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Effects of Tolaasin Inhibitory Factors on Tolaasin Peptide Channel Evaluated by Competition with Zn^{2+}

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Tolaasin is a 1.9 kDa bacterial lipodepsipeptide toxin and forms membrane pores causing brown blotch disease on the cultivated mushrooms. Molecular multimerization of tolaasin is required to form membrane channel. Previously, we showed that various chemicals inhibit the multimerization of tolaasins and thus prevent from brown blotch disease. These chemicals named tolaasin-inhibitory factor (TIF) were applicable to control the disease. Various candidates were chosen from different food additives and they were successful for disease control at 1-100 μM. Interestingly, after tolaasin molecules formed channels on the erythrocyte membrane and hemolysis started, further hemolyses were stopped as soon as TIF's were added. The added TIF was not washed by centrifugation and addition of fresh HBS solution. These result imply that TIF's are able to inhibit the preformed tolaasin channels. TIF may inhibit tolaasin-induced hemolysis either by plugging the channel pores or by the dissociation of tolaasin multimers. Zn^{2+} is a potent tolaasin inhibitor known to bind to tolaasin channel. In order to characterize the TIF-induced inhibition, competition effect of Zn^{2+} and TIF on tolaasin channel activity was investigated. When Zn^{2+} and TIF-9 were added simultaneously, no additive effects were observed at various concentration combinations. However, in the combinations of Zn^{2+} plus either TIF-11 or TIF-16, no additive effect was measured and the inhibitory effect of Zn^{2+} was reduced in the presence of low concentration of Zn^{2+}. In these experiments, the inhibition of hemolysis was dominated by Zn^{2+}concentration. Based on these results, we suggest that TIF has high affinity to tolaasin channel than Zn^{2+} although Zn^{2+} is a strong inhibitor.

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Effects of Tolaasin Inhibitory Factors on Tolaasin-Induced Blotch Formation and Hemolysis

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Tolaasin, 1.9 kDa peptide toxin, is produced by *Pseudomonas tolaasii*. It forms pores on the membrane and thus destroys cellular structure, causing brown blotch disease on the cultivated mushroom. Previously, we showed that the tolaasin-induced pore formation required the molecular multimerization and the multimerization of tolaasin was blocked by the treatment of various tolaasin-inhibitory factors (TIF). This was successful to prevent from causing brown blotch disease. Various TIF's blocked effectively the tolaasin-induced hemolysis at 1-100 μM. The tolaasin-induced hemolysis was dependent on the temperature and pH; however, TIF always inhibited hemolysis. In the pitting test using mushrooms of Agaricus bisporus, tolaasin treatment caused brown blotches on the surface of mushroom and the addtion of TIF at 0.1-10 mM completely blocked the blotch formation by tolaasin. Since TIF's were selected from various food additives, the effect and mechanism will be different on the tolaasin-induced hemolysis. In this study, we have measured the synergic effect of various TIF's on the inhibition of tolaasin toxicity. When TIF-9 and TIF-11 were added, tolaasin-induced hemolysis was effectively inhibited. In the presence of both TIF's, the inhibition was increased to 2.5 times compared to the inhibition obtained by any one of them. Similar synergic effect was also measured by the additions of TIF-9 and TIF-16. The inhibition was also increased more than 3 times. However, TIF-10 and TIF-11 did not show any synergic effect. These results showed that there are synergic effects among various TIF's and the effect was dependent on the type and concentration of TIF.

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A Biochemical Characterization of the Major Peptides from the Venom of the Giant Neotropical Hunting Ant *Dinoponera Australis*

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Venom from the ''false tocandira'' *Dinoponera australis*, a giant Neotropical hunting ant,paralyzes small invertebrate prey and induces a myriad of systemic effects in large vertebrates. HPLC/DAD/MS analyses revealed that the venom has over 75 unique proteinaceous components with a large diversity of properties ranging in size, hydrophobicity, and overall abundance. The six most abundant peptides, demonstrative of this diversity and hereafter referred to as *Dinoponeratoxins*, were *de novo* sequenced by exact mass precursor ion selection and Edman degradation. The smallest peptide characterized, Da-1039, is hydrophilic and has similarities to vasoactive peptides like kinin and bombesin. The two largest and most abundant peptides, Da-3105 and Da-3177, have a 92.9% identity in a 28 residue overlap and share ~50 of their sequence with ponericin G2 (an antimicrobial from another ponerine ant *Pachycondyla goeldii*). One peptide, Da-1585, is a hydrophilic cleavage product of an amphipathic peptide, Da-2501. The most hydrophobic peptide, Da-1837, is amidated (a PTM observed in one half of the major peptides) and shares homology with poneratoxin, a sodium channel modifier found in the bullet ant *Paraponera clavata.* This study is the first examination of potential pharmacophores from venom of the genus Dinoponera (Order: Hymenoptera).

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Phloretin Affects the Voltage Gating of Alpha-Hemolysin Channel

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We studied the effect of dipole modifiers on the channel forming activity of *Staphylococcus aureus* alpha-hemolysin in phosphocholine bilayers bathed in 1 M KCl (pH 7.5). We manipulate bilayer dipole potential (V_d) adding phloretin or phloridzin to reduce (V_d), and 6-ketocholestanol and RH 421 to increase V_d. In the absence of dipole modifiers, an alpha-hemolysin pore fluctuates between high (~1 nS) and low (~0.1nS) conductance states at transmembrane voltage $V \geq |100|$ mV. Addition of 20 μM phloretin after the channel formation induces transition of the channel to low-conductance state at $V \geq |25|$ mV. Adding phloretin before channel incorporation does not influence its voltage gating. Additions of phloridzin (20 μM) and RH 421 (10 μM) in the membrane bathing solution before or after channel formation, or addition of 6-ketocholestanol (50 mol.%) into the membrane composition do not affect the channel gating. We conclude that variation in V_d induced by dipole modifiers does not influence alpha-hemolysin pore gating and the effect of phloretin is likely to be attribute to specific interaction. It is also likely that the binding site for phloretin becomes accessible after alpha-toxin incorporation into the membrane and subsequent pore formation. We suggest that phloretin binding reduces the energy barrier for conformation transition of alpha-hemolysin pore to low-conductance state. The nature of specific interaction between phloretin and alpha-hemolysin channel is discussed. The work is supported in part by RFBR (project 09-04-00883), the Program of Presidium of the RAS ''Molecular and Cell Biology'', the Grant of Administration of St.-Petersburg for young scientists and State Contract (FAE П1372).

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Peptide-Gating of Hynacs Shares Features with Proton-Gating of ASICs

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Recently we have identified five new ion channel subunits (HyNaC1-5) from *Hydra magnipapillata* that belong to the ENaC/DEG family of ion channels. We could show that the ion channel made out of HyNaC2/3/5 is gated directly by two endogeneous neuropeptides, the HydraRFamides I and II. Prominent human members of the ENaC/DEG family are the proton-gated ASICS, which are involved in pain sensation, learning, memory and salt taste. Here we present our results showing that peptide gating of HyNaCs shares features with proton gating of ASICs. We found that similar to ASICS, HyNaCs were opened by low Ca^{2+} concentrations with a concentration of ~15 μM leading to half maximal activation. Moreover, the apparent peptide affinity was increased in solutions containing 10 μM Ca^{2+} compared to 2 mM. Application of HydraRFamides in low Ca^{2+} solution lead to a potentiation of currents indicating that divalent cations block the open channel. In ASICS, Asp_{432} is involved in this open channel block by Ca^{2+}. Introducing this mutation in HyNaCs almost completely abolished Ca^{2+} induced currents and the apparent peptide affinity became independent of Ca^{2+} concentration. This mutation also diminished the potentiating effect of low Ca^{2+} on peptide activated currents. These results show that Ca^{2+} is an important modulator of peptide-gating of HyNaCs, like it is on proton-gating of ASICs, and that the Ca^{2+} binding site is conserved between HyNaCs and ASICs.

Protons, which gate ASICs, had a dual effect on HyNaC2/3/5 currents. First, application of pH 5 inhibited open channels. Second, in solutions nominally free of divalent cations strongly increased current amplitude after washout of protons uncovered an activation of the channel by low pH. In summary these results indicate that the gating mechanism is similar between the evolutionary old HyNaCs and ASICs.

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Mapping the β-Scorpion Toxin Receptor Site on Voltage-Gated Sodium Channels

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Voltage-gated sodium channels are molecular targets of β-scorpion toxins, which enhance excitability by shifting the voltage dependence of activation

to more negative potentials. These effects result from a voltage sensor trapping mechanism, in which toxins trap the voltage sensor in its activated conformation. Determinants of β-scorpion toxin (CssIV) binding and action on sodium channel (Nav1.2) are located in the S1-S2 and S3-S4 extracellular linkers in the voltage-sensing module in domain II. To completely map these regions, we made substitutions for previously unstudied amino acid residues and examined modulation by $CssIV^{E15A}$, a highly active toxin derivative. Of 11 positions studied in IIS1-S2, only one significantly altered the toxin effect from wild-type by reducing binding to the resting state and almost abolishing trapping activity. In IIS3-S4, five positions surrounding a previously identified key binding determinant, G845, define a hotspot of high impact residues. Three of these substitutions reduced toxin binding and voltage-sensor trapping. The other two, V843A and E844N, increased voltage-sensor trapping approximately 4-fold and decreased apparent EC_{50}. The rate of voltage sensor trapping upon depolarization was unchanged for V843A and increased approximately 2.5-fold for E844N. The rate at which the toxin releases the voltage sensor upon repolarization was increased 2.2-fold for the V843A but was unchanged for E844N. Thus $CssIV^{E15A}$ interacts with a short segment of IIS1-S2 and a broader region of DIIS3-S4. The bidirectional effects of mutations on toxin efficacy suggest that native residues make both positive and negative interactions with the toxin. Substitutions that increase toxin effects do so by increasing affinity of resting channels for the toxin and further increasing the relative affinity of the activated voltage-sensor for the toxin. These results provide further support for the voltage sensor-trapping model.

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A Cytotoxic Peptide from a Marine Sponge, Polytheonamide B; II. Properties for Ion Conduction and Voltage Dependent Gating

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A 48-mer peptide from a marine sponge *Theonella swinhoei*, polytheonamide B (pTB), is highly cytotoxic against eukaryotic cells. Alternating D- and L-chirals throughout the molecule suggest formation of β-helix, which was supported by channel activity having high permeability to Cs^+. The 48-mer β-helix is long enough to span the membrane and was shown to form the channel by single peptide. In this study we evaluated the ion conduction and gating properties of pTB channel by use of planar lipid bilayer technique. Single-channel *I-V* curve in CsCl solution exhibited weak outward rectification. Concentration-dependency of single-channel conductance of pTB channel was examined. For Cs^+ permeation, clear saturation in unitary conductance was observed in the concentration range up to 3.0 M, which was contrast to gramicidin A (gA) channel. Gating manner of pTB channel was characteristic. Fast transition between open and closed state was observed, suggesting that the structural changes of single pTB molecule directly link to the gating. In addition to asymmetrical single-channel conductance, distinctive voltage-dependent gating was observed in single-channel and macroscopic current traces of pTB channels. On the basis of the structure of pTB channel, mechanism of ion conduction and voltage-dependent gating will be discussed.

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Physalia physalis Poison Depolarizes Beta Cell Membrane and Increases Insulin Secretion

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Peptide toxins isolated from marine cnidarians are considered as useful tools for studying ionic channels and promising drugs for therapeutics. Hemolytic and cardiotoxic activities have been described in Physalia physalis venom, and two toxins with anticholinergic and antiglutamatergic effects have been isolated in its high molecular weight fraction.
In this work, we explored some crude extract of P. physalis and its low molecular weight fractions on ionic currents and insulin secretion of pancreatic rat beta-cells. P. physalis specimens were collected at the north littoral of La Habana, Cuba. Crude extract were purified by a gel filtration (Superdex 200). Mass spectrometry MALDI-TOF and RP-HPLC (C18 column) were performed on each fraction collected from gel filtration. The biological activity on insulin secretion was tested in a reverse hemolytic plaque assay on primary cultures of pancreatic beta cells from male Wistar rats.
In basal glucose (5.6 mM), the crude extract (25 protein ug/mL) increased by 77% the average immunoplaque area, which is directly proportional to insulin secreted by isolated cells, without disrupting of beta cells or erythrocytes integrity. Low molecular fractions from gel filtration did not exceed of 20 kDa, and most of them eluted before the 20 % of acetonitrile on RP-HPLC. These fractions increased both, the percentage of insulin-secreting cells and the average immunoplaque area at a basal glucose level, suggesting a direct effect on TRP-type channels that are responsible for beta cell depolarization.
Physalia physalis poison contains polar bioactive compounds capable of enhance the secretory behavior of pancreatic beta cells from male Wistar rats by depolarizing the membrane, in non stimulant glucose conditions.
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Insights on Channel-Like Activity of Membrane Bound Alpha-Synuclein

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Alpha-synuclein (syn) is a natively unfolded protein with the ability to acquire secondary structure upon interaction with membranes or with itself. It is linked to Parkinson's disease (PD) by two evidences: the accumulation of amyloid fibrils of the protein and the autosomal dominant forms of the disease (A53T, A30P and E46K mutants). Both the biological role of this protein as the mechanisms involved in the ethiopathogenesis of PD are still unknown. The protein is located at the presynaptic terminal of neurons in all the Central Nervous System, where it exists free in the citosol or bound to synaptic vesicles. The membrane binding causes the formation of an amphipatic alpha-helix in the first part of the protein, which lies at the membrane surface without crossing the bilayer. A recent paper by Zakharov et al. (Biochemistry, 2007) reports that upon the application of a potential a channel like activity of syn can be recorded. Authors suppose that the helices of the first hundred amino acid of syn can pass the membrane bilayer to compose a pore only upon the application of a potential. Both the mechanism and the biological implication of this behaviour are still unknown and potentially of interest for the role that syn channel may play. In this study we extended this approach to syn deletion mutants. Furthermore, the effect of monomer topology in the construction of the purported channels have been explored thought the design of several types of syn dimers. A comparative analysis of the electrophysiological properties of these constructs will be presented and discussed.

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Developing a Functional Screening Assay of Small Molecules That Can Reduce Leakage of Liposomes Induced by Amyloid-Beta Peptides

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Alzheimer's disease (AD), an ultimately fatal neurodegenerative disorder, is characterized by the presence of plaques containing fibrillar aggregates of amyloid-beta (Aβ) peptides. These peptides, with 39-43 amino acids, especially Aβ(1-40) and Aβ(1-42), are the major components of plaques formed in the brain of patients with AD and are considered to be pathologically important. Both peptides aggregate rapidly in aqueous solution to form Aβ oligomers as well as Aβ fibrils over time. Increasing evidence indicates that Aβ peptides, especially in their oligomeric state, play an important role in pathogenesis of AD. One possible pathogenic mechanism of these Aβ oligomers is the formation of pores through neuronal membranes, resulting in cell death. This research examined the effect of Aβ on permeabilization of liposomes by monitoring the change of fluorescence intensity of pH-sensitive dye which was encapsulated in the liposomes due to the leakage of protons. These experiments showed that the effect of Aβ depended on the lipid composition and on the aggregation state of Aβ. The developed liposome leakage assay makes it possible to screen several potential drug candidates for their ability to inhibit or reduce the leakage of liposomes.

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Efficacious in vivo Electrophysiological Screening of Neuromodulatory Compounds: Using Drosophila to Evaluate the Activity of Conotoxins

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Finding compounds that affect neuronal function is central for the development of probes or potential therapeutic agents. We have devised an efficacious