the channel. This suggests that the residue Ala\(^{1529}\) may be involved in the process of opening the channel pore. We envision that the P-loop of domain IV, which contains residue A1529, undergoes some kind of movement during the opening process of the channel. The replacement of alanine 1529 by aspartic acid might interfere with this motion, thus rendering the channel susceptible to undergo the molecular rearrangement that is reflected by ultra-slow inactivation.

The notion that the P-loop in DIV may participate in channel gating is supported by the finding that this part of the channel is extraordinarily flexible (21, 58, 59). This flexibility may be mediated by two glycines in close proximity to residue Ala\(^{1529}\) (Gly\(^{1530}\) and Gly\(^{1533}\)). Glycine residues are considered to allow for a high degree of protein backbone flexibility (60). Contrary to P-loop DIV, P-loops in DII and III contain only one glycine (Gly\(^{754}\) and Gly\(^{1238}\), respectively), and P-loop of DI does not contain any glycine. This underscores a potentially unique role of P-loop in DIV as a flexible part of the channel.

Complementary support for a possible role of the DIV P-loop as a part of the gating machinery of the channel comes from the demonstration that the adjacent DIV S6 segment plays an important role in fast inactivation (61–64), slow inactivation (64–66), and in binding of batrachotoxin (BTX) and local anesthetics (64, 67, 68).

BTX is a potent neurotoxin that stabilizes the open state of Na\(^+\) channels. As a result the channels open persistently, activation is shifted in the hyperpolarized direction, deactivation is slowed, and inactivation is prevented (69). Furthermore, the ionic selectivity of BTX-modified channels is substantially reduced, which indicates that the selectivity filter may be involved in the action of the drug. Finally, BTX binding has been shown to produce U-shaped voltage dependence of Na\(^+\) channel availability (39). Thus, BTX-modified channels share a number of properties with channels carrying the mutation DIV-A1529D. This suggests that both Ala\(^{1529}\) and the binding site of BTX may be mechanistically linked to structures involved in the gating machinery. This notion is supported by a recent report demonstrating that the residue DIV-S6 Val\(^{1583}\) is implicated in BTX binding and in channel gating (64). According to a recent model of the Na\(^+\) channel, the DIV-S6 residue Val\(^{1583}\) may be in reasonable proximity to the DIV-P-loop residue Ala\(^{1529}\) to allow for interaction between the two residues during a rearrangement associated with channel gating (70, 71). In this context it is noteworthy that the DIV P-loop may not only be extremely flexible, as discussed above, but may protrude further into the pore than P-loops of domains I–III, based on electrical distance measurements (19). Thus, it is not unreasonable to assume an interaction of the P-loop residue Ala\(^{1529}\) with residues located in DIV-S6.

Similar to BTX binding, the local anesthetic block is well known to be linked to channel gating. A mutual relationship appears to exist between the local anesthetic block and the conformation of the outer channel vestibule because outer pore mutations influence the access of local anesthetics to their binding pocket (71, 72), and local anesthetic binding is associated with a structural rearrangement of the outer channel vestibule (73). Furthermore, recent studies suggest that the mechanism of action of the local anesthetic lidocaine involves transitions along the activation pathway (74–76). These findings accord well with the idea that DIV-S6 and the DIV P-loop may be linked to activation gating.

Tetrodotoxin and saxitoxin are known to block the Na\(^+\) channel by binding to its outer vestibule/selectivity filter region and thereby occluding the pore (77). Makielski et al. (78) characterized a “post-repolarization” block by these toxins and proposed that the toxins had a higher affinity for a pre-open state that was accessed briefly during depolarization and for a more prolonged period during recovery (78). Satin et al. (79) further showed that the DI vestibule residue, which is the greatest determinant of isofrom differences in toxin affinity (Tyr\(^{493}\) in \(\mu\)1), influences the rate constants for recovery through the pre-open higher affinity state. This reinforces the idea that some residues in the channel vestibule are involved in the
conformational changes associated with the pre-open state.

This idea gains further support from recent structural data regarding the bacterial KscA K⁺ channel. In KscA the transmembrane helices, TM2, which are structurally equivalent to S6, undergo a structural rearrangement during activation (80). Specifically, TM2 rotates in a counterclockwise direction while swinging away from the permeation pathway, thus increasing the diameter of the inner vestibule. A similar model has been proposed for the mechanism of activation in voltage-gated K⁺ channels (81, 82). In the voltage-gated Na⁺ channel, the inner vestibule is considered to be lined by the S6 segments of domains I–IV (83). As mentioned above, it is plausible that the P-loop residue Ala1529 may be in close proximity to the binding pocket for BTX and local anesthetics in S6. Thus, molecular motions of the DIV S6 segment during activation gating may well be transmitted to the adjacent P-loop residues and vice versa.

In summary, we propose that the mobility of the P-loop in DIV allows for participation of this structure in the complex rearrangement of S6 segments during channel opening. The mutation DIV-A1529D may interfere with this complex rearrangement prior to channel opening. As a result the outer channel vestibule undergoes a dynamic rearrangement that forms the basis of the propensity of DIV-A1529D to enter the ultra-slow inactivated state at voltages near the threshold for channel opening, thus accounting for the U-shaped voltage dependence of ultra-slow inactivation.

Acknowledgments—We thank Yu Huang, Bei Li, Gayle Tunkovich, and Anton Karel for technical assistance. Thanks are due to Dr. Denis McMaster (Peptide Synthesis Laboratory, University of Calgary Faculty of Medicine) for providing the peptide, α-CTX R13Q.

REFERENCES
1. Nuss, H. B., Balser, J. R., Orzios, D. W., Lawrence, J. H., Tomasielli, G. F., and Marban, E. (1996) J. Physiol. (Lond.) 494, 411–429.
2. Kambouris, N. G., Haste, I. S., Stepanovic, S., Marban, E., Tomasielli, G. F., and Balser, J. R. (1998) J. Physiol. (Lond.) 512, 683–705.
3. Cummins, T. R., and Sigworth, F. J. (1996) Biophys. J. 71, 227–236.
4. Wang, S., and Wang, G. K. (1996) Pflugers Arch. Eur. J. Physiol. 432, 692–699.
5. Passow, D. G., and Tare, D. (1997) Biophys. J. 71, 3098–3109.
6. Todi, H., Dudley, S. J., Kyle, J. W., French, R. J., and Fozzard, H. A. (1999) Biophys. J. 76, 1335–1345.
7. Fox, J. M. (1997) Biophys. Acta 226, 232–244.
8. Fiedlerich, I. A., Frieden, A., and Gutnick, M. J. (1999) J. Physiol. (Lond.) 503, 339–348.
9. Cannon, S. C. (1996) Trends Neurosci. 19, 31–30.
10. Pa, J., Balser, J. R., and Boyden, P. A. (1998) J. Gen. Physiol. 112, 311–320.
11. Zhu, W., and Stuhmer, W. (1997) J. Gen. Physiol. 110, 197–207.
12. Stuhmer, W., Conti, F., Suzuki, H., Wang, X. D., Noda, M., Yahagi, N., Kubo, H., and Numa, S. (1989) Nature 339, 597–603.
13. Paton, D. E., West, J. W., Catterall, W. A., and Goldin, A. L. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10905–10909.
14. West, J. W., Paton, D. E., Scheuer, T., Wang, Y., Goldin, A. L., and Catterall, W. A. (1992) Proc. Natl. Acad. Sci. U. S. A. 89, 10910–10914.
15. Todi, H., Hilib, K., Dudley, S. C., Kudla, O., French, R. J., Kyle, J. W., and Fozzard, H. A. (2000) Biophys. J. 78, 83 (abstr.)
16. Lipkind, G. M., and Fozzard, H. A. (1994) Biophys. J. 66, 1–13.
17. Chaimovich, N., Perez-Garcia, M. T., Tomasielli, G. F., and Marban, E. (1996) J. Physiol. (Lond.) 491, 51–59.
18. Chaimovich, N., Perez-Garcia, M. T., Ranjan, R., Marban, E., and Tomasielli, G. F. (1996) Neuron 16, 1037–1047.
19. Perez-Garcia, M. T., Chaimovich, N., Marban, E., and Tomasielli, G. F. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 300–304.
20. Tsafrir, R. G., Li, R. A., and Backx, P. H. (1997) J. Gen. Physiol. 109, 463–475.
21. Fawaz, I., Moczydlowski, E., and Schild, L. (1996) Biophys. J. 71, 3110–3125.
22. Cha, A., Ruben, P. C., George, A. L., Fujimoto, E., and Bezanilla, F. (1999) Science 283, 73–87.
23. Horn, R., Ding, S., and Gruber, H. J. (2000) J. Gen. Physiol. 116, 416–476.
24. Mitrovic, N., George, A. L., and Horn, R. (2000) J. Gen. Physiol. 115, 707–718.
25. Passow, D. G., and Tare, D. (1999) Methods Enzymol. 294, 575–605.
26. Isom, L. D., De Jongh, K. S., Patton, D. E., Reber, B. F., Offerd, J., Charbonneau, H., Walsh, K., Goldin, A. L., and Catterall, W. A. (1992) Science 256, 839–842.
27. Patton, D. E., Ison, L. L., Catterall, W. A., and Goldin, A. L. (1994) J. Biol. Chem. 269, 17649–17652.
28. Nuss, H. B., Chaimovich, N., Perez-Garcia, M. T., Tomasielli, G. F., and Marban, E. (1995) J. Gen. Physiol. 106, 1171–1191.
29. Chang, S. Y., Satin, J., and Fozzard, H. A. (1996) Biophys. J. 70, 2581–2592.
30. Eckert, R., and Chad, J. E. (1984) Progr. Biophys. Mol. Biol. 44, 215–237.
31. Zeng, J. Y., Potts, J. F., Fozzard, J. S., Agnew, W. S., and Sigworth, F. (1991) Neuron 7, 775–785.
32. Cunnon, S. C., McClatchey, A. I., and Gasell, J. F. (1993) Pflugers Arch. Eur. J. Physiol. 423, 155–157.
33. Ukomadu, C. A., Zgierski, G. W., and Fozzard, H. A. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3440–3445.
The Selectivity Filter of the Voltage-gated Sodium Channel Is Involved in Channel Activation
Karlheinz Hilber, Walter Sandtner, Oliver Kudlacek, Ian W. Glaaser, Eva Weisz, John W. Kyle, Robert J. French, Harry A. Fozzard, Samuel C. Dudley and Hannes Todt

J. Biol. Chem. 2001, 276:27831-27839.
doi: 10.1074/jbc.M101933200 originally published online May 29, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M101933200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 81 references, 26 of which can be accessed free at http://www.jbc.org/content/276/30/27831.full.html#ref-list-1