595-Pos

Divergent Pharmacological Properties of SCN1A Splice Variants

Christopher H. Thompson, Kristopher M. Kahlig, Alfred L. George Jr.
Vanderbilt University, Nashville, TN, USA.

Voltage-gated sodium channels undergo alternative mRNA splicing. In the human neuronal Nav1.1 channel encoded by SCN1A, a common genetic variant affecting an intron splice donor site alters the proportion of transcripts that incorporate the canonical exon 5 (exon 5A) or an alternative (exon 5N) encoding portions of the S3 and S4 segments of domain 1. Epileptic subjects with this genetic variant require lower doses of anticonvulsant drugs such as phenytoin compared with individuals lacking this variant. Because this genetic variant is associated with a larger proportion of exon 5N containing transcripts in brain, we hypothesized that differences in function and pharmacology of Nav1.1 channels containing either exon 5N or 5A account for the observed divergence in anticonvulsant dose requirements. To examine differences in drug efficacy of SCN1A splice variants, we performed whole-cell recording on tsA201 cells transiently co-transfected with either Nav1.1-5A or Nav1.1-5N and two accessory subunits (β1,β2). We examined voltage-dependence of activation, steady-state inactivation, and recovery from fast inactivation and observed no significant differences between splice variants. We also measured both steady-state block and use-dependent block (10Hz) by phenytoin, carbamazepine, and lamotrigine. Nav1.1-5N channels exhibited greater steady-state block by phenytoin(100μM) ($16 \pm 5\%$ vs. $2 \pm 6\%$) and lamotrigine(200μM) ($25 \pm 4\%$ vs. $14 \pm 2\%$) compared to Nav1.1-5A. Additionally, Nav1.1-5N exhibited greater use-dependent block by phenytoin ($39 \pm 5\%$ vs. $24 \pm 4\%$) and lamotrigine ($29 \pm 6\%$ vs. $18 \pm 2\%$). We tested cells stably transfected with either Nav1.1-5A or Nav1.1-5N and both β subunits using an automated planar patch clamp system (Patchliner, Nanion Inc.) to perform concentration-response curves to determine steady-state and inactivated state affinities for lamotrigine. Similar to conventional patch clamp experiments, lamotrigine exhibited greater steady-state and inactivated state affinity for Nav1.1-5N than Nav1.1-5A. These results suggest SCN1A transcripts containing the alternative exon 5N encode channels that are more sensitive to multiple anticonvulsant drugs.

596-Pos

From Plastic-Bottle-Toxin to Sodium Channel Blocker: A New Role for Bisphenol A

Angelika Lampert[1,2], Andrias O. O'Reilly[3], B. A. Wallace[3], Christian Alzheimer[1,2].
[1]FAU Erlangen, Erlangen, Germany, [2]Univ. of Kiel, Physiology, Kiel, Germany, [3]Univ. of London, Dept. Crystallography, London, United Kingdom.

Bisphenol A has reached public attention due to its presence in food and beverages following leaching from plastic containers.. It is a monomer that is polymerized to manufacture polycarbonate plastic food wrappings and it is detectable in the blood of human populations in developed countries (Palanza et al. 2008, Environmental Research). In animal studies and in vitro bisphenol A was shown to have estrogenic effects. Data link bisphenol A exposure to a variety of diseases including miscarriage, menstrual pain and cardiovascular syndromes.

The human heart voltage-gated sodium channel hNav1.5 was expressed in HEK293t cells to determine the effect of bisphenol A on sodium channel function.

With whole-cell patch clamp analysis, we show that bisphenol A reduces the peak sodium current through hNav1.5 from a holding potential of −120 mV with an EC50 of 54 ± 8 μM This concentration is considerably higher than has been found in beverages from plastic bottles. However, compared to other known sodium channel blockers, such as lamotrigine or lidocaine, its efficacy is approximately ten-fold higher.

Bisphenol A shifts steady-state fast inactivation to more hyperpolarized potentials, whereas voltage-dependence of activation is unaffected. As with local anesthetics, bisphenol A binds preferentially to the inactivated state. The association time constant, as determined by a single exponential fit of peak current decline induced by 30 μM bisphenol A is approximately 14 times faster than that induced by 300 μM lidocaine.

In conclusion, we have determined that bisphenol A has blocking effects on hNav1.5 sodium currents that are more pronounced than those of known blockers, such as lidocaine or lamotrigine.

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597-Pos

Characterization of the Reverse Use Dependent Block of Voltage Gated Sodium Channels

Caterina Virginio, Elisa Ballini, Laura Aldegheri, Laura Castelletti, AnnaMaria Capelli, Wolfgang Jarolimek.
GlaxoSmithKline, Verona, Italy.

Some clinically used drugs interact *via* state-dependent inhibition of voltage-gated sodium channels. For example, cocaine, procaine or lidocaine preferentially interact with, and stabilize the inactivated conformation of the channel. Upon repetitive high frequency activation they cause a progressive inhibition during the pulse train which is termed use-dependent inhibition.

Here we describe compounds that show the opposite behaviour, ie the inhibition is diminished during the pulse train.

To adequately determine the state-dependent interactions of drugs with sodium channels, we developed a high-throughput electrophysiological assay using the IonWorks(r) Quattro(tm) PPC platform. Compounds were tested against the brain Nav1.3 sodium channel expressed in CHO cells. A train of 10 depolarizing voltage steps from −90mV to 0mV for 20ms (10Hz frequency) was applied before and after compound addition. To evaluate the tonic block, inhibition of the peak current at the first pulse was measured while the use-dependent block was determined as the inhibition at the 10th pulse. Lidocaine shows the expected use-dependent inhibition. Surprisingly, we found compounds with the opposite profile: the compound with the most pronounced effect blocked the 1st and 10th pulses by 72.3 ± 6.1 % and 42.9 ± 6.9 % (mean ± SD, n=5) at 10uM. In a second instance these compounds were tested against the cardiac Nav1.5 and the peripheral nervous and neuroendocrine systems Nav1.7 observing similar effects. An in-depth comparison between use-dependent and reverse use-dependent blockers was performed for parameters such as voltage-dependent activation and inactivation, recovery from inactivation, and frequency dependency. These data provide biophysical insights in the mechanism of reverse use-dependent inhibition for Nav channels.

598-Pos

Insights on the Mechanisms of the Fast Blockade of TTX-R Na+ Channels by Eugenol

João L. Carvalho-de-Souza, Antonio Carlos Cassola.
University of São Paulo, São Paulo, Brazil.

OBJECTIVES. It was previously shown that eugenol, a phenylpropene, blocks fast and reversibly voltage-gated Na+ channels (NaV), but little concern was given to the blocker binding to different conformational states of channel molecule. Here we reported a detailed analysis of state-dependent effects of eugenol on tetrodotoxin-resistant (TTX-R) NaV isoforms, comparing them to those of lidocaine, a reference blocker.

METHODS. TTX-R Na+ currents were recorded in dorsal root ganglia neurons from newborn Wistar rats with whole-cell configuration of patch clamp technique. Tetrodotoxin-sensitive Na+ currents were blocked by TTX 100nM in the extracellular solution.

RESULTS and CONCLUSIONS. A dose-dependent fast blockade due to eugenol was observed in 0.2Hz time series depolarizations from a holding potential of −110 mV to a 0 mV pulse. This tonic blockage is due to eugenol binding to the closed state. The IC50 was 2.28 ± 0.10mM for eugenol compared to 0.44 ± 0.08mM for lidocaine. The tonic NaV blockade was more effective when the membrane was held at more depolarized, still sublimiar, holding potentials. This observation indicates a higher affinity of eugenol for closed substates dwelled at less hyperpolarized potentials. No consistent evidences for additional binding to open state were observed. A displacement of steady-state inactivation curve to more negative potentials, associated with a slower recovery from fast inactivation under eugenol indicates that this molecule also binds to fast inactivated state. For currents undergoing slow inactivation, a consistent reduction by eugenol indicates that the phenylpropene additionally binds to the slow inactivated state. A frequency-dependent blocking effect of eugenol on NaV was observed, but the effect is smaller than that induced by lidocaine. In conclusion, eugenol binds to several isoforms of TTX-R NaV and to the different states of the proteins, leading to a channel blockage.

599-Pos

A Residue (W756) in the P-Loop Segment of Sodium Channel is Critical for Primaquine Binding

Eduardo M. Salinas[1], Evelyn Martinez-Morales[1], Lourdes Millan-PerezPeña[2], Thomas F. Scior[3].
[1]Instituto de Fisiologia-BUAP, Puebla, Mexico, [2]Centro de Quimica-BUAP, Puebla, Mexico, [3]Facultad de Ciencias Quimicas-BUAP, Puebla, Mexico.

The scope of our study on wild-type and mutant-types of the voltage-dependent sodium channel in rat skeletal muscle (Nav 1.4) was to examine the possible binding site of primaquine (PQ) using a combined design and experimental approach. We applied a standard voltage-clamp in oocytes and in-silico methods, mainly protein modeling and ligand docking. Previously, we demonstrate that PQ blocks the voltage-dependent sodium current in rat myocytes, and these block is concentration-dependent and voltage-independent fashion. Direct site mutagenesis in the P-loop segment (W756C, W1239C and W1531A at the

outer tryptophan-rich lip, as well as D400C, E758C, K1237C and A1529C of the DEKA locus) helped us to identify residues playing a key role in aminoquinoline binding. In full agreement with our computed results, tryptophan W756 is crucial for the reversible blocking effects of PQ. W756C abolished the blocking effect of PQ in voltage-clamp assays.

600-Pos

Defining the Voltage Sensor Properties and Pharmacology of Nav1.9

Frank Bosmans[1], Michelino Puopolo[2], Marie-France Martin-Eauclaire[3], Bruce P. Bean[2], Kenton J. Swartz[1].
[1]NIH, Bethesda, MD, USA, [2]Harvard Medical School, Boston, MA, USA, [3]CNRS, Marseille, France.

The voltage-activated sodium channel Nav1.9 is preferentially expressed in DRG neurons where it is believed to play an important role in pain perception. However, progress in revealing the gating characteristics and pharmacological sensitivities of Nav1.9 has been slow because attempts to express this channel in a heterologous expression system have been unsuccessful. Here we use a protein engineering approach to study the contributions of the four Nav1.9 voltage sensors to channel function. We define individual S3b-S4 paddle motifs within each voltage sensor and show that these structural motifs sense changes in membrane voltage and determine the kinetics of voltage sensor activation. Toxins from tarantula and scorpion venom interact with each of these four motifs and can be used as pharmacological tools to alter Nav1.9 currents in native DRG neurons. Our results provide answers to fundamental questions on the functional role of the four voltage sensors in Nav1.9 and may be useful in developing new strategies to combat pain.

Voltage-gated K Channels-Permeation

601-Pos

KcsA Barium Permeation Blocked by External Potassium

Kene N. Piasta, Christopher Miller.
Brandeis University, Waltham, MA, USA.

Block by Ba^{2+} is a distinctive property of K^+ channels, and in a few cases this block can be used as a tool to determine the affinity for various ions at specific sites in the selectivity filter. We measure the discrete block of single E71A KcsA channels, a non-inactivating mutant, with micromolar concentrations of internal Ba^{2+} and find at high concentrations of external K^+ the block time distribution is described by a double exponential. This suggests there are two Ba^{2+} sites in the selectivity filter, fitting well with the published Ba^{2+} containing structure of KcsA where a Ba^{2+} ion resides approximately in S2 and S4. Utilizing a kinetic analysis of the blocking events, we can determine the occupancy of K^+ and other cationic monovalent ions in an extracellular site, presumably S1, and thus determine selectivity for this particular site. Our kinetic data has shown KcsA has an unusually high selectivity for K^+ over Na^+ with a ddG^0 of -5.6 kcal mol^{-1}.

602-Pos

The Role Of Oligo-(R)-3-Hydroxybutyrates in the *Streptomyces lividans* KcsA Channel

Alexander Negoda, Elena Negoda, Rosetta N. Reusch.
Michigan State University, East Lansing, MI, USA.

The *Streptomyces lividans* potassium channel KcsA is a tetramer of four polypeptides, each covalently modified by oligo-(R)-3-hydroxybutyrate (cOHB), which envelop a core molecule of inorganic polyphosphate (polyP). It has been proposed that the polyanion, polyP, attracts, binds and conducts K^+ in response to an electrochemical stimulus whilst the polypeptides govern access to polyP and regulate its selectivity. The function of cOHB, however, has been undefined.Digestion of KcsA with CNBr yields a 6.7 kDa fragment that contains most of the ion pathway (residues 97-154). This fragment was shown to contain cOHB by Western blot and chemical assays. The conjugation sites for cOHB were determined to be on S102 and S129. The effects of the single mutations S102, S129 and the double mutation S102:S129 on channel activity were examined. Wild-type KcsA, incorporated into planar lipid bilayers of POPC:POPE:POPG (3:3:1) between aqueous solution of 200 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4 *cis* and 20 mM KCl, 5 mM $MgCl_2$, 20 mM Hepes, pH 7.4 *trans* forms well-structured channels of 147 pS conductance. Under the same conditions, S102G exhibits irregular conductance with a major conductance state of 75 pS and a minor conductance state of 103 pS, S129G exhibits frequent but unsuccessful attempts to insert into the bilayer, and S102:S129 exhibits no channel activity whatsoever. The results suggest that cOHB-modification of KcsA polypeptides is essential for normal channel function.

603-Pos

Potassium Channel Block by a Tripartite Complex of Neutral Ligands with a Potassium Ion

Pavel I. Zimin[1], Bojan Garic[2], Heike Wulff[1], Boris S. Zhorov[2].
[1]UC Davis, Davis, CA, USA, [2]McMaster University, Hamilton, ON, Canada.

Potassium channels are targets for medically important drugs of large chemical diversity. While classical hydrophilic cations like tetraethylammonium block Kv channels with a stoichiometry of 1:1, many uncharged lipophilic (neutral) compounds exhibit Hill coefficients of 2. An example is the alkoxypsoralen PAP-1, which blocks Kv1.3 channels in lymphocytes with an IC50 of 2 nM and constitutes a potential drug for autoimmune disease such as type-1 diabetes, multiple sclerosis, rheumatoid arthritis, and psoriasis. The atomic mechanism of Kv channel block by ligands like PAP-1 is unknown. We first studied structure-activity relationships of PAP-1 derivatives and found that the carbonyl group in PAP-1's coumarin ring is indispensable, but does not accept an H-bond from the channel. We next demonstrated that block by PAP-1 is voltage-dependent, a feature expected for cationic but not neutral ligands. We then employed molecular modeling to predict the PAP-1 receptor and arrived at a model in which the carbonyl groups of two PAP-1 molecules coordinate a potassium ion in the permeation pathway, while the hydrophobic phenoxyalkoxy side-chains extend into the intrasubunit interfaces between helices S5 and S6 and reach the L45 linker. We tested the model by generating 58 point mutants involving residues in and around the predicted receptor, and then determined their biophysical properties and sensitivity to block by PAP-1. We found excellent agreement between the atomistic model and the results of experimental studies. Besides the known drug-binding locus in the inner pore, which is rather conserved between different Kv channels, the PAP-1 receptor involves loci where sequence homology is low. These loci constitute attractive targets for the design of subtype-specific potassium channel drugs and offer new directions for structure-based drug design.
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604-Pos

Effect of Peptide Toxins on C-Type Inactivation of a Mutant Human Voltge-Gated Potassium Channel (*hKv1.3)*

Azadeh Nikouee Ghadikolaei, Stephan Grissmer.
Institute of Applied Physiology, Ulm, Germany.

Current through wt *h*Kv1.3channels is characterized by the typical C-type inactivation and the high affinity block by scorpion peptide toxins. These toxins belong to a short peptide toxin family with high similarities in 3D shape and sequence and are thought to block current through *h*Kv1.3 channels by interacting with the outer vestibule of the channel thereby physically occluding the channel pore. In this study we measured current through *h*Kv1.3_V388C mutant channels, which inactivated faster compared to the wt channels and recovery from inactivation was also slower. In our experiments we examined the effect of CTX, NTX, KTX, AgTX2 and MgTX, each at a concentration of 100 nM, on current through mutant channels. KTX and AgTX2 did not or hardly block peak current through the mutant channel at 100 nM, respectively, indicating a loss of affinity by a factor of >100 due to the mutation in the channel. In addition, KTX and AgTX2 did not change the inactivation time course of the current through the mutant channels. In contrast, MgTX, even at a concentration of 10 nM, almost completely blocked peak current, indicating similar affinities of MgTX to mutant and wt channels. Interestingly, CTX and NTX did not block peak current through the mutant channels, however, almost completely abolished inactivation. This is different from the effect of TEA that is known to prevent inactivation while blocking current through the channel. We conclude that CTX and NTX bind to the external vestibule of the channel thereby preventing structural rearrangements of the outer vestibule that normally occur when channels inactivate.
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605-Pos

Alprenolol Inhibits Herg Potassium Channels

Seung Ho Lee, Hyang Mi Lee, Bok Hee Choi.
Chonbuk National University Medical School, Jeonju, Republic of Korea.

The action of alprenolol, a non-selective beta blocker as well as 5-HT receptor antagonist, on the cloned cardiac human ether-a-go-go-related gene (HERG) channels was investigated using the whole-cell patch-clamp technique. Alprenolol reduced HERG whole-cell currents in a reversible concentration-dependent manner, with an IC_{50} value and a Hill coefficient of $12.3 \pm 0.8 \in 1/4M$ and 0.98 ± 0.06, respectively. Alprenolol affected the channels in the activated and inactivated states but not in the closed states. The alprenolol-induced blockade of HERG was found to be use-dependent, exhibiting a more rapid onset and a greater steady-state block at the higher frequencies of activation.