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ORIGINAL ARTICLE

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A tryptophan residue (W736) in the amino-terminus of the P-segment of domain II is involved in pore formation in $Na_v1.4$ voltage-gated sodium channels

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Abstract Voltage-gated Na channels comprise four homologous domains each consisting of six transmembrane segments (S1–S6) linked by loops. The linkers between segments S5 and S6 in each domain (P-loops), denoted as SS1-SS2, form the pore of the channel. It is believed that the SS1 region of the P-loops dips into, while the SS2 region exits out of the membrane. We have reported previously that residues A728 and D730 (in SS1 of domain II) contribute to the external vestibule of the pore of the rat skeletal muscle Na channel ($Na_v1.4$). In this study, we examined the role of a conserved neighbouring tryptophan residue at position 736 (W736) in the pore formation. The W736 residue of $Na_v1.4$ was replaced by a cysteine using site-directed mutagenesis. Complementary RNAs encoding the wild-type and mutant channels were injected into *Xenopus laevis* oocytes and macroscopic Na^+ currents measured using the two-microelectrode voltage-clamp technique. The W736C mutant showed increased channel sensitivity to externally applied Cd^{2+} and methanethiosulphonate-ethyltrimethylammonium (MTSET). Furthermore, micromolar concentrations of Cd^{2+} reduced single-channel current amplitude in the $Na_v1.4$/W736C mutant without affecting its voltage dependence. However, only small differences in tetrodotoxin and μ-conotoxin GIIIA affinity were observed between the wild-type and mutant channels. Replacing Na^+ with other cations – K^+, Li^+, Cs^+ or NH_4^+ – did not change the ion permeation sequence of the $Na_v1.4$/W736C mutant channel. The results suggest that W736 contributes to the formation of the pore, close to the mouth of the channel, but is not part of the selectivity filter.

Keywords Sodium channel · Skeletal muscle · $Na_v1.4$ · *Xenopus* oocytes · μ-Conotoxin · Tetrodotoxin

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Introduction

Voltage-gated Na channels are transmembrane proteins that conduct Na^+ selectively at a high rate (10^6 ions/s) through a narrow pore [12]. They are central to the mechanisms of cellular and tissue excitability. The channels are formed by a complex of an α-subunit and one or more tissue-specific β-subunits ($\beta1$–$\beta3$). Cloning and expression studies have confirmed that the α-subunit forms the main functional component and the pore of the channel [4, 7]. The accessory β-subunits regulate expression and gating, but not the permeation characteristics, of the α-subunit.

Numerous isoforms of the α-subunit have been cloned from different species and tissue types. Their primary structure is highly conserved and consists principally of four homologous domains (DI–DIV) linked by loops, with each domain being composed of six α-helical transmembrane segments (S1–S6). Structure-function studies have indicated that the loops between S5 and S6 (P-loops) form the pore and the selectivity filter of the channel [11, 17], while the S4 segments act as voltage sensors [19] and the DIII-DIV linker is the inactivation gate [16, 22].

Each P-loop is composed of two "short segments" (SS1 and SS2, respectively), that span part of the plasma membrane [9, 10, 20]. Structure studies predict that the SS1 segments enter and the SS2 segments exit the membrane from the external side to form the pore of the channel. Cysteine-scanning studies in conjunction with Cd^{2+} and cysteine modifiers, (methanthiosulphonate, MTS), have been used successfully to identify the residues lining the SS2 pore segment [17, 23]. In these experiments, mutating successive residues of SS2 in all four domains has shown that the cysteine mutants remain accessible to blockade by Cd^{2+} and modification by MTS [17]. Furthermore, successive residues in the P-loop can bind Cd^{2+} co-ordinately with residues of adjacent domains [3, 21]. These studies hence suggest that the SS2 region is flexible but highly selective. Unlike SS2, little is known about the amino acids that form the SS1 segment

and the role of this segment in ion permeation has not been investigated.

In this study we investigated the role of a tryptophan residue (W736) in the SS1 segment in pore formation of $Na_v1.4$ [8]; this residue is highly conserved among all cloned Na channels. The W736 residue (Fig. 1A) was replaced by a cysteine and the sensitivity of the mutant channel to externally applied Cd^{2+} and MTS reagents was examined. The currents of the W736C mutant were reduced by extracellular Cd^{2+} and methanethiosulphonate-ethyltrimethylammonium (MTSET), the sulphhydryl-directed reagent, suggesting that this residue is located near the narrow part of the pore.

The data are consistent with our previous finding that the segment between the C-terminus of the S5 and W736 of the SS1 is involved in the formation of the outer vestibule of the channel [5]. However, our findings are not concordant, at least in DII, with models of the Na^+ channel pore that propose that SS1 is buried within the channel and that the side chain moieties of the SS1 are not exposed within the external vestibule [9, 10, 20]. Our data will serve as a foundation for delineating the amino acids forming the SS1 of the P-loop of DII.

Materials and Methods

Mutagenesis

The oligonucleotide-directed mutagenesis kit QuickChange (Stratagene, La Jolla, Calif., USA) was used to replace W736 with cysteine in the rat skeletal muscle Na channel gene (*$Na_v1.4$*) provided by Dr. G. Mandel (Department of Neurobiology, SUNY at Stony Brook). Mutant Na channels were constructed using the following mutagenic sense and antisense primers: Nav1.4/R735C: 5'-GAC TGC AAC CTG CCT TGC TGG CAC ATG AAC GAC-3' and 5'-GTC GTT CAT GTG CCA GCA AGG CAG GTT GCA GTC-3'; Nav1.4/W736C: 5'-GAC TGC AAC CTG CCT CGC TGC CAC ATG AAC GAC TTC-3' and 5'-GAA GTC GTT CAT GTG GCA GCG AGG CAG GTT GCA GTC-3'. Nav1.4/H737C: 5'-C AAC CTG CCT CGC TGG TGC ATG AAC GAC TTC TTC-3' and 5'-GAA GAA GTC GTT CAT GCA CCA GCG AGG CAG GTT G-3'. The underlined nucleotides represent mutated sites. Mutations were confirmed by fluorescent dideoxy terminator sequencing at the automated sequencing facility of Laval University, Québec, Québec. Complementary RNA (cRNA) was prepared by the T7 mMessage mMachine kit (Ambion, Austin, Tex., USA).

Expression in *Xenopus* oocytes

Female *X. laevis* were anaesthetized with 1.5 mg/ml tricaine (Sigma, Oakville, Ontario, Canada) and two or three ovarian lobes removed surgically. Follicular cells surrounding the oocytes were removed by incubating at 22 °C for 2.5 h in calcium-free oocyte medium (in mM) 82.5 NaCl, 2.5 KCl, 1 $MgCl_2$, 5 HEPES (pH 7.6) containing 2 mg/ml collagenase (Sigma). The oocytes were washed first in calcium-free medium and with a 50% diluted solution of Leibovitz's L-15 medium (Gibco Life Technologies, Burlington, Ont., Canada) enriched with 15 mM HEPES, 5 mM L-glutamine, supplemented with 10 mg/ml gentamycin (pH 7.6) and stored in this medium until use. Stage V–VI oocytes were selected and microinjected with 50 ng cRNA encoding wild-type $Na_v1.4$, $Na_v1.4$/R735C $Na_v1.4$/W736C or $Na_v1.4$/H737C. cRNAs encoding these wild-type and mutant Na channels were co-injected with cRNA encoding the β_1-subunit. Oocytes were incubated at 18 °C and used for experiments after 3–5 days.

Electrophysiology

The macroscopic Na^+ currents from cRNA-injected oocytes were measured using the two-microelectrode voltage-clamp technique. The oocytes were impaled with electrodes containing 3 M KCl (<2 MΩ) and voltage-clamped with an OC-725 Oocyte clamp (Warner Instruments, Hamden, Conn., USA). The Ringer bath solution contained (in mM): 90 NaCl, 2 KCl, 1.8 $CaCl_2$, 2 $MgCl_2$, 5 HEPES (pH 7.6). Peak currents were measured at –20 mV for 40 ms each 5 s from a holding potential of –100 mV. Currents were filtered at 1.55 kHz with an 8-pole Bessel filter and sampled at 10 kHz. Data were acquired and analysed using appropriate software (pCLAMP v. 7, Axon Instruments, Union City, Calif., USA). Cadmium ($CdCl_2$) at various concentrations, MTSET (1 mM), tetrodotoxin (TTX, 300 nM), μ-conotoxin GIIIA (μ-CTX, 300 nM) and dithiothreitol (DTT, 1 mM) were added to the bath solution. Cadmium, TTX, μ-CTX GIIIA and DTT were from Sigma; MTSET was purchased from Toronto Research Chemicals (North York, Ont., Canada). For the concentration/response curves, the $[Cd^{2+}]$ eliciting half-maximal blockade (IC_{50}) was determined by least-squares fits to the following function: $\frac{I}{I_o} = \frac{1}{1+\left(\frac{[Cd^{2+}]}{IC_{50}}\right)^n}$, where I is the current in the presence and I_o that in the absence of Cd^{2+}. In the presence of TTX or μ-CTX, the reduction of current amplitude from a depolarization to a test voltage of –20 mV from a holding potential of –100 mV was defined as the fractional block (F_B). Assuming a single, high-affinity binding site, the IC_{50} (toxin concentration at which 50% of the channels are blocked) for toxin inhibition is given by $[\mu\text{-CTX}](1\text{-}F_B)/F_B$ [6, 15]. The kinetics of Na channel blockade by μ-CTX and block reversal upon washout are well described by single exponential functions [5, 6]. Two processes define the equilibrium between the toxin-bound and toxin-unbound channel. First, the association rate constant k_{on} is toxin-concentration dependant and defined as $\frac{\frac{1}{\tau_{on}}-\frac{1}{\tau_{off}}}{[\mu-CTX]}$, where τ_{on} and τ_{off} are the time constants of toxin binding and unbinding, respectively, and are obtained from the single exponential functions describing the time course of the binding and unbinding reactions. The dissociation rate constant k_{off} is $1/\tau_{off}$. A confirmation of the IC_{50} value is given by the dissociation constant, $K_d = k_{off}/k_{on}$.

To test the selectivity of the channels, Na^+ in the Ringer solution was replaced iso-osmotically with the chloride salts of K^+, Li^+, Cs^+ and sulphate salt of NH_4^+.

For single-channel recording, patch electrodes were made from Corning 8161 glass (Dow Corning, Midland, Mich., USA) coated with Silgard to reduce their capacitance. The bath solution contained (in mM) 100 K-aspartate, 50 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose and 10 K-HEPES (pH 7.4). Both bath and patch pipette solutions contained 50 μM fenvalerate (a gift from Dupont Pharmaceutical, Wilmington, Del., USA) to inhibit fast inactivation and to simplify the measurement of unitary Na^+ currents [2]. The patch pipette solution contained (in mM) 150 NaCl, 10 TEA-Cl, (to block endogenous K^+ channels), 2 KCl, 1.5 $CaCl_2$, 1 $MgCl_2$, 10 glucose, 10 Na-HEPES (pH 7.4). Records were obtained using Axopatch 200B amplifier (Axon Instruments). Data were acquired using the Digidata 1200 acquisition system (Axon Instruments). Data were acquired, stored and analysed using pClamp v. 7. Single-channel currents were filtered at 5 kHz and sampled at 100 kHz.

Parameter estimates are given as means±SEM.

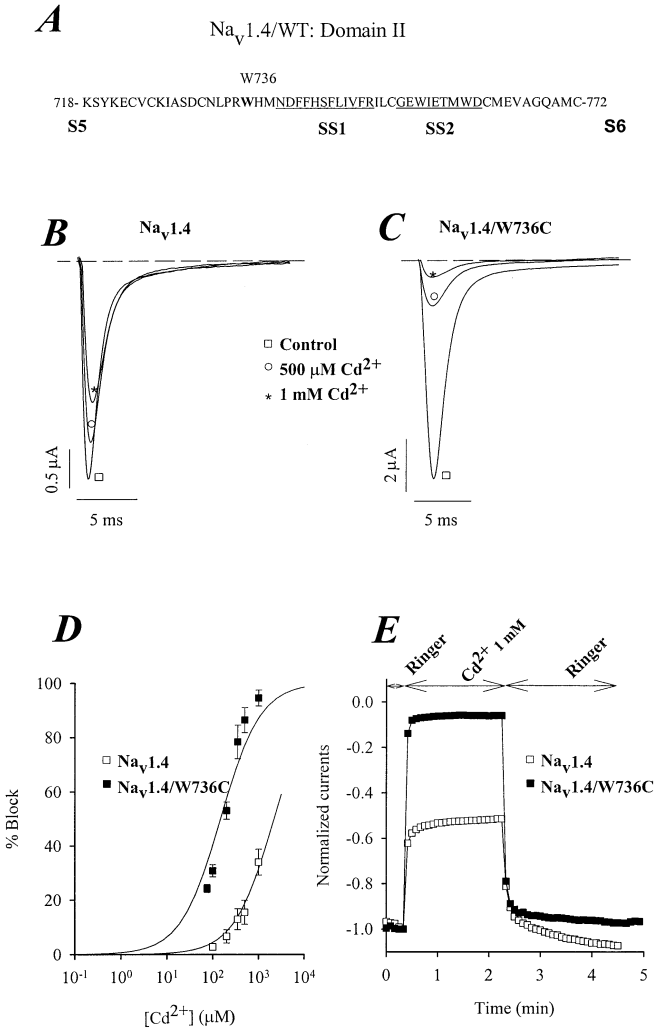

Fig. 1 **A** Amino acid sequence of the S5-S6 loop of domain II of the voltage dependent Na channel Na$_v$1.4. The sequences corresponding to the short segments SS1 and SS2 are labelled [9, 20]. The *bold letter* "*W*" represents tryptophan at position 736. **B**, **C** Comparative effects of group IIb cations (here cadmium) applied extracellularly on the wild-type Na$_v$1.4 (**B**) and on the mutant channel Na$_v$1.4/W736C (**C**). Superimposed current records during steps from –100 mV to –20 mV in Ringer solution (*control*) and at steady-state after exposure to different [Cd^{2+}]. **D** Concentration/response relations for blockade of the macroscopic Na currents for wild-type Na$_v$1.4 (■) and Na$_v$1.4/W736C (❑) by Cd^{2+}. The normalized relative maximal Na$^+$ currents are shown as functions of [Cd^{2+}]. Means±SEM; *n*=3–5 experiments on separate oocytes per concentration. **E** Reduction of Na channel currents through Na$_v$1.4 (■) and Na$_v$1.4/W736C (❑) channels by 1 mM extracellular Cd^{2+} followed by Ringer solution washout

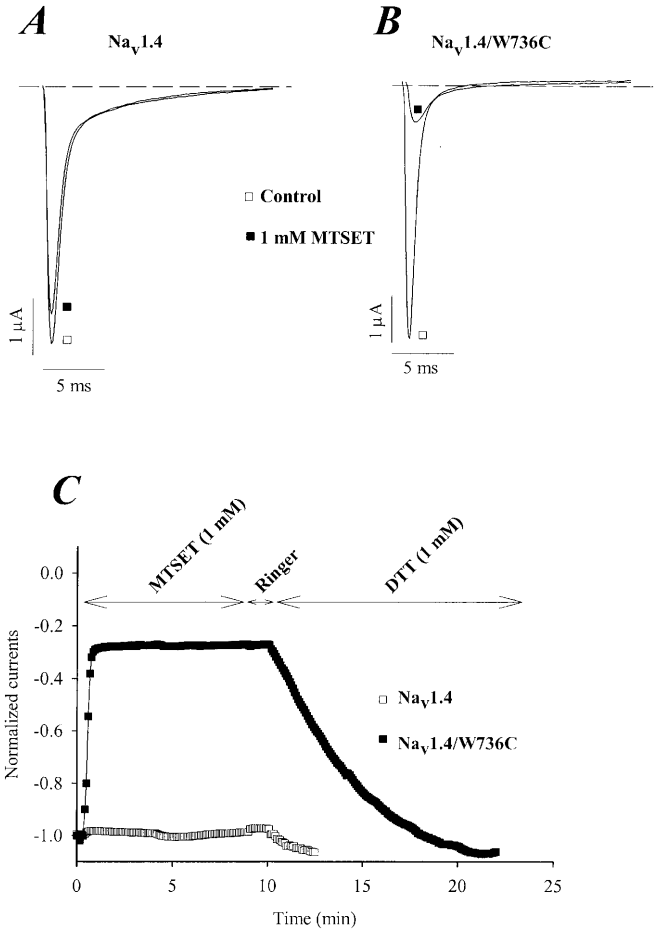

Fig. 2A–C Effect of methanethiosulphonate-ethyltrimethylammonium (*MTSET*) on Na$_v$1.4/wild-type and Na$_v$1.4/W736C mutant channels. **A** Wild-type Na$_v$1.4 and **B** Na$_v$1.4/W736C. Superimposed current records during steps from –100 mV to –20 mV in Ringer solution (*control*) and at steady-state effect of MTSET. **C** Reduction of Na channel current by exposure of wild-type Na$_v$1.4 (■) and Na$_v$1.4/W736C (❑) channels to extracellular MTSET followed by Ringer solution washout and application of dithiothreitol (*DTT*, 1 mM). Normalized current records are from –100 mV holding potential to –20 mV test pulse

Results

Effects of Cd^{2+} on Na$_v$1.4/wild-type and Na$_v$1.4 mutant channels

Large Na currents were recorded from Na$_v$1.4/W736C mutant (Fig. 1C) indicating that the synthesis and expression of the channel were not impaired significantly by the mutation. A small effect of Cd^{2+} was observed on wild-type Na$_v$1.4 (Fig. 1B) (IC$_{50}$ 2,189±257 μM, *n*=4; Fig. 1D). The mutant channel had a higher affinity for Cd^{2+} (IC$_{50}$ 147.9±17.3 μM, *n*=4; Fig. 1D). Figure 1E illustrates the effects of 1 mM Cd^{2+} on Na$_v$1.4 and Na$_v$1.4/W736C. Mutation of W736 adjacent residues Na$_v$1.4/R735C and Na$_v$1.4/H737C resulted in no currents being expressed (data not shown).

Effects of MTSET on Na$_v$1.4/wild-type and Na$_v$1.4/W736C mutant channels

MTS reagents were used to study the involvement of the W736C in pore formation. MTS reagents covalently modify the sulphydryl group of cysteine. When the cysteine side chain faces the aqueous side of the pore, steric hindrance resulting from sulphydryl modifications,

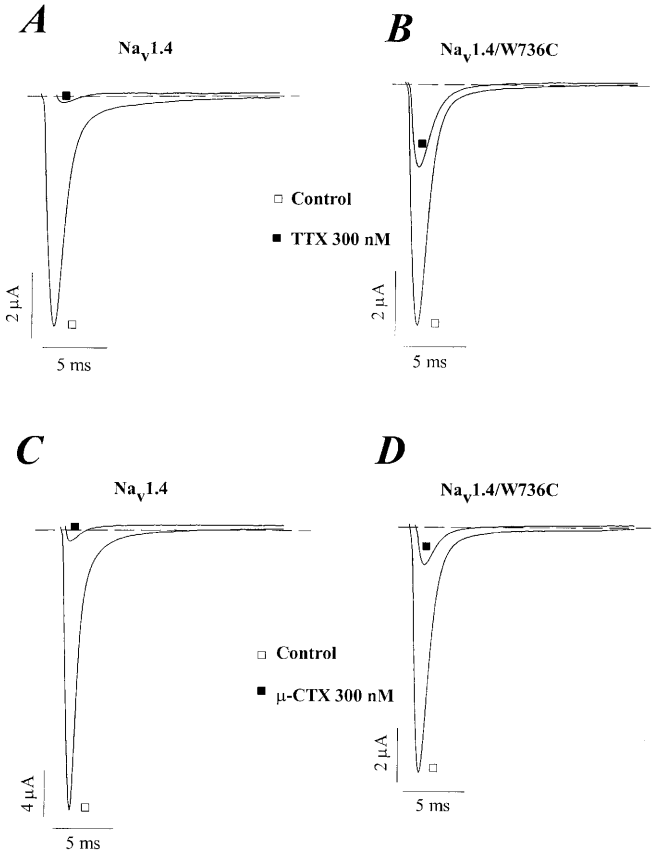

Fig. 3 Effects of tetrodotoxin (*TTX*, 300 nM, **A**, **B**) or µ-conotoxin GIIIA (*µ-CTX*, 300 nM, **C**, **D**) on $Na_v1.4$/wild-type (**A**, **C**) and $Na_v1.4$/W736C mutant (**B**, **D**) channels. Superimposed current records during steps from a holding potential of –100 mV to a test potential of –20 mV in Ringer solution (*control*) and at steady-state effect of TTX or µ-CTX GIIIA

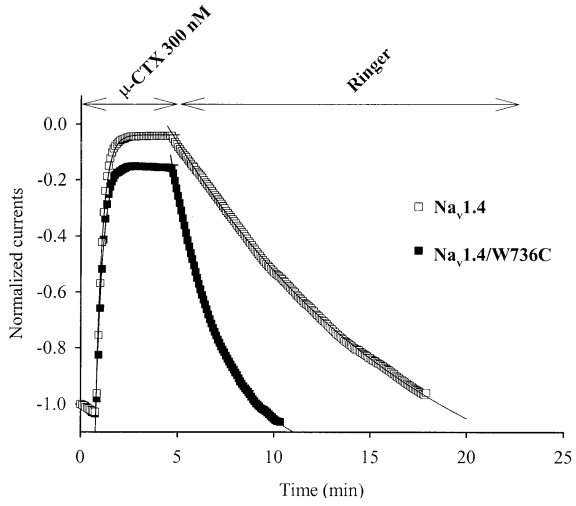

Fig. 4 Kinetics of the effect of 300 nM µ-CTX GIIIA applied extracellularly on wild-type $Na_v1.4$ (■) and $Na_v1.4$/W736C (❑) channels followed by a washout (Ringer). Normalized current records are from steps from a holding potential of –100 mV to a test pulse of –20 mV. The *solid line* is the exponential fit obtained using the mean values for the time constants (τ_{on} and τ_{off}) in the text

electrostatic interactions, or both, can diminish Na^+ conductance. The covalent modification of cysteine residues by MTS reagents can be reversed by a reducing agent such as dithiothreitol (DTT).

Figure 2 shows that $Na_v1.4$/W736C was accessible to the positively charged MTSET. Wild-type $Na_v1.4$ Na currents were inhibited only slightly by 1 mM MTSET (Fig. 2A). $Na_v1.4$/W736C mutant Na currents were reduced by 79.20±2.10%, n=9 (Fig. 2B). Figure 2C also shows that the MTSET reaction was reversed by a reducing agent (1 mM DTT) but not by the Ringer solution without MTSET. DTT slowly reduced the cysteine-MTSET bonds on the W736C mutant and restored current flow through the Na channel (Fig. 2C).

Effects of TTX and µ-CTX GIIIA on $Na_v1.4$/wild-type and $Na_v1.4$/W736C mutant channels

The role of W736 in pore formation was tested further using pore-blocking toxins. The guanidinium-containing toxin TTX (applied externally, in nanomolar concentrations) blocks neuronal and skeletal muscle isoforms of Na channels. We thus compared the effects of TTX (300 nM) affinity on wild-type and W736C mutant $Na_v1.4$ channels (Fig. 3A and B). Mutant $Na_v1.4$/W736C was 16-fold less sensitive to block by TTX than $Na_v1.4$, (IC_{50} 175.7±8.2 nM, n=5 vs. 11.4±3.5 nM n=5) (Fig. 3A and B), indicating that this amino acid weakens TTX binding.

µ-CTX (GIIIA) is another useful probe that specifically blocks the skeletal isoform at a site partially overlapping that of TTX [6, 15]. We exposed mutant and wild-type channels to µ-CTX (300 nM) to reveal any difference in toxin binding. When 300 nM µ-CTX was applied (Fig. 3C and D), the mutated channel showed 82.1±1.9% block, n=5, resulting in an IC_{50} of 59.3±9.4 nM (Fig. 3D) and was slightly less sensitive than the wild-type (98.2±1.2% block, n=3, IC_{50} 6.7±3 nM) (Fig. 3C). The mutant channel was almost 9-fold less sensitive to µ-CTX block than wild-type $Na_v1.4$.

µ-CTX association and dissociation rate constants (k_{on} and k_{off}) were also determined. Figure 4 shows that µ-CTX dissociated more rapidly from the mutant than from the wild-type channel ($\tau_{off}^{Na_v1.4/W736C}$ was 167.6±14 s, corresponding to a k_{off} of $6.4\pm0.6\cdot10^{-3}$ s^{-1}, n=10; $\tau_{off}^{Na_v1.4}$ was 544.7±58 s, corresponding to a k_{off} of $2.1\pm0.3\cdot10^{-3}$ s^{-1}, n=9). There was little difference in the kinetics of the blocking reaction: $\tau_{on}^{Na_v1.4/W736C}$ was 18.2±1 s, corresponding to a k_{on} of $1.68\pm0.12\cdot10^5$ M^{-1} s^{-1}, n=10 and $\tau_{on}^{Na_v1.4}$ was 15.3±1 s, corresponding to a k_{on} of $2.19\pm0.21\cdot10^5$ M^{-1} s^{-1}, n=9). However, there was a 3-fold increase in k_{off} for µ-CTX binding, which may explain the slight reduction in the affinity of the W736C mutant. It is noteworthy that IC_{50} values measured in steady-state conditions are in agreement with the values calculated from k_{on} and k_{off}.

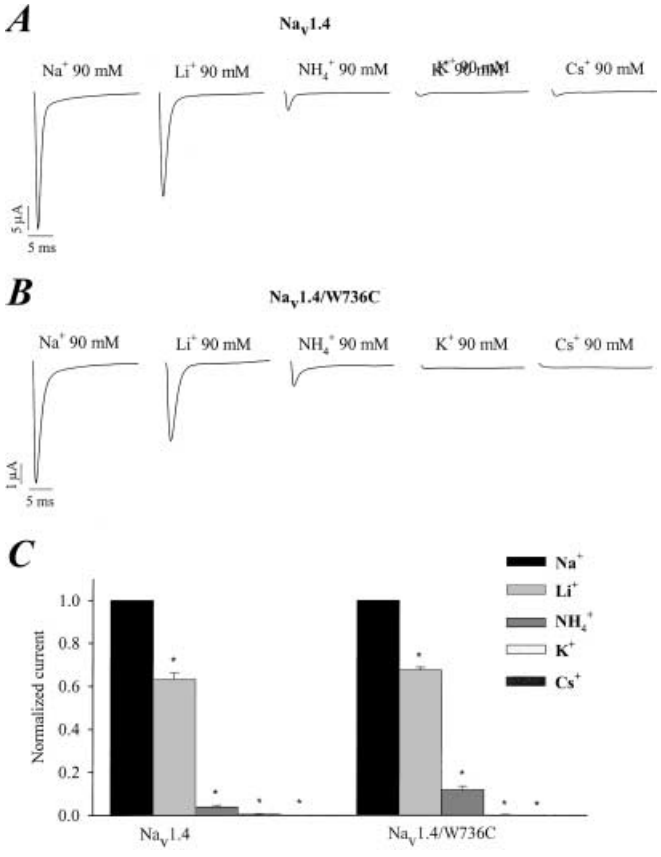

Fig. 5A–C Na$_v$1.4/W736C mutation does not alter selectivity of the channel. Comparative selectivity between wild-type Na$_v$1.4 (**A**) and Na$_v$1.4/W736C (**B**) in presence of different cations at 90 mM. Normalized current records obtained from a holding potential of –100 mV to a test pulse of –20 mV. **C** Summary of experiments of the type shown in **A** and **B**. **P*<0.05 vs. Na current

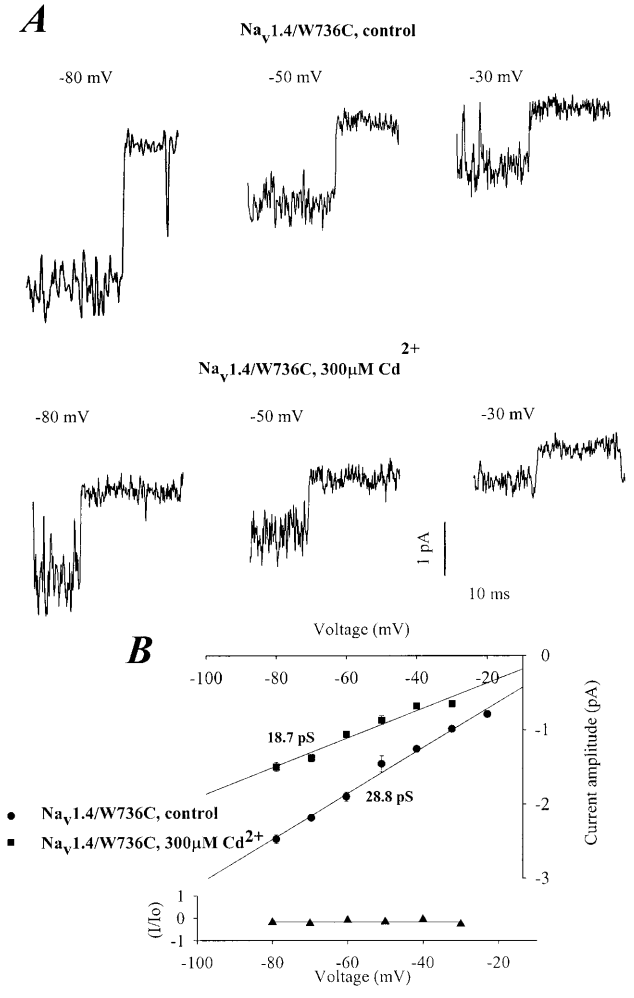

Fig. 6A, B Effect of Cd^{2+} on Na$_v$1.4/W736C single-channel currents. Single-channel Na currents obtained in cell-attached patches recorded from a holding potential of –100 mV to various voltage steps (–80, –50 and –30 mV) with (**A**, *top*) and without 300 µM Cd^{2+} (**A**, *bottom*). **B** Single-channel current/voltage relationship (*upper panel*) and the fractional block as a function of voltage (*lower panel*). The *solid lines* shown the linear regression through the data with the indicated conductances

Effect of Na$_v$1.4/W736C mutation on ion selectivity

Skeletal muscle Na channels are highly selective for Na^+. We exposed the mutant channel to Ringer solutions in which Na^+ had been replaced by K^+, Li^+, Cs^+ or NH_4^+ to test for changes in the cation permeation sequence (Fig. 5). No notable changes were found in the Na$_v$1.4/W736C permeation sequence, indicating that the mutant channel (Fig. 5B) is as selective for Na^+ as Na$_v$1.4/WT (Fig. 5A). There was no difference between Na$_v$1.4/WT and Na$_v$1.4/W736C in the sequence of ion permeation, which was $Na^+ > Li^+ > NH_4^+ > K^+ = Cs^+$. This is consistent with the lack of change in ionic selectivity in the presence of the mutation.

Effect of Cd^{2+} on single-channel currents

Single-channel recordings were obtained in the cell-attached configuration. Cd^{2+} (300 µM) reduced the unitary current amplitude of the Na$_v$1.4/W736C mutant Na channel (Fig. 6A). The reduction in unitary Na currents was more apparent at more negative voltages, however with no obvious voltage-dependence as shown in the bottom panel of Fig. 6B, in which the fractional block (I/I_0) is shown as a function of voltage.

Discussion

Cysteine-scanning mutagenesis and the accessibility of the cysteine mutant to divalent cation blockers such as Cd^{2+} and sulphhydryl-directed MTS reagents such as MTSET have been used extensively to study the role of amino acids residues in pore formation in many ion channels, including voltage-gated Na channels [1, 2, 13, 18]. In this study we used a similar approach to investigate the role of W736 in pore formation of Na$_v$1.4, a rat skeletal muscle Na channel. This is a continuation of previous work in which we identified two

residues in domain II (DII) involved in μ-CTX binding: alanine[728] and aspartate[730] [5]. Our previous data also showed that these residues do not contribute to the pore region but rather to the outer vestibule of the channel. In this study we found that the W736 residue contributes to the structural external vestibule and may be located close to the narrow part of the pore. This residue is conserved in all cloned Na channels and is located outside the proposed SS1 region of DII [9, 10, 20]. In fact, the W736 residue is located three amino acids away from the N-terminal side of the SS1 region. The role of W736 in forming the outer vestibule is consistent with the close proximity of this residue to the SS2 segment, which includes residues important for selectivity and toxin binding.

$Na_v1.4$/W736C Na currents were inhibited by Cd^{2+}, which occludes the permeation pathway. This is in contrast to the $Na_v1.4$/WT channel, which was relatively insensitive to Cd^{2+}. We also modified the sulphhydryl groups of cysteine residues covalently by MTSET to verify that the W736 side chain faces the interior of the pore. MTSET diminished the conductance of the $Na_v1.4$/W736C channel. Inhibition by externally applied Cd^{2+} and MTSET are consistent with the conclusion that this residue is located within close proximity to the external narrow part of the pore.

W736 contributes to the structure of the pore and is involved in toxin binding

We have shown previously that the segment that includes residues 728 and 730 contributes to μ-CTX binding but does not appear to be located near the pore [5]. We have shown here that Cd^{2+} and MTSET interaction with the W736C mutant inhibits currents. These data support the conclusion that the native tryptophan may be located in close proximity to the pore. The W736 mutation had a slight effect on TTX and μ-CTX binding, indicating that this residue is accessible from the outer vestibule of the channel. This also suggests that the amino acids that form the SS1 of domain II are neighbours to the W736 residue.

W736 is not responsible for channel selectivity

The conclusion that W736 is not responsible for the channel's selectivity is based on two observations. First, the reversal potential of Na currents obtained from current/voltage (*I*/*V*) curves in control Ringer solution was not affected by the mutation and was comparable to the wild-type channel (data not shown), indicating that this residue is not part of the selectivity filter. Secondly, replacing the Na^+ charge carrier by K^+, Li^+, Cs^+ or NH_4^+ significantly diminished current flow for both mutant and wild-type channel and did not affect the known sequence of ion permeation for Na channels: $Na^+ > Li^+ > NH_4^+ > K^+ = Cs^+$. The non-involvement of W736 residue in the channel's selectivity was not surprising since several other pore residues also have been shown not to be involved in the selectivity filter [14].

Single-channel analysis

Our single-channel data revealed that the W736 residue is not located deep in the pore. This conclusion is based on the minimal or absent voltage dependence of blockade by Cd^{2+} as was shown by plotting the fractional block as a function of voltage. These data are consistent with the small changes in TTX and μ-CTX binding, suggesting that this residue is not located deep inside the pore.

Is the W736 residue part of SS1?

The two short segments SS1 and SS2, located between S5 and S6 of each domain, span the plasma membrane [9]. This P-loop dips into (SS1) and out of (SS2) the membrane, forming the pore lining. Whilst many residues of SS2 have been shown to contribute to the formation of the pore, the contribution of the residues in SS1 is not known. This transmembrane region is believed to provide the ionic pathway through the voltage-gated Na channel. As shown in Fig. 1A, the W736 residue is located outside what is generally considered to be the SS1 region. However, our results show that the W736 is implicated in pore formation. This suggests that the SS1 region is longer than suggested and extends to residues neighbouring W736 and that W736, along with 728 and 730, contribute to the structure of the pore to the external vestibule of the channel (see also [5]). It is noteworthy that the arginine[735] and histidine[737] mutants did not generate any currents; one possibility is that the mutations in the Na channels affect the structure of the pore. The effect of DTT on both cysteine mutants was also tested in these mutant channels to eliminate the possibility that the introduced cysteines bridge with intra-channel cysteines (data not shown), that could lock up the ion permeation pathway. DTT did not restore Na currents, suggesting that intra-channel cysteines are not responsible for the absence of Na currents in the R735 and H737 cysteine mutants. Further studies are required to determine the structure of this region of $Na_v1.4$ Na channel.

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