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Mitogenic Activation and Proliferation of T Lymphocytes in TRPM7 Kinase-Dead Mutant Mice

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LY2087101 ([2-[(4-Fluorophenyl)amino]-4-methyl-5-thiazolyl]-3-thienylmethanone) was identified as a positive allosteric modulator (PAM) of nicotinic acetylcholine receptors (nAChR) in a high-throughput screen at Eli Lilly and Company. LY2087101 potentiated α7 and α4β2 nAChRs but not α3β2 nAChR (Broad et al. 2008, *J. Pharmacol. Exp. Ther., 318*: 1108). Mutational analyses and computer docking simulation with α7 nAChR predicted that PNU120596 and LY2087101 bind to a common site within the transmembrane domain (Young et al. 2008, *PNAS 105*:14686). In this study, we use mutational analyses and two-electrode voltage-clamp recording from *Xenopus* oocytes to begin pharmacological characterization of LY208101 interaction with α4β2 nAChR and to identify the molecular determinants of LY2087101 selectivity for α4β2 vs. α3β4 nAChRs. LY2078101 potentiated ACh-induced currents of low ACh sensitivity (α4)3(β2)2 nAChR and high ACh sensitivity (α4)2(β2)3 nAChR with similar potency. However, the maximum potentiation at 10 μM LY2087101 was higher in (α4)3(β2)2 than (α4)2(β2)3 nAChR. Mutational analyses of amino acids contributing to extracellular subunits interface (e.g. α4His114, α4Gln122, and α4Thr124, known selectivity determinants for the (α4)3(β2)2 nAChR selective PAM NS9283; Olsen et al 2013, JBC 288: 35977) had no effect on LY2087101 potentiation. Within the transmembrane domain, mutation at α4Ser285 but not α4Thr261 within the M1 helix significantly reduced LY2087101 potentiation of α4β2 nAChR α4Ser285 and α4Thr261 are equivalent to α7Ser222 and α7Ala225, respectively; two important positions for potentiation of α7 nAChR by LY2087101 and PNU120596. α4Thr261 projects to intra-subunit pocket within the α4 transmembrane helix bundle whereas α4Ser285 is exposed to intra-subunit pocket and inter-subunit space. Ongoing mutational analyses within the M1 transmembrane domain will define the contribution of intra-subunit vs. inter-subunit binding sites on LY2087101 potentiation of α4β2 nAChR.

Ion Channel Regulatory Mechanisms

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High Membrane Permeability for Melatonin

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The pineal gland is the endocrine organ in the brain that synthesizes and secretes the circulating night hormone melatonin throughout the night. How is this hormone secreted? Is it by calcium-dependent vesicular exocytosis or by simple diffusion across the pinealocyte plasma membrane? Experiments were designed to compare the cell membrane permeability to three indoleamines: melatonin and its precursors N-acetyl serotonin (NAS) and serotonin. The three experimental approaches were (i) to measure the concentration of indoleamine amperometrically in the bath while cells were being loaded by a whole-cell pipette, (ii) to measure the rise of intracellular indoleamine fluorescence as the compound was perfused in the bath, and (iii) to measure the rate of quenching of intracellular fura-2 dye fluorescence as indoleamines were perfused extracellularly in the bath. These measures showed that permeabilities of melatonin and NAS are high (both are uncharged), whereas that for serotonin (mostly charged) is much lower: melatonin > NAS ≫ serotonin. Comparisons are made with predictions of solubility-diffusion theory and compounds of known permeability, and a diffusion model is made to simulate all the measurements. The bottom line is that melatonin equilibrates across the plasma membrane in 3.5 s and has a membrane permeability of ~1.7 μm/s. Thus it and NAS will be ''secreted'' from pineal cells by membrane diffusion within seconds of synthesis. Circumstances are suggested when serotonin and possibly catecholamines (mostly charged) may also appear in the extracellular space passively by membrane diffusion from processes with high surface-to-volume ratio.

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Locking the Asymmetric open Conformation of MG^{2+} Channel Cora with a Synthetic Antibody Fragment

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Mg^{2+} is the most abundant divalent ion in living cells. Cells have adapted sophisticated mechanisms to regulate, store and transport Mg^{2+} across the membrane. In prokaryotes, the major uptake pathway for Mg^{2+} is the ion channel CorA. X-ray structures of closed state CorA at high Mg^{2+} concentrations reveal a symmetric homopentameric assembly where Mg^{2+} ions are bound in between the cytoplasmic domains inter-locking the channel. Recent data obtained by single particle cryo-EM suggest that at low Mg^{2+} concentrations, the open state CorA adopts an asymmetric conformation in which one of the cytoplasmic subunits 'sways away' from the axis of conductance pathway.

Here, we have identified high affinity synthetic antibody fragments (sAB) using a novel phage display protocol. sAB C12 was generated against nanodiscs-incorporated CorA under free Mg^{2+} conditions. Interestingly, even in the presence of Mg^{2+} the stoichiometry of sAB C12 binding to CorA remains at 1:5 (Fab:CorA), consistent with the asymmetric nature of the open state. Further, the crystal structure of the soluble domain of CorA in complex with sAB C12 shows that the sAB binds to a region of CorA not accessible in the closed state of the channel. Based on these results, additional analysis of C12-CorA interaction using biochemical, CW-EPR, and oocyte TEVC experiments confirm that the channel is locked in the open, conductive state. It is expected that sAB C12 complex can be used to gain further insights into this unique asymmetric conformation and to elucidate the molecular basis of Mg^{2+} permeation and gating in CorA.

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Mitogenic Activation and Proliferation of T Lymphocytes in TRPM7 Kinase-Dead Mutant Mice

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Calcium and magnesium ions have been implicated in T lymphocyte proliferation in response to antigen recognition. Specifically, it is believed that calcium and magnesium elevations in the cytoplasm are necessary for efficient T cell proliferation. Research over the past two decades has focused on identifying the plasma membrane ion channels responsible for governing Ca^{2+} influx in lymphocytes and Orai-STIM, Kv1.3 and KCa3.1 channels were shown to be crucial for persistent calcium mobilization. Pharmacologic or genetic suppression of calcium influx gives rise to lymphoproliferative defects. In addition to Ca^{2+}, several studies have demonstrated that Mg^{2+} influx positively regulates proliferation. TRPM7 is a channel-kinase highly expressed in T cells and reported to conduct both Ca^{2+} and Mg^{2+}. TRPM7 channels give rise to outwardly rectifying currents inhibited by cytosolic Mg^{2+}. TRPM7 protein appears to be necessary for murine thymocyte development, while small reductions in splenic T lymphocyte numbers were observed in TRPM7-deleted mice. Deletion of TRPM7 in a chicken B cell line resulted in severe proliferation defects. In this work we examine the role of TRPM7 kinase in T-cell blastogenesis and proliferation using a recently reported kinase-dead mutant mouse (Kaitsuka et al, Sci. Rep. 2014). Our goal was to compare the effects of extracellular Ca^{2+}/Mg^{2+} between wildtype and kinase-dead mutant mouse splenic T cells stimulated with PMA/ionomycin. Increasing Ca^{2+} or Mg^{2+} stimulated proliferation in both wildtype and kinase-dead mouse lymphocytes but with significant differences. Using a Coulter counter we also measured the diameters of resting and activated T cells in response to changes in external Ca^{2+}/Mg^{2+}. The differences between WT and mutant T cell blastogenesis and proliferation parameters are discussed in the context of controversies regarding the role of TRPM7 channel and kinase function in cellular Mg^{2+} homeostasis.

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Dynamic Interplay of Calmodulin and Fibroblast Growth Factor Homologous Factors in Regulating NA Channels

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In recent years, the ubiquitous Ca^{2+}-binding protein calmodulin (CaM), and the non-canonical intracellular fibroblast growth factor homologous factors (FHF) have emerged as two potent modulators of voltage-gated sodium channels with profound implications for the excitability of neuronal, cardiac, and skeletal muscle cells. Curiously, both regulatory molecules associate with the Na channel through a well-conserved channel carboxy-terminus with CaM preassociating to a canonical IQ motif and FHF interacting with the closely apposed preIQ - EF hand surface. Even so, the interaction of these auxiliary proteins is thought to evoke distinct and independent channel regulatory effects. CaM interaction with the Na channel confers a rapid Ca^{2+}-dependent feedback regulation (*Cell* **157**(7):1657) while FHF appear to alter various voltage-dependent properties of the canonical channel fast inactivation gate (*Cell. Mol. Life Sci.* **59**:1067). Here, we demonstrate a novel role of FHF to allosterically suppress Ca^{2+}/CaM-dependent regulation of skeletal muscle Na channels ($Na_V1.4$). In the absence of FHF, the wild-type $Na_V1.4$ channels exhibit rapid and robust Ca^{2+}-dependent inhibition mediated by a resident CaM. However, co-expression of FHF1 entirely abolishes Ca^{2+}-dependent inhibition of these channels. Similarly, co-expression of various isoforms of FHF (FHF2-4) with $Na_V1.4$ results in a graded suppression of Ca^{2+}/CaM regulation. These results argue that Ca^{2+}-regulation