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Potent modulation of the voltage-gated sodium channel $Na_v1.7$ by OD1, a toxin from the scorpion *Odonthobuthus doriae*

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Running title: Scorpion toxin OD1 is a potent modulator of $Na_v1.7$

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ABSTRACT

Voltage-gated sodium channels are essential for the propagation of action potentials in nociceptive neurons. $Na_v1.7$ is found in peripheral sensory and sympathetic neurons and involved in acute and inflammatory pain. $Na_v1.8$ and $Na_v1.3$ are major players in nociception and neuropathic pain, respectively. In our effort to identify isoform-specific and high-affinity ligands for these channels, we investigated the effects of OD1, a scorpion toxin isolated from the venom of the scorpion *Odonthobuthus doriae*. $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$ channels were coexpressed with β_1-subunits in *Xenopus* oocytes. Na^+ currents were recorded with the two-electrode voltage-clamp technique. OD1 modulates $Na_v1.7$ at low nanomolar concentrations: (1) Fast inactivation is dramatically impaired, with an EC_{50} value of 4.5 nM. (2) OD1 substantially increases the peak current at all voltages. (3) OD1 induces a substantial persistent current. $Na_v1.8$ was not affected by concentrations up to 2 micromolar, whereas $Na_v1.3$ was only sensitive to concentrations higher than 100 nM. OD1 impairs the inactivation process of $Na_v1.3$ with an EC_{50} value of 1127 nM. Finally, the effects of OD1 were compared with a classical α-toxin, AahII from *Androctonus australis hector* and a classical α-like toxin, BmK M1 from *Buthus martensii* Karsch. At a concentration of 50 nM, both toxins affected $Na_v1.7$. $Na_v1.3$ was sensitive to AahII, but not to BmK M1, whereas $Na_v1.8$ was not affected by both toxins. In conclusion, the present study shows that the scorpion toxin OD1 is a potent modulator of $Na_v1.7$, with a unique selectivity pattern.

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Voltage gated sodium channels (VGSC) are the signature channels of excitable cells. The channels are large and complex proteins that open transiently upon membrane depolarization, giving the upstroke of the action potential. They consist of a pore forming α-subunit and auxiliary β-subunits. To date, ten mammalian α-subunits ($Na_v1.1$-$Na_v1.9$, Na_x) and 4 β-subunits have been cloned. The β-subunits modulate the localization, expression and functional properties of α –subunits (Catterall et al., 2005). Different α-subunits have distinct electrophysiological and pharmacological properties and they are targeted by a large variety of chemically different toxins from animal venoms and plants (Wang and Wang, 2003). Many loss- as well as gain-of-function mutations of α-subunits have been identified in human conditions characterized with epilepsy, seizures, ataxia and increased sensitivity to pain (Meisler and Kearney, 2005).

Physiological and pharmacological evidence has demonstrated a critical role for VGSCs in many types of pain syndromes (Wood et al., 2004). Two VGSCs, $Na_v1.8$ and $Na_v1.9$, are expressed selectively in damage-sensing peripheral neurons, while a third channel, $Na_v1.7$, is found predominantly in sensory and sympathetic neurons and being implicated with inflammatory pain. An embryonic channel, $Na_v1.3$, is also upregulated in damaged peripheral nerves and associated with increased electrical excitability in neuropathic pain states. A combination of antisense and knock-out studies support a specialized role for these VGSCs in pain pathways, and pharmacological studies with some peptidyl toxins suggest that isoform-specific antagonists should be feasible and therefore could be useful analgesics (Wood et al., 2004).

In addition to its role in inflammatory pain (Nassar et al., 2004; Yeomans et al., 2005), $Na_v1.7$ has been implicated in other pathophysiological conditions. Recent work has shown

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that autosomal dominant erythermalgia is associated with mutations in this VGSC (Dib-Hajj et al., 2005; Drenth et al., 2005; Michiels et al., 2005; Waxman and Dib-Hajj, 2005). Furthermore, functional expression of $Na_v1.7$ has been linked with strong metastatic potential in prostate cancer and this channel has been suggested to be a functional diagnostic marker (Diss et al., 2005).

Despite its clinical importance, the pharmacological characterization of $Na_v1.7$ is not very elaborate and a selective high-affinity modulator is still missing. The local anesthetic and class I anti-arrhythmic lidocaine, a well-known VGSC modulator, decreases $Na_v1.7$ currents in a frequency-dependent matter, with an EC_{50} value of 450 μM (Chevrier et al., 2004). In addition, recent work showed that the T-type Ca^{2+} channel antagonist mibefradil is a state-dependent VGSC modulator, blocking $Na_v1.2$, $Na_v1.4$, $Na_v1.5$ and $Na_v1.7$ at low micromolar concentrations (McNulty and Hanck, 2004). Two tarantula peptides, ProTx-I and ProTx-II, isolated from *Thrixopelma pruriens*, inhibit activation of $Na_v1.2$, $Na_v1.5$, $Na_v1.7$ and $Na_v1.8$ in the nanomolar range (Middleton et al., 2002). Heinemann and coworkers demonstrated that the scorpion α-toxins Lqh-2 and Lqh-3 from *Leiurus quinquestriatus hebraeus*, previously described as potent modulators of $Na_v1.5$ (Chen and Heinemann, 2001) and $Na_v1.4$ (Chen et al., 2000), impair the inactivation process of $Na_v1.7$ at low nanomolar concentrations (Chen et al., 2002).

In the field of scorpion toxinology, the Asian scorpion *Buthus martensii* Karsch has received notable attention with reference to pain. From its venom, a number of analgesic peptides have been reported, for most of which it can be assumed that they could target VGSCs by virtue of their primary structure homology and similar scaffold with so called

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long-chain sodium channel toxins (Goudet et al., 2002). They are: BmK ITAP, an excitatory insect-selective toxin (Xiong et al., 1999) and BmK dITAP3, a depressant insect-selective toxin (Guan et al., 2001a), with an analgesic effect in mice, BmK AGAP, an antitumor analgesic peptide showing inhibitory effect on both visceral and somatic pain (Liu et al., 2003) and BmK Ang P1 (Guan et al., 2001b), BmK AngM1 (Cao et al., 2004), BmK AS (Chen and Ji, 2002), BmK IT2 (Wang et al., 2000), BmK I1, BmK I4 and BmK I6 (Guan et al., 2000), all peptides for which analgesic properties in mice have been demonstrated. These findings indicate that scorpion toxins can be a valuable source of potential analgesics.

In the present study we investigated the effect of the recently discovered scorpion toxin OD1 (Figure 1), on 3 VGSCs implicated in pain sensation, namely $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$. Most scorpion neurotoxins targeting VGSCs are single chain polypeptides composed of 60-70 amino acids cross-linked by 4-disulfide bridges. They comprise two main groups: α- and β-toxins (Possani et al., 1999). Scorpion α-toxins bind to site 3 and slow down the inactivation process. According to their different pharmacological and binding properties, the α-toxins can be further divided into three subgroups, classical α-, α-like and insect α-toxins. Classical α-toxins (*e.g.* AahII, Lqh-2) are highly toxic to mammals, whereas the insect α-toxins (*e.g.* LqhαIT) are highly toxic to insects. The α-like toxins (*e.g.* Lqh-3, BmK M1) act on both mammals and insects (Gordon et al., 1996; Goudet et al., 2002; Rodriguez de la Vega RC and Possani, 2005). OD1 is the first toxin isolated from the Iranian yellow scorpion *Odonthobuthus doriae* and was recently characterized as an α-like toxin. Jalali *et al.* showed that the inactivation process of the insect channel, para/tipE, was

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severely hampered by 200 nM of OD1 (EC_{50} = 80 ± 14 nM) while $Na_v1.2/\beta_1$ still was not affected at concentrations up to 5 μM**.** $Na_v1.5/\beta_1$ was only influenced at micromolar concentrations (Jalali et al., 2005).

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MATERIALS AND METHODS

Sodium channel expression in Xenopus laevis oocytes

For *in vitro* transcription, rβ_1/pSP64T was first linearized with *Eco*RI. Next, capped cRNA's were synthesized from the linearized plasmid using the large-scale SP6 mMESSAGE-mMACHINE transcription kit (Ambion, U.S.A.). The hNav1.8/pBSTA, rNav1.7/pBSTA, rNav1.3/pNa3T and the hβ_1/pGEM-HE vector were linearized with respectively, *Not*I, *Sac*II, *Not*I and *Nhe*I, and transcribed with the T7 mMESSAGE-mMACHINE kit (Ambion, U.S.A.).

The harvesting of oocytes from anaesthetized female *Xenopus laevis* frogs was as previously described (Tytgat et al., 1997). Oocytes were injected with 50 nl of cRNA at a concentration of 1 ng nl^{-1} using a Drummond micro-injector (U.S.A.). The solution used for incubating the oocytes contained (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4), supplemented with 50 mg l^{-1} gentamycin sulphate and 180 mg l^{-1} theophyllin (except for $Na_v1.7$).

Electrophysiological measurements

Sodium currents were recorded using the *Xenopus laevis* expression system. Two-electrode voltage-clamp recordings were performed at room temperature (18° – 22° C) using a GeneClamp 500 amplifier controlled by a pClamp data acquisition system (Molecular Devices, Axon instruments, USA). Whole-cell currents from oocytes were recorded 2-4 days after injection. Current and voltage electrodes had resistances as low as possible (0.2 – 1 MΩ) and were filled with 3 M KCl. Currents were sampled at 5 kHz and filtered at 1 kHz

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using a four-pole low-pass Bessel filter. Leak subtraction was performed using a -P/4 protocol. To eliminate the effect of the voltage drop across the bath-grounding electrode, the bath potential was actively controlled. Voltage records were carefully monitored on an oscilloscope (Hameg).

The bath solution (ND-96 solution) was composed of (in mM): NaCl 96, KCl 2, $CaCl_2$ 1.8, $MgCl_2$ 2 and HEPES 5 (pH 7.4). Toxins were added directly to the recording chamber from a stock solution in ND-96, to obtain the desired final concentration. Immediately after adding the toxin stock solution at some distance from the oocyte, the bath solution was mixed in order to obtain a homogenous final concentration within a few seconds.

For activation protocols, 100 ms test depolarisations ranging from -45 mV to +70 mV were applied from a holding potential of -100 mV in 5 mV increments, at an interval of 5 s. Voltage-dependent steady-state inactivation was determined by means of a double-pulse protocol in which a conditioning pulse was applied from a holding potential of -100 mV to a range of potentials from -90 (or -70) mV to 0 (or -5) mV in 5 mV increments for 50 ms, immediately followed by a test pulse to 0 (or -5) mV. The peak current amplitudes during the tests were normalized to the amplitude of the first pulse and plotted against the potential of the conditioning pulse. The voltage dependence of the relative current (activation and fast inactivation) was fit by a Boltzmann function. Recovery from fast inactivation was examined using a standard double-pulse protocol. A 25 ms conditioning pulse to 0 mV was used to fully fast-inactivate the channel. The membrane was then hyperpolarized to -100 mV from 0 to 160 ms, and then the recovery was monitored by measuring the relative peak Na^+ current elicited by a second pulse to 0 mV. The interval between the pulses was 5 s. The recovery of the peak amplitude was fitted with a double exponential.

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Data were analyzed in Winascd (Guy Droogmans, KULeuven, Belgium) and in Origin (Originlab Corporation, USA).

Quantification of toxin effects

To assay the complex effects of the toxin, two parameters were determined. (1) The increased influx of Na^+ ions at different voltages was quantified by measuring the area under the current-voltage curve (AUC) in control conditions and in the presence of the toxin. (2) The degree of fast inactivation was assayed by measuring the peak current as well as the current amplitude at 30 ms after the start of the depolarization. The ratio I_{30ms}/I_{peak} gives an estimate for the fast inactivation process and similar parameters have been used by others as a measure of fast inactivation (Chen et al., 2002).

The dose dependence for toxin-induced effects was measured by plotting the parameter I_{30ms}/I_{peak} as a function of toxin concentration. The concentration dependence was described with the following dose-response equation [1]:

$$\text{effect} = A_1 + \frac{A_2 - A_1}{1 + \left(\frac{EC_{50}}{[\text{toxin}]} \right)^p}$$

where p is the Hill coefficient, [toxin] the toxin concentration and EC_{50} the concentration of half-maximal effect. A_1 is the offset and was determined from experiments in the absence of toxin ([toxin] = 0). For fitting the data, the A_1 value was fixed to the value obtained under control conditions. The value A_2 is measure for the maximal effect of the toxin, and was determined from the fits.

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RESULTS

Differential effects of OD1 on $Na_v1.7$, $Na_v1.3$ and $Na_v1.8$

Figure 2 shows representative whole cell Na^+ currents recorded from oocytes expressing $Na_v1.7$, $Na_v1.3$ and $Na_v1.8$ in control conditions (upper panels) and in the presence of 50 nM ($Na_v1.7$), 100 nM ($Na_v1.3$) and 1000 nM ($Na_v1.8$) OD1 (middle panels). Na^+ currents were evoked by applying a series of depolarizing voltage steps between -45 and +70 mV in 5 mV increments. The lower panels show the corresponding I-V curves in control conditions (filled squares) and in the presence of OD1 (open squares).

Compared to control conditions, 50 nM OD1 has dramatic effects on $Na_v1.7$ currents: (1) the inactivation is impaired, (2) there is a substantial persistent current at the end of the 100 ms voltage step and (3) there is an increase in inward peak current. This last effect is visible at all voltages, and was quantified by measuring the area under the I-V curve (AUC). 50 nM OD1 increases the AUC with 170 ± 10 % (n = 4). The impairment of the inactivation process becomes clear by comparing the current amplitude at 30 ms (vertical arrow) for control conditions and in the presence of 50 nM OD1. The inset shows the corresponding I_{30ms}-voltage relation. At 50 nM OD1, $Na_v1.3$ and $Na_v1.8$ were not affected. $Na_v1.8$ is completely insensitive to 1000 nM OD1, whereas $Na_v1.3$ was unaffected by concentrations up to 100 nM.

$Na_v1.3$ is affected by higher concentrations of OD1. The modulation of $Na_v1.3$ by 500 nM OD1 is less dramatic, but similar to the effects of 50 nM OD1 to $Na_v1.7$: there is an increase in peak current, a larger persistent current at the end of the test pulse and an impairment of the fast inactivation process.

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We used this last parameter to quantify and compare the effects of OD1 on these two VGSCs. Figure 3 shows the relative I_{30ms}/I_{peak} in function of increasing concentrations of OD1 for $Na_v1.7$ (squares) and $Na_v1.3$ (circles). Each data point represents the mean ± SEM (n = 3-6). The dotted line represents the best fit of the data to the dose-response equation described above. The EC_{50} value and Hill coefficient were, respectively, 4.5 ± 0.2 nM and 1.5 ± 0.1 for $Na_v1.7$ and 1127 ± 263 nM and 1.1 ± 0.2 for $Na_v1.3$. These values demonstrate that OD1 displays a selectivity for $Na_v1.7$.

Effects of OD1 on the gating of $Na_v1.7$ and $Na_v1.3$

The availability of Na^+ channels upon depolarization is dependent on the cell membrane resting potential. Fewer channels become available as the resting membrane potential progressively moves towards more depolarized voltages. This effect is due to the accumulation of channels in the nonconducting inactivated state. Experimentally, this phenomenon was measured using constant conditioning pulses to voltages between -90 and 0 mV. The fraction of available current left was measured by standard test pulses (0 mV for $Na_v1.7$ and -5 mV for $Na_v1.3$). The normalized currents were then plotted against the conditioning voltage in the absence and presence of OD1 (Figure 4, upper panel $Na_v1.7$, lower panel $Na_v1.3$). 50 nM OD1 does not significantly shift the $V_{1/2}$ of inactivation for $Na_v1.7$ ($p < 0.05$) (Table 1). However, OD1 has clear effects on the completeness of the inactivation. At 0 mV, the availability was 0.53 ± 0.65 % in control conditions, but 9.4 ± 0.5 % in the presence of 50 nM OD1. For $Na_v1.3$, 500 nM OD1 induced a non-significant small depolarizing shift of 3 mV in the $V_{1/2}$ of inactivation ($p < 0.05$). $V_{1/2}$ and slope factors are depicted in Table 1. As for $Na_v1.7$, the inactivation of $Na_v1.3$ in the presence of OD1 is

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less complete. The percentage of available channels at -5 mV was 3.3 ± 0.5 % in control conditions and 13.9 ± 1 % in the presence of 500 nM OD1.

The effect of OD1 on the activation of the two channels was also investigated. The activation curves were derived from the I-V curves. The activation curves of $Na_v1.7$ and $Na_v1.3$ in the absence and presence of OD1 were plotted against voltage (Figure 4, upper panel $Na_v1.7$, lower panel $Na_v1.3$). For $Na_v1.7$, 50 nM OD1 caused a 3 mV hyperpolarizing shift of the midpoint of activation. This shift was not significant ($p < 0.05$, Table 1), but became larger with higher concentrations of OD1 (data not shown). For $Na_v1.3$, 500 nM OD1 did not affect the activation process (Table 1).

Effect of OD1 on the recovery from fast inactivation of $Na_v1.7$

Next, we examined the recovery from fast inactivation in the absence and presence of OD1. Figure 5 shows the fraction of recovered $Na_v1.7$ channels for control conditions and in the presence of 100 nM OD1 (mean ± SEM, n = 3). The two time constants (see inset) are 8.8 ± 0.2 ms and 46.8 ± 5.3 ms for the control condition and 1.8 ± 0.2 and 11.8 ± 0.3 ms in the presence of OD1, indicating that OD1 accelerates the recovery from fast inactivation of $Na_v1.7$.

Comparison of OD1 effects with AahII and BmK M1

To investigate whether the multiple effects were typical for OD1, we compared OD1 with a classical α-toxin, AahII from *Androctonus australis hector* (Rochat et al., 1972), and a classical α-like toxin, BmK M1 from *Buthus martensii* Karsch (Ji et al., 1996) (Fig. 1). Figure 6A and D show $Na_v1.7$ current traces in response to a 100 ms voltage step to 0 mV

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in control conditions and in the presence of 50 nM AahII or 50 nM BmK M1. Like OD1, both AahII and BmK M1 induce (1) an increase in peak current, (2) an impairment of the inactivation process and (3) a large persistent current at the end of 100 ms voltage step, indicating that these 3 effects are not unique to OD1. The absolute values of the parameter I_{30ms}/I_{peak} were 0.17 ± 0.01 for 50 nM OD1, 0.18 ± 0.02 for 50 nM AahII and 0.12 ± 0.01 for 50 nM BmK M1. Those values represent the mean ± SEM of at least 3 experiments. For comparison, in control conditions this value was 0.048 ± 0.009 (n = 9), implying that the ratio I_{30ms}/I_{peak} increases 3.6 fold for 50 nM OD1, 3.8 fold for 50 nM AahII and 2.5 fold for 50 nM BmK M1. Fig. 6B and E show the corresponding current-voltage relation recorded from the same oocyte as in Fig. 6A and D respectively. At a concentration of 50 nM, both toxins substantially increase the area under the I-V curve. For this representative example, the increase in the AUC was 200 % for AahII and 160 % for BmK M1.

The effects of 50 nM AahII and BmK M1 on the gating of $Na_v1.7$ are depicted in the lower panels (Fig. 6 C and F). Table 2 (AahII) and Table 3 (BmK M1) show the corresponding $V_{1/2}$ values and slope factors, as determined by fitting the data points to the Boltzman equation. The activation process of $Na_v1.7$ is not affected by 50 nM of AahII or BmK M1, whereas the steady-state inactivation is only modified by AahII. The latter causes a significant ($p < 0.05$) depolarizing shift of 5.3 mV in the $V_{1/2}$. Similar to OD1, both toxins reduce the completeness of steady-state inactivation. The percentage of available channels at -5 mV was 14.5 ± 3.4 % in the presence of 50 nM AahII and 9.7 ± 0.4 % the presence of 50 nM BmK M1.

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In addition, we also investigated the effects of 50 nM of AahII and BmK M1 on $Na_v1.3$ and $Na_v1.8$. At this concentration, $Na_v1.8$ was not affected by AahII ($n \geq 5$), nor BmK M1 ($n \geq 4$). In addition, concentrations up to 500 nM were tested and did not induce any change in $Na_v1.8$ currents. $Na_v1.3$ however is sensitive to 50 nM AahII. Figure 7A shows $Na_v1.3$ current traces in response to a 100 ms voltage step to -5 mV in control conditions and in the presence of 50 nM AahII. AahII induces (1) an increase in peak current, (2) an impairment of the inactivation process and (3) a large persistent current at the end of 100 ms voltage step. The absolute values of the parameter I_{30ms}/I_{peak} were 0.23 ± 0.02 in control and 0.70 ± 0.01 in the presence of 50 nM AahII ($n \geq 4$), resulting in a 3.1 fold increase of the I_{30ms}/I_{peak} ratio. Fig. 7 B shows the corresponding current-voltage relation recorded from the same oocyte. At a concentration of 50 nM, AahII increases the AUC with 240 %. The effects on the activation and steady-state inactivation curves of $Na_v1.3$ is depicted in Fig. 6C. The corresponding $V_{1/2}$ values and slope factors, as determined by fitting the data points to the Boltzman equation, are shown in Table 2. 50 nM AahII causes a 8.7 mV hyperpolarizing shift in the $V_{1/2}$ for activation. The $V_{1/2}$ for steady-state inactivation was not affected, but the completeness of steady-state inactivation changed dramatically in the presence of 50 nM AahII. The percentage of available channels at -5 mV increased from 3.7 ± 0.5 % in control conditions to 53.9 ± 2.9 % in the presence of 50 nM AahII.

Nav1.3 was not sensitive to 50 nM BmK M1. Figure 7D shows $Na_v1.3$ current traces in response to a 100 ms voltage step to -5 mV in control conditions and in the presence of 50 nM BmK M1. Fig. 7E shows the current-voltage curve recorded from the same oocyte, demonstrating that BmK M1 does not affect this channel. Higher concentrations (up to 500 nM) did not induce significant changes in $Na_v1.3$ currents (data not shown). The activation and steady-state inactivation curves are depicted in Fig. 7F. The corresponding $V_{1/2}$ values

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and slope factors, as determined by fitting the data points to the Boltzman equation, are shown in Table 3. BmK M1 at a concentration of 50 nM did not induce any significant ($p < 0.05$) in the activation or inactivation parameters.

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DISCUSSION

The aim of this study was to examine the effects of the recently discovered scorpion toxin OD1 (Fig. 1), on 3 VGSCs involved in pain sensation, namely $Na_v1.3$, $Na_v1.7$ and $Na_v1.8$. We showed that they had different sensitivities to OD1. $Na_v1.7$ was 250-fold more sensitive to OD1 than Nav1.3, whereas $Na_v1.8$ was not affected at the tested concentrations. The EC_{50} value for modulation of $Na_v1.7$ is 4.5 nM, demonstrating that OD1 is one of the most potent ligands for $Na_v1.7$ described at present.

OD1 was recently characterized as an α-like toxin. Jalali *et al.* showed that the inactivation process of the insect VGSC, para, was severely hampered by 200 nM of OD1. However, the mammalian VGSCs $Na_v1.2$ and $Na_v1.5$ were not at all affected by concentrations up to 1 μM (Jalali et al., 2005). Unlike OD1, the other toxins known as $Na_v1.7$ modulators do not exhibit this selectivity pattern. Heinemann and coworkers demonstrated that the scorpion toxin Lqh-3 (Fig. 1) from *Leiurus quinquestriatus hebraeus* impairs fast inactivation of $Na_v1.7$ with an EC_{50} value of 14 nM, but that $Na_v1.5$ is even more sensitive (EC_{50} 2.5 nM). Lqh-2 (Fig. 1) was shown to restrain the fast inactivation process of $Na_v1.7$ with an EC_{50} value of 32 nM, whereas the values for $Na_v1.2$ and $Na_v1.5$ were 1.8 nM and 12 nM, respectively (Chen and Heinemann, 2001; Chen et al., 2002). In addition, the tarantula peptides ProTx-I and ProTx-II inhibit the activation of $Na_v1.7$ as well as other VGSCs ($Na_v1.2$, $Na_v1.5$ and $Na_v1.8$) with IC_{50} values below 100 nM (Middleton et al., 2002).

This work demonstrates that OD1 affects the gating of $Na_v1.7$ at low nanomolar concentrations, resulting in (1) an impairment of fast inactivation, (2) a marked increase in peak Na^+ influx and (3) a substantial persistent Na^+ current, compared to control conditions. This non-inactivating current is reflected by the failure of steady state availability to reach zero, even at positive potentials. As a consequence, in the presence of OD1, channels will

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conduct much more inward Na^+ current than in the absence of the toxin. To examine whether these multiple effects are typical for OD1, it was compared with a classical α-toxin, AahII (Fig. 1) from *Androctonus australis hector* (Rochat et al., 1972), and a classical α-like toxin, BmK M1 (Fig. 1) from *Buthus martensii* Karsch (Ji et al., 1996). Our data show that these multiple effects on $Na_v1.7$ are not exceptional for OD1, since the effects of AahII and BmK M1 were similar to OD1. However, in contrast to those toxins, OD1 is unique in its selectivity for $Na_v1.7$ over $Na_v1.2$, $Na_v1.3$ and $Na_v1.5$. Unlike OD1, AahII is a potent modulator of rat brain sodium channels and it was shown that 0.5 nM of this toxin has dramatic effects on the inactivation process (Gordon et al., 1996). Our data demonstrate that 50 nM AahII severely hampers fast inactivation of $Na_v1.3$. In addition, BmK M1 impairs the inactivation of $Na_v1.5$ channels with an EC_{50} value of 195 nM (native toxin) (Goudet et al., 2001) or 500 nM (recombinant BmK M1) (Liu et al., 2005).

Some scorpion neurotoxins show specificity for insect or mammalian VGSCs and others are able to discriminate between VGSCs subtypes. This selectivity is attributed to differences in active sites on the toxins and to variations in receptor binding sites on distinct VGSCs. All scorpion α-toxins bind to receptor site 3, which involves the extracellular loop between segments S3 and S4 of domain IV of the VGSC (Rogers et al., 1996) and the extracellular loops S5-S6 of domain I and IV (Tejedor and Catterall, 1988; Thomson and Catterall, 1989). Mutagenesis within the external linker S3-S4 in domain IV of rat $Na_v1.2$ identified a negatively charged residue, Glu1613 as a major determinant that affects the binding (Rogers et al., 1996). The authors propose that non-acidic residues in the extracellular loops S5-S6 of domain I and IV may contribute to α-scorpion toxin binding by providing unique determinants that are involved in the interactions between the toxin

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and the channel. In order to understand the pharmacological selectivity pattern of OD1, we compared the amino acid sequences of receptor site 3 of $Na_v1.7$ with $Na_v1.2$, $Na_v1.3$, $Na_v1.5$ and the insect sodium channel para. Aligning the sequences, using the ClustalW algorithm, did not reveal striking differences in S5-S6 of domain I and S3-S4 of domain IV. In S5-S6 of domain IV, $Na_v1.7$ and para have an asparagine at position 1674, whereas $Na_v1.2$, $Na_v1.3$ and $Na_v1.5$ have an aspartic acid. It is not unlikely that this asparagine contributes to the sensitivity of $Na_v1.7$ and para to OD1. Finally, it is interesting to note that $Na_v1.8$, that seems to be resistant to all tested scorpion α-toxins, has an uncharged hydrophobic amino acid, alanine, at the corresponding position of Glu1613 in $Na_v1.2$ in the S3-S4 extracellular loop of domain IV. This might explain the resistance of $Na_v1.8$ to scorpion α-toxins.

VGSCs open when the membrane potential is depolarized and close on repolarization, but also on continuous depolarization by a process termed inactivation, which leaves the channel refractory, i.e. unable to open again for a period of time. For the process of fast inactivation this time is of the millisecond range, but it can last much longer (up to seconds) in a different slow type of inactivation. Fast inactivation is highly vulnerable and is known to be affected by many agents, including toxins (Ulbricht, 2005). Our data show that on the one hand OD1 impairs the process of fast inactivation of $Na_v1.7$, on the other hand it accelerates the recovery from fast inactivation. This is in accordance with the work of Hank and coworkers, who demonstrated that the site 3 sea anemone toxin Anthopleurin B prolongs the macroscopic inactivation and increases the rate of whole-cell recovery of cardiac and neuronal VGSCs (Benzinger et al., 1997). They propose that the non-

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inactivating current in the presence of toxin arises from an O ⇆ I equilibrium that partially favors the open state, but that the overall rate of I → O recovery is still not sufficiently large to cause appreciable numbers of channels to recover through the open state during repolarization. Nonetheless, toxin treatment does augment recovery from inactivation. The authors suggest that both of these observations can be rationalized under the assumption that the toxin destabilizes the terminal inactivated state of the channel. Open-state inactivation is slowed, producing the well-known prolongation of macroscopic inactivation and increase in mean open time. This slowing, combined with a possible augmentation of the open-state recovery rate I → O, produces the observed plateau current. Finally, destabilization of the final inactivated state enhances the recovery from closed state inactivation (Benzinger et al., 1997). We assume the effects of OD1 on $Na_v1.7$ could be explained in a similar way. The increase in peak current observed in the presence of OD1 would then be compatible with an enhanced recovery from closed state inactivation.

The VGSC $Na_v1.7$ has been implicated in several pathophysiological conditions, as acute inflammatory pain (Nassar et al., 2004), erythermalgia (Dib-Hajj et al., 2005; Drenth et al., 2005; Michiels et al., 2005), and prostate cancer (Diss et al., 2005). Mainly because of its role in pain, $Na_v1.7$ has become a interesting therapeutic target. At first one would logically think about ion channel blockers, but looking at certain sodium channelopathies suggest that there may be other ways to inhibit the propagation of action potential trains in neurons. Mutations in VGSC genes have been identified as the cause of epilepsy, periodic paralysis, muscle stiffness (myotonia), or cardiac arrhythmia. For the majority of these, the mutations produce missense substitutions that result in functional channels with subtle changes in the voltage dependence of channel opening and closing (gating) (Cannon, 2002). Extensively

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studied examples of sodium channelopathies are the autosomal dominantly inherited forms of myotonia and periodic paralysis. Missense mutations in *SCN4A*, the Na^+ channel α subunit of skeletal muscle, cause predominantly gain-of-function defects wherein inactivation is partially disrupted, or in a few cases activation is enhanced. The end result is that mutant channels conduct more inward Na^+ current than wild-type ones. Interestingly, the aberrant inward current can either result in pathologically enhanced excitability (small persistent Na^+ currents 1%–2% of the peak cause bursts of repetitive muscle fiber discharges producing sustained myotonic stiffness), whereas slightly more severe defects of inactivation (>3%) induce a loss of muscle excitability manifest as flaccid weakness due to prolonged depolarization-induced reduction in Na^+ channel availability (Cannon, 2000). We think this last situation might be comparable to the modulation of $Na_v1.7$ by OD1.

In correlation with proinflammatory, hyperalgesic agents such as serotonin, prostaglandin E_2 (PGE_2) and adenosine, which cause abnormal bursting activity in primary sensory neurons, OD1 causes a dose-dependent increase in the amplitude of Na^+ currents, accompanied by a left-ward shift in the voltage-dependence of activation (Gold et al., 1996). However, in sharp contrast to the aforementioned hyperalgesic agents, OD1 impairs the fast inactivation process and results in an incomplete inactivation in steady state conditions. We presume that the persistent inward Na^+ current may lead to a sustained depolarisation of the cell membrane *in vivo*. As a consequence, the remaining $Na_v1.7$ channels that were not affected by OD1 would be trapped in the inactivated state, resulting in the loss of electrical excitability of nociceptor neurons. A similar mechanism was proposed to explain feeling of numbness described after contact of skin with the VGSC modulator batrachotoxin (Bosmans et al., 2004).

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In conclusion, the present study shows that the scorpion toxin OD1 is a potent modulator of $Na_v1.7$. Low nanomolar concentrations of this toxin impair the steady-state fast inactivation process, enhance the recovery from fast inactivation, increase the peak Na^+ current and give rise to a substantial persistent Na^+ current. At these concentrations, other mammalian VGSCs ($Na_v1.2$, $Na_v1.3$, $Na_v1.5$ and $Na_v1.8$) were not affected.

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Footnotes

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FIGURE LEGENDS

Figure 1. Comparison of amino acid sequence of OD1 with BmK M1, AahII, Lqh-2 and Lqh-3. Alignment is based on cystine residues (indicated in bold) using ClustalW (http://www.ebi.ac.uk/clustalw/). % Similarity is shown relative to OD1.

Figure 2. Differential effects of OD1 on $Na_v1.7$, $Na_v1.3$ and $Na_v1.8$ Na^+ channels heterologously expressed in *Xenopus* oocytes. Representative whole cell Na^+ currents of oocytes expressing $Na_v1.7$ (left), $Na_v1.3$ (middle) and $Na_v1.8$ (right) in control conditions and in the presence of different concentrations of OD1. Horizontal arrows indicates zero current. Currents were elicited by depolarizing steps between -45 and +70 mV in 5 mV increments from a holding potential of -100 mV. Corresponding current-voltage relationship for control conditions (filled squares) and in the presence of OD1 (open squares) are depicted in the lower part of the Figure. Vertical arrows ($Na_v1.7$) indicate time at 30 ms. The inset shows the corresponding current-voltage relation at that certain point in time.

Figure 3. $Na_v1.7$ and $Na_v1.3$ have different sensitivities to OD1. Dose-response curves for OD1 on $Na_v1.7$ (squares) and $Na_v1.3$ (circles) channels obtained by plotting the relative I_{30ms}/I_{peak} values in function of the toxin concentrations, fitted with equation [1] yielding an EC_{50} value and Hill coefficient. For $Na_v1.7$ these values were, respectively, 4.5 ± 0.2 nM and 1.5 ± 0.1. For $Na_v1.3$ they were 1127 ± 263 nM and 1.1 ± 0.2. Each data point is an average from three to six experiments.

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Figure 4. Effect of OD1 on the activation and steady-state inactivation curves of $Na_v1.7$ (upper panel) and $Na_v1.3$ (lower panel). Activation curves (squares) were derived from the same family of currents used for the current-voltage curves (Fig. 2) using the standard procedure (see Methods). Steady-state inactivation curves (circles) were determined using conditioning pulses to voltages between -90 and 0 mV and a standard test pulse to 0 mV for $Na_v1.7$ or -5 mV for $Na_v1.3$. Test currents were normalized and plotted against the conditioning voltage. The steady-state properties for $Na_v1.7$ and $Na_v1.3$ in the absence of OD1 (filled squares and circles) are shown on the same graph as in the presence of OD1 (open squares and circles). Each data point is an average of at least three experiments. The dashed lines are Boltzmann fits. See Table 1 for $V_{1/2}$ values and slope factors.

Figure 5. Effect of OD1 on the recovery of fast inactivation of $Na_v1.7$. Recovery from fast inactivation examined using a standard double-pulse protocol. A 25 ms conditioning pulse to 0 mV to fully fast-inactivate the channel, followed by a hyperpolarizing step to -100 mV for variable duration (0 to 160 ms). Recovery was monitored by measuring the relative peak Na^+ current elicited by a second pulse to 0 mV and plotted as a function of the interval between the pulses. The average of at least three experiments is depicted for control conditions (filled squares) and in the presence of 100 nM OD1 (open squares). The recovery of the peak amplitude was fitted with a double exponential. The inset shows the time constants for control conditions (filled bar) and in the presence of 100 nM OD1 (open bar).

Figure 6. Effects of AahII and BmK M1 on $Na_v1.7$. Top panels show representative whole cell Na^+ currents of an oocyte expressing $Na_v1.7$ channels, in response to a 100 ms voltage

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pulse to V_{max} (0 mV). Solid lines represent control currents, dashed lines represent the current in the presence of 50 nM AahII (A) and BmK M1 (D). Arrow indicates zero current. Panels B and E show the corresponding current-voltage relationships in control conditions (filled squares) and in the presence of toxin (open squares), recorded from the same oocyte as in A and D, respectively. Panels C and F depict the activation and steady-state inactivation curves in control conditions (filled symbols) and in the presence of toxin (open symbols). Activation curves (squares) were derived from the same family of currents used for the current-voltage relationships. Steady-state inactivation curves (circles) were determined using conditioning pulses to voltages between -90 and 0 mV and a standard test pulse to 0 mV. Test currents were normalized and plotted against the conditioning voltage. Each data point is an average of four experiments. The dashed lines are Boltzmann fits. See Table 2 and 3 for $V_{1/2}$ values and slope factors.

Figure 7. Effects of AahII and BmK M1 on $Na_v1.3$. Top panels show representative whole cell Na^+ currents of an oocyte expressing $Na_v1.3$ channels, in response to a 100 ms voltage pulse to V_{max} (-5 mV). Solid lines represent control currents, dashed lines represent the current in the presence of 50 nM AahII (A) and BmK M1 (D). Arrow indicates zero current. Panels B and E show the corresponding current-voltage relationships in control conditions (filled squares) and in the presence of toxin (open squares), recorded from the same oocyte as in A and D respectively. Panels C and F depict the activation and steady-state inactivation curves in control conditions (filled symbols) and in the presence of toxin (open symbols). Activation curves (squares) were derived from the same family of currents used for the current-voltage relationships. Steady-state inactivation curves (circles) were determined using conditioning pulses to voltages between -90 and -5 mV and a standard

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test pulse to -5 mV. Test currents were normalized and plotted against the conditioning voltage. Each data point is an average of four experiments. The dashed lines are Boltzmann fits. See Table 2 and 3 for $V_{1/2}$ values and slope factors.

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TABLE 1

Effects of OD1 on fast activation and inactivation parameters for $Na_v1.7$ and $Na_v1.3$

		Activation			Inactivation		
		$V_{1/2}$ (mV)	***Slope***	***n***	***$V_{1/2}$ (mV)***	***Slope***	***n***
$Na_v1.7$	***Control***	-15.1 ± 0.5	4.2 ± 0.2	4	-44.8 ± 0.7	11.3 ± 0.4	4
	OD1 50 nM	-17.4 ± 0.4	4.1 ± 0.2	4	-46.7 ± 0.4	10.0 ± 0.3	4
$Na_v1.3$	***Control***	-18.3 ± 0.6	3.2 ± 0.1	3	-24.8 ± 1.1	8.3 ± 0.4	3
	OD1 500 nM	-17.8 ± 0.5	3.2 ± 0.2	3	-21.6 ± 0.9	8.4 ± 0.3	3

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TABLE 2

Effects of AahII on fast activation and inactivation parameters for $Na_v1.7$ and $Na_v1.3$

		Activation			Inactivation		
		$V_{1/2}$ (mV)	***Slope***	***n***	***$V_{1/2}$ (mV)***	***Slope***	***n***
$Na_v1.7$	***Control***	-16.3 ± 0.5	3.6 ± 0.3	4	* -48.4 ± 0.5	10.4 ± 0.4	4
	AahII 50 nM	-18.1 ± 0.5	4.1 ± 0.2	4	* -43.1 ± 0.4	9.2 ± 0.2	4
$Na_v1.3$	***Control***	* -19.6 ± 0.8	4.6 ± 0.4	4	-31.6 ± 0.2	7.5 ± 0.1	4
	AahII 50 nM	* -28.2 ± 0.9	3.2 ± 0.5	4	-32.9 ± 1.6	8.2 ± 0.6	4

* $p < 0.05$

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TABLE 3

Effects of BmK M1 on fast activation and inactivation parameters for $Na_v1.7$ and $Na_v1.3$

		Activation			Inactivation		
		$V_{1/2}$ (mV)	***Slope***	***n***	***$V_{1/2}$ (mV)***	***Slope***	***n***
$Na_v1.7$	***Control***	-17.9 ± 0.7	4.4 ± 0.2	4	* -46.4 ± 0.8	8.9 ± 0.5	4
	BmK M1 50 nM	-18.5 ± 0.3	3.6 ± 0.1	4	* -44.1 ± 0.7	8.3 ± 0.3	4
$Na_v1.3$	***Control***	-15.7 ± 0.3	3.7 ± 0.2	4	-31.2 ± 0.2	7.4 ± 0.2	4
	BmK M1 50 nM	-14.4 ± 0.3	4.4 ± 0.3	4	-29.4 ± 0.1	7.8 ± 0.1	4

* $p < 0.05$


OD1 GVRDAYIADDKNCVYTCAS-NGYCNTECTKNGAESGYCQWIGRYGNACWCIKLPDEVPIRIPG-KCR 100 %
BmK M1 -VRDAYIAKPHNCVYECAR-NEYCNDLCTKNGAKSGYCQWVGKYGNGCWCIELPDNVPIRVPG-KCH 75 %
AaH II -VKDGYIVDDVNCTYFCGR-NAYCNEECTKLKGESGYCQWASPYGNACYCYKLPDHVRTKGPG-RCN 60 %
Lqh-2 -IKDGYIVDDVNCTYFCGR-NAYCNEECTKLKGESGYCQWASPYGNACYCYKLPDHVRTKGPG-RCR 60 %
Lqh-3 -VRDGYIAQPENCVYHCFPGSSGCDTLCKEKGGTSGHCGFKVGHGLACWCNALPDNVGIIVDGVKCH 44 %

Figure 1

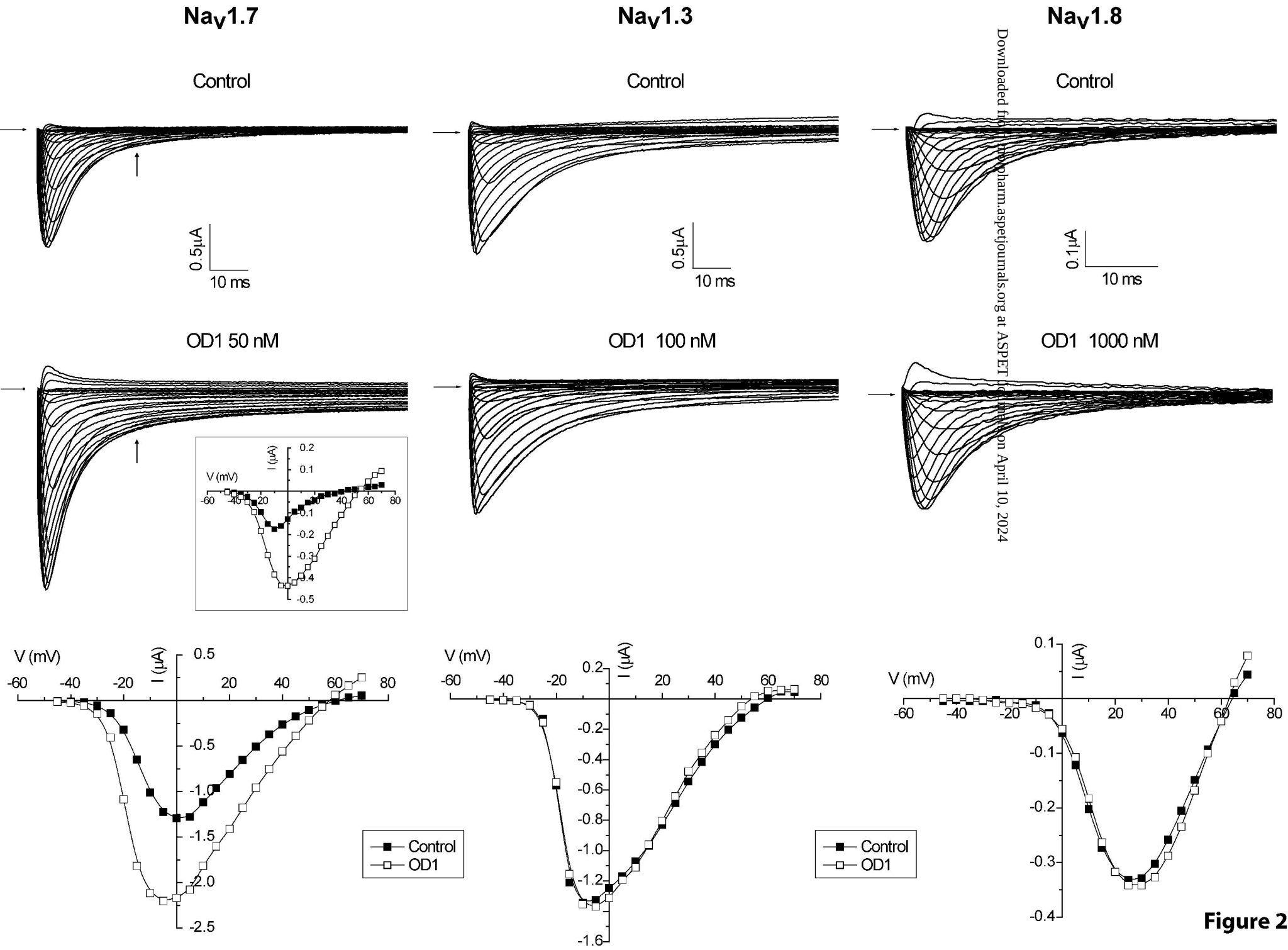


Figure 2

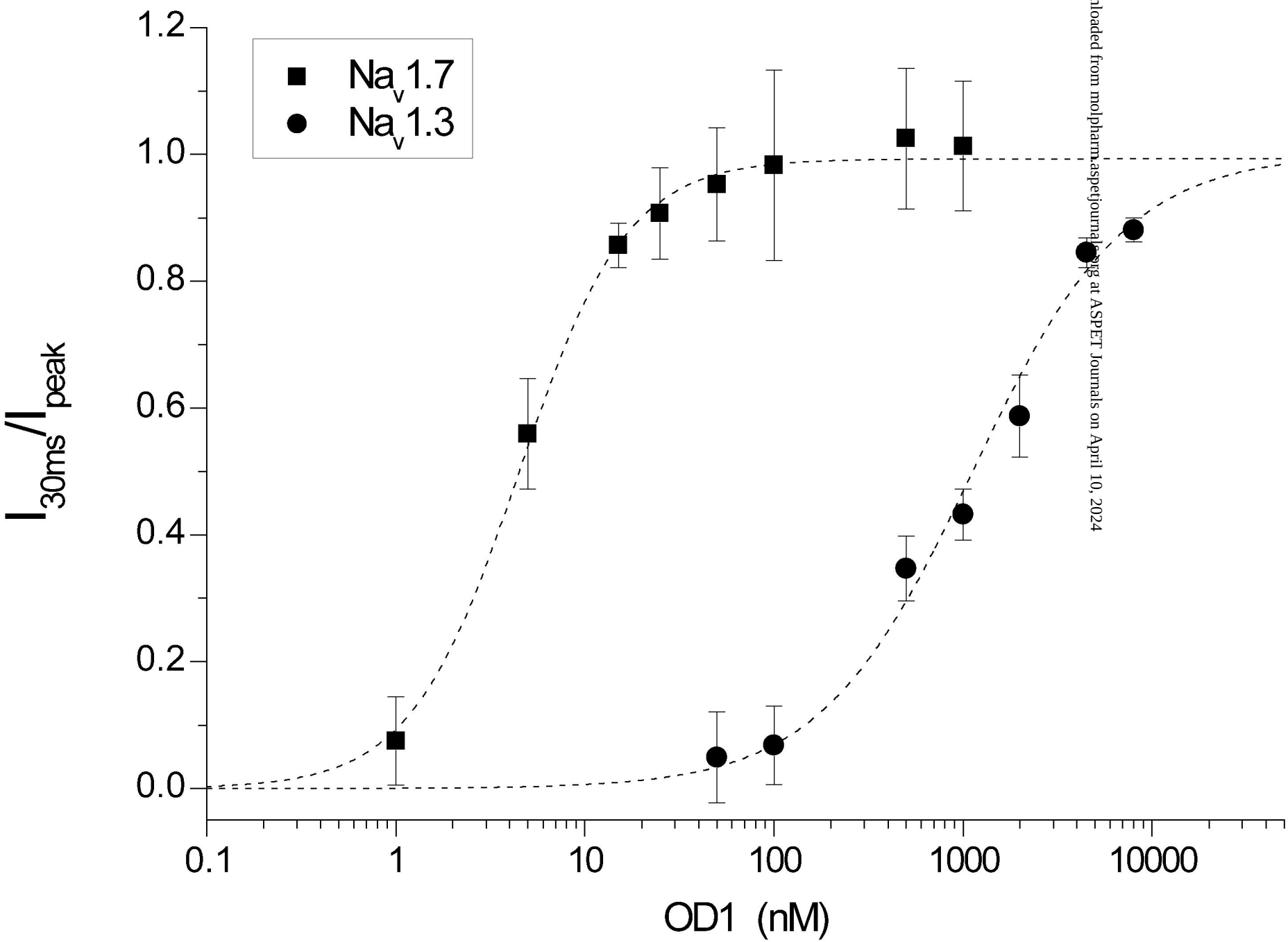
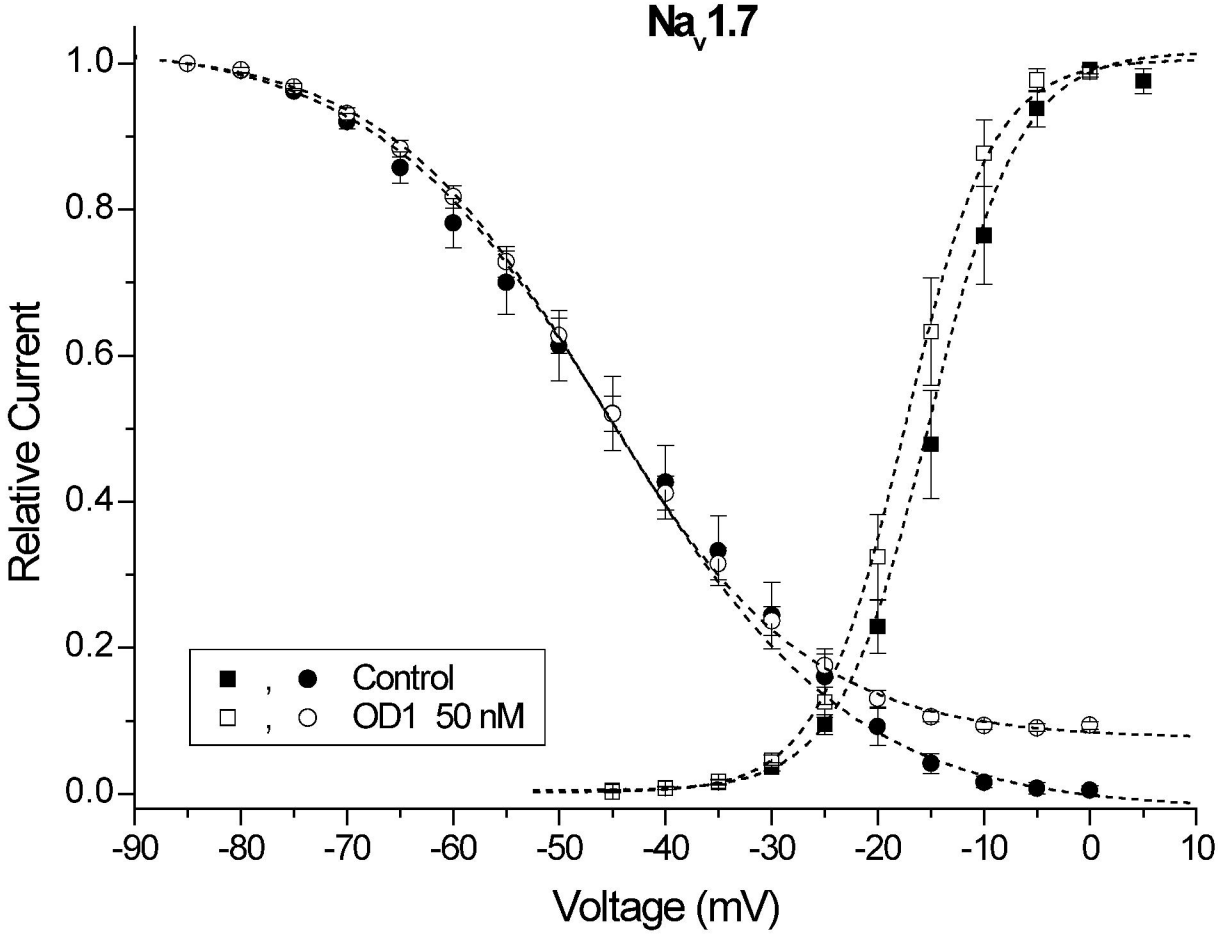


Figure 3


Na$_v$1.7
Relative Current
Voltage (mV)
Control
OD1 50 nM

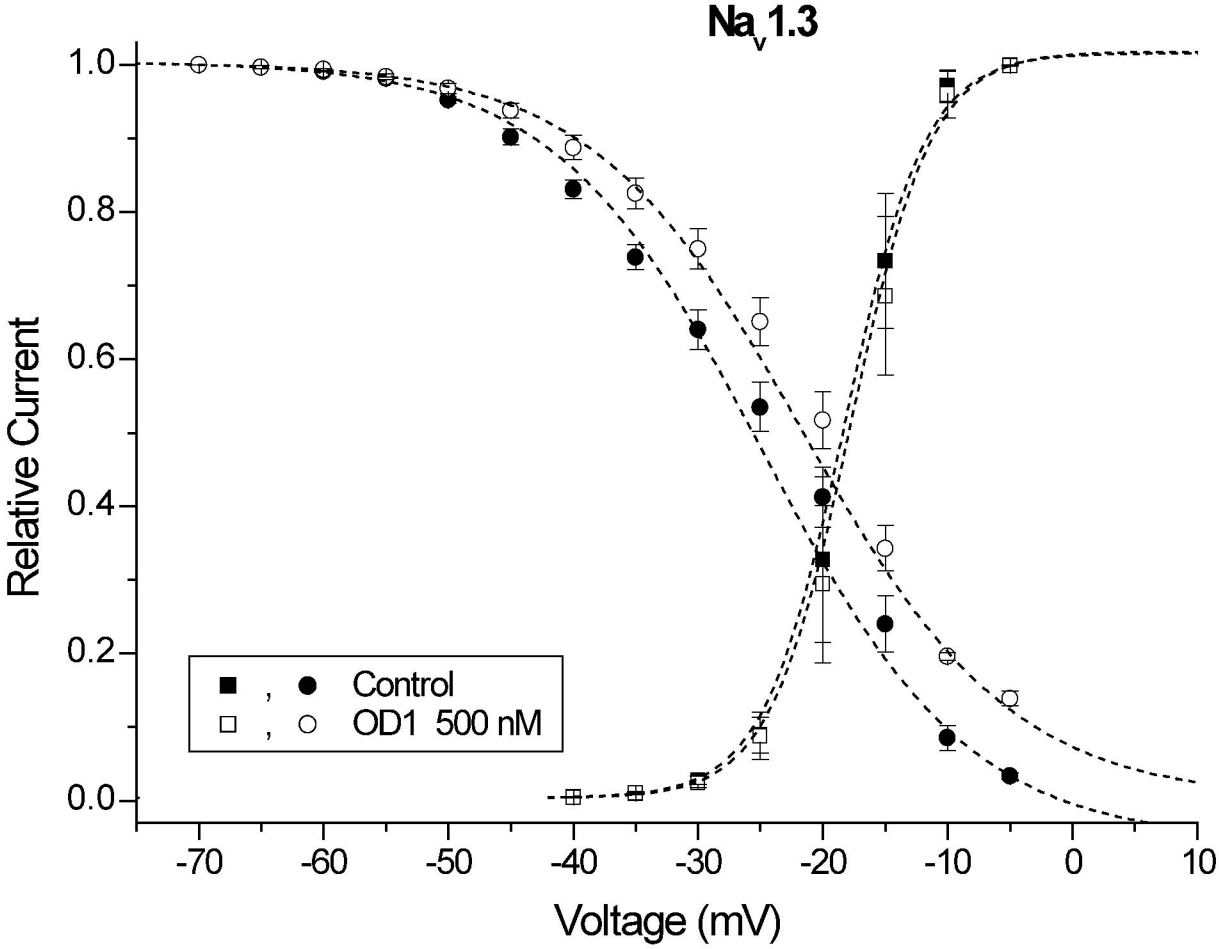

Na$_v$1.3
Relative Current
Voltage (mV)
Control
OD1 500 nM

Figure 4

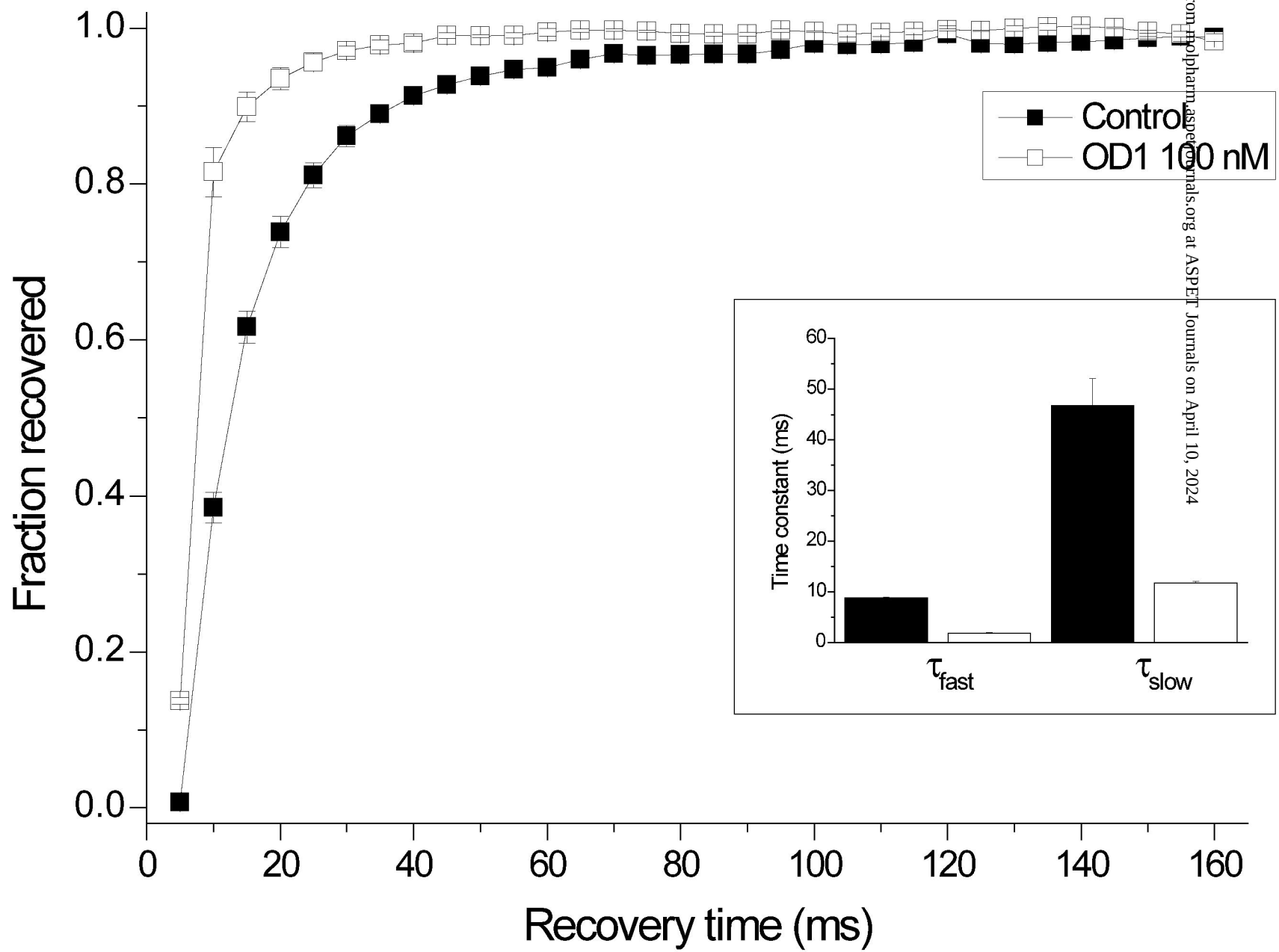


Figure 5

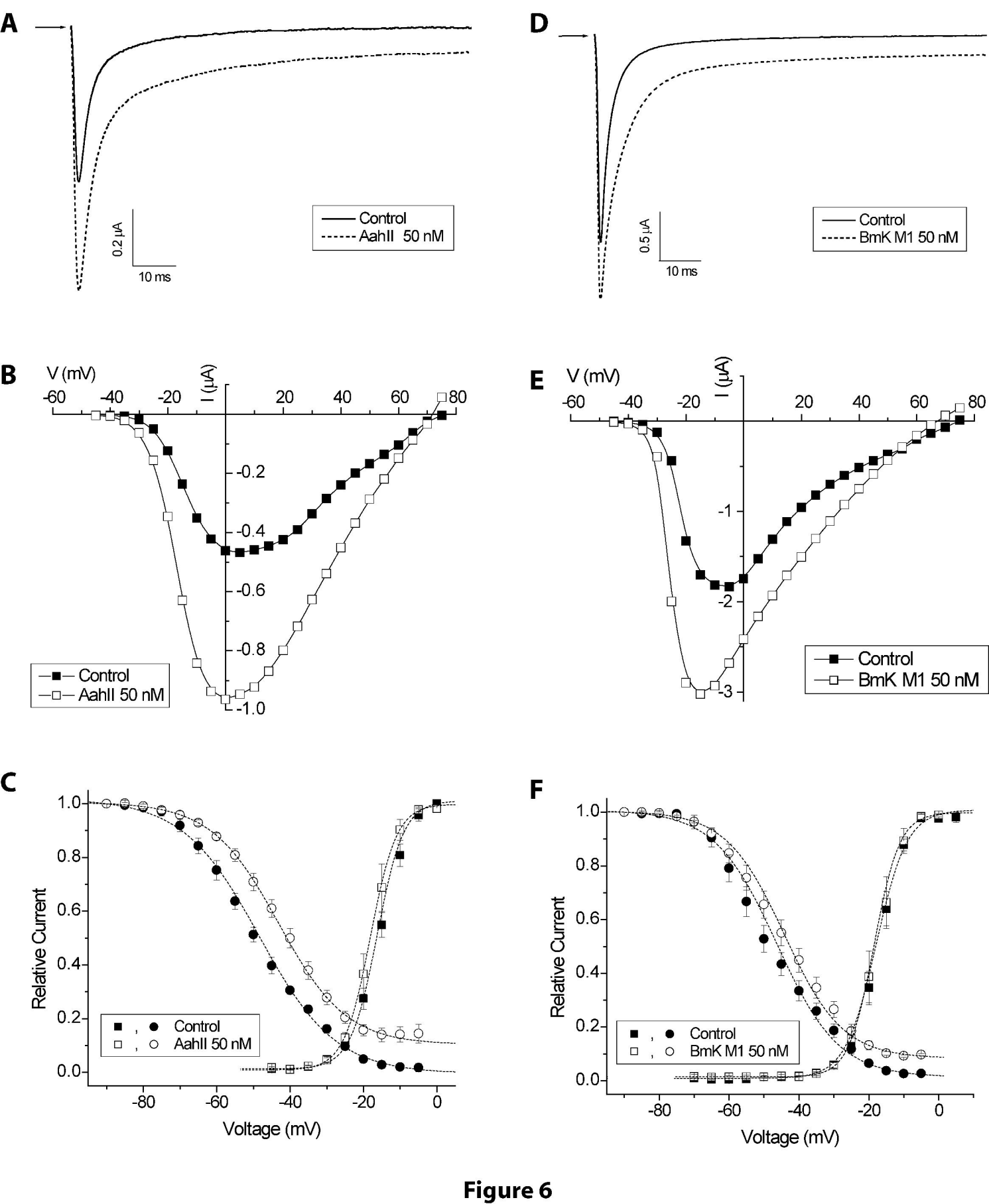

A
0.2 μA
10 ms
Control
AahII 50 nM
D
0.5 μA
10 ms
Control
BmK M1 50 nM
B
V (mV)
I (μA)
-60 -40 -20 20 40 60 80
-0.2 -0.4 -0.6 -0.8 -1.0
Control
AahII 50 nM
E
V (mV)
I (μA)
-60 -40 -20 20 40 60 80
-1 -2 -3
Control
BmK M1 50 nM
C
Relative Current
1.0 0.8 0.6 0.4 0.2 0.0
-80 -60 -40 -20 0
Voltage (mV)
Control
AahII 50 nM
F
Relative Current
1.0 0.8 0.6 0.4 0.2 0.0
-80 -60 -40 -20 0
Voltage (mV)
Control
BmK M1 50 nM

Figure 6

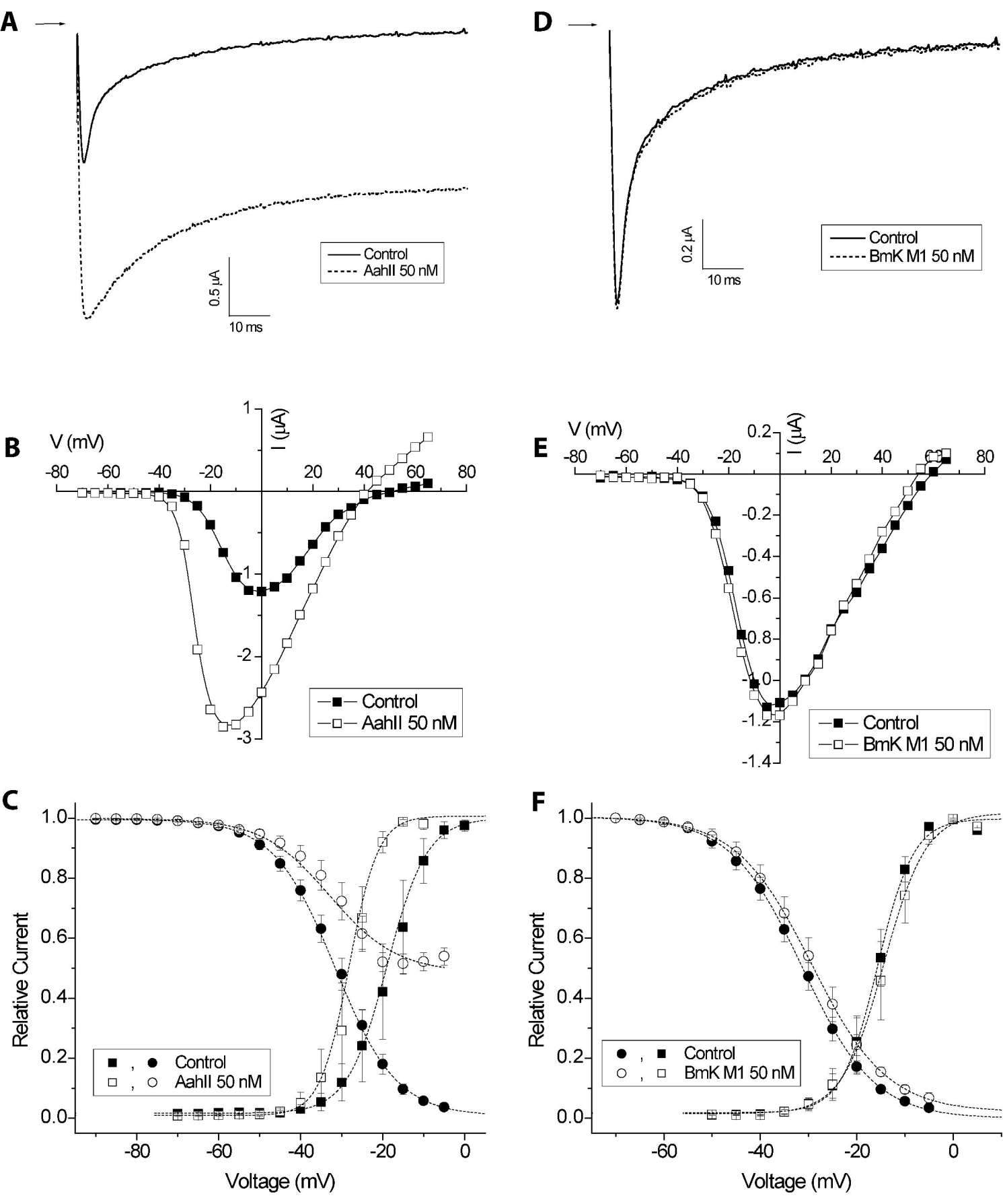

A
Control
AahII 50 nM
0.5 μA
10 ms
D
Control
BmK M1 50 nM
0.2 μA
10 ms
B
V (mV)
I (μA)
Control
AahII 50 nM
E
V (mV)
I (μA)
Control
BmK M1 50 nM
C
Relative Current
Voltage (mV)
Control
AahII 50 nM
F
Relative Current
Voltage (mV)
Control
BmK M1 50 nM

Figure 7