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Single channel study of deltamethrin interactions with wild-type and mutated rat $Na_V1.2$ sodium channels expressed in *Xenopus* oocytes

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ABSTRACT

Single channel analysis was used to compare the electrophysiological properties of wild-type (WT) and I874M mutant (M874) rat $Na_V1.2$ channels expressed in *Xenopus* oocytes and their modulation by the pyrethroid deltamethrin. In the absence of pyrethroid, histograms of channel open times were best-fit by single exponentials. The open time constants at −40 mV for WT (0.53 ± 0.05 ms) and M874 (0.65 ± 0.08 ms) channels were significantly different and both decreased with depolarisation. At ≥100 nM deltamethrin, WT open time histograms at −40 mV were best-fit by two exponentials (time constants, 0.49 ± 0.03 ms ($\tau_{o,fast,WT}$) and 5.2 ± 0.5 ms ($\tau_{o,slow,WT}$). The population of long-duration openings and $\tau_{o,slow,WT}$ increased when the concentration of deltamethrin was raised, but $\tau_{o,fast,WT}$ was unaffected. Qualitatively similar results were obtained for the M874 channel, but with ≥10 nM deltamethrin. Deltamethrin also caused a negative shift in the relationships between channel opening probability (P_{op}) and membrane potential and first latency and membrane potential, suggesting that the pyrethroid binds to the closed channel. Selectivity for Na was increased by the pyrethroid (10 μM, WT; 1 μM, mutant).

1. Introduction

The energetic coupling between membrane potential and the activation gate is the defining characteristic of a voltage-gated Na channel and one which leads to depolarisation-induced influx of Na ions. Voltage-gated Na channels in mammalian brain comprise one α-subunit and two β-subunits, although expression of the former is sufficient to provide voltage-sensitive Na gating in vitro (Catterall, 1992). The α-subunit is a 260 kDa protein comprising four homologous domains (D) connected by intracellular linkers, each domain containing six membrane-spanning segments (S) with a re-entrant loop between each S5–S6. It is generally accepted that the activation gate is formed by the four uncharged S6 segments, whilst the voltage sensor is formed by the positively charged S4 segments (Catterall, 1986). Voltage-dependent activation seemingly arises through the outward movement of the four S4 segments, whereas deactivation results from a voltage-dependent return to their original positions. Upon depolarisation, Na channels are considered to undergo voltage-dependent transitions through multiple closed states before opening (McPhee et al., 1998). The open channel then converts either quickly ("fast" open-state inactivation) or slowly ("slow" open-state inactivation) to an inactivated, non-conducting state in which it remains until the membrane is repolarised and the channel is deactivated. There is strong evidence that S4–S5 linkers are involved in "fast" open-state inactivation, their cytoplasmic domains possibly forming part of the docking site for an inactivation particle (Holmgren et al., 1996; McPhee et al., 1998), i.e. the IFM motif from the cytoplasmic loops between DIII and DIV (Catterall, 2000). This inactivated state can also be reached through one or more of the closed states (closed-state inactivation).

Voltage clamp studies have shown that some mammalian Na channels are sensitive to low concentrations (10–100 nM) of pyrethroids (Tabarean and Narahashi, 1998, 2001; Tatebayashi and Narahashi, 1994), but the majority respond only to >1 μM. The effects of the pyrethroid deltamethrin on single Na channel properties have been investigated in a mammalian neuroblastoma cell line (Chinn and Narahashi, 1986, 1989) and on rat hippocampal neurons (Motomura and Narahashi, 2000, 2001). We have used single channel analysis further to compare the electrophysiological properties of wild-type (WT) and I874M mutant (M874) rat $Na_V1.2$ channels expressed in *Xenopus* oocytes and their interactions with deltamethrin. Previously, we have shown in voltage clamp experiments that substitution of isoleucine for methionine at position 874 (I874M) in the rat $Na_V1.2$ channel α-subunit causes a 100-fold increase in sensitivity to the pyrethroid deltamethrin

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(Vais et al., 2000). We show here that deltamethrin affects channel opening probability, channel opening rate, channel open time and ion selectivity of both channels, and channel openings occur after repolarisation because of a reduction in deactivation. Tabarean and Narahashi (2001) obtained evidence that the gating charge of deltamethrin-modified Na channels is immobilised by deltamethrin and that modification of "fast" inactivation alone cannot account for the observed effects of the pyrethroid. Our single channel data support this general conclusion and specifically show that the slower open-closed kinetics and slower kinetics of "fast" inactivation of the M874 channel contribute to its higher sensitivity to the pyrethroid.

2. Materials and methods

2.1. Construction of rat brain $Na_V1.2$ channel α-I874M mutant

A plasmid containing the rat brain $Na_V1.2$ channel cDNA (pVA2580) was kindly provided by Dr. C. Labarca (California Institute of Technology). The mutation I874M was introduced into the gene using the QuikChange site-directed mutagenesis kit (Stratagene Cloning Systems, UK). Wild-type and mutant plasmids were linearised with Cla1 and T7 transcripts synthesised using the mMESSAGE mMACHINE kit (Ambion). Transcripts were dissolved in sterile water at a final concentration of 1 μg/μl and stored at −80 °C until required.

2.2. Oocyte preparation

Oocytes were obtained by ovariectomy of female *Xenopus laevis* (Xenopus I, The Northside, Ann Arbor, MI, USA) anesthetized with 2 g/L MS-222. The animals were humanely killed after oocyte collection. This protocol was approved by the Committee on Animal Resources, University of Nottingham. Oocytes were prepared for injection by treating fragments of ovary with 0.2 mg/ml collagenase (type 1A, Sigma) in Ca^{2+}-free solution (in mM): 96 NaCl, 2 KCl, 1 $MgCl_2$, 5 HEPES; adjusted to pH 7.6 with NaOH for 1–2 h. After thoroughly rinsing, released oocytes were mechanically de-folliculated. cRNA transcripts of either WT or M874 channels (1 μg/μl) were mixed with rat β1 transcripts (1 μg/μl) and RNase-free water, the final mixture having a 1:1:3 ratio by volume. Oocytes were injected with 50 nl of the mixture, and then incubated at 19 °C for 1–5 days in ND-96 GPT solution (in mM: 96 NaCl, 2 KCl, 1 $MgCl_2$, 1.8 $CaCl_2$, 2.5 Na-pyruvate, 0.5 theophylline, 5 HEPES; 50 mg/ml gentamicin, pH 7.5) prior to experimentation. Before patch-clamping, an oocyte was immersed for 20 min in a hypertonic solution (in mM: 200 K-aspartate, 20 CsCl, 2 $MgCl_2$, 10 HEPES, 5 EGTA; pH adjusted to 7.4 with CsOH) and its vitelline membrane removed manually (Choe and Sackin, 1997).

2.3. Electrophysiological measurements

Patch pipettes were pulled from borosilicate glass (1B150F-4, World Precision Instruments, Inc.) and fire-polished to a final tip diameter of less than 1 μm. High sodium saline (in mM: 240 NaCl, 5 HEPES, 2 $CaCl_2$, 1 $MgCl_2$, adjusted to pH 7.4 with NaOH) was used in the bath for outside-out patches and the pipette for inside-out patches; low sodium saline (in mM: 2.4 NaCl, 100 K-asparate, 10 HEPES, 20 CsCl, 5 EGTA, adjusted to pH 7.2 with CsOH) was used in the pipette for outside-out patches and the bath for inside-out patches. Pipette resistances were 7–10 MΩ. All solutions were filtered immediately before use through a membrane of 0.2 μm pore size (Sartorius Vivascience AG Hanover Germany). Patches were excised from oocytes 1–3 days post-injection of RNA; patches were difficult to form using older oocytes. Recordings were made with an EPC-10 patch-clamp amplifier (HEKA) controlled by a computer running Pulse and PulseFit software (HEKA) for data acquisition and analysis. A P/4 procedure was applied to subtract linear leak and capacitive transient currents from the raw data. Recordings were filtered at 2.9 kHz (−3 dB, 4 pole Bessel filter). The sampling rate was 40 kHz during 50 ms sweeps and 5 kHz during 400 ms sweeps. The root mean square (RMS) noise was usually between 0.5 pA and 1.0 pA. All experiments were conducted at room temperature (20–24 °C). Single channel openings were detected using standard half-amplitude threshold analysis. Kinetic analysis and graphing was done with WinEDR V2.4.8, PATCH V1, ORIGIN50 and Graphpad Prism 5 software. Most recordings were made using inside-out patches. Single channel conductances (G) were determined from:

$$G = \frac{I}{V_m - V_{I,rev}}$$

where I is the single channel current, V_m is the membrane potential and $V_{I,rev}$ is the single channel current reversal potential. Na (116 mV) and K (−116 mV) equilibrium potentials were estimated from:

$$E = \frac{RT}{nF} \log_e \frac{[ion_o]}{[ion_i]}$$

where ion_o and ion_i are the activities of either Na or K outside and inside a patch, R is the gas constant, F the Faraday constant and T the absolute temperature. The relative permeabilities of Na and K ions were determined from current–voltage characteristics using:

$$V_{rev} = \frac{(G_{Na}/G_K)(E_{Na}) + E_K}{(G_{Na}/G_K) + 1}$$

where V_{rev} is the linear extrapolated reversal potential of the single Na channel current, E_{Na} and E_K are the Na and K equilibrium potentials and G_{Na} and G_K are Na and K conductances. The number of channels in a patch was estimated using the maximum likelihood method of Patlak and Horn (1982) and data were corrected accordingly. Data were compared using a two-tail t-test.

2.4. Chemicals

Deltamethrin, a Type-II pyrethroid, was obtained from Dr. B. Khambay (Rothamsted Research). All other chemicals were purchased from Sigma (Poole, Dorset, UK). Stock solutions of deltamethrin were prepared in acetone and added to the bath saline to give the required concentration of deltamethrin. The concentration of acetone in the bath saline never exceeded 0.1% and it had no effect on the properties of the Na channels. Qualitatively and quantitatively similar data were obtained when the pyrethroid was applied to either face of a patch.

3. Results

Single Na channel currents were obtained following depolarisation (from −100 mV to −40 mV) in ~70% of patches excised from oocytes that had exhibited peak whole-cell Na currents >2 μA with a 2-electrode voltage clamp (Hamill et al., 1981). Addition of 200 nM TTX to the external solution bathing outside-out patches completely abolished the single channel currents, either before or after exposure of patches to deltamethrin, thus strongly supporting the view that they were openings of voltage-gated Na channels. Na channel openings could be readily distinguished from those of endogenous stretch-activated (SA) channels that have been observed previously in patches excised from *Xenopus* oocytes (Methfessel et al., 1986). SA channels are cation-selective, have a conductance of 28 pS in frog saline, open at −100 mV and do not inactivate. Initially, gadolinium was used to