

RESEARCH PAPER

Actions of veratridine on tetrodotoxin-sensitive voltage-gated Na⁺ currents, Na_v1.6, in murine vas deferens myocytesHai-Lei Zhu¹, Richard D. Wassall², Maki Takai³, Hidetaka Morinaga⁴, Masatoshi Nomura⁴, Thomas C. Cunnane² and Noriyoshi Teramoto¹¹Department of Pharmacology, ³Department of Surgery and Science, ⁴Department of Medicine and Bioregulatory Science, Graduate School of Medical Sciences, Kyushu University, Fukuoka, Japan and ²Department of Pharmacology, University of Oxford, Mansfield Road, Oxford, UK**Background and purpose:** The effects of veratridine, an alkaloid found in *Liliaceae* plants, on tetrodotoxin (TTX)-sensitive voltage-gated Na⁺ channels were investigated in mouse vas deferens.**Experimental approach:** Effects of veratridine on TTX-sensitive Na⁺ currents (I_{Na}) in vas deferens myocytes dispersed from BALB/c mice, homozygous mice with a null allele of Na_v1.6 (Na_v1.6^{-/-}) and wild-type mice (Na_v1.6^{+/+}) were studied using patch-clamp techniques. Tension measurements were also performed to compare the effects of veratridine on phasic contractions in intact tissues.**Key results:** In whole-cell configuration, veratridine had a concentration-dependent dual action on the peak amplitude of I_{Na}: I_{Na} was enhanced by veratridine (1–10 μM), while higher concentrations (≥30 μM) inhibited I_{Na}. Additionally, two membrane current components were evoked by veratridine, namely a sustained inward current during the duration of the depolarizing rectangular pulse and a tail current at the repolarization. Although veratridine caused little shift of the voltage dependence of the steady-state inactivation curve and the activation curve for I_{Na}, veratridine enhanced a non-inactivating component of I_{Na}. Veratridine caused no detectable contractions in vas deferens from Na_v1.6^{-/-} mice, although in tissues from Na_v1.6^{+/+} mice, veratridine (≥3 μM) induced TTX-sensitive contractions. Similarly, no detectable inward currents were evoked by veratridine in Na_v1.6^{-/-} vas deferens myocytes, while veratridine elicited both the sustained and tail currents in cells taken from Na_v1.6^{+/+} mice.**Conclusions and implications:** These results suggest that veratridine possesses a dual action on I_{Na} and that the veratridine-induced activation of contraction is induced by the activation of Na_v1.6 channels.*British Journal of Pharmacology* (2009) **157**, 1483–1493; doi:10.1111/j.1476-5381.2009.00301.x; published online 22 June 2009**Keywords:** channel enhancer; dual action; Na_v1.6; smooth muscle-type Na⁺ channels; tetrodotoxin; veratridine**Abbreviations:** I_{Na}, voltage-gated Na⁺ currents; *med*, motor end-plate disease; NMDG, N-methyl-D-glucamine; PSS, physiological salt solution; TEA, tetraethylammonium; TTX, tetrodotoxin; V_{half}, 50% activation or inactivation potential

Introduction

Veratridine is an alkaloid toxin isolated from the rhizomes of *Veratrum album* and the sabadilla seeds of Mexican *Schoenocaulon officinale*. Using electrophysiological techniques, several studies have been performed to clarify the mechanisms underlying the action of veratridine on fast transient

inward voltage-gated Na⁺ currents (I_{Na}) in a wide variety of tissues such as skeletal muscle (Campos *et al.*, 2004), neurons (Rando, 1989), cardiac muscle (Nánási *et al.*, 1994); and *Xenopus* oocytes expressed with cloned Na⁺ channel (Na_v) genes (μ1, namely Na_v1.4; Wang *et al.*, 2000; channel nomenclature follows Alexander *et al.*, 2008). These studies show an increase of I_{Na} as veratridine concentrations are increased. It has been reported that veratridine binds to the D1 homologous domain of the S6 transmembrane segment in α subunits of voltage-gated Na⁺ channels (Wang and Wang, 2003b). When veratridine binds to these sites, Na⁺ channels are shifted towards long-lasting open states with a reduction in

Correspondence: Dr Noriyoshi Teramoto, Department of Pharmacology, Graduate School of Medical Sciences, Kyushu University, 3-1-1 Maidashi, Higashi Ward, Fukuoka, 812-8582, Japan. E-mail: noritera@med.kyushu-u.ac.jp
Received 30 October 2008; revised 19 January 2009; accepted 19 March 2009

single-channel conductance, thereby altering voltage dependency and Na⁺ permeability (Wang and Wang, 2003a). It is well known that veratridine enhances the channel activity of most Na_v1.X isoforms including Na_v1.1, Na_v1.2, Na_v1.3, Na_v1.4, Na_v1.5, Na_v1.6 and Na_v1.7 (reviewed by Catterall *et al.*, 2005).

Recently, we reported the biophysical and molecular properties of tetrodotoxin (TTX)-sensitive I_{Na} recorded in smooth muscle cells, freshly dispersed from mouse vas deferens, showing that the molecular identity of the only voltage-gated Na⁺ channel functional in these cells is the Na_v1.6 channel, encoded by the *Scn8a* gene (Zhu *et al.*, 2008). This system therefore provides an ideal opportunity to investigate the actions of veratridine on a single functional TTX-sensitive voltage-gated Na⁺ channel, Na_v1.6, in native smooth muscle cells.

In the present study, we have shown that veratridine caused a dual action on the peak amplitude of I_{Na}, depending on its concentrations, and that veratridine induced a sustained inward current during the depolarizing rectangular pulse. We also found a tail current at the repolarization of the pulse, exhibiting a Na⁺ permeability and sensitivity to TTX. Furthermore, we have demonstrated that veratridine induces TTX-sensitive phasic contractions in the isolated vas deferens from wild-type littermates (Na_v1.6^{+/+}), and that veratridine caused no contractions in tissues from Na_v1.6-null mice (Na_v1.6^{-/-}). Finally, using Na_v1.6-null mice and wild-type littermates, we show that the detectable inward currents evoked by veratridine were due to I_{Na} mediated by Na_v1.6 channels.

Methods

Preparation of the vasa deferentia

All animal experiments were approved by the animal care and use committee of Faculty of Medicine, Kyushu University (Fukuoka, Japan). Male BALB/c mice (8–10 weeks) or C3HeB/FeJ mice (2–3 weeks) were killed by cervical dislocation. Efforts were made to minimize the number of animals used and their suffering; several types of experiment were carried out on aliquots of cells from any one animal.

Contraction studies

For tension measurements, a modified Krebs solution was used (in mM): Na⁺ 137, K⁺ 5.9, Mg²⁺ 1.2, Ca²⁺ 2.5, Cl⁻ 133.7, HCO₃⁻ 15.4, H₂PO₄⁻ 1.2 and glucose 11.5 which was bubbled with 97% O₂ and 3% CO₂. Tissue strips were prepared as described previously (see Teramoto and Brading, 1996; Cuprian *et al.*, 2005). The mucosa was removed from the tissues and isometric contraction of the longitudinal smooth muscle layer was measured using a force transducer (Minebea Co. Ltd., Nagano, Japan). An initial tension equivalent to 0.5 g weight was applied to the tissues and they were allowed to equilibrate for approximately 2–3 h at 37°C. Isometric contractions of the longitudinal muscle layer were sampled (100 Hz) and digitized using Chart 5.0 software (AD Instruments, Castle Hill, Australia) and an IBM computer (VAIO PCV-HX51B, SONY, Tokyo, Japan). The area under the tension

trace was measured (expressed as mN min mg⁻¹) and compared with control values.

Electrophysiology

Mouse vas deferens single smooth muscle cells isolated from the intact tissue were dissociated freshly by the gentle tapping method (described previously in Teramoto and Brading, 1996; Zhu *et al.*, 2008) and stored in cold physiological salt solution (see below) at 4–5°C. After isolation, relaxed spindle-shaped cells were stored on ice in the room, and used within 3–4 h. Patch-clamp experiments (conventional whole-cell configuration) were performed at room temperature (21–23°C) and the data recording system used was essentially the same as that described previously (Zhu *et al.*, 2008). The whole-cell current data were low-pass filtered at 5 kHz by an eight-pole Bessel filter, sampled at 0.1 ms and analysed on a PowerMac G4 computer (Apple Computer Japan, Tokyo, Japan) using Chart v5.5.6 (AD Instruments Pty Ltd., Castle Hill, Australia). A method for pharmacological subtraction of the leak and the capacitive currents was performed. By subtracting the current in the presence of TTX (1 μM) and Cd²⁺ (100 μM) from that in the presence of Cd²⁺, a TTX-sensitive component was obtained (see Zhu *et al.*, 2008). No electronic compensation for either series resistance or membrane capacitance was applied for the pharmacological subtraction of the membrane currents. However, both series resistance and membrane capacitance were measured in BALB/c mice vas deferens myocytes (series resistance 19.4 ± 6.6 MΩ; membrane capacitance, 49.3 ± 10.6 pF; *n* = 53 cells, 23 different animals) and C3HeB/FeJ mice vas deferens myocytes (series resistance, 15.4 ± 5.8 MΩ; membrane capacitance, 13.1 ± 2.8 pF; *n* = 9 cells, 6 different animals). Due to the different ages between BALB/c mice (8–10 weeks) and C3HeB/FeJ mice (2–3 weeks), the size of the smooth muscle cells was different.

Current–voltage relationships were obtained from measurement of the peak amplitude of voltage-gated Na⁺ currents (I_{Na}) within the first 10 ms after application of depolarizing pulses (see Figure 3 and see Figure 7).

Steady-state inactivation of the current was assessed using a two-step protocol in which the cells were stepped from -70 mV to a range of voltages between -110 and 30 mV for a period of 20 s before a 200 ms step to -10 mV. The peak amplitude of I_{Na} evoked by each test pulse was measured. The peak amplitude of I_{Na} with application of the conditioning pulse at -110 mV was normalized to unity. The lines were drawn by fitting the data to the Boltzmann's equation in the least-squares method;

$$I = (I_1 - I_2) / (1 + \exp((V - V_{\text{half}})/k)) + I_2$$

where *I*, *I*₁, *I*₂, *V*, *V*_{half} and *k* are the relative amplitude of I_{Na} observed at various amplitudes of the conditioning pulse (*I*) and the amplitude of the inactivating component of current (*I*₁), the amplitude of the non-inactivating component (*I*₂), the amplitude of the conditioning pulse (*V*), and where the amplitude of I_{Na} was reduced to half (*V*_{half}), and the slope factor (*k*) respectively (see Figure 5A).

Activation curves were derived from the current–voltage relationships. Conductance (*G*) was calculated from the

equation $G = I_{Na}/(E_m - E_{Rev-Na})$, where I_{Na} is the peak current elicited by depolarizing test pulses to 40 mV from a holding membrane potential of -70 mV and E_{Rev-Na} is the reversal potential for Na⁺. E_{Rev-Na} was estimated by extrapolation of each current-voltage relationship to the current axis. G_{max} is the maximal Na⁺ conductance (calculated at potentials above -10 mV). Values for G/G_{max} were plotted against membrane potential as relative amplitudes.

Solutions and drugs

The composition of the bath solution (physiological salt solution) for patch-clamp experiments was (mM): Na⁺ 140, K⁺ 5, Mg²⁺ 1.2, Ca²⁺ 2, glucose 5, Cl⁻ 151.4, HEPES 10, titrated with Tris base to pH 7.35–7.40. The composition of the pipette solution was (mM): Cs⁺ 130, tetraethylammonium (TEA⁺) 10, Mg²⁺ 2, Cl⁻ 144, glucose 5, EGTA 5, ATP 5, HEPES 10, titrated with Tris base to pH 7.35–7.40. Occasionally, 20 mM NaCl was added to the above pipette solution to estimate the reversal potential of tail currents (i.e. see results in Figure 4). Cells were allowed to settle in the small experimental chamber (80 μL in volume) which was superfused by gravity throughout the experiments at a rate of 2 mL min⁻¹ from a solution reservoir. TTX citrate (Sankyo Co. Ltd., Tokyo, Japan) was dissolved in deionized water to produce a stock solution of 1 mM, aliquoted and stored frozen (-20°C) until required. All other drugs were purchased from Sigma-Aldrich (Sigma-Aldrich Japan K.K., Tokyo, Japan).

Nav1.6-null mice

Heterozygous *Scn8a*-motor end-plate disease (*med*) mice maintained on a C3HeB/FeJ background were obtained from The Jackson Laboratories (Bar Harbor, ME, USA; see Zhu *et al.*, 2008). The *med* mutation produces complete loss of Nav1.6 (Ensembl ID: ENSG00000196876) expression (Alexander *et al.*, 2008). In order to compare animals homozygous for *med* (Nav1.6^{-/-}) with wild-type (Nav1.6^{+/+}) littermates, genotyping was performed with individual pups from the intercrosses of heterozygote (Nav1.6^{+/-}) mice, and then Nav1.6^{-/-} or Nav1.6^{+/+} mice were selected for further examination. Genotyping of Nav1.6 was performed by PCR as described previously (Kohrman *et al.*, 1996; Zhu *et al.*, 2008). PCR amplification was achieved with 35 cycles of 94°C for 30 s, 58°C for 30 s and 72°C for 30 s using the following specific primers: for the wild-type allele, 5'-GGA GCA AGG TTC TAG GCA GCT TTA AGT GTG-3' and 5'-GTC AAA GCC CCG GAC GTG CAC ACT CAT TCC-3' and for the mutant allele, 5'-TCC AAT GCT ATA CCA AAA GTC CC-3' and 5'-GGA CGT GCA CAC TCA TTC CC-3'. PCR products were separated on a 1.5% agarose gel, allowing the resolution of a 230-base-pair product for the wild-type allele and a 194-base-pair product for the mutant allele.

Data analysis

Statistical comparisons were performed using Student's *t*-test for paired observations, taking $P < 0.05$ as significant. Statistical analyses were also performed with ANOVA tests (two-way

with replication) unless otherwise stated. Measurements are presented as mean ± standard deviation (SD).

Results

Dual action of veratridine on the peak amplitude of voltage-gated Na⁺ currents in murine *vas deferens* myocytes

Patch-clamp experiments were performed to investigate the effects of veratridine on TTX-sensitive Na⁺ channels in myocytes freshly dissociated from BALB/c mice *vas deferens*. Using a conventional whole-cell configuration, a rectangular depolarizing voltage step pulse to -10 mV from a holding potential of -70 mV elicited a fast transient inward Na⁺ current (I_{Na}) in the presence of 100 μM Cd²⁺ (described previously in Zhu *et al.*, 2008). Figure 1A shows the representative current traces of the membrane currents in the absence (i.e. control) and presence of veratridine (1–100 μM). Veratridine (1–10 μM) enhanced the peak amplitude of I_{Na} ($P < 0.05$) although higher concentrations of veratridine (30–100 μM) reduced the peak amplitude of I_{Na} (30 μM, $91 \pm 12\%$; $n = 5$ cells, 2 different animals, $P < 0.05$; 100 μM, $61 \pm 10\%$; $n = 17$ cells, 7 different animals, $P < 0.05$), showing a dual action when the peak amplitude of I_{Na} was normalized as one just before the application of veratridine (Figure 1B).

Higher concentrations of veratridine (≥ 3 μM) promoted the appearance of both a sustained inward component during the test depolarization, and a slowly deactivating tail current at repolarization. The amplitude of the veratridine-induced sustained inward current became stable during the depolarizing step pulse and increased in a concentration-dependent manner (Figure 1C). The decay of the veratridine-induced tail current was well fitted by a single exponential. The time constant of the current decay (τ) was prolonged from 0.9 ± 0.2 s (veratridine 3 μM, $n = 6$ cells, 5 different animals) to 2.2 ± 0.2 s (veratridine 100 μM, $n = 17$ cells, 7 different animals) in a concentration-dependent manner (Figure 1C). Note that the veratridine-induced sustained inward current and the veratridine-induced tail current were reversibly abolished by additional application of TTX (1 μM).

Figure 2A shows the time course of the effects of veratridine (100 μM) on the peak amplitude of I_{Na} , the mean amplitude of the sustained inward current during the last 20 ms, and the peak amplitude of the tail current respectively. Application of veratridine (100 μM) gradually induced the sustained inward current and the tail current, while, in contrast, the peak amplitude of I_{Na} was inhibited by veratridine (100 μM). In order to identify the veratridine-induced membrane currents as Na⁺ currents, extracellular Na⁺ concentration was changed to an iso-osmotic solution of TEA⁺ in the presence of veratridine (100 μM). When extracellular Na⁺ was replaced with TEA⁺, the peak amplitude of I_{Na} was readily suppressed (see Figure 2A, (iii)). Similarly, the sustained inward current and the tail current markedly decreased, and 2 min later, both veratridine-induced membrane currents were abolished. On reintroduction of Na⁺ and removal of TEA⁺, the membrane currents recovered to the control level (Figure 2, $n = 5$ cells, 3 different animals). Similar results were obtained when the same amount of Na⁺ was replaced with Tris⁺ or N-methyl-D-glucamine (NMDG⁺) (data not shown).

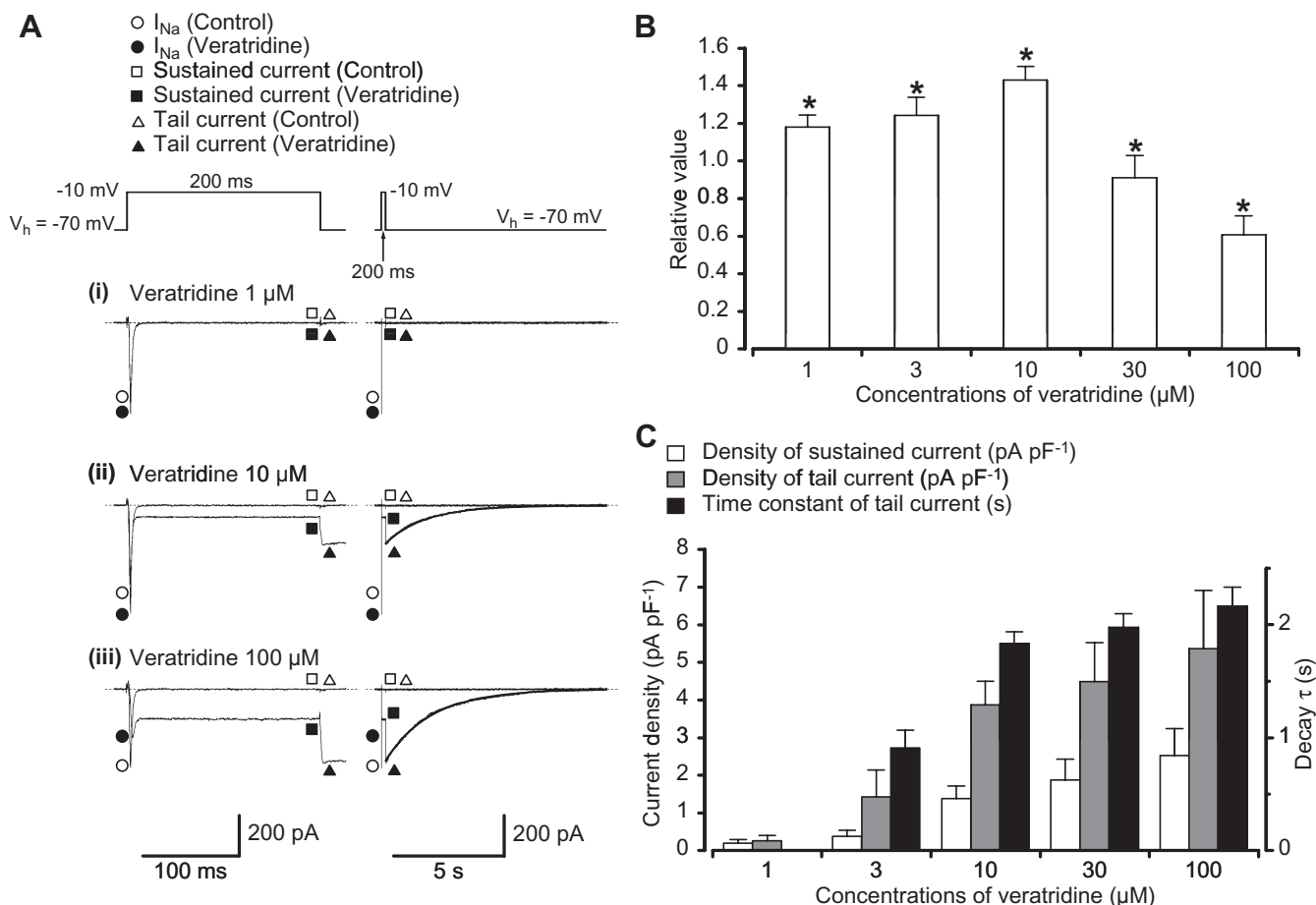


Figure 1 Effects of veratridine on the membrane currents in myocytes from mouse vas deferens using a conventional whole-cell configuration. (A) Representative current traces in the absence (control) and presence of veratridine (1 (i), 10 (ii) and 100 μM (iii)) using the same cell are shown at the left panel. The tail currents are also shown as an expanded trace at the right panel. The tail currents were well fitted by an exponential (10 μM, $\tau = 1.8$ s; 100 μM, $\tau = 2.1$ s). Membrane currents were evoked by a depolarizing rectangular pulse (200 ms duration; 20 s interval) from a holding potential of -70 to -10 mV. (B) Dual action of veratridine (1–100 μM) on the peak amplitude of I_{Na} . The relative values of the peak amplitude were expressed when the peak amplitude of I_{Na} in the absence of veratridine (control) was normalized as one. (C) Veratridine (1–100 μM) increased the amplitude of the sustained current (measuring the mean value of the last 20 ms of the test pulse) and the peak amplitude of the tail current in a concentration-dependent manner. The time constant of tail current decay (τ) was prolonged when the concentrations of veratridine were increased. The ordinate at the left side shows the current density of I_{Na} (pA pF⁻¹). The time constant of the tail current decay (τ) was also indicated at the right side. Each column shows the mean of 5–17 cells (13 different animals) with \pm SD shown by vertical lines. Asterisk indicates a statistically significant difference and changes were considered significant at $P < 0.05$ (ANOVA test).

Effects of veratridine on I_{Na}

As shown in Figure 3A, at potentials more positive than -40 mV, the evoked I_{Na} reached a peak and then gradually decayed. The peak amplitude was obtained at approximately -10 mV and the amplitude was reduced at more positive potentials, demonstrating a voltage dependency. In the absence of veratridine (control), no tail currents were evoked after the rectangular depolarization pulses (Figure 3A). In the presence of 100 μM veratridine, the peak amplitude of I_{Na} was reduced, shifting the maximum value to approximately 0 mV. Figure 3B shows the current–voltage relationships in the absence and presence of 100 μM veratridine. The maximum amplitude of the veratridine-induced sustained inward current was obtained approximately at -20 mV, reducing the peak value at more positive potentials (Figure 3C). In contrast, the peak amplitude of the veratridine-induced tail current gradually increased and the amplitude became stable at 0 mV (Figure 3D).

Ion selectivity of veratridine-induced tail currents

In order to make a rough estimation of the ion selectivity of the slow veratridine-induced tail currents, a double pulse protocol [conditioning pulse, rectangular pulse (200 ms duration) from -70 mV to -10 mV; test pulse, 15 s duration, see inset, Figure 4A] was performed when the external concentration of Na⁺ was 140 mM and the pipette Na⁺ concentration was 20 mM. Figure 4A shows the current traces of the veratridine-induced tail currents at the indicated membrane potentials (from -130 mV to 70 mV). As shown in Figure 4B, the peak amplitude of the veratridine-induced tail currents was plotted against the test potentials, estimating the reversal potential. As the current voltage relationship was linear, the reversal potential was estimated to be 48.5 ± 1.6 mV ($n = 5$ cells, 2 different animals). This value was close to the theoretical Na⁺ equilibrium potential calculated on the basis of the Nernst equation ($E_{Na} = 50.0$ mV).

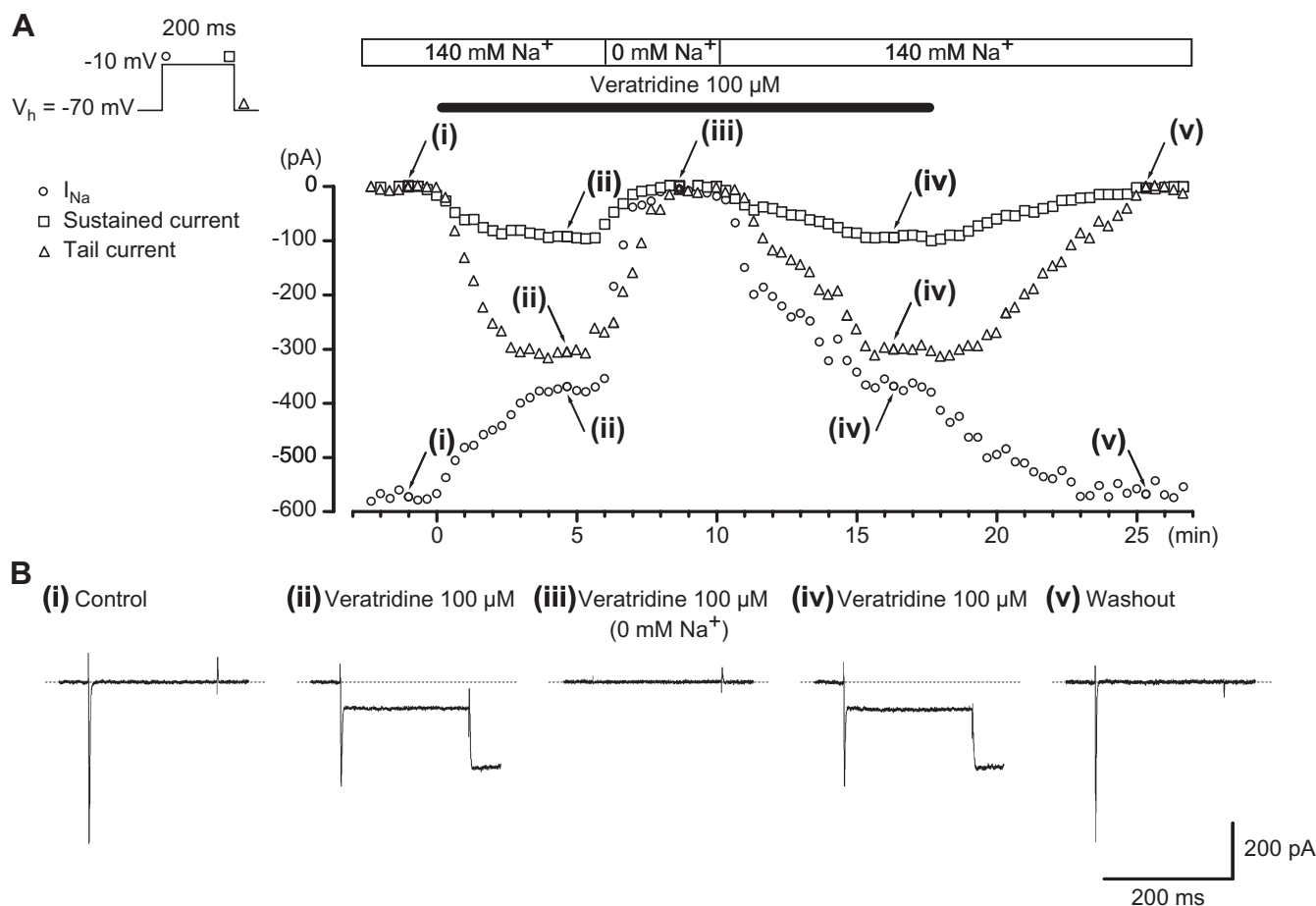


Figure 2 The time course of the effects of veratridine (100 μM) on three different parameters of the membrane currents (the peak amplitude of I_{Na}, the mean value of the sustained current (the mean value of the last 20 ms of the test pulse) and the peak amplitude of the tail current) elicited by repetitive depolarizing pulses (200 ms duration; 20 s interval) from -70 to -10 mV. (A) In the presence of 100 μM veratridine, the extracellular Na⁺ was reversibly replaced with TEA⁺. (B) Original current traces before (control, (i)) and after (ii) application of veratridine (100 μM), as indicated in (A). (iii) indicates a current trace after extracellular Na⁺ was replaced with several iso-osmotic solutions (TEA⁺) in the presence of veratridine. On removal of Na⁺-free solution, the membrane current was recovered (iv). (v) indicates a current trace after the removal of veratridine (100 μM).

Effects of veratridine on inactivation and activation curves of I_{Na}
The voltage dependency of inactivation was investigated before and after application of veratridine (100 μM) using the experimental protocol shown in Figure 5A (conditioning pulse duration, 20 s; holding membrane potential, -70 mV). In the absence of veratridine (control), inactivation of I_{Na} occurred with conditioning pulses positive to -50 mV [the 50% inactivation potentials (V_{half}); control, V_{half} = -45.9 ± 4.0 mV, n = 5 cells, 2 different animals]. In the presence of veratridine (approximately 5 min later), the voltage-dependent inactivation curve for the peak amplitude of I_{Na} was not shifted significantly (V_{half} = -45.3 ± 3.7 mV; n = 5 cells, 2 different animals; P > 0.05) although the non-inactivated component was enhanced [0.99 ± 0.02 (n = 5 cells, 2 different animals) of the peak I_{Na} inactivated in control whereas 0.89 ± 0.09 (n = 5 cells, 2 different animals) of peak I_{Na} inactivated in the presence of veratridine; P < 0.05, ANOVA test]. The activation curves obtained from the current-voltage relationships in Figure 3B, fitted to the Boltzmann equation, are shown in Figure 5A. In the presence of veratridine (100 μM), the activation curve for the peak amplitude of I_{Na} was not significantly

shifted (the 50% activation potentials (V_{half}); control, V_{half} = -23.7 ± 2.7 mV, n = 5 cells, 3 different animals; 100 μM veratridine, V_{half} = -21.9 ± 2.9 mV, n = 5 cells, 3 different animals; P > 0.05). As shown in Figure 5B, current traces of the test pulses at the indicated conditioning pulse potentials were detected in the absence and presence of 100 μM veratridine. In the presence of veratridine, the peak-matched traces are also demonstrated at the indicated conditioning pulse potentials when the peak amplitude of I_{Na} evoked by the test pulse with no conditioning pulse was superimposed on that in the absence of veratridine (i.e. control).

Effects of veratridine on muscle tone of intact vas deferens in Nav1.6-null mice (med mouse) lacking the expression of Na⁺ channel gene, Scn8a

As described previously in Zhu *et al.* (2008), the only functional voltage-gated Na⁺ channel present in mouse vas deferens when depolarizing pulses were applied was the Na_v1.6 channel. The effects of veratridine on the muscle tone of vas deferens were thus compared between homozygous mice with

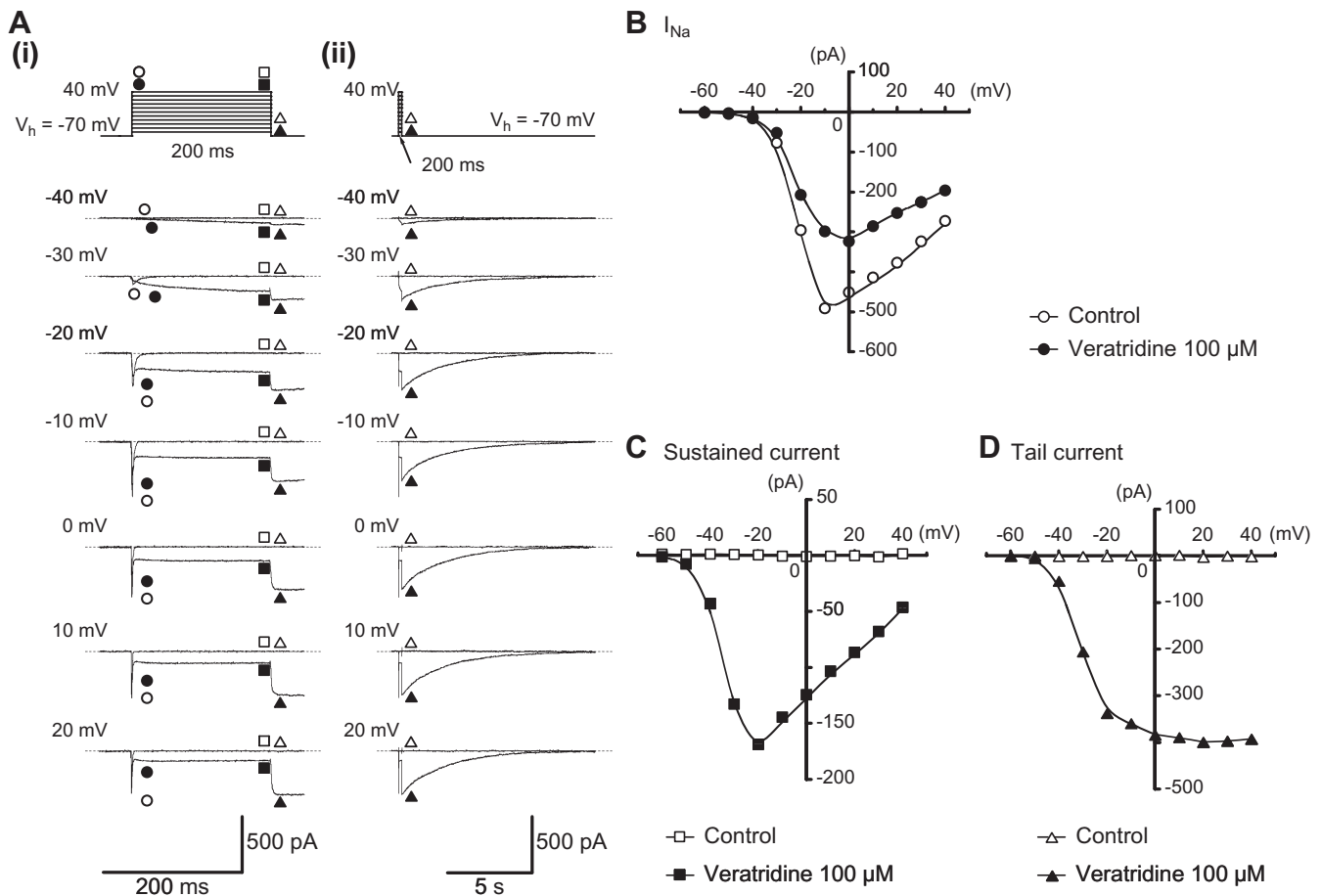


Figure 3 Effects of veratridine (100 μM) on I_{Na} in myocytes from mouse vas deferens. (A) (i) Original current traces were superimposed before (control) and after application of 100 μM veratridine at indicated pulse potentials. (ii) The decay of the tail current traces in the absence and presence of 100 μM veratridine superimposed at a slower time frame of (i). (B) Current–voltage relationships of the peak amplitude of transient I_{Na} obtained in the absence (control) or presence of 100 μM veratridine. The peak amplitude of I_{Na} was measured as the largest amplitude of the first 10 ms of the test pulse. (C and D) Current–voltage relationships of the 100 μM veratridine-induced sustained current (measured mean amplitude of the last 20 ms of the test pulse) and tail current (the peak amplitude). All curves were drawn by eye.

a null allele of $\text{Na}_v1.6$ (*med* mice, $\text{Na}_v1.6^{-/-}$; Burgess *et al.*, 1995) and wild-type mice ($\text{Na}_v1.6^{+/+}$). Under normal conditions, the vas deferens from wild-type mice does not show detectable spontaneous contractions. In vas deferens from $\text{Na}_v1.6^{+/+}$ mice, the application of low concentrations of veratridine ($\leq 1 \mu\text{M}$) did not cause any change to the isometric tension recorded from the tissue compared with control. However, application of higher concentrations of veratridine ($\geq 3 \mu\text{M}$) resulted in a gradual increase in phasic contractions ($n = 4$, 3 different animals; see Figure 6A). The integrated area of the veratridine-induced spontaneous contractions (the last 1 min duration) increased in a concentration-dependent manner ($n = 4$, 3 different animals, Figure 6C). In some preparations, these contractions were superimposed upon an increase in the basal tension in the tissue. These phasic contractions were abolished by the additional application of 1 μM TTX ($n = 6$ tissues, 3 different animals), suggesting that veratridine was having its effects through stimulation of Na⁺ channels (Figure 6A). Furthermore, in vasa deferentia from $\text{Na}_v1.6^{-/-}$ mice, cumulative application of veratridine (1–30 μM) caused no spontaneous contractions (Figure 6B and C). Figure 6C summarizes the difference in the veratri-

dine (3 and 10 μM)-induced contractions between tissues from $\text{Na}_v1.6^{+/+}$ and $\text{Na}_v1.6^{-/-}$ mice ($P < 0.05$, ANOVA test).

The effects of veratridine on the membrane currents in $\text{Na}_v1.6^{-/-}$ myocytes

To ensure that veratridine was only activating a current mediated by the $\text{Na}_v1.6$ channel, membrane currents in smooth muscle cells from the vasa deferentia of $\text{Na}_v1.6^{-/-}$ mice were investigated ($n = 5$ cells, 4 different animals). As shown in Figure 7A, in $\text{Na}_v1.6^{+/+}$ cells, TTX-sensitive I_{Na} were evoked by application of depolarizing rectangular pulses from a holding potential of -70 mV ($n = 4$ cells, 2 different animals), which showed similar electrophysiological and pharmacological properties with those recorded in BALB/c mouse vas deferens cells. Similarly, in $\text{Na}_v1.6^{+/+}$ type cells, veratridine (100 μM) induced both the sustained currents and the tail currents although the peak amplitude of I_{Na} was inhibited. Application of veratridine (100 μM) to tissues from $\text{Na}_v1.6^{-/-}$ mice caused no detectable inward membrane currents in dispersed smooth muscle cells from the vas deferens (Figure 7B). These results imply that the veratridine-induced membrane currents

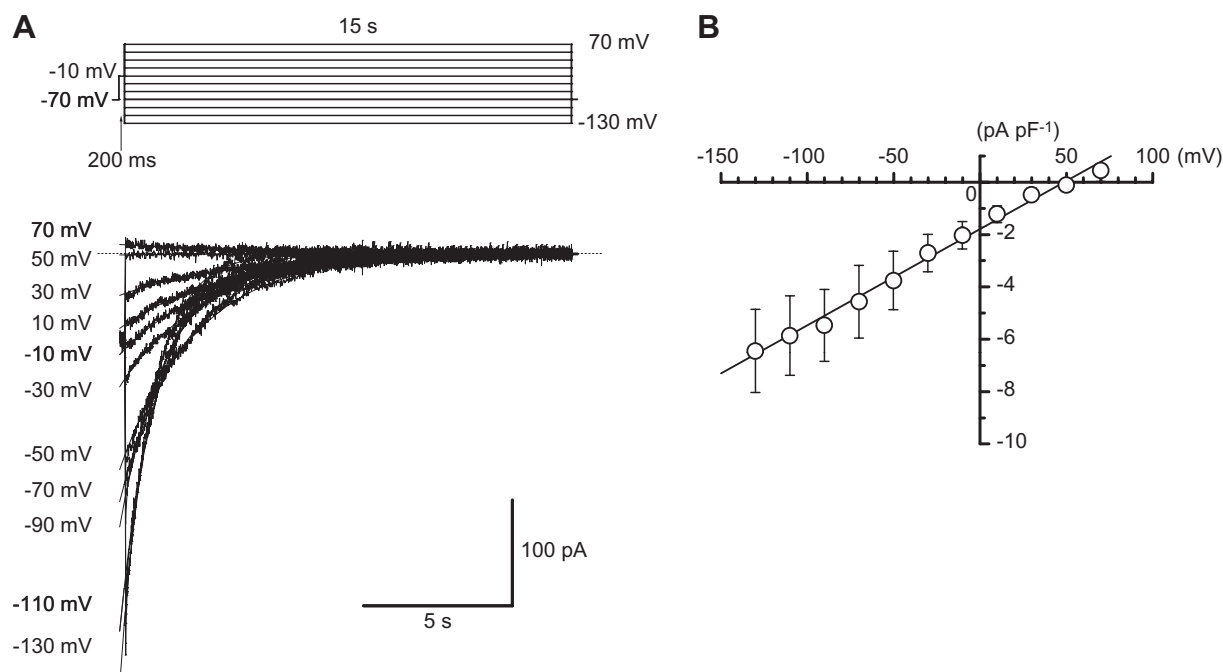


Figure 4 Ion selectivity of the veratridine-induced tail currents in myocytes from mouse vas deferens. (A) Current traces at the indicated membrane potentials. After an initial depolarization to -10 mV from a holding potential of -70 mV, the cell was repolarized to different test potentials (from -130 to 70 mV). (B) The peak amplitude of the veratridine-induced tail currents was plotted against the test potentials. The reversal potential was estimated to be 48.5 ± 1.6 mV.

observed in vas deferens smooth muscle cells are closely related to the activation of Na_v1.6, and that no other voltage-gated Na⁺ channels are contributing to the membrane currents evoked by veratridine.

Discussion

In the present study, it has been demonstrated that veratridine possesses a dual action on I_{Na} in freshly dispersed smooth muscle cells from mouse vas deferens, depending on its concentrations. The present work also establishes that the veratridine-induced contractions, sustained currents and tail currents are TTX-sensitive. Finally, these currents and contractions appear to link to the activation of Na_v1.6 channels.

Veratridine-induced contractions

Veratridine has been used as a potent voltage-gated Na⁺ channel activator in many excitable tissues, and like the other lipid soluble neurotoxins, batrachotoxin and aconitine, is thought to target the type 2 receptor site (D1-S6) of the α subunit of the voltage-gated Na⁺ channel, causing depolarization of the resting membrane potential and repetitive action potential firing (see Wang and Wang, 2003b). We have previously shown that smooth muscle cells of the mouse vas deferens express Na_v1.6 as the sole functional channel present in these cells, which was activated by veratridine (Zhu *et al.*, 2008), and so the actions of veratridine on the longitudinal layer of the intact vas deferens were investigated. Veratridine (10 μ M) induced rhythmical contractions in all preparations of wild-type littermates (i.e. Na_v1.6^{+/+}) but not Na_v1.6-null mice (i.e. Na_v1.6^{-/-}), similar to the contractions induced by

veratridine (a mixture of alkaloid esters, including veratridine, cevadine, cevine, cevadilline, and sabadine) detected by Chen *et al.* (2002). Furthermore, the veratridine-induced contractions were TTX-sensitive, suggesting that Na_v1.6 channels were involved.

Dual action of veratridine on the peak amplitude of I_{Na}

The effects of veratridine on the peak amplitude of I_{Na} have been well-examined in a wide variety of excitable cells (reviewed by Wang and Wang, 2003b). Veratridine (30–300 μ M) enhanced the peak amplitude of I_{Na} in a concentration-dependent manner [frog myelinated nerve (Rando, 1989); rat ventricular myocytes (Zong *et al.*, 1992)], while in contrast, veratridine (100–166 μ M) reduced the peak amplitude of I_{Na} in other tissues [frog skeletal muscle (Sutro, 1986); N18 neuroblastoma cells (Barnes and Hille, 1988); rat skeletal muscle (Wang and Wang, 2003a)]. Especially, in cultured vascular and visceral smooth muscles, veratridine (100 μ M) mainly inhibited the peak amplitude of I_{Na} [cultured rat myometrial cells (Martin *et al.*, 1990); cultured human coronary myocytes (Quignard *et al.*, 1996; Boccara *et al.*, 1999); cultured human uterine leiomyosarcoma cell lines (Kusaka and Sperelakis, 1994); descending vasa recta (Zhang *et al.*, 2005)]. Thus, it seems that the effects of veratridine vary between molecular types of voltage-gated Na⁺ channels, depending on the subtypes of Na⁺ channels present (neuron, Na_v1.2; skeletal muscle, Na_v1.4; cardiac myocytes, Na_v1.5 etc. reviewed by Catterall *et al.*, 2005). In the present experiments, we have shown a bell-shaped relationship between the concentrations of veratridine and the peak amplitude of I_{Na} (acting mainly on Na_v1.6; see also Zhu *et al.*, 2008) in murine vas deferens

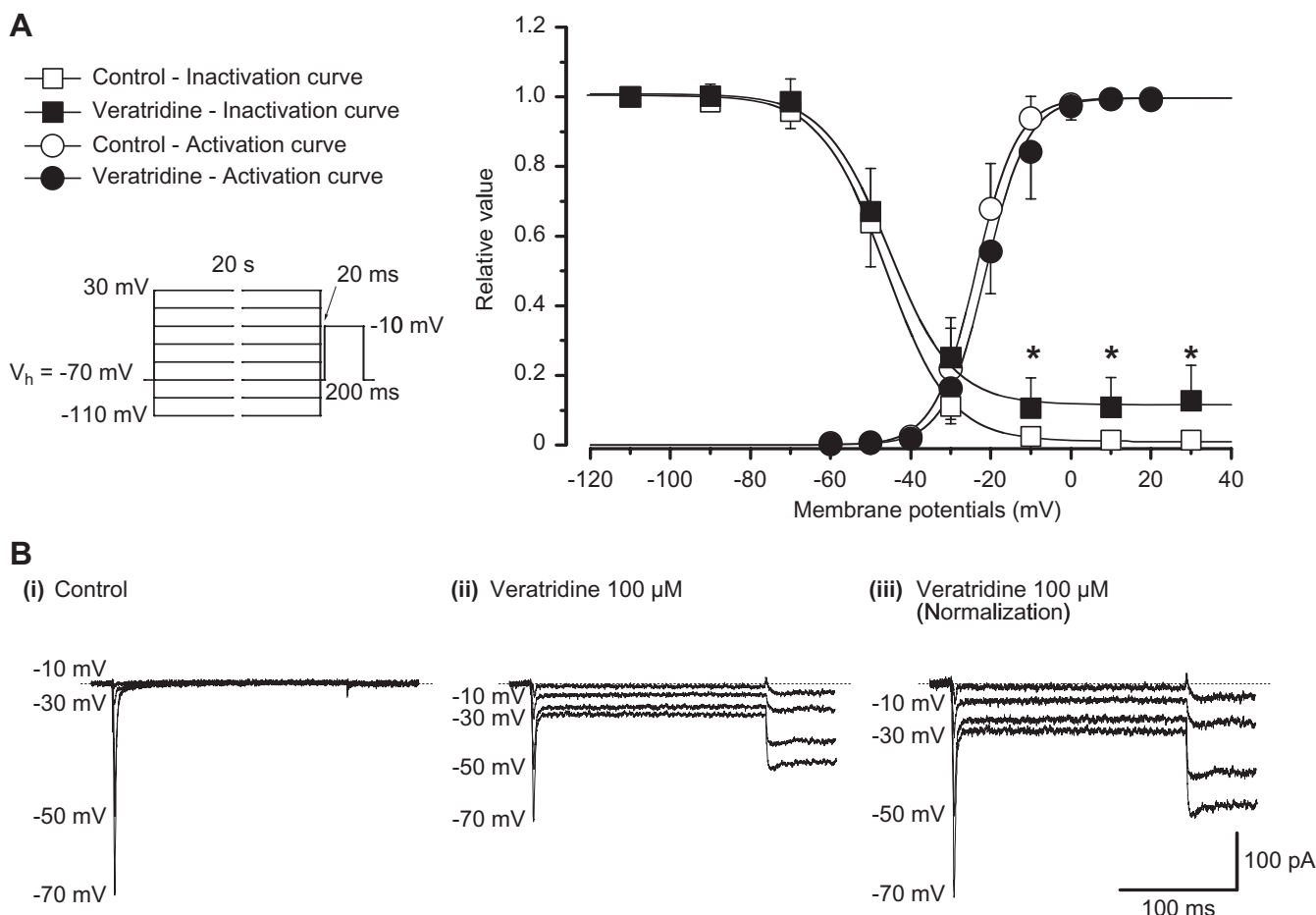


Figure 5 Effects of veratridine on the voltage-dependent activation and inactivation of I_{Na} in myocytes from mouse vas deferens. (A) The steady-state inactivation curves in the absence or presence of veratridine were drawn using the following values: $I_1 = 0.99$, $I_2 = 0.01$, $V_{half} = -45.8$ mV and $k = 8.0$ in control; $I_1 = 0.88$, $I_2 = 0.12$, $V_{half} = -45.5$ mV and $k = 8.0$ in the presence of veratridine for the peak amplitude of I_{Na} . The activation curves in the absence or presence of veratridine were drawn using the following values: $I_{max} = 1.0$, $V_{half} = -23.7$ mV and $k = 5.2$ in control; $I_{max} = 1.0$, $V_{half} = -21.2$ mV and $k = 5.2$ in the presence of veratridine for the peak amplitude of I_{Na} . Each symbol indicates the mean of five observations with \pm SD shown by vertical lines. Some of the SD bars are less than the size of the symbol. (B) Original current traces before (control, (i)) and after application of 100 μ M veratridine (ii) at the indicated conditioning pulse potentials. (iii) I_{Na} from (ii) scaled to match the peak amplitude of the current with no conditioning pulse in the absence of veratridine (i.e. control) are shown.

myocytes, reaching maximum enhancement at a concentration of around 10 μ M. These results indicate that veratridine possesses a dual effect on the peak amplitude of I_{Na} in myocytes from mouse vas deferens (i.e. in native cells expressing the sole functional voltage-gated Na⁺ channel, $Na_v1.6$), depending on its concentration. As veratridine (100 μ M) inhibited the peak amplitude of I_{Na} in cultured vascular and visceral smooth muscles, this is the first report to show smooth muscle type Na⁺ currents which were activated by veratridine at concentrations less than 10 μ M. Moreover, as veratridine is a potent agonist for voltage-gated Na⁺ channels with long-lasting opening times, some reports indicate that veratridine (100 μ M) alters the ion selectivity of I_{Na} , particularly in skeletal muscle preparations [see, e.g. rat skeletal muscle (Garber and Miller, 1987); frog muscle fibre (Leibowitz *et al.*, 1986)]. However, in the present set of experiments, when extracellular Na⁺ concentration was changed to an iso-osmotic solution of TEA⁺ in the presence of 100 μ M veratridine, the peak amplitude of I_{Na} was abolished, and on reintroduction of Na⁺ and removal of TEA⁺, the peak amplitude of I_{Na} readily recovered to the control level. These

results suggest that I_{Na} is carried mainly by Na⁺, without altering the ion selectivity even in the presence of veratridine. Moreover, it has also been reported that veratridine reduced the size of single-channel conductances to about one quarter of that in control (Barnes and Hille, 1988). It is possible that the reduction of the peak amplitude of I_{Na} when high concentrations of veratridine are applied (as observed in this study) is related to a decrease in single-channel conductance.

Veratridine-induced membrane currents in murine vas deferens

In vascular and visceral smooth muscles, two types of the veratridine-induced membrane currents can be detected; namely, the sustained currents during the depolarizing step pulses, which are thought to be due to incomplete fast inactivation, and the tail currents at repolarization, which are thought to be due to the toxin-bound Na⁺ channels remaining open for a prolonged period at the holding potential (Martin *et al.*, 1990; Kusaka and Sperelakis, 1994; Wang and Wang, 2003a). In murine vas deferens myocytes, application of

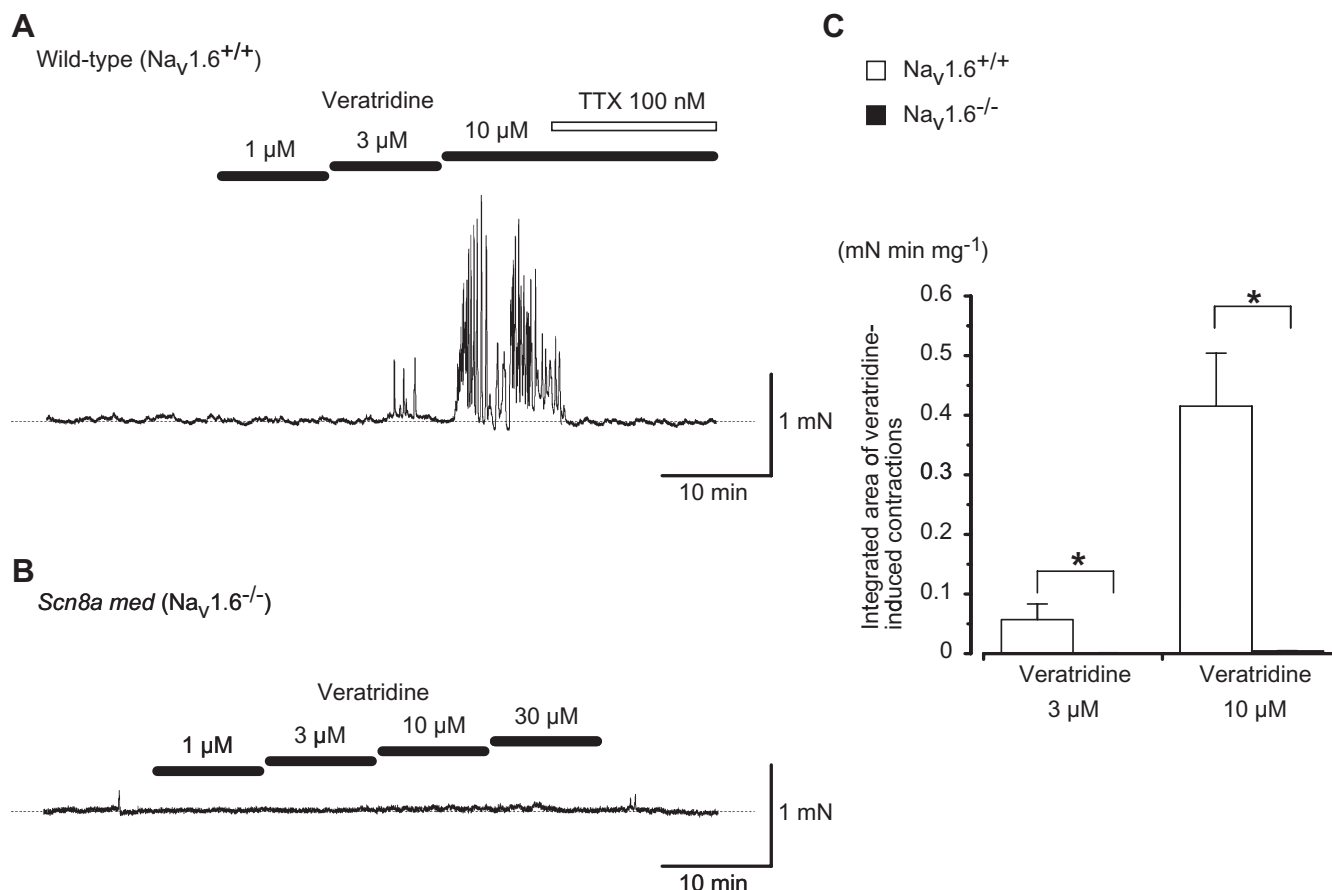


Figure 6 Veratridine-induced contractions involve activation of $\text{Na}_v1.6$ channels. (A) Veratridine-induced contractions in $\text{Na}_v1.6^{+/+}$ vas deferens. The cumulative application of veratridine resulted in rhythmical contractions which were abolished rapidly by subsequent application of tetrodotoxin (TTX) (100 nM). The dashed line indicates the mean resting tone of vas deferens. (B) Trace showing how the application of veratridine (1–30 μM) resulted in little change to baseline tension of vas deferens from a $\text{Na}_v1.6^{-/-}$ mouse. The dashed line indicates the mean resting tone of vas deferens. (C) Overall results showing veratridine (3 and 10 μM)-induced contractions in vasa deferentia from $\text{Na}_v1.6^{+/+}$ and $\text{Na}_v1.6^{-/-}$ mice. The integrated areas of the veratridine-induced contractions (the last 1 min duration) were calculated (mN min mg^{-1}). Each column indicates the mean + SD shown by vertical lines. Asterisk indicates a statistically significant difference and changes were considered significant at $P < 0.05$ (ANOVA test).

veratridine ($\geq 3 \mu\text{M}$) also induced sustained currents and tail currents. In the present study, both veratridine-induced membrane currents were abolished by additional application of 1 μM TTX in the presence of Cd^{2+} (showing sensitivity to TTX), and were reversibly suppressed by substitution of Na^+ with TEA^+ , Tris^+ and NMDG^+ (showing a selective Na^+ permeability). After long depolarizing pulses (≥ 15 s), these currents were inactivated, thereby suggesting that voltage-dependent components were involved. Using $\text{Na}_v1.6^{-/-}$ mice, neither sustained nor tail veratridine-induced membrane currents were detected in $\text{Na}_v1.6^{-/-}$ vasa deferentia, while both the tail and sustained veratridine-induced membrane currents were recorded in tissues from $\text{Na}_v1.6^{+/+}$ mice. Taken together, these results show unequivocally that the veratridine-induced membrane currents are mediated by TTX-sensitive voltage-gated Na^+ channels (i.e. $\text{Na}_v1.6$).

Kinetics studies of veratridine-induced I_{Na}

As veratridine shifted activation curves to more negative potentials by 40–90 mV, producing the sustained currents and the tail currents, it is generally believed that veratridine modi-

fies the activation gating kinetics of Na^+ channels, thereby allowing them to open more easily and to stay open longer (Leibowitz *et al.*, 1986; Hille, 1992; Sunami *et al.*, 1993). However, in the present experiments, the activation and inactivation curves for the peak amplitude of I_{Na} in mouse vas deferens were not shifted in the presence of 100 μM veratridine. Similarly, veratridine (100 μM) caused no shift of the activation and inactivation curves for the peak amplitude of I_{Na} in cultured smooth muscle cells (human uterine leiomyosarcoma cell lines, Kusaka and Sperelakis, 1994). In contrast, in the presence of veratridine, some I_{Na} remained as a sustained component during the depolarization step pulses from -50 mV to 40 mV. Furthermore, veratridine-induced tail currents were evoked after the rectangular step pulses. These results suggest that veratridine may modify the gating of $\text{Na}_v1.6$, allowing the channels to stay open longer after depolarization.

Molecular properties of the veratridine-induced I_{Na} in $\text{Na}_v1.6^{+/+}$ murine vas deferens

Saleh *et al.* (2005) reported that two different types of voltage-gated Na^+ channel proteins (i.e. $\text{Na}_v1.6$ and $\text{Na}_v1.7$) were

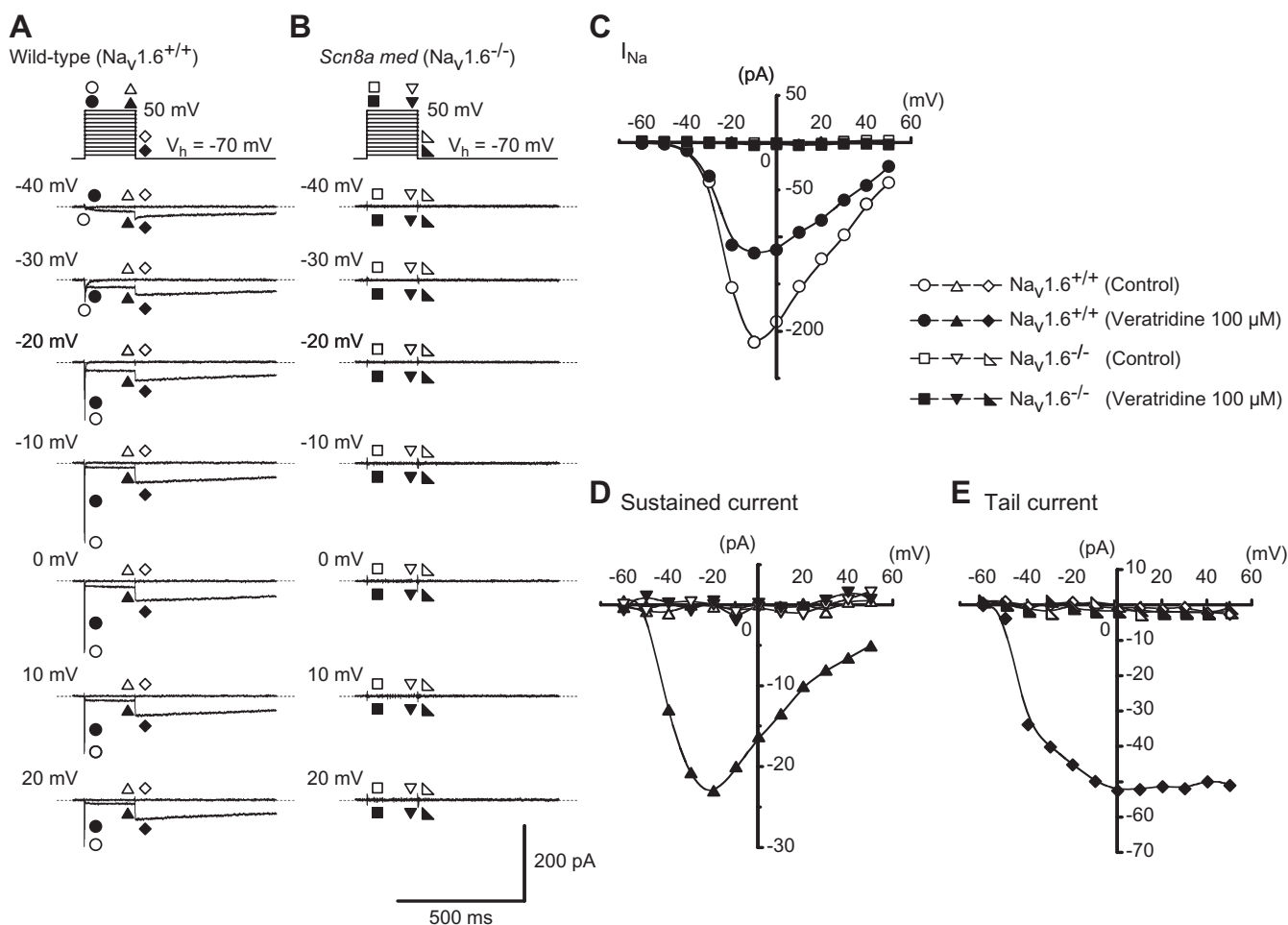


Figure 7 Comparative studies of the effects of veratridine (100 μM) on membrane currents in myocytes from vasa deferentia of $\text{Na}_v1.6^{+/+}$ and $\text{Na}_v1.6^{-/-}$ mice. (A) In $\text{Na}_v1.6^{+/+}$ cells, representative traces of the membrane currents in the absence and presence of veratridine at the indicated membrane potentials. (B) In $\text{Na}_v1.6^{-/-}$ cells, representative traces of the membrane currents in the absence and presence of veratridine at the indicated membrane potentials. (C) The current–voltage relationships in $\text{Na}_v1.6^{+/+}$ and $\text{Na}_v1.6^{-/-}$ myocytes. The current amplitude was measured as the peak amplitude of I_{Na} (within the first 10 ms of the depolarizing test pulse) and the peak amplitude of the tail current at each membrane potential. The mean amplitude of the veratridine-induced sustained currents was measured at the last 20 ms of the rectangular pulse. (D and E) Current–voltage relationships of the veratridine-induced sustained current (measured mean amplitude of the last 20 ms of the test pulse) and tail current (the peak amplitude) in $\text{Na}_v1.6^{+/+}$ and $\text{Na}_v1.6^{-/-}$ myocytes. The curves were drawn by eye.

localized in murine portal vein by use of immunohistological studies and they concluded that there were multiple types of voltage-gated Na⁺ channels in vascular smooth muscle. However, recently, we have reported that the molecular identity of the voltage-gated Na⁺ channels responsible for the TTX-sensitive I_{Na} in murine vas deferens myocytes is primarily $\text{Na}_v1.6$ (Zhu *et al.*, 2008). Furthermore, using $\text{Na}_v1.6^{-/-}$ mice, I_{Na} were not detected in dispersed smooth muscle cells from the vas deferens, while TTX-sensitive I_{Na} were recorded in $\text{Na}_v1.6^{+/+}$ (Zhu *et al.*, 2008). As veratridine has been used classically as a voltage-gated Na⁺ channel agonist, it is generally accepted that veratridine targets several types of voltage-gated Na⁺ channels, including $\text{Na}_v1.1$, $\text{Na}_v1.2$, $\text{Na}_v1.3$, $\text{Na}_v1.4$, $\text{Na}_v1.5$, $\text{Na}_v1.6$ and $\text{Na}_v1.7$ (Catterall *et al.*, 2005). In the present experiments, application of veratridine caused no further detectable membrane currents in $\text{Na}_v1.6^{-/-}$ tissues, while veratridine induced the sustained currents and the tail currents in $\text{Na}_v1.6^{+/+}$. These results strongly suggest that the sustained currents and the tail currents in mouse vas deferens

are primarily due to activation of $\text{Na}_v1.6$ channels, confirming the presence of $\text{Na}_v1.6$ as the predominant functional channel in mouse vas deferens.

In conclusion, we found that veratridine suppressed the peak amplitude of I_{Na} but induced sustained currents and the tail currents through $\text{Na}_v1.6$ channels. The present study also provides novel evidence that the $\text{Na}_v1.6$ channel is the major TTX-sensitive Na⁺ channel expressed in mouse vas deferens smooth muscle cells, and that the preparation could provide a unique experimental system for the study of $\text{Na}_v1.6$ channel function in native cells.

Acknowledgements

This work was supported by a Grant-in-Aid from the Japan Science and Technology Agency (15-B01-2008 to Noriyoshi Teramoto), a Grant-in-Aid for Exploratory Research from the Japanese Society for the Promotion of Science (19650733 to

Noriyoshi Teramoto) and a Grant-in-Aid for Scientific Research (B) from the Japanese Society for the Promotion of Science (20300178 to Noriyoshi Teramoto). This work was also supported by the Goho Life Sciences International Fund (2008 to Noriyoshi Teramoto). Dr Noriyoshi Teramoto was also supported by a grant from the Japanese Society for Scientist Exchange Program between the Japan Society for the Promotion of Science and The Royal Society (2006-1-36-RS to Noriyoshi Teramoto). Dr Hai-Lei Zhu was awarded by a Grant-in-Aid from the Japan Society for the Promotion of Science (FY2007 JSPS Postdoctoral Fellowship for Foreign Researcher, P 07196 to Noriyoshi Teramoto). Dr Thomas C. Cunnane is supported by the Wellcome Trust (069768) and Mr Richard D. Wassall is a British Pharmacological Society A.J. Clark Student. Part of this study has been communicated to the 81st Annual Meeting of the Japanese Pharmacological Society in an abstract form (Zhu and Teramoto, 2008).

Conflict of interest

None.

References

- Alexander SPH, Mathie A, Peters JA (2008). Guide to Receptors and Channels (GRAC), 3rd edition (2008 revision). *Br J Pharmacol* **153** (Suppl. 2): S1–S209.
- Barnes S, Hille B (1988). Veratridine modifies open sodium channels. *J Gen Physiol* **91**: 421–443.
- Boccarda G, Choby C, Frapier JM, Quignard JF, Nargeot J, Dayanithi G *et al.* (1999). Regulation of Ca²⁺ homeostasis by atypical Na⁺ currents in cultured human coronary myocytes. *Circ Res* **85**: 606–613.
- Burgess DL, Kohrman DC, Galt J, Plummer NW, Jones JM, Spear B *et al.* (1995). Mutation of a new sodium channel gene, Scn8a, in the mouse mutant 'motor endplate disease. *Nat Genet* **10**: 461–465.
- Campos FV, Moreira TH, Beirão PS, Cruz JS (2004). Veratridine modifies the TTX-resistant Na⁺ channels in rat vagal afferent neurons. *Toxicol* **43**: 401–406.
- Catterall WA, Goldin AL, Waxman SG (2005). International union of pharmacology. XLVII. Nomenclature and structure-function relationships of voltage-gated sodium channels. *Pharmacol Rev* **57**: 397–409.
- Chen YF, Lin YT, Tan TW, Tsai HY (2002). Effects of veratrine and paeoniflorin on isolated mouse vas deferens. *Phytomedicine* **9**: 296–301.
- Cuprian AM, Solanki P, Jackson MV, Cunnane TC (2005). Cholinergic innervation of the mouse isolated vas deferens. *Br J Pharmacol* **146**: 927–934.
- Garber SS, Miller C (1987). Single Na⁺ channels activated by veratridine and batrachotoxin. *J Gen Physiol* **89**: 459–480.
- Hille B (1992). Modifiers of gating. In: Hille B (ed.). *Ionic Channels of Excitable Membranes*, 2nd edn. Sinauer Associates: Sunderland, MA, pp. 445–471.
- Kohrman DC, Harris JB, Meisler MH (1996). Mutation detection in the med and medJ alleles of the sodium channel Scn8a. Unusual splicing due to a minor class AT-AC intron. *J Biol Chem* **271**: 17576–17581.
- Kusaka M, Sperelakis N (1994). Veratridine actions on two types of fast Na⁺ channels in human uterine leiomyosarcoma cells. *Eur J Pharmacol* **271**: 387–393.
- Leibowitz MD, Sutro JB, Hille B (1986). Voltage-dependent gating of veratridine-modified Na channels. *J Gen Physiol* **87**: 25–46.
- Martin C, Arnaudeau S, Jmari K, Rakotoarisoa L, Sayet I, Dacquet C *et al.* (1990). Identification and properties of voltage-sensitive sodium channels in smooth muscle cells from pregnant rat myometrium. *Mol Pharmacol* **38**: 667–673.
- Nánási PP, Varró A, Lathrop DA, Bryant SH (1994). Effects of veratridine on Na and Ca currents in frog skeletal muscle. *Gen Pharmacol* **25**: 1661–1666.
- Quignard JF, Ryckwaert F, Albat B, Nargeot J, Richard S (1997). A novel tetrodotoxin-sensitive Na⁺ current in cultured human coronary myocytes. *Circ Res* **80**: 377–382.
- Rando TA (1989). Rapid and slow gating of veratridine-modified sodium channels in frog myelinated nerve. *J Gen Physiol* **93**: 43–65.
- Saleh S, Yeung SY, Prestwich S, Pucovsky V, Greenwood I (2005). Electrophysiological and molecular identification of voltage-gated sodium channels in murine vascular myocytes. *J Physiol* **568**: 155–169.
- Sutro JB (1986). Kinetics of veratridine action on Na channels of skeletal muscle. *J Gen Physiol* **87**: 1–24.
- Sunami A, Sasano T, Matsunaga A, Fan Z, Swanobori T, Hiraoka M (1993). Properties of veratridine-modified single Na⁺ channels in guinea pig ventricular myocytes. *Am J Physiol* **264**: H454–H463.
- Teramoto N, Brading AF (1996). Activation by levromakalim and metabolic inhibition of glibenclamide-sensitive K channels in smooth muscle cells of pig proximal urethra. *Br J Pharmacol* **118**: 635–642.
- Zhang Z, Cao C, Lee-Kwon W, Pallone TL (2005). Descending vasa recta pericytes express voltage operated Na⁺ conductance in the rat. *J Physiol* **567**: 445–457.
- Zhu HL, Teramoto N (2008). Modulation of voltage-gated Na⁺ currents in the murine vas deferens by veratridine. *J Pharmacol Sci* **106** (Suppl. I): 249P, P3J–19.
- Zhu HL, Aishima M, Morinaga H, Wassall RD, Shibata A, Iwasa K *et al.* (2008). Molecular and biophysical properties of voltage-gated Na⁺ channels in murine vas deferens. *Biophys J* **94**: 3340–3351.
- Zong XG, Dugas M, Honerjäger P (1992). Relation between veratridine reaction dynamics and macroscopic Na current in single cardiac cells. *J Gen Physiol* **99**: 683–697.
- Wang GK, Wang SY (2003a). Veratridine block of rat skeletal muscle NaV1.4 sodium channels in the inner vestibule. *J Physiol* **548**: 667–675.
- Wang SY, Wang GK (2003b). Voltage-gated sodium channels as primary targets of diverse lipid-soluble neurotoxins. *Cell Signal* **15**: 151–159.
- Wang GK, Quan C, Seaver M, Wang SY (2000). Modification of wild-type and batrachotoxin-resistant muscle $\mu 1$ Na⁺ channels by veratridine. *Pflügers Arch* **439**: 705–713.