Voltage-gated Na Channels in Muscle

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Cloning and Sequence Analysis of the Voltage-Gated Muscle Na^+ Channel from the Poison Dart Frog *Phyllobates aurotaenia*

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Poison dart frogs of the genus *Phyllobates* secrete lipophilic alkaloid toxins through their skin that were used by Colombian Amerindians to poison the tips of blowdarts. One of the most potent toxins identified is batrachotoxin (BTX) which is an activator of voltage-gated Na^+ channels. BTX causes sustained opening of these channels by shifting the voltage-dependent activation to more hyperpolarized potentials and by disabling both fast and slow inactivation. It also alters pore conductance and selectivity. Endogenous Na^+ channels of the poison arrow frog have been proposed to be insensitive to lethal amounts of BTX. In this project we aim to identify what confers BTX insensitivity to Na^+ channels of the host frog *Phyllobates aurotaenia*, therefore we cloned its skeletal muscle Na_V channel. Total RNA from skeletal muscle of *Phyllobates aurotaenia* was isolated and cDNA was obtained with degenerate primers.
The 1819 amino acids sequence shares 72% sequence identity with the rat Na^+ channel $Na_V1.4$, and 73% with that of the snake *Thamnophis sirtalis*. The TMs are extremely well conserved (87%) with absolute conservation of S4 in all domains. The N-and C-termini as well as the cytoplasmic linkers between domains are more divergent. The D3-D4 linker containing the IFM motif is highly conserved except for Q1348E and K1350P. The DEKA-motif is also absolutely conserved as are the GGGS gating hinge and the QGFS motifs. BTX is thought to bind in the pore region, from the selectivity filter ring to the pore lining S6 TMs. We have identified two S to A mutations flanking the gating-hinge in domains 1 and 3 that may participate in toxin-insensitivity of the *Phyllobates* channel by impairing the binding of BTX. Supported by NIH GM68044(AMC) and GM30376(FB) and by COLCIENCIAS1106-12-13836(LF).

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Effect Of Temperature On Slow Inactivation Of WT And R1448H Mutant Sodium Channels

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Mutations in the voltage-gated sodium channel $hNa_v1.4$ have been correlated to various muscle diseases, such as Paramyotonia Congenita (PC), a disease clinically characterized by attacks of muscle stiffness and long lasting weakness mainly triggered by exposure to cold. Mutations causing PC share a common gating defect: slowed inactivation from the open state that mainly accounts for the disease symptoms. Previous studies show only slight effects of PC mutations on slow inactivation and its temperature dependence and significance remain unclear.
Therefore, we performed a detailed study of WT and R1448H, a typical PC mutation, gating in a broad temperature range (10 - 30°C) by performing whole-cell patch-clamp experiments on HEK-293 cells, stably expressing $hNa_v1.4$. Our focus was especially on the transitions from and into the slow inactivated state. Cooling slows entry and recovery from slow inactivation and shifts the steady-state inactivation to depolarized potentials. R1448H does not change the temperature dependency of slow inactivation, but shifts the steady-state slow inactivation curve about 5mV to hyperpolarized potentials at all investigated temperatures. This shift is mainly caused by a slowed recovery from slow inactivation together with an unchanged entry into slow inactivation.
In WT, the cooling induced depolarizing shift of the steady-state slow inactivation curve prevents channels from entering the slow inactivated state to maintain excitability in the cold. This effect is diminished in the mutant, which may contribute to cold induced paralysis.

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Use-Dependent Block of Voltage-Gated Sodium Channels by Orphenadrine through Binding at the Local Anesthetic Receptor

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Orphenadrine is most often used against muscle spasm and rigidity, and pain of various etiologies. The drug may act on multiple targets, including muscarinic, histaminic, and NMDA receptors. Its structure recalls that of local anesthetics, with an aromatic hydrophobic moiety linked to a protonable tertiary ammine terminal. Thus we wondered whether orphenadrine may also block voltage-gated sodium channels. We tested the drug on whole-cell sodium currents recorded using patch-clamp in HEK293 cells expressing the skeletal muscle (Nav1.4), cardiac (Nav1.5) and neuronal (Nav1.1 and Nav1.7) subtypes of human sodium channels. The results indicate that orphenadrine inhibits sodium channels in a concentration, voltage and frequency dependent manner. Introducing the F1586C mutation in hNav1.4 reduced two-fold the tonic block at the holding potential (hp) of -120 mV and almost zeroed the use-dependent block at 10 Hz, indicating that orphenadrine binds to the same receptor as the local anesthetics. Channel state-dependent affinities of orphenadrine were calculated using specific protocols. At the hp of -180 mV, the entire population of channels are closed and ready to open in response to depolarization. Thus determination of tonic block at this hp allows the determination of drug affinity for resting channels, which was 160 μM. The affinity for inactivated sodium channels (KI) was calculated according to the modulated receptor hypothesis, which forecasts that the apparent affinity measured at a particular hp depends on the proportion of resting and inactivated channels at this hp. The calculated KI value was 2.2 μM. Use-dependent reduction of sodium currents at the hp of -100 mV was enhanced by orphenadrine at 0.1 μM, a clinically relevant concentration. This study suggests that blockade of sodium channels may contribute to clinical efficacy of orphenadrine (Supported by Telethon-Italy grant GGP04140).

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Molecular Determinants of Coupling Between the Domain III Voltage-sensor and Local Anesthetic Binding Site in the Skeletal Sodium Channel

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Sodium channels are a major target for many toxins and drugs including local anesthetics (LA). Gating current (*Sheets and Hanck, J. Gen. Physiol.; 121(2), 2003*) and fluorescence measurements (*Muroi and Chanda, Biophysical Society Meeting, 2008*) show that LA binding to the pore mainly stabilizes the voltage-sensor of domains III of sodium channel in an activated conformation. The midpoints of the fluorescence-voltage (F-V) curves of probes attached to the voltage-sensor of domain III are left shifted by as much as 50 mV upon LA binding. How does the binding of LA to the pore affect the movement of the S4-domain III voltage-sensor? To identify the molecular determinants of interaction between the voltage-sensor and LA binding site, we systematically substituted tryptophan residues in the S4-S5 linker and S6 of domain III of skeletal Na^+ channel and examined the effect of these substitutions on the movement of DIII-voltage sensor in the absence and presence of LA by voltage-clamp fluorometry technique. A number of mutations in the S4-S5 linker and S6 either showed a significantly diminished or no shift in the F-V curve upon LA binding, suggesting that those residues are involved in coupling the voltage sensor and LA binding site of the sodium channel.
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Local Anesthetic Block of Mutant Rat Skeletal Muscle Na^+ Channels Lacking Fast Inactivation: Evidence for Activation Gate Trapping

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Local anesthetics (LAs) inhibit voltage-gated Na+ channels in a complex time-, voltage-, and state-dependent fashion that may involve a ''guarded intrapore receptor'' where closed channel gates (*m* - activation, and *h* - inactivation) preclude LA escape via the inner channel mouth leading to trapping and long-lived, drug blocked states. Recent evidence discounts the involvement of the *h* gate in this mechanism, but *m* gate trapping may contribute importantly to use-dependent block. Critical steps in *m* gate trapping are the initial binding of a local anesthetic molecule to the intrapore receptor followed by *m* gate closure upon deactivation. Consequently, the mechanism predicts that the fraction of long-lived blocked channels is directly related to the probability of intrapore receptor occupancy immediately preceding *m* gate closure.
To test this prediction we explored the relationship between open channel block and the long-lived drug blocked states in a mutant Na+ channel lacking fact inactivation. Disabling fast inactivation in this manner provided for the study of open channel blockade and use-dependent block in the absence of a functional *h* gate. We studied macro- and microscopic currents of a mutant rat skeletal muscle Na^+ channel (μ1, Nav 1.4) lacking fast inactivation through the triple point mutation (IFM/QQQ) in the III-IV interdomain (QQQ) expressed in *Xenopus* oocytes. Lidocaine (LIDO) caused use-dependent block of QQQ involving a long-lived drug blocked state (recovery τ=0.12s, -100mV). LIDO produced time dependent reduction of non-inactivating macroscopic currents and discrete and rapid block of single channel currents both of which report