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Distinct modulating effects of TipE-homologs 2–4 on *Drosophila* sodium channel splice variants

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ABSTRACT

The *Drosophila melanogaster* TipE protein is thought to be an insect sodium channel auxiliary subunit functionally analogous to the β subunits of mammalian sodium channels. Besides TipE, four TipE-homologous proteins (TEH1–4) have been identified. It has been reported that TipE and TEH1 have both common and distinct effects on the gating properties of splice variants of the *Drosophila* sodium channel, $DmNa_v$. However, limited information is available on the effects of TEH2, TEH3 and TEH4 on the function of $DmNa_v$ channel variants. In this study, we found that TEH2 increased the amplitude of peak current, but did not alter the gating properties of three examined $DmNa_v$ splice variants expressed in *Xenopus* oocytes. In contrast, TEH4 had no effect on peak current, yet altered the gating properties of all three channel variants. Furthermore, TEH4 enhanced persistent current and slowed sodium current decay. The effects of TEH3 on $DmNa_v$ variants are similar to those of TEH4, but the data were collected from a small portion of oocytes because co-expression of TEH3 with $DmNa_v$ variants generated a large leak current in the majority of oocytes examined. In addition, TEH3 and TEH4 enhanced the expression of endogenous currents in oocytes. Taken together, our results reveal distinct roles of TEH proteins in modulating the function of sodium channels and suggest that TEH proteins might provide an important layer of regulation of membrane excitability *in vivo*. Our results also raise an intriguing possibility of TEH3/TEH4 as auxiliary subunits of other voltage-gated ion channels besides sodium channels.

1. Introduction

Voltage-gated sodium channels are responsible for the initiation and propagation of action potentials in almost all excitable cells. Mammalian sodium channels are composed of a pore-forming α subunit and one or more β subunits. The sodium channel α subunit is composed of four homologous domains (I–IV), each containing six transmembrane segments (S1–S6). The S1–S4 segments serve as a voltage-sensing module. The S5 and S6 segments and the reentrant loops connecting them form the inner pore. In response to membrane depolarization, the S4 segments moves outward which initiates the channel activation process (Catterall, 2012). Within a few milliseconds, sodium channels close or inactivate. This fast inactivation is mediated by an inactivation particle formed by residues in the linker connecting domains III and IV which physically occludes the inner pore, preventing sodium ions from flowing into the cell (Catterall, 2012). Under prolonged depolarization, sodium channels enter into another inactivation state called the slow inactivated state. Unlike fast inactivation where recovery takes tens of milliseconds, recovery from slow inactivation requires seconds to minutes of membrane repolarization to return to a resting state (Goldin, 2003; Vilin and Ruben, 2001). The processes of sodium channel activation, fast inactivation and slow inactivation play critical roles in regulating membrane excitability (Goldin, 2003).

In mammals, there are at least nine sodium channel isoforms with different gating properties and expression patterns in various cell types, tissues, and developmental stages (Catterall, 2012; Goldin et al., 2000; Yu and Catterall, 2003). There are four genes encoding sodium channel β subunits (β1–β4). The β subunits are small transmembrane proteins that possess an extracellular immunoglobulin (Ig) domain, a single transmembrane segment, and a short intracellular C-terminal domain. They modulate gating properties and expression of sodium channels (Patino and Isom, 2010). In addition to their roles in channel modulation, β subunits also function as cell adhesion molecules interacting with both cytoskeleton and extracellular matrix, regulating cell migration and cellular aggregation (Brackenbury and Isom, 2011; Patino and Isom, 2010).

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In contrast, most insects species have only one sodium channel gene that encodes the α subunit equivalent of mammalian sodium channels (Dong, 2010; Dong et al., 2014), such as *para*, also known as *DmNa*$_v$, in *Drosophila melanogaster* (Loughney et al., 1989). Instead, insects take advantage of alternative splicing and RNA editing to generate a large collection of sodium channel variants, such as DmNa$_v$ in *D. melanogaster*, which exhibit diverse gating properties (Dong, 2007; Dong et al., 2014; Lin et al., 2009; Olson et al., 2008). *Drosophila* lacks any orthologs of mammalian β subunits (Littleton and Ganetzky, 2000). However, there is a small transmembrane protein, TipE, which is critical for sodium channel expression and neuronal activities, and functions as an auxiliary subunit for insect sodium channels (Feng et al., 1995; Warmke et al., 1997). *TipE*$^-$ mutant flies exhibit a temperature-sensitive paralytic phenotype, similar to sodium channel mutants (Feng et al., 1995), and [H^3]saxitoxin binding studies of insect embryonic neurons indicate that *tipE*$^-$ mutant flies have reduced sodium channel density (Jackson et al., 1986). Consistently, whole-cell patch recording indicates that sodium current is decreased about 40%–60% in dissociated embryonic neurons of *tipE*$^-$ mutant flies (O'Dowd and Aldrich, 1988). TipE also increases peak current of sodium channels transiently expressed in *Xenopus* oocytes (Derst et al., 2006; Feng et al., 1995; Wang et al., 2013; Warmke et al., 1997).

In addition to *TipE*, there are four *TipE*-homologous genes (*TEH1–4*) in *D. melanogaster* and three to four orthologs in other insect species (Derst et al., 2006). *TEH1* is exclusively expressed in the nervous system, while transcripts of *TipE* and *TEH2–4* are detected in both neuronal and non-neuronal tissues (Derst et al., 2006). All four TEH proteins differentially modified one DmNa$_v$ variant expressed in *Xenopus* oocytes (Derst et al., 2006). A recent study compared the modulation of three different DmNa$_v$ splice variants by TipE or TEH1 and revealed that TEH1 extensively modified the functional properties of all three variants, whereas TipE only modified the gating of one of the variants (Wang et al., 2013).

Whereas TEH2–4 have been shown to modulate the expression and some gating properties of a DmNa$_v$ variant, the effects of TEH2, TEH3, or TEH4 on the gating properties of different DmNa$_v$ splice variants remain unknown. In this study, we examined the modulating effects of TEH2–4 on gating properties of three variants: DmNa$_v$9-1, DmNa$_v$22, and DmNa$_v$26 expressed in *Xenopus* oocytes. Our data revealed strikingly distinct effects of TEH2, TEH3 and TEH4 on the expression and function of these variants. In addition to modulating sodium channel gating properties, TEH3 and TEH4 enhanced an outward current from an endogenous channel in *Xenopus* oocytes. Our results suggest that TEH proteins provide an important layer of regulation of membrane excitability *in vivo*. Our results also raise an intriguing possibility of TEH3 and TEH4 as auxiliary subunits of other voltage-gated ion channels besides sodium channels.

2. Materials and methods

2.1. Xenopus oocyte expression system

Oocytes were obtained surgically from female *Xenopus laevis* (Nasco, Ft. Atkinson. WI) and incubated with 1 mg/ml Type IA collagenase (Sigma Co., St. Louis, MO) in Ca^{2+}-free ND-96 medium (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, and 5 mM HEPES, pH 7.5). Following digestion, any follicle membrane still attached to the oocytes was physically removed with forceps. Isolated oocytes were incubated in ND-96 medium supplemented with 1.8 mM $CaCl_2$ supplemented with 50 mg/ml gentamicin, 5 mM pyruvate, and 0.5 mM theophylline (Goldin, 1992). Healthy stage V-VI oocytes were used for cRNA injection. *TEH2–4* cRNA or H_2O (as control) was injected alone or in combination with *DmNa*$_v$ cRNA at a 1:1 ratio.

2.2. Electrophysiological recording and analysis

Methods for two-electrode recording and data analysis were similar to those described previously (Tan et al., 2005). The borosilicate glass electrodes were filled with filtered 3 M KCl in 0.5% agarose and had a resistance of 0.5–1.0 MΩ. The recording solution was ND-96 (96 mM NaCl, 2.0 mM KCl, 1.0 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 10 mM HEPES, pH adjusted to 7.5 with NaOH). Sodium currents were measured with a Warner OC725C oocyte clamp amplifier (Warner Instrument, Hamden, CT) and processed with a Digidata 1440 (Axon Instruments Inc., Foster City, CA). Data were sampled at 50 kHz and filtered at 2 kHz. Leak currents were corrected by p/4 subtraction (Bezanilla and Armstrong, 1977).

The voltage dependence of sodium channel activation was calculated by measuring the peak current at test potentials ranging from −80 mV to +65 mV in 5-mV increments and divided by $(V - V_{rev})$, where V is the test potential and V_{rev} is the reversal potential for sodium ion. Peak conductance values were normalized to the maximal peak conductance (G_{max}) and fitted with a two-state Boltzmann equation of the form $G/G_{max} = [1 + \exp(V - V_{1/2})/k]^{-1}$, in which V is the potential of the voltage pulse, $V_{1/2}$ is the voltage for half-maximal activation, and k is the slope factor.

The voltage dependence of sodium channel fast inactivation was determined by using 100-ms inactivating pre-pulses ranging from −120 mV to 0 mV in 5 mV increments from a holding potential of −120 mV, followed by test pulses to −10 mV for 20 ms. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude and plotted as a function of the pre-pulse potential. Data were fitted with a two-state Boltzmann equation of the form $I/I_{max} = [1 + (\exp(V - V_{1/2})/k)]^{-1}$, in which I is the peak sodium current, I_{max} is the maximal current evoked, V is the potential of the voltage pre-pulse, $V_{1/2}$ is the half-maximal voltage for inactivation, and k is the slope factor.

The voltage dependence of sodium channel slow inactivation was measured with 60 s conditioning pulses ranging from −100 mV to 0 mV in 10 mV increments, followed by repolarization to a holding potential of −120 mV for 100 ms to remove fast inactivation, and at last a −10 mV test pulse for 20 ms. The peak current amplitude during the test depolarization was normalized to the maximum current amplitude and plotted against the pre-pulse potential. Data were fitted with a two-state Boltzmann equation as above for fast inactivation.

Development of fast inactivation was measured by holding oocytes at −120 mV, followed by a depolarization to −45 mV for 0–80 ms, and then a −10 mV test pulse for 20 ms to measure the fraction of sodium current inactivated during the pre-pulse. The peak current during the test pulse was divided by the peak current which has a pre-pulse duration of 0 ms and plotted as a function of duration time of pre-pulse. Time constant (τ) was calculated by fitting the plot with a single exponential decay function and plotted as a function of the development pulse voltage (−40 mV, −45 mV or −50 mV).

Recovery from fast inactivation was tested with a conditioning depolarization of −10 mV for 100 ms, which will drive all sodium channels into the fast inactivated state, then repolarization to −70 mV for 0–20 ms followed by a 20-ms test pulse to −10 mV. The peak current during the test pulse was divided by the peak current during the inactivating pulse and plotted as a function of the duration of the repolarization pulse. Time constant (τ) of recovery from fast inactivation was calculated by fitting the plot with a single exponential function and plotted as a function of the recovery voltage (from −120 mV to −50 mV in 10- mV increments).