A single serine residue confers tetrodotoxin insensitivity on the rat sensory-neuron-specific sodium channel SNS

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Abstract Sensory neurons express a sodium channel (SNS) that is highly resistant to block by tetrodotoxin ($IC_{50} = 60$ μM). SNS is 65% homologous to the cardiac sodium channel, in which a single hydrophilic residue in the SS2 segment is critical for tetrodotoxin resistance. By site-directed mutagenesis, we have substituted phenylalanine for serine at the equivalent position in SNS: this mutated (S356F) SNS channel is functionally similar to wild-type SNS when expressed in *Xenopus* oocytes, but is potently blocked by tetrodotoxin and saxitoxin with IC_{50}s of 2.8 nM and 8.2 nM, respectively. These data provide clues to the rational design of selective blockers of SNS with potential as analgesic drugs.
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1. Introduction

Voltage-gated sodium channels (VGSC) are membrane glycoproteins formed by a large α-subunit of 260 kDa, a β1-subunit of 36 kDa and, in the case of brain VGSCs, a covalently associated β2-subunit of 33 kDa [1–3]. α-Subunit cRNA is sufficient to produce functional VGSCs in *Xenopus* oocytes[4–6], but the expression of such channels is enhanced by co-expression of the accessory β1- and β2-subunits, which also affect the kinetics of recombinant channels [3]. The β1 subunit accelerates the time course of sodium channel activation and inactivation by shifting the channel population to a fast gating mode when co-expressed with rat brain VGSC α-subunits in *Xenopus* oocytes [3]. The β2-subunits may regulate not only the level of expression but also the localisation of the functional channel because they contain extracellular immunoglobulin domains that are structurally similar to those found in the cell adhesion molecule, contactin [2].

VGSC α-subunits consist of four repeated homologous domains (DI-IV), each containing six potential α-helical transmembrane segments (S1-6), intracellular loops connecting the four domains and intracellular N- and C-terminals. Domains critical for the function of the channel are highly conserved across VGSCs and include the S4 voltage-sensors, the loop between domains III and IV (involved in inactivation [2]) and the SS1 and SS2 segments in the extracellular loops between S5 and S6 (which form the channel vestibule and ion selectivity filter) [7–9].

Tetrodotoxin (TTX) is a potent blocker of most VGSCs and is often used as pharmacological tool to define the biological role of such channels. However, a number of TTX-insensitive VGSCs have been identified in heart [10], sensory neurons [11], denervated skeletal muscle [12] and glia [13]. The corresponding α-subunits for TTX-insensitive VGSCs have been cloned from heart [14] and sensory neurons [5,6] and, when heterologously expressed, reproduce the TTX resistance of the native channels. The site of TTX binding is in the channel vestibule, where a number of acidic residues are important for the toxin binding[15]; these residues are also important for ion permeation and are highly conserved in both TTX-sensitive and TTX-resistant α-subunits. Site-directed mutagenesis showed that another SS2 residue determines TTX sensitivity; this is a hydrophilic Cys^{374} in the heart channel and an aromatic Tyr or Phe in TTX-sensitive VGSCs [16,17]. In the present study we show that, in the sensory neuron specific VGSC SNS, mutagenesis of the equivalent amino acid residue (356) from hydrophilic Ser to aromatic Phe gives rise to a recombinant channel highly sensitive to TTX and STX. These findings confirm the importance of hydrophobic interactions between TTX or STX and aromatic residues in the channel atrium (see the Lipkind-Fozzard model of TTX-binding to the sodium channel pore [18]).

2. Materials and methods

cDNA for the rat SNS α-subunit [5] was cloned into the mammalian expression vector pRK5 (*Hind*III–*Kpn*I) sites before site-directed mutagenesis was performed with the oligonucleotide 5′-GGCGCTC-CCAAAAGTCCTGCGTCA-3′ to convert the serine in position 356 into a phenylalanine [19]. This mutation was confirmed by DNA sequence analysis using dideoxy sequencing on both strands. The mutated SNS cDNA was sub-cloned as the *Hind*III–*Kpn*I fragment into the oocyte expression vector pSP64GL. The resulting plasmid was linearised with *Xba*I; for cRNA transcription with SP6 polymerase using 1 mM 7-methylGpppG [5]. cRNA (70 ng) was injected into stage IV *Xenopus* oocytes 7–14 days before recording.

Oocytes were impaled with 3 M KCl electrodes (0.5–1 MΩ) and perfused at 1–2 ml/min with modified Ringer solution containing (mM): NaCl 115, KCl 2.5, HEPES 10, $MgCl_2$ 1.8, $CaCl_2$ 1, pH 7.2, at a temperature of 19.5–20.5°C. Voltage commands were applied and currents were recorded under two-electrode voltage-clamp, using an Axoclamp-2B amplifier (Axon Instruments) driven by a PC with a CED-1401plus interface (Cambridge Electronic Design) and WCP software (courtesy of John Dempster, University of Strathclyde, Glasgow). Currents were filtered at 3 kHz and sampled at 20 kHz. Digital leak subtraction of the current records was carried out using a P/4 protocol. Oocytes in which hyperpolarizing commands elicited time-dependent currents were discarded.

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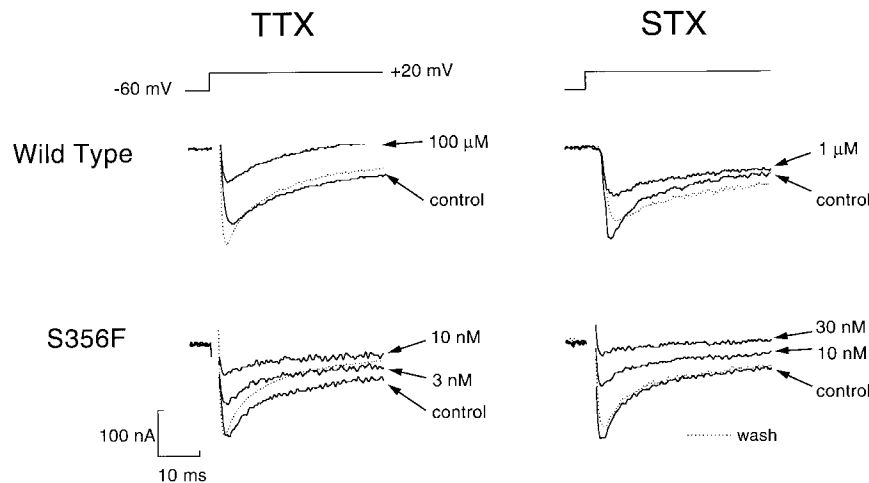[1]The first two authors contributed equally to this work.

Fig. 1. Effect of TTX and STX on the peak Na current of oocytes expressing wild-type SNS and S356F SNS. Inward currents were elicited by a step from −60 mV to +20 mV (0.1 Hz; leak-subtracted and filtered at 0.5 kHz) in control conditions, after toxin application (10 min for SNS and 20 min for the mutant) and after wash (dotted traces) in control Ringer solution (10 min for SNS, 30 min for the mutant). Left: Effect of TTX on wild-type (top; 100 μM) and mutant (bottom; 3 and 10 nM) SNS currents. Right: Effect of STX on wild-type (top; 1 μM) and mutant (bottom; 10 and 30 nM) SNS currents.

3. Results

Expression of the SNS mutant S356F in oocytes resulted in voltage-dependent sodium currents that were similar to those produced by the wild-type construct in the long time required for expression, in the I/V relation (data not shown) and in the expression level. In the current series of experiments, a depolarizing 80 mV command from a holding potential of −60 mV evoked a I_{Na} of 174.4 ± 22.8 nA and 218.5 ± 52.7 nA from wild-type SNS and S356F SNS-injected oocytes (mean ± SEM; $n = 8$ and 7, respectively).

Fig. 1 shows the profound effect of the S356F mutation on the channel sensitivity to block by guanidinium toxins. As shown by Akopian et al. [5], oocyte-expressed wild-type SNS channels are similar to native TTX-resistant DRG sodium channels in that they are only affected by TTX concentrations equal to or greater than 10 μM (Fig. 1, top left); the TTX IC_{50} is 100 μM for DRG [20] and 60 μM for recombinant SNS (Fig. 2, top). Also in agreement with the results on native DRG channels, we found STX to be more potent than TTX, producing significant block on wild-type SNS at concentrations as low as 300 nM (Fig. 1, top right; Fig. 2, bottom). The S356F mutation increased the channel sensitivity to TTX by approximately 21 000-fold and that to STX by approximately 100-fold (Table 1 and Fig. 2).

In recombinant cardiac sodium channels, the equivalent (C374Y) mutation not only increases the sensitivity to guanidinium toxins, but also eliminates the use-dependence of the block by TTX, leaving unchanged the use dependence of the block by STX. TTX-resistant SNS channels, native or recombinant, differ from the cardiac sodium channels in that current amplitude declines with repetitive trains even in the

absence of a blocker and even at moderate rates of stimulation: 20 pulses at 0.1 Hz decreased the peak I_{Na} by $9.2 \pm 2.5\%$ and $14 \pm 3.8\%$ in wild-type and S356F SNS ($n = 13$ and 8, respectively). In the presence of 100 μM TTX or 1 μM STX, the reduction in wild-type SNS currents during the train of 20 pulses was $18.0 \pm 7.0\%$ and $16.3 \pm 2.2\%$ ($n = 5$ and 8, respectively). Any further reduction in peak current during the train of command pulses in the presence of blockers was therefore too small and variable to allow us to determine whether the mutation affected additional use-dependent components of toxin block.

4. Discussion

The site of block of the guanidine-containing marine toxins TTX and STX was first suggested to be the ion selectivity filter of sodium channels by Hille [21], who had earlier demonstrated that VGSCs were permeant to guanidinium ions, and that carboxyl groups are required for toxin binding. This proposal is consistent with the fact that both permeant and blocking ions compete with toxin binding, and chemical modification or pH titration of carboxyl groups alters both conductance and toxin binding. A number of acidic residues present in the SS2 segment were found by site-directed mutagenesis to be important for channel block by the toxins [2,15]. However, a comparison of the pore region of the TTX-sensitive type II VGSC and TTX-resistant heart channel demonstrated only two differences in amino acid residues: N377 and C374 of the heart channel are, respectively, R and Y in the brain type II channel (heart channel numbering). The first of these two residues does not appear to be important for TTX binding, as TTX block is unaffected by the R377N mutation

Table 1
IC_{50} of TTX and STX on peak I_{Na} of wild-type SNS and S356F mutant SNS

	Wild type SNS IC_{50}	S356F SNS IC_{50}	nH
TTX	59.4 ± 7.2 μM	2.83 ± 0.36 nM	0.809 ± 0.09
STX	757.2 ± 102.4 nM	8.17 ± 0.27 nM	0.839 ± 0.100

IC_{50} and Hill slopes (nH) are the result of the fits to the dose-response curves shown in Fig. 2. $n = 2$–8 oocytes for each concentration.
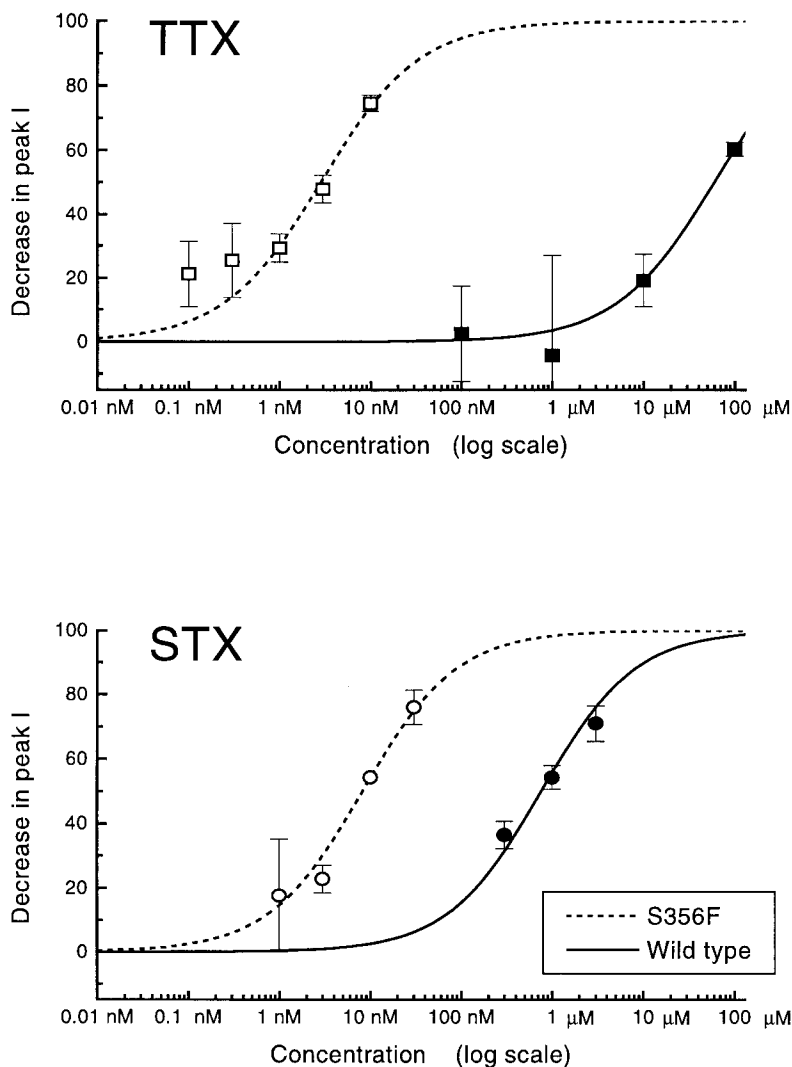
Fig. 2. Effect of the S356F mutation on the sensitivity of SNS Na current to guanidinium toxins. A: Dose-response curves for the effect of TTX on SNS and S356F SNS. B: Dose-response curves for the effect of STX on SNS and S356F SNS. Data points shown ($n = 2$–8) were normalized to the peak Na currents in control conditions; dose-response curves were fitted by the Hill equation ($100^*[\text{toxin}]-nH)/([\text{toxin}]-n-H+\text{IC}_{50}-nH$) with the constraint of parallelism for the two dose-response curves for each toxin (CvFit software, courtesy of David Colquhoun, University College London). Note how the leftward shift in toxin IC_{50} produced by the mutation was more than 2 orders of magnitude greater for TTX than for STX.

in heart channels or the reverse mutation in type II channels [15,16]. This leaves C374. A Cys residue in the pore accounts for the heart channel sensitivity to block by Cd and Zn ions [22]; a Cys is involved also in the binding of guanidinium toxins, as covalent modification with the SH-reactive agent iodoacetamide diminishes both STX and Zn block [23]. That the critical -SH is indeed the one in position 374 in SS2 was confirmed by the high sensitivity to TTX and the reduction in heavy metal block conferred by the C374Y mutation in the cardiac channel [16]; the converse mutation Y385C in the type II brain channel caused TTX-insensitivity and increased heavy metal block to occur [24]; finally, domain-swop experiments between the cardiac and type II channels showed that TTX resistance co-segregated with the cardiac domain I region [25].

Knowing the significance of the aromatic residue at the equivalent positions to 374, and the role of negatively charged glutamate and aspartate groups has allowed models of the atrium of the channel, and the sites of interaction with STX
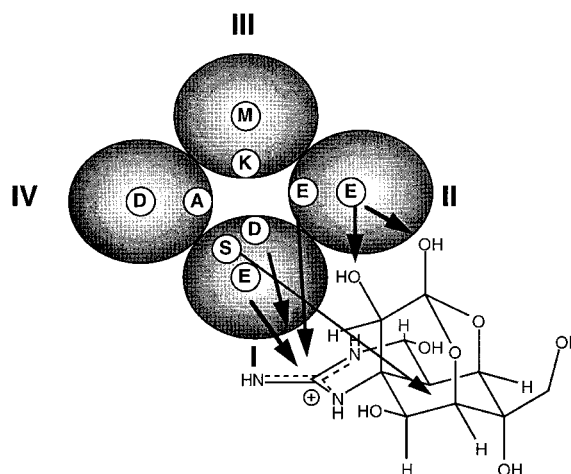

Fig. 3. Critical residues present in the atrium of the voltage-gated sodium channel SNS, and their interactions with tetrodotoxin, based on the modelling studies of Lipkind and Fozzard [18].

and TTX to be developed by Lipkind and Fozzard [18] and Guy and Durell [26], which suggest either a β-sheet hairpin or a helix–loop–helix structure for the SS1–SS2 segments. Essential elements of the structure for toxin binding have also been identified, and these comprise the positively charged guanidinium group and the hydroxyl residues present at C9 and C10 of TTX. Using energy minimisation paradigms and data gleaned from the solution structure of soluble proteins, Lipkind and Fozzard proposed that the SS1–SS2 regions are β-hairpin structures and that acidic residues in the channel pore interact with the C9 and C10 carboxyl groups of TTX whilst the positively charged guanine moiety interacts with acidic residues in the pore (see Fig. 3). In this model, the residue at position 374 forms with an uncharged nonpolar region in the blocker molecule either a relatively weak van der Waals interaction (in the case of Cys) or a much stronger hydrophobic interaction (in the case of Tyr or Phe); the energy gain with a mutation from Cys to Tyr is calculated as -5 kcal/mol, which agrees with the observed shift in TTX potency [18]. SNS channels have a Ser in the equivalent position and therefore the bond with the nonpolar region of TTX is formed by a hydroxyl, rather than a sulphydryl group. The lower blocker potency when oxygen is involved, rather than sulphur, supports the idea that the interaction between this residue and the blocker is of a hydrophobic rather than electrostatic nature (and hence dominated by dispersion) [27].

In the case of STX, a smaller gain in potency was observed following the S356F mutation; however, the binding sites for TTX and STX overlap but are not identical, and indeed diverge at Cys^{374}, as suggested by the fact that covalent modification of this residue by methane sulfonate reagents is inhibited by TTX, but not STX. The toxin receptor complexes for STX and TTX may be conformationally distinct and the hydrophobic interaction less significant for STX than for TTX [28].

Our findings suggest that the atrium of the sensory neuron specific TTX-resistant SNS channel is similar to that of the heart channel and the hydrophilic Ser residue 356 abolishes a hydrophobic interaction that would normally result in high-affinity binding to TTX. The accessibility of this residue from the pore is one of the given points for both the Lipkind-Fozzard and the Guy and Durell models of the VGSC channel vestibule [18,26] and is confirmed by the cysteine mutagenesis study by Chiamvimonvat et al. (which also suggests that the SS1–SS2 segments are quite flexible, do not have a α-helix or β-sheet structure and that they reach different depths in the pore[29]). The observation that SNS is the only known sodium channel with a Ser in the vestibule has implications for the development of selective blockers of this channel, which may play a pivotal role in the transfer of nociceptive information. Thus bradykinin induced depolarisation of dorsal horn neurons via the activation of DRG sensory neurons is TTX-insensitive [30] A specific SNS channel blocker might have therefore selective analgesic activity. A TTX-like molecule with a polar ring structure containing a COOH group rather than a hydrophobic region might be expected to show a

selective affinity for the SNS channel. As the Lipkind-Fozzard model has been well defined in terms of atomic co-ordinates [18] molecular modelling of guanidine-based channel blockers may prove a fruitful approach to developing selective blockers of SNS.

References

[1] W.A. Catterall, Physiol. Rev. 72 (1992) S15–S48.
[2] H.A. Fozzard, D.A. Hanck, Physiol. Rev. 76 (1996) 887–926.
[3] L.L. Isom, D.S. Ragsdale, K.S. De Jongh, R.E. Westenbroek, B.F. Reber, T. Scheuer, W.A. Catterall, Cell 83 (1995) 433–442.
[4] M. Noda, T. Ikeda, T. Kayano, T. Suzuki, H. Takeshima, M. Kurasaki, T. Takahashi, S. Numa, Nature 320 (1986) 188–192.
[5] A.N. Akopian, L. Sivilotti, J.N. Wood, Nature 379 (1996) 257–262.
[6] L. Sangameswaran, S.G. Delgado, L.M. Fish, B.D. Koch, L.B. Jakeman, G.R. Stewart, P. Sze, J.C. Hunter, R.M. Eglen, R.C. Herman, J. Biol. Chem. 271 (1996) 5953–5956.
[7] W. Stuhmer, F. Conti, H. Suzuki, X. Wang, M. Noda, N. Yahagi, H. Kubo, S. Numa, Nature 339 (1989) 597–603.
[8] W.A. Catterall, Annu. Rev. Pharmacol. Toxicol. 20 (1980) 15–43.
[9] W.J. Thomsen, W.A. Catterall, Proc. Natl. Acad. Sci. USA 86 (1989) 10161–10165.
[10] K. Shigenobu, N.J. Sperelakis, Mol. Cell. Cardiol. 3 (1971) 271–286.
[11] Y. Matsuda, S. Yoshida, T. Yonezawa, Brain Res. 154 (1978) 69–82.
[12] M.M. White, L.Q. Chen, R. Kleinfield, R.G. Kallen, R.L. Barchi, Mol. Pharmacol. 39 (1991) 604–608.
[13] S. Bevan, S.Y. Chiu, P.T. Gray, J.M. Ritchie, Proc. R. Soc. Lond. B 225 (1985) 299–313.
[14] R.B. Rogart, L.L. Cribbs, L.K. Muglia, D.D. Kephart, M.W. Kaiser, Proc. Natl. Acad. Sci. USA 86 (1989) 8170–8174.
[15] H. Terlau, S.H. Heinemann, W. Stuhmer, M. Pusch, F. Conti, K. Imoto, S. Numa, FEBS Lett. 293 (1991) 93–96.
[16] J. Satin, J.W. Kyle, M. Chen, P. Bell, L.L. Cribbs, H.A. Fozzard, R.B. Rogart, Science 256 (1992) 1202–1205.
[17] J. Satin, J.T. Limberis, J.W. Kyle, R.B. Rogart, H.A. Fozzard, Biophys. J. 67 (1994) 1007–1014.
[18] G.M. Lipkind, H.A. Fozzard, Biophys. J. 66 (1994) 1–13.
[19] Yuckenberg, P.D., Witney, F., Geisselsoder, J. and McClary, J. (1991) in: Directed mutagenesis: a practical approach (McPherson, M.J. ed.), pp. 27–48, Oxford University Press, Oxford.
[20] M.L. Roy, T. Narahashi, J. Neurosci. 12 (1992) 2104–2111.
[21] B. Hille, Biophys. J. 15 (1975) 615–619.
[22] D. DiFrancesco, A. Ferroni, S. Visentin, A. Zaza, Proc. Roy. Soc. Lond. (B) 223 (1985) 475–484.
[23] L. Schild, E. Moczydlowski, Biophys. J. 59 (1991) 523–537.
[24] P. Backx, D. Yue, E. Lawrence, E. Marba, G. Tomaselli, Science 257 (1992) 248–271.
[25] L.Q. Chen, M. Chahine, R.G. Kallen, R.L. Barchi, R. Horn, FEBS Lett. 309 (1992) 253–257.
[26] H.R. Guy, S.R. Durell, Soc. Gen. Physiol. Ser. 50 (1995) 1–16.
[27] G. Filippini, A. Gavezzotti, Acta Cryst. B 49 (1993) 868–880.
[28] G.E. Kirsch, M. Alan, H.A. Hartmann, Biophys. J. 67 (1994) 2305–2315.
[29] N. Chiamvimonvat, M.T. Pérez-García, R. Ranjan, E. Marban, G.F. Tomaselli, Neuron 16 (1996) 1037–1047.
[30] S. Jeftinija, Brain Res. 665 (1994) 69–76.