# Voltage-gated Na Channels in Muscle

**1263-Pos Board B107**

**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.
Support: National Institutes of Health, AHA and Shaw Scientific Award.

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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 $\tau$=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

occupancy of the intrapore receptor. The fraction of long-lived states was tightly linked with the degree of receptor occupancy. The findings provide strong support for the *m* gate trapping of LIDO.

**1268-Pos Board B112**

**Molecular Determinants of μ-Conotoxin KIIIA Block of Voltage-Gated Sodium Channels**

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mu-Conotoxin (muCTX) KIIIA is of special interest both functionally and structurally because (1) it blocks neuronal voltage-gated sodium ($Na_v$) channels involved in pain signalling (Zhang et al., 2007, J. Biol. Chem.) and (2) unlike previously discovered muCTXs (most >22 amino acids), KIIIA has only 16 amino acids, missing amino acids in the N-terminal section. We have performed preliminary molecular dynamics simulations of muCTX KIIIA docking to a model of the $Na_v1.4$ outer vestibule (Choudhary et al, 2007, Channels). The results are consistent with a possible binding orientation in $Na_v1.4$ with K7 facing down into the pore, interacting with the outer ring charges (E403 & E758) in domains I and II. To exam this possible orientation, single-channel bilayer recordings from rat brain (preparation includes $Na_v1.1$, 1.2, 1.3 and 1.6) and rat skeletal muscle (muscle, predominantly $Na_v1.4$) preparations demonstrated that when lysine-7 (K7) is neutralized, channels show an increase in fractional residual current ($f_{res}$) upon KIIIA[K7A] addition (brain, $48 \pm 3\%$ & muscle, $45 \pm 9\%$) compared to wild type KIIIA (brain, $19 \pm 3\%$, muscle $19 \pm 3\%$). The wild-type non-zero $f_{res}$ hints that the lack of N-terminal residues or the use of a lysine residue (instead of arginine) to occlude the pore in KIIIA leads to incomplete toxin block, suggesting KIIIA has a ''looser'' interaction with the channel, with the key basic residue, K7, playing a smaller role in toxin block than in GIIIA and PIIIA. This data is supported by whole-cell experiments looking at KIIIA and KIIIA[K7A] interactions with multiple $Na_v$ isoforms. The single-channel and whole-cell data suggest KIIIA binds to the outer vestibule with the lysine at position 7 blocking current through the pore, similar to R13 in GIIIA (skeletal muscle specific) and R14 in PIIIA (blocks both skeletal and neuronal channels).

**1269-Pos Board B113**

**ProTxII Interacts Specifically with the Domain II Voltage Sensor of $Na_V1.4$ Modifying Gating Without Immobilization**

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ProTxII, a peptide extracted from the venom of the tarantula *Thrixopelma pruriens*, binds to multiple voltage-gated sodium channel isoforms. In $Na_V1.2$ ProTxII reduces the sodium conductance and decreases total gating charge (Sokolov et al., Mol. Pharm. 73:1020). In this isoform the toxin's effect could be partially reversed with strong depolarization indicating interactions with one or more of the voltage sensors. Mutagenesis data further implicated the domain II voltage sensor in the toxin-channel interaction in $Na_V1.2$. Here we have shown that, as was seen for $Na_V1.2$, ProTxII (2-5μM) produced a decrease in maximum conductance (~60%) and a decrease in total gating charge (~20%) in the $Na_V1.4$ isoform expressed in Xenopus oocytes. Unlike $Na_V1.2$, however, these effects on $Na_V1.4$ could not be reversed with strong depolarization. We also discovered a single residue, S660, located at the S3-S4 linker/S4 boundary of DII, which when mutated, renders this channel insensitive to toxin. We used site-specific fluorescent measurements to determine the effect of ProTxII on the movement of individual voltage sensors. In the presence of ProTxII the voltage dependence of the fluorescent signal of DIIS4 was modified, but not eliminated, indicating that this voltage sensor is not completely immobilized upon toxin binding. The fluorescent signals measured from domains I and III were not significantly affected by ProTxII implicating a specific interaction with DII in producing the effect of ProTxII on $Na_V1.4$. Supported by GM30376 (FB) and NS061535-01(GBE).

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**Tryptophan Scanning Mutagenesis to Identify the Residues Involved in Coupling between the Pore and DIII Voltage-Sensor of a Sodium Channel**

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In response to membrane depolarization, voltage-gated ion channels undergo a structural rearrangement that moves the voltage sensing segments in the electric field and initiates a series of conformational transitions that ultimately opens the channel pore. The mechanism of coupling between the voltage-sensing domain and pore domain remains poorly understood. To characterize the molecular basis of this coupling, we have systematically substituted tryptophan residues in the S4-S5 region (from C-terminus of S4 to N-terminus of S5) and C-terminus of S6 of domain III in the skeletal muscle sodium channel. The effects of these perturbations on the movement of the voltage-sensor were monitored by using a site-specific fluorescent reporter on S4 of domain III using voltage-clamp fluorometry. Conformational changes in the pore were tracked by measuring the inward sodium currents. Our study identifies a number of mutants, which stabilize the voltage-sensors in the activated conformation while destabilizing the open pore conformation relative to the wild type. We suggest that the residues at these positions play an important role in coupling the voltage-sensor of domain III to the pore of the sodium channel.
Support: National Institutes of Health, American heart Association and Shaw Scientist Award.

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**Double Mutant Perturbation Analysis Reveals High Conformational Stability Of The Domain IV S6 Segment Of The Voltage-gated Na Channel**

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The S6 segment of domain IV (DIV-S6) of the voltage-gated Na channel is considered to be a key player in gating and local anesthetic drug block. Thus, some mutations in DIV-S6 substantially alter the channel's inactivation properties. In order to get a comprehensive picture of the kinetic role of DIV-S6 in fast inactivation we performed a cysteine scanning analysis of sites 1575-1591 in the DIV-S6 of the rNav1.4 channel. In addition, we produced the same cysteine replacements in the background of the mutation K1237E. K1237 is located in the P-loop of domain III and mutations at this site have dramatic effects both on permeation and gating properties. Hence, K1237E most likely causes a complex conformational change of the channel. We sought to explore whether K1237E changes the pattern of gating perturbations by the serial cysteine replacements in DIV-S6. The constructs were expressed in *Xenopus laevis* oocytes and studied by means of two electrode voltage-clamp. The half-point of availability following a 50 ms conditioning prepulse (V05) was -44 ± 1 mV and -51 ± 1 mV in wild-type and K1237E, respectively ($P < 0.001$) . Most serial amino acid replacements in DIV-S6 produced shifts in V05, both in wild-type and in K1237E background, ranging from +17 ± 1 mV to -9 ± 2 mV. A plot of the shifts in V05 by single DIV-S6 mutants relative to wild-type versus the shifts in V05 by double mutants relative to K1237E showed a significant positive correlation ($R = 0.72$, $P = 0.002$). This indicates that the general pattern of gating perturbations in DIV-S6 is not affected by K1237E, suggesting a high conformational stability of the DIV-S6 segment during the fast inactivated state. Support: FWF P21006-B11

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**Charge Immobilization From The Open And Closed States Of Voltage-Gated Sodium Channels**

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We compared the immobilization of voltage sensor gating charge during open- and closed-state fast inactivation in skeletal muscle sodium channels. To do this we compared charge movement and its immobilization using ionic and gating current recordings in the cut open oocyte configuration. Charge movement and its immobilization were steeply voltage dependent at subthreshold voltages. Kinetics of charge immobilization during open- or closed-state fast inactivation were more rapid than the concomitant decay of ionic current. The extent of charge immobilized was correlated with the completion of closed-state fast inactivation at the most negative pulse commands, and reached a maximum of 2/3 of the ON gating charge. Anthopleurin-A decreased the votlage dependence of charge immobilization compared to wild type channels. Whereas anthopleurin slowed open-state fast inactivation, the toxin accelerated currrent decrement at voltages for which only closed-state fast inactivation was possible. Anthopleurin selectively accelerated remobilzation of the gating charge in fast inactivated channels without slowing the onset or decreasing the extent of charge immobilized. This work was supported by NIH P20RR016454 to ISU.

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**Structure-activity Relationship of Primaquine and Sodium Channel rNav 1.4**

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Mutations in the putative selectivity filter region of the voltage-gated $Na^+$ channel, the so-called DEKA-motif not only affects selectivity and the