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Time- and state-dependent effects of methanethiosulfonate ethylammonium (MTSEA) exposure differ between heart and skeletal muscle voltage-gated Na^+ channels

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ABSTRACT

The substituted-cysteine scanning method (SCAM) is used to study conformational changes in proteins. Experiments using SCAM involve site-directed mutagenesis to replace native amino acids with cysteine and subsequent exposure to a methanethiosulfonate (MTS) reagent such as methanethiosulfonate ethylammonium (MTSEA). These reagents react with substituted-cysteines and can provide functional information about relative positions of amino acids within a protein. In the human heart voltage-gated Na^+ channel hNav1.5 there is a native cysteine at position C373 that reacts rapidly with MTS reagents resulting in a large reduction in whole-cell Na^+ current (I_{Na}). Therefore, in order to use SCAM in studies in this isoform, this native cysteine is mutated to a non-reactive residue, e.g., tyrosine. This mutant, hNav1.5-C373Y, is resistant to the MTS-mediated decrease in I_{Na}. Here we show that this resistance is time- and state-dependent. With relatively short exposure times to MTSEA (<4 min), there is little effect on I_{Na}. However, with longer exposures (4–8 min), there is a large decrease in I_{Na}, but this effect is only found when hNav1.5-C373Y is inactivated (fast or slow) — MTSEA has little effect in the closed state. Additionally, this long-term, state-dependent effect is not seen in human skeletal muscle Na^+ channel isoform hNav1.4, which has a native tyrosine at the homologous site C407. We conclude that differences in molecular determinants of inactivation between hNav1.4 and hNav1.5 underlie the difference in response to MTSEA exposure.

1. Introduction

The substituted-cysteine scanning method (SCAM) can be used to study conformational changes in proteins [1]. Experiments using SCAM involve site-directed mutagenesis to replace native amino acids with cysteine and subsequent exposure to a methanethiosulfonate (MTS) reagent such as methanethiosulfonate ethylammonium (MTSEA). If the substituted cysteine is "accessible" to the applied MTS reagent, its sulfhydral side chain (−SH) can form a covalent disulfide bond with sulfur in the MTS reagent. If this reaction produces a measurable effect on the function of the protein of interest (e.g., changes in current responses to voltage stimuli in voltage-gated ion channels) then conclusions about protein conformation can be made.

During SCAM experiments, we and others have previously shown that the wild-type human heart isoform of the voltage-gated Na channel (hNav1.5) responds rapidly with a decrease in whole-cell current (I_{Na}) when exposed to extracellular methanethiosulfonate (MTS) reagents [2,3]. This rapid response (seconds) is eliminated with site-directed mutagenesis replacing cysteine at site C373 in the outer pore of wild-type hNav1.5, e.g., with tyrosine in hNav1.5-C373Y [2,3]. Interestingly, the C373Y site mutation in hNav1.5-C373Y is analogous to the MTS-resistant skeletal muscle Na channel hNav1.4 [2], in which tyrosine is found at the C373-homologous site (Y401 in rat Nav1.4, Y407 in human Nav1.4). The C373 site in hNav1.5 is also of interest because this is the site that renders hNav1.5 resistant to tetrodotoxin (TTX) block, a resistance that is abolished with the same C to Y substitution as above [3,4].

While studies with SCAM often find effects within a relatively short MTS exposure time (seconds to minutes), there is little information about effects of longer MTS exposure times, e.g., >4 min. In this study, we characterize the effects of long-term exposure (up to 8 min) of hNav1.5-C373Y to extracellular MTSEA. While ≈4 min of MTSEA exposure results in little effect on I_{Na} in hNav1.5-C373Y, whether in the closed, fast-inactivated, or slow-inactivated state [2,3], we demonstrate here that exposure to MTSEA for greater than 4 min produces a significant reduction in current in this mutant. However, this effect only occurs when the channels are in an inactivated state (slow or fast), demonstrating that this effect is both time- and state-dependent. Additionally, while hNav1.5-C373Y responds to MTSEA in a time- and state-dependent manner, the skeletal muscle isoform hNav1.4 does not, suggesting that there are isoform specific structural determinants of inactivation that underlie this difference.

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2. Materials and methods

2.1. Site-directed mutagenesis

Site-directed mutagenesis to produce hNav1.5-C373Y was carried out using a modification of a 2-stage modification of the Quick Change XL Site directed Mutagenesis Kit protocol as previously described [2].

2.2. Transient transfection

Plasmid expression constructs containing wild-type hNav1.4, wild-type hNav1.5, and mutant hNav1.5-C373Y cDNA clones (10 μg) were transiently transfected into HEK cells using calcium phosphate precipitation as previously described [5,6]. The transfection solution included 2 μg of a plasmid encoding cell surface antigen CD8 (OriGene, Rockville, MD, USA) and cells were selected for recording based on positive immunoreaction with anti-CD8 Dynabeads (Invitrogen).

2.3. Electrophysiology

Using standard patch clamp techniques [7], we recorded whole-cell Na^+ current (I_{Na}) from wild-type hNav1.4, wild-type hNav1.5, and mutant hNav1.5-C373Y in transiently transfected HEK cells. Recordings were performed at room temperature (20–22 °C) and were initiated ≈5 min after whole-cell recording was established unless otherwise noted. Glass micropipettes (Drummond Scientific, Broomall, PA, USA) were pulled on a Model P-97 Flaming-Brown puller (Sutter Instruments, Novato, CA, USA) and had resistances from 0.5 to 1.5 MΩ. The recording solutions in mM were: extracellular — 65 NaCl, 85 choline-Cl, 2 $CaCl_2$, and 10 HEPES, titrated to pH 7.4 with TMA-OH; intracellular — 100 NaF, 30 NaCl, 10 EGTA, and 10 HEPES, titrated to pH 7.2 with CsOH. The advantage of the outward Na^+ gradient has been presented previously [8]. During recordings, the cells were continuously bathed with extracellular solution via a gravity-fed superfusion system at a rate of approximately 0.1–0.2 ml/min. For all recordings, series resistance was compensated at 80% and leak currents were subtracted based on five hyperpolarizing pulses. Any endogenous K^+ currents were blocked with Cs^+ in the pipette, and HEK cells express no native Ca^{++} current [9]. Recordings were performed 1 to 3 days post-transfection. The holding potential (V_{hold}) for all experiments was −160 mV. A test pulse to +50 mV (4 or 6 ms) was used to record peak available Na^+ current (I_{Na}).

2.4. Application of cysteine modification agent methanethiosulfonate ethylammonium (MTSEA)

The cysteine modification agent MTSEA (Toronto Research Chemicals, Toronto, Ontario, Canada) was dissolved in water (15 mM) on the day of the experiment and kept on ice in the dark before final dilution into extracellular solution to 1.5 mM immediately prior to use. MTSEA was applied extracellularly via the gravity fed superfusion system. We used MTSEA and extracellular application because MTSEA can enter and cross the membrane and access transmembrane and intracellular protein sites [10].

For intracellular application, MTSEA was included in the pipette solution. Because this method essentially exposes the channels to MTSEA once whole-cell access has been established, it is not possible to absolutely control the start and stop of MTSEA exposure, and the data is difficult to directly compare with results from extracellular application, where exposure time is more easily and accurately controlled. Also, MTSEA exposure (extracellular or intracellular) of longer than 10 min often resulted in a degradation of cell morphology, increased holding current, and loss of electrical recording. We believe that this toxic effect of MTSEA is probably due to its accumulation in the membrane and subsequent disruption of membrane integrity.

We performed additional experiments using the membrane-impermeant MTS-reagent methanethiosulfonate ethyltrimethylammonium (MTSET), which, interestingly, was much better tolerated by the HEK cells (e.g., good cell morphology and stable holding current for 20 min or more) compared with MTSEA. We used MTSET in an effort to determine if any effects depended on which side of the membrane the MTS reagent was applied.

MTS modification was determined by monitoring effects on whole-cell current with a 4- or 6-ms test pulse to +50 mV. In all protocols, the test pulse was immediately preceded by a 100-ms step to −180 mV to minimize potential effects of voltage shifts in fast inactivation. For closed-state accessibility the test pulse was from V_{hold} every 40 s. For fast-inactivated state accessibility, we used a 30-s train of 100 ms depolarizing pulses to 0 mV with 100-ms repolarization between each pulse, then 10-s at V_{hold}, then the test pulse. We also used a train with 10-ms depolarizations to 0 mV, with a 40-ms repolarization between each pulse, followed by the 10-s at V_{hold} prior to the test pulse. The data from these two protocols were not different and were pooled for analysis. For slow-inactivated state accessibility we used a 30-s prepulse to 0 mV and a 10-s interpulse to V_{hold} before each test pulse. For extracellular MTSEA or MTSET, currents were normalized to the test pulse immediately preceding the onset of MTS application.

2.5. Data collection and analysis

Data were collected using an Axopatch 200B amplifier (filtered at 5 kHz) and pCLAMP 10 software (Molecular Devices, Sunnyvale, CA, USA). Analysis of data was performed with pCLAMP and Origin software (MICROCAL Software, Inc., Northampton, MA, USA). Differences were considered significant at $p<0.05$ (ANOVA). Grouped data are presented as mean ± S.E.M.

3. Results

3.1. In the closed state, hNav1.5-C373Y is resistant to time-dependent MTSEA-mediated decrease in whole cell Na^+ current (I_{Na})

We characterized the baseline electrophysiology of the mutant hNav1.5-C373Y in a previous study [2]. This mutant, with a tyrosine (Y) substituted for a cysteine (C) in the outer pore region, is used for MTS studies because the native C373 in wild-type hNav1.5 reacts rapidly (seconds) with MTS reagents when in the closed state, which greatly reduces I_{Na} (by 50.3 ± 0.1% at 40 s, and 85.3 ± 0.02% at 120 s, n = 4; Fig. 1) [2,3]. This response also occurs when wild-type hNav1.5 is in the fast inactivated (reduced by 87.1 ± 0.01% at 120 s, n = 3) or slow inactivated (reduced by 85.3 ± 0.02% at 120 s, n = 3) states (data not shown). Substitution of tyrosine at C373 in hNav1.5-C373Y eliminates this rapid response to MTS reagents [2,3] and I_{Na} is only slightly decreased even after 8 min of MTSEA exposure in the closed state (by 11.5 ± 0.02%; n = 12; Fig. 1). Tyrosine was chosen because this is the residue at the homologous site Y407 in the human skeletal muscle isoform hNav1.4, which is resistant to the MTSEA-mediated reduction in I_{Na} (7.5 ± 0.02% at 8 min, n = 4; Fig. 1).

Intracellular exposure of hNav1.5-C373Y to MTSEA in the closed state produced a modest decrease in I_{Na} over 8 min of whole-cell recording (21.6 ± 0.1% reduction, n = 5; data not shown). These data were normalized to a test pulse ≈30 s after rupture of the membrane.

3.2. In the inactivated state, but not the closed state, long-term MTSEA exposure (8 min) reduces I_{Na} in hNav1.5-C373Y

In our previous study, the hNav1.5-C373Y mutant was exposed to MTSEA for a relatively short 4 min in the closed, fast-inactivated, or slow-inactivated state with little effect [2]. Here we show that a longer exposure time to extracellular MTSEA (8 min) in hNav1.5-C373Y produces a significant decrease in I_{Na} in a state-dependent manner

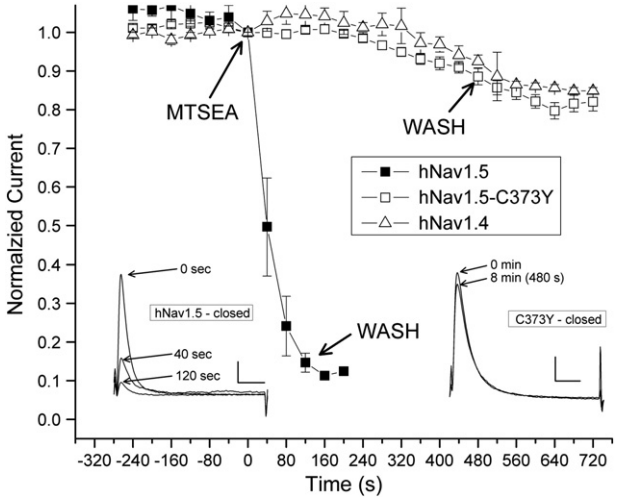

Fig. 1. The rapid response to MTSEA in wild-type hNav1.5 is eliminated in the mutant hNav1.5-C373Y. The effect is not seen in wild-type hNav1.4. The cells were exposed to MTSEA at V_{hold} of −160 mV (closed state) with a test pulse to +50 mV every 40 s. Insets, representative traces. Scale bars, 2 nA, 1 ms.

(Fig. 2). Specifically, while this decrease in I_{Na} *is not* seen when hNav1.5-C373Y is in the closed state (see Section 3.1, Fig. 1, Fig. 2), this effect *is* seen when hNav1.5-C373Y is fast-inactivated or slow-inactivated (reduced by 51.9 ± 0.06% at 8 min, n = 8, and 47.8 ± 0.05% at 8 min, n = 14, respectively; $p<0.001$ compared with closed state; Fig. 2). Additionally, the reduction in I_{Na} is not reversed by washout of MTSEA (Fig. 2), suggesting that the effect is due to direct MTSEA covalent bonding to the channels, and not the result of an indirect, non-specific effect.

Although it is more difficult to control exposure time with intracellular application via inclusion in the recording pipette, we did observe that intracellular MTSEA exposure over 8 min reduced I_{Na} when the channels were fast-inactivated or slow-inactivated (by 49.1 ± 0.01%, n = 3, and 46.3 ± 0.07%, n = 5, respectively; data not shown). The inactivation data were normalized to a test pulse immediately prior to the beginning of the inactivation protocols, which started around 1–2 min after going whole-cell, i.e., total MTSEA exposure time for the intracellular inactivation experiments totaled 8 min, with ≈1–2 min in the closed state before beginning the inactivation protocols.

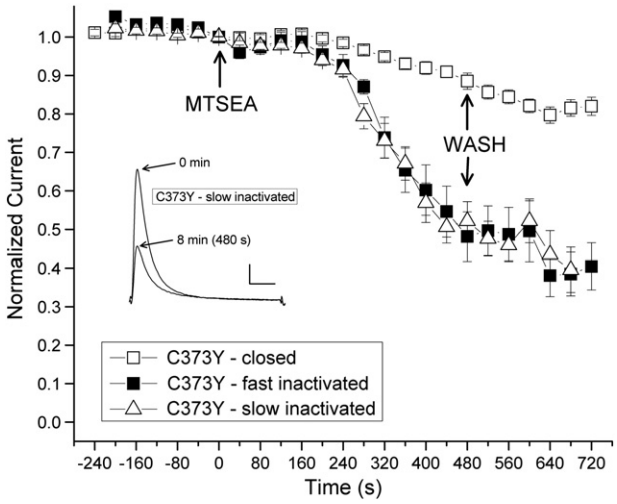

Fig. 2. MTSEA exposure >4 min decreases whole cell current when hNav1.5-C373Y is in the inactivated state (fast or slow). For closed-state accessibility, a test pulse (+50 mV) from V_{hold} (−160 mV) every 40 s. For fast-inactivated state accessibility, a 30-s train of 100 ms depolarizing pulses to 0 mV with 100-ms repolarization between each pulse, then 10 s at V_{hold}, then the test pulse, or a train with 10-ms depolarizations to 0 mV, with a 40-ms repolarization between each pulse, followed by 10 s at V_{hold} prior to the test pulse. For slow-inactivated state accessibility, a 30-s prepulse to 0 mV and a 10-s interpulse to V_{hold} before each test pulse. Inset, representative traces. Scale bars, 2 nA, 1 ms.

3.3. Methanethiosulfonate ethyltrimethylammonium (MTSET) does not produce the time- and state-dependent effect seen with MTSEA

We also performed these experiments with 8 min of extracellular exposure to methanethiosulfonate ethyltrimethylammonium (MTSET), which does not cross the membrane and is "bulkier" than MTSEA [11]. With extracellular application of MTSET, we found no significant reduction in I_{Na} in the closed (8.8 ± 0.03%, n = 5), fast-inactivated (10.8 ± 0.05%, n = 4), or slow-inactivated (8.8 ± 0.03%, n = 5) states (data not shown). Inclusion of MTSET in the pipette also did not show the reduction in I_{Na} (closed, 8.1 ± 0.04%, n = 3; fast-inactivated, 12.6 ± 0.03%, n = 7; slow-inactivated, 5.8 ± 0.03%, n = 4; data not shown).

3.4. Four minutes of MTSEA exposure in the closed state is sufficient to allow for the state-dependent reduction of I_{Na} in hNav1.5-C373Y

To ask whether the long-term, MTSEA-mediated effect was dependent on the kinetic state of hNav1.5-C373Y during the entire exposure time of 8 min, we exposed hNav1.5-C373Y to MTSEA for 4 min while in the closed state, and then, with the continuous flow of MTSEA, put hNav1.5-C373Y into either the fast- or slow-inactivated state for an additional 4 min (total of 8 min MTSEA exposure). We reasoned that this would help determine whether hNav1.5-C373Y must be in an inactivated state the whole time for the effect to occur, or whether the effect becomes evident once MTSEA reaches the site of modification. The results are shown in Fig. 3. While there is little reduction in I_{Na} during the first 4 min in the closed state (8.1 ± 0.01%), once hNav1.5-C373Y is inactivated (fast or slow) after the first 4 min of MTSEA, there is a rapid and sharp reduction in I_{Na} over the next 4 min (fast-inactivated by 61.4 ± 0.04% at 8 min, n = 4, and slow-inactivated by 61.6 ± 0.04% at 8 min, n = 5; $p<0.001$ for both compared with closed state; Fig. 3).

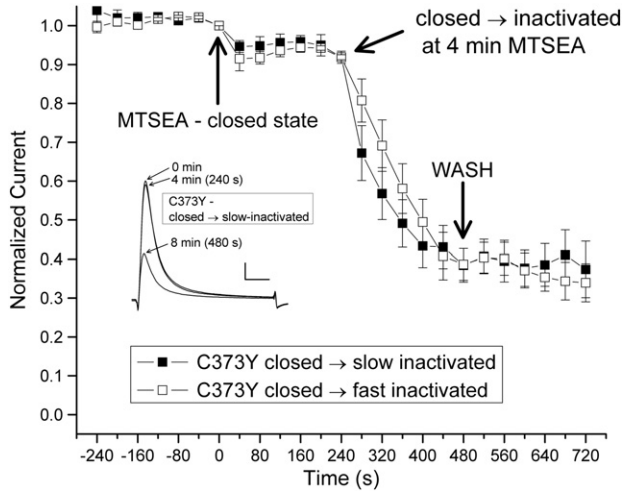

Fig. 3. MTSEA effect is not dependent on kinetic state during the first 4 min of exposure. Cells were exposed to MTSEA for 4 min in the closed state and then put in an inactivated state (fast or slow) for 4 additional minutes (total exposure of 8 min). Once the cells are inactivated, there is a large and rapid decrease in current in response to MTSEA. For closed-state accessibility, a test pulse (+50 mV) from V_{hold} (−160 mV) every 40 s. For fast-inactivated state accessibility, a 30-s train of 100 ms depolarizing pulses to 0 mV with 100-ms repolarization between each pulse, then 10 s at V_{hold}, then the test pulse, or a train with 10-ms depolarizations to 0 mV, with a 40-ms repolarization between each pulse, followed by 10 s at V_{hold} prior to the test pulse. For slow-inactivated state accessibility, a 30-s prepulse to 0 mV and a 10-s interpulse to V_{hold} before each test pulse. Inset, representative traces. Scale bars, 2 nA, 1 ms.

This result demonstrates that the first 4 min of MTSEA exposure is independent of the kinetic state with regard to producing the MTSEA-mediated effect. Because there is no effect during the initial 4 min regardless of kinetic state [2], this suggests that it takes about 4 min for the MTSEA to get to the site of modification, and that hNav1.5-C373Y must be inactivated for the effect to take place after this time.

3.5. The time- and state-dependent MTSEA-mediated reduction of I_{Na} seen in hNav1.5-C373Y does not occur in hNav1.4

The long-term (8 min), inactivation-dependent effect of extracellular MTSEA observed in hNav1.5-C373Y is not seen in wild-type human skeletal muscle Na channel hNav1.4 when compared with hNav1.5-C373Y. In fast-inactivated hNav1.4, I_{Na} was reduced by $21.3 \pm 0.05\%$, compared with 51.9% in fast-inactivated hNav1.5-C373Y ($n = 5$; $p < 0.01$; Fig. 4). In slow-inactivated hNav1.4, I_{Na} was reduced by $16.8 \pm 0.06\%$ compared with 47.8% in slow-inactivated hNav1.5-C373Y ($n = 8$; $p < 0.001$; Fig. 4). This suggests that there are isoform-specific differences in molecular determinants of inactivation between these two isoforms that mediate the differential MTSEA effect.

4. Discussion

We have shown that relatively long exposure to MTSEA (8 min) reduces I_{Na} in the modified heart Na^+ channel hNav1.5-C373Y in a state- and time-dependent manner, and that this effect is not seen in the hNav1.4 skeletal muscle isoform.

4.1. Time-dependence of MTSEA effect in hNav1.5-C373Y

Our previous work demonstrated resistance to MTSEA in hNav1.5-C373Y with exposure of 4 min [2]. Here we find that continuing the MTSEA exposure up to 8 min produces a significant reduction in I_{Na} in this mutant, but only when hNav1.5-C373Y is in an inactivated state (fast or slow). This effect is not found when hNav1.5-C373Y is in the closed state. We attribute this long-term effect to a time-dependent diffusion of MTSEA to an undetermined molecular site, presumably a cysteine residue(s), where it would produce this effect.

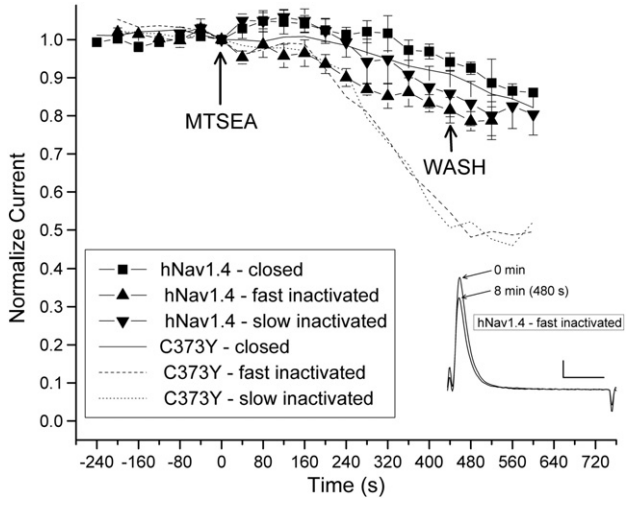

Fig. 4. The inactivation-dependent, MTSEA-mediated reduction of current is not found in hNav1.4. The data for C373Y (line graphs) and closed hNav1.4 are from Fig. 2. For closed-state accessibility, a test pulse (+50 mV) from V_{hold} (−160 mV) every 40 s. For fast-inactivated state accessibility, a 30-s train of 100 ms depolarizing pulses to 0 mV with 100-ms repolarization between each pulse, then 10 s at V_{hold}, then the test pulse, or a train with 10-ms depolarizations to 0 mV, with a 40-ms repolarization between each pulse, followed by 10 s at V_{hold} prior to the test pulse. For slow-inactivated state accessibility, a 30-s prepulse to 0 mV and a 10-s interpulse to V_{hold} before each test pulse. Inset, representative traces. Scale bars, 2 nA, 1 ms.

Additional experiments with the membrane-impermeant MTSET did not produce the effect when applied extracellularly, suggesting that the MTSEA-mediated reduction in I_{Na} is dependent on MTSEA's ability to enter and/or cross the membrane [2,10].

We also performed experiments with MTS reagents included in the recording pipette, thereby exposing the intracellular side of the channels to MTS. Although it is difficult to accurately control the time of exposure to MTS with this method, we obtained similar results as with extracellular application. That is, MTSEA reduced I_{Na} in the inactivated states, while MTSET did not. The reagent MTSET is membrane impermeant and is often used to determine accessibility from one side of the membrane or the other [10,12,13]. Therefore, the intracellular MTSET results suggest that the site of modification is not found in the intracellular portions of the channels. However, calculated molecular dimensions compute the volume of MTSET at 173 $Å^3$ and MTSEA at 113 $Å^3$ [11]. Therefore, another possibility would be that MTSET is too "bulky" to gain access to sites of modification. In contrast, MTSEA, which is smaller and membrane permeant, can more easily diffuse to protein modification sites, whether at intracellular regions, transmembrane segments, or pore areas of the channels [10,11]. The difference between MTSEA and MTSET would also suggest that MTSEA can illuminate potentially significant molecular rearrangements during inactivation processes that are not evident with MTSET application.

4.2. State-dependence of long-term MTSEA effect in hNav1.5-C373Y — fast and slow inactivation

An interest in slow inactivation is what originally led us to uncover the effect of relatively long exposure to MTSEA in hNav1.5-C373Y. Initial experiments using slow inactivation protocols demonstrated state-dependence of long-term MTSEA exposure, i.e., it occurred in the slow-inactivated state, but not the closed state. During control experiments, we also discovered that this long-term effect occurs when hNav1.5-C373Y is fast inactivated. One interpretation of this result is that because fast inactivation occurs first, then channels that are slow inactivated are potentially also still fast inactivated. Therefore, the MTSEA effect during the slow inactivation protocol may simply reflect an earlier fast-inactivation modification, and no further effect resulting from entry into the slow-inactivated state. This interpretation is consistent with results from studies demonstrating that disruption of fast inactivation in voltage-gated Na^+ channels does not eliminate slow inactivation, suggesting that fast and slow inactivation occur via different molecular mechanisms and are distinct inactivation states [14–17]. However, these same studies showed that disruption or removal of fast inactivation accelerates slow inactivation, suggesting that these processes are "linked" or "coupled" and hence share molecular determinants [14–17]. Hence, it would not be surprising if the MTSEA-mediated effect in fast- and slow-inactivated hNav1.5-C373Y in this study share molecular components, and that the inactivation-dependence of the MTSEA effect is mediated by conformational determinants that are common to both fast and slow inactivation.

A possible way to look at coupling, or lack thereof, between fast and slow inactivation would be to create mutants in which inactivation (fast or slow) is disrupted, and to expose these mutants to MTSEA. For example, a mutant with disrupted fast inactivation (but with normal slow inactivation kinetics) may show a diminished response to MTSEA when fast inactivated, but without any change in the response during slow inactivation. A conclusion from such an experiment would be that MTSEA produces the same effect (decreased I_{Na}) during inactivation (either fast or slow), but that there are distinct molecular rearrangements and modifications associated with each state, each of which could react with MTSEA and reduce I_{Na}. Alternatively, if the mutant also shows a reduced response to MTSEA

during slow inactivation, it would suggest modification of a molecular determinant common to both fast and slow inactivation.

While we believe that the MTSEA-mediated reduction in I_{Na} is probably due to modification of mechanism(s) of inactivation, an alternative explanation would be that activation mechanisms are affected. For example, it has been demonstrated that local anesthetics, which block current flow through Navs, produce their effect, at least in part, by inhibiting the movement of S4 voltage-sensors once activation has occurred, thereby stabilizing Navs in an inactivation state [18]. Another activation-dependent mechanism of Nav inhibition which is found with some neurotoxins would be "trapping" of S4 segments in the resting state, thereby impeding activation [19]. Therefore, although the specific mechanism for the reduction in I_{Na} in this study is not known, it could be due to a combination of molecular modifications of gating kinetics, including inactivation, activation, allosteric effects, or simply a physical blocking of the pore.

4.3. Time-dependence of MTSEA effect is independent of kinetic state for the first 4 min of exposure

Another interesting result from this study comes from the experiments that demonstrated that MTSEA exposure for the first 4 min is independent of kinetic state, i.e., the inactivation-dependent decrease in I_{Na} recorded over minutes 4–8 occurs whether or not the first 4 min of MTSEA exposure is in closed, fast-, or slow-inactivated hNav1.5-C373Y. One possible explanation for this result is that the site(s) of modification is not readily accessible to MTSEA. For example, if the modification is occurring intracellularly, e.g., in the intracellular loops between segments and/or domains, the MTSEA would need to cross the membrane in order to access these sites. While MTSEA can cross the membrane, it does not do so very rapidly [10]. This would explain the relatively long time it takes to produce the MTSEA effect. Once the MTSEA has reached the site, molecular rearrangement and accessibility associated with inactivation would allow modification, resulting in decreased I_{Na}.

4.4. Different Nav isoforms, different responses to MTSEA — hNav1.5-C373Y vs hNav1.4

The long-term, state-dependent MTSEA-mediated reduction in I_{Na} that we discovered in heart hNav1.5-C373Y is not found in the skeletal muscle hNav1.4 isoform. This result suggests that structural or kinetic differences in inactivation between the two Nav isoforms are responsible for this differential response to MTSEA. A possible difference is that there are cysteine(s) in hNav1.5-C373Y that are not found in hNav1.4. Sequence comparison between hNav1.5-C373Y and hNav1.4 locates eleven cysteines in hNav1.5-C373Y that are absent in hNav1.4, ten of which are found in the intracellular loops D1–D2 or D2–D3. If the MTSEA response is dependent on reaction with one or more of these cysteines during inactivation, it could explain the differential response of the heart vs. skeletal muscle isoform. This hypothesis could be addressed using site-directed mutagenesis to replace these cysteines to determine which one(s), if any, are responsible for the isoform-specific differences.

An intriguing potential outcome from studies of hNav1.5-C373Y mutants in which cysteines have been replaced would be that a specific mutant may show an altered, e.g., reduced, response to MTSEA, but only in one of the inactivated states, i.e., either fast or slow. This result would suggest that there are specific molecular determinants of the MTSEA-mediated effect that differ between fast and slow inactivation that include interactions at the mutated cysteine(s) residue.

In conclusion, we have demonstrated that long-term exposure to MTSEA reduces I_{Na} in hNav1.5-C373Y in a time- and inactivated state-dependent manner, and that this effect is not found in the Nav isoform hNav1.4. We propose that isoform-specific conformational determinants of inactivation underlie the difference between these isoforms.

Acknowledgements

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