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Mexiletine-responsive Erythromelalgia Due To A New $Na_V1.7$ Mutation Showing Use-dependent Block

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Inherited erythromelalgia (IEM), characterized by episodic burning pain and erythema of the extremities, is produced by gain-of-function mutations in sodium channel $Na_v1.7$, which is preferentially expressed in nociceptive and sympathetic neurons. Most patients do not respond to pharmacotherapy, although a few patients have been reported as showing partial relief with lidocaine or mexiletine. We report here a new IEM $Na_v1.7$ mutation and its favorable response to mexiletine. SCN9A exons from the proband were amplified and sequenced. Whole-cell patch-clamp analysis was used to characterize wild-type and mutant $Na_v1.7$ channels in mammalian cells. A 7-year-old girl, with a two-year history of symmetric burning pain and erythema in her hands and feet, was diagnosed with erythromelalgia. Treatment with mexiletine reduced the number and severity of pain episodes. We identified a single nucleotide substitution (T2616G) in exon 15, not present in 200 ethnically-matched control alleles, which substitutes valine 872 by glycine (V872G) within DII/S5. V872G shifts activation by -10 mV, slows deactivation, and generates larger ramp currents. We observed a stronger use-dependent inhibition by mexiletine of V872G compared to wild-type channels. The $Na_V1.7$/V872G mutation provides a molecular basis for DRG hyperexcitability which can produce pain. While most IEM patients do not respond to pharmacotherapy, this patient displayed a favorable response to mexiletine, which appears to be due to use-dependent block of mutant channels. Continued relief from pain, even after mexiletine was discontinued in this patient, might suggest that early treatment may slow the progression of the disease.

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Biophysical Characterization Of Duloxetine Activity On Voltage-gated Sodium Channels Involved In Pain Transmission

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Duloxetine, an inhibitor of the serotonin-norepinephrine reuptake system, is widely used for the treatment of major depression and has also been found to be effective in reducing neuropathic pain. This latter effect is believed to be related to the role of the two neurotransmitters in inhibiting pain signals at the level of spinal and supraspinal neural circuits. However the possibility that duloxetine also acts through alternative mechanisms has not yet been investigated. In this study we focused on the potential effect of duloxetine on neuronal isoforms of voltage-gated sodium channels (NaV1.3 and NaV1.7) that have a pivotal role in the generation and propagation of pain signals. Conventional whole-cell patch-clamp recordings were performed on CHO-cells expressing hNaV1.3 and hNaV1.7. Duloxetine exerted a concentration-dependent resting block, measured by the reduction of the sodium current elicited by a single depolarizing step from -100mV to 0mV for 10 ms, with IC50 values of 5 and 16 microM for NaV1.3 and NaV1.7, respectively. These values were halved at holding potentials that reduce channel availability of both channels by 50%, indicating a preference for the inactivated state of sodium channels. Furthermore we observed that 10 microM duloxetine induced a 10mV negative shift of the steady state inactivation curves, and preliminary results indicate that this effect is correlated with stabilization of slow inactivation. Finally, for both sodium channel isoforms, an additional 20% inhibition over the resting block was observed at concentrations close to the IC50 when the stimulation frequency was increased up to 10Hz. Thus, the antidepressant duloxetine exhibits most of the state-dependent mechanisms of a classic sodium channel blocker. These results may add valuable information about the pharmacological spectrum of duloxetine, an issue of high clinical impact considering its increasingly widespread use.

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Biophysics Of Inhibition Of hNav1.3 And hNav1.7 Channels By A-803467

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It has been recently published that a small molecule, A-803467, selectively inhibits hNav1.8 sodium channel subtype and is also efficacious in a variety of pre-clinical pain models (ref. 1). While A-803467 blocks $hNa_v1.8$ with an IC50 value of 8 nM (at half-maximal inactivation), it also inhibits other sodium channel subtypes with a selectivity ratio of 30-1000-fold. In particular, it inhibits $hNa_v1.3$ and $hNa_v1.7$ channels, which have connection to pain sensation in animal models (ref. 2), with IC_{50} values around 1 μM. We suggest that inhibition of these channels may also contribute to analgesic efficacy of A-803467. We therefore were interested in studying biophysical parameters of interaction of A-803467 with $hNa_v1.7$ and $hNa_v1.3$ channels. We present the data on the affinity of binding of the drug to the resting and inactivated states of the channels, as well as the on-rate of binding to inactivated channels, retardation of repriming and use-dependent block of trains of depolarizing pulses. The biophysical profile of A-803467 suggests interaction with a site distinct from the ''classical'' local anaesthetic receptor site in Nav channels. This profile is compared with the parameters measured for other clinically relevant analgesics.

(1) Jarvis MF, et al. Proc Natl Acad Sci USA 2007; 104:8520-85.
(2) Dib-Hajj SD, et al. Trends in Neurosciences 2007; 30 (11):555-63.

1292-Pos Board B136

Counting EGFP-Marked Ion-Channels of Voltage-Clamped Oocytes to Deduce the Single Channel Gating Charge

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Recently we indirectly determined the gating charge of single Na-channels (qNa) to be about 6 e (Gamal El-Din et al., 2008). We compared the ratios of total fluorescence intensity to total gating charge (Ft/Qt) of EGFP marked K- and Na-channels expressed in Xenopus laevis oocytes. This allowed to deduce qNa from the better known K-channels (qK = 13-14 e) without the need to know the fluorescence intensity of a single EGFP (Fs). Since we used EGFP to mark and count both channel types, Fs cancelled out in the comparison.
We now attempt to determine and apply Fs to obtain the total number of channels (Nt = Ft/Fs) and from that q = Qt/Nt directly. As outlined in the above cited study, Ft was extrapolated to the whole oocyte's surface from a defined region along the circumference in order to minimize contributions from submembraneous channels and cytoplasmic autofluorescence. Micrographs of EGFP solutions of known concentration in a hemocytometer chamber provided a figure for Fs. However, this figure cannot simply be transferred to the situation of the oocyte images because it holds true only for EGFP molecules in frontal areas, accessible for the entire opening of the microscope objective. At lateral areas of the oocyte the fluorescence intensity is attenuated. The attenuation was determined by measuring the fluorescence intensity of control oocytes whose surface was strongly and homogeneously stained with a fluorescent dye such that autofluorescence from the cytoplasm could be neglected. Comparison of the extrapolation from either the lateral or the frontal area to the total fluorescence of the oocyte (Ft) gave an attenuation factor of 3 to 6 depending on the optics. This factor was used to obtain the number of EGFP marked channels.

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Measuring The Contribution Of S4 Charges On Gating Currents Of A Sodium Channel

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Upon membrane depolarization, conformational changes in the S4 voltage sensors results in the opening of the voltage-gated sodium channel. The movement of the positively charged residues on the four distinct voltage-sensors in the membrane electric field generates a measurable transient current referred to as the gating current. The ON gating currents of the Na+ channel has two kinetic components and the fast component correlates well with the kinetics of fluorescence changes of probes attached to the voltage sensors of domains I, II, and III. The slow component of the gating current, however, matches with the fluorescence kinetics of probes on domain IV voltage-sensor (Chanda and Bezanilla JGP 2002 120; 629-45). In an attempt to specify the molecular origin of the slow and fast components of the gating current, we neutralized the first three charged residues (Q3 mutants) in each of the four voltage-sensing segments of the sodium channel. Our data supports the hypothesis that the voltage-sensor of domain IV primarily contributes to the slow component of the gating current and provides support to the notion that the movement of this voltage sensor is slower than the opening of the pore.

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Gateless Gating Model vs. Gated Pore Model: Phase Pinning of Guanidinium Toxins in Sodium Channels

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Unlike the gated pore model, which assumes the existence of a movable gate within the molecule, the gateless gating model (H. R. Leuchtag, *Voltage-Sensitive Ion Channels,* Springer 2008) (VSIC) explains ion-channel gating as a phase transition. Under this model, the closed channel conformation imposed

by the resting electric field is a compact, ordered phase with behavior, such as critical temperature, hysteresis, fractional dispersion exponent (constant phase angle) and Curie-Weiss law, similar to that of phases seen in ferroelectric liquid crystals (VSIC, pp. 355-383). In the absence of a toxin molecule, threshold membrane depolarization indirectly brings about a stochastic phase transition to a less ordered phase in which S4 segments expand by the mutual repulsion of their positively charged residues. The resulting wider pitch of permeation pathway α helices elastically linked to the S4s permits ion replacement in the interloop H bonds and the subsequent percolation of permeant ions through the channel (VSIC, 477f, 506f). With an externally applied tetrodotoxin (TTX) or analog molecule complexed in the channel, however, the ordered phase is pinned by the toxin, inhibiting the transition to the ion-conducting phase (VSIC, 76, 382f). Phase pinning by impurities is an established effect in ferroelectric liquid crystals. In the toxin, a guanidinium group, $H_2N^+{=}C(NH_2)_2$, a highly resonant, planar, positive ion, is active in pinning the closed phase. The fact that guanidinium is also found in ferroelectric crystals such as guanidinium aluminum sulfate hexahydrate suggests that TTX enhances the spontaneous polarization of the resting phase. This explanation by the gateless gating model of specific toxin action is based on physical principles; in contrast, the phrase ''TTX blocks the pore'' offered by the gated pore model is vague and at the macroscopic rather than molecular scale.

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Self-organized Models of Selectivity in Ca and Na Channels

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A simple pillbox model with two adjustable parameters accounts for selectivity of both DEEA Ca channels and DEKA Na channels in many solutions of different composition and concentration. Only side chains are different in the model of Ca and Na channels. Parameters are the same for both channels in all solutions. 'Pauling' radii are used for ions. No information from crystal structures is used. Side chains are over-approximated as spheres. Predicted properties of Na and Ca channels are very different. How can a simple model give such powerful results when chemical intuition says that selectivity depends on the precise relation of ions and side chains? We use Monte Carlo simulations of this model that determine the most stable — the lowest free energy — structure of ions and side chains. Structure is the computed consequence of the forces in this model. Forces are steric repulsion and electrostatic attraction of ions crowded into a small space, modified by protein polarization. The relationship of ions and side chains varies with ionic solution and is very different in Na and Ca channels. Selectivity is a consequence of the 'induced fit' of side chains to ions and depends on flexibility (entropy) of side chains as well as their location. The model captures the relation of side chains and ions well enough to account for selectivity of both Na and Ca channels in the many conditions measured in experiments. Evidently, the structures in the real Na and Ca channels responsible for selectivity are self-organized, at their free energy minimum, close to the positions computed in our model. Oversimplified models are enough to account for selectivity if the models calculate the 'most stable' structure, as it changes from solution to solution, and mutation to mutation.

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Design, Production and Characterisation of a Thermally-stable Mutant of the Bacterial Voltage Gated Sodium Channel Nachbac

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NaChBac from *B. halodurans* is a bacterial homologue of the eukaryotic voltage-gated sodium channels which has been expressed and purified from *E. coli*. We have previously shown (Nurani et al (2008) Biochemistry 31:8114-8121) that this membrane protein,, purified from E. coli, forms a mostly helical, tetrameric detergent-solubilisable protein that is capable of binding the drug mibefradil and inducing sodium flux when reconstituted into vesicles. The tetrameric quaternary structure of NaChBac differentiates it from the single-chain eukaryotic sodium channels.

The aim of the present study was to produce a more thermally-stable version of this ion channel which would be suitable for a wide range of structural and functional studies. Using molecular modelling techniques, we have designed a mutant, G219S, which incorporates a serine instead of a glycine at the proposed site which is proposed to form the hinge which enables opening and closing of the channel. The aim was to reduce flexibility and ''lock'' the protein in a single state. Mutant protein was cloned, expressed and purified from E. coli and compared with the wild type protein isolated in the same manner. Whilst it had a similar secondary structure, thermal melting curves monitored by circular dichroism spectroscopy indicated that the mutant was considerably more stable than the wild type protein, although it is still capable of binding mibefradil. Thus the protein produced had the properties as designed and is a particularly suitable candidate for new structural, functional and drug binding studies.
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Ion Pair Formation During Activation of the NaChBac Voltage Sensor

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S4 transmembrane segments of voltage-gated ion channels move outward upon depolarization initiating a conformational change that opens the pore. Formation of ion pairs between gating-charge-carrying arginine residues in S4 and negatively charged amino acid residues in neighboring transmembrane segments is an essential feature of the *sliding helix* model of gating (Catterall, 1986; Guy and Seetharamulu, 1986; Yarov-Yarovoy et al., PNAS, 2006). We studied NaChBac mutants in which E70 in the S2 segment and the fourth gating charge of S4 (R4) were replaced with cysteines. As previously reported for D60:R3 (DeCaen et al. PNAS, 2008), activation of the E70C:R4C reduced I_{Na} irreversibly but had no effect on WT or single mutants. Application of the reducing agent β-mercaptoethanol restored I_{Na}, suggesting reversal of disulfide bond formation between E70 and R4. The voltage dependence of disulfide locking matched the voltage dependence of activation ($V_{1/2} \approx$ -75 mV). Fast deactivation was blocked, and the loss of current upon repolarization was slowed to the rate of inactivation ($\approx$330ms). Evidently, depolarization drives movement of the S4 segment that allows disulfide locking of R4C and E70C, and this activated state of the voltage sensor signals opening of the pore and then inactivation of the channel. These data suggest that gating charge R4 forms an ion pair with E70 during activation and that the side chains of these residues approach within ~2 Å, as required for rapid formation of disulfide bonds in the E70C:R4C mutant. This new molecular interaction allows further refinement of the ROSETTA sliding helix model of gating (see adjacent poster by Yarov-Yarovoy et al). Supported by NIH Grants T32 GM07270 (PGD) and R01 NS157561 (WAC).

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Structural Modeling of Intermediate States of the Gating Pore of NaChBac

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Voltage-gated sodium channels initiate action potentials in excitable cells. Despite progress in determining the structures of voltage-gated potassium channels, the high-resolution structure of the voltage-gated sodium channels remains unknown. We used the Rosetta-Membrane method (Yarov-Yarovoy et al. Proteins 62, 1010, PNAS 103, 7292) and experimental data suggesting proximity between E70 in S2 and R4 in S4 during activation of NaChBac to construct structural models of intermediate states during channel gating. The structure of the Kv1.2-Kv2.1 chimera channel in the open state (Long et al. (2007) Nature 450, 376) was used as a template and proximity between Cβ atoms of E70 in S2 and R4 in S4 was favored during modeling. The resulting structural models suggest a molecular mechanism of the voltage-dependent activation of NaChBac in which S4 rotates clockwise (as viewed from the extracellular side of the membrane) and translates outward, as proposed in the 'sliding helix' model of gating, while gating-charge-carrying arginines in S4 sequentially interact with negatively charged residues in the S1, S2, and S3 segments. Transition through a local 3-10 helical conformation of a short segment of S4 containing two gating-charge-carrying arginines in the narrow part of the gating pore is required for simultaneous interaction with their ion pair partners during activation. The side chain of highly conserved F67 in S2 is oriented sideways away from the gating pore to allow the long side chains of arginines in S4 to pass through the middle of the gating pore. Outward motion of the S4 segment is coupled to lateral movement of the S4-S5 linker and movements of the S5 and S6 segments that open the intracellular gate of the pore-forming module. Supported by NIMH Grant K01 MH67625 (to V.Y.-Y.) and NIH Grant R01 NS15751 (to W.A.C.).

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Biophysical And Pharmacological Profiling Of Multiple Na_V Subtypes On QPatch HT

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The voltage dependent sodium channel is responsible for the upstroke and directed propagation of action potentials in nerve and muscle cells, and is therefore a central ion channel in excitable tissues. The implication of voltage gated sodium channels in pain mediation, and diseases such as epilepsy and cardiac arrythmia has made them very important targets for drug discovery. Nine