Ligand-gated Channels: Glutamate Receptors

2521-Pos Board B491

A Mutant δ2 Ionotropic Glutamate Receptor Exhibits Dual Regulation by Phosphoinositides

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The δ2 glutamate receptor (GluRδ2) is considered a member of the ionotropic glutamate receptor family, although a specific ligand that activates the wild-type receptor has yet to be identified. GluRδ2 is enriched in the parallel fiber-Purkinje cell (PF-PC) synapse, but the precise physiological role of the receptor is still unclear. A naturally-occurring single point mutant in the third transmembrane domain of the receptor (A654T), named Lurcher ($GluR\delta2^{Lc}$), exhibits constitutive activity.

Our previous preliminary results suggested that the δ2 glutamate receptor - Lurcher mutant is inhibited by direct interactions with phosphatidylinositol 4,5-bisphosphate (PIP_2). Here we show that pre-incubation with wortmannin affects the activity of the receptor in a concentration-dependent manner, leading to an inhibition of the channel in the low micromolar range. This suggests that phosphatidylinositol 3-kinase (PI3K) could also be involved in the regulation of the δ2 glutamate receptor. We further investigate the involvement of phosphoinositides in the regulation of δ2 glutamate receptor using more specific PI3K and PI4K inhibitors as well as soluble stereoisomers of different phosphoinositides.

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Engineering light-gated glutamate receptors

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Ionotropic glutamate receptors are the major neurotransmitter receptor found at excitatory synapses in the central nervous system. We have developed a light-gated ionotropic glutamate receptor (LiGluR) that, when introduced into neurons, enables remote control of their activity. Light regulation is conferred by a nanoscale photoswitch consisting of a glutamate analog that is covalently tethered to the glutamate receptor through a photoisomerizable azobenzene moiety. The photo-switch requires a single amino-acid substitution in the glutamate receptor as a point for covalent attachment; thus LiGluR can be genetically targeted to neurons of interest.

We show that optical stimulation can be structured in designed spatial and temporal patterns, with action potentials generated by 1-5 millisecond long pulses of light. By changing the location of the amino-acid substitution, modifying the properties of the photo-switch, or applying this strategy to other glutamate receptors, we show that this approach represents a toolbox of options for precisely manipulating glutamate receptors and neural activity with light.

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Design Of A Potassium Selective, Light-gated Glutamate Receptor

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A major goal in molecular neuroscience is to understand structure-function relationships of glutamate receptor ion channels (iGluRs) and their role in behavior. In the mammalian central nervous system, iGluRs form non-selective cation channels that generate depolarizing potentials in fast excitatory synaptic processes. Having earlier introduced the light-activated glutamate receptor LiGuR, we went one step further and applied two types of structure-based design to develop a potassium selective, light-gated ion channel. Simple and fast Monte-Carlo simulations allow the rational design of optically-controlled proteins in general and LiGluR variants in particular. Using homology modeling we modified the transmembrane pore-region of LiGluR to create a potassium selective channel that responds to glutamate and light. We demonstrate that rapid pulses of light can hyperpolarize HEK-293 cells and neurons expressing this novel channel, which can be used as a tool in glutamate receptor screening applications and may enable optical neuronal inhibition.

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NMDA Receptor Subunit Arrangement Probed By LRET

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NMDA receptors are unique among the different subtypes of the ionotropic glutamate receptors since they require two agonists, glycine and glutamate, to bind to the receptor for the channel to open. They are heteromeric receptors composed of glycine binding subunits, such as NR1 and glutamate binding subunits, such as NR2, with the NR1 and NR2 subunits forming a dimer of dimers. While the crystal structures of the isolated ligand binding domain suggest that the dimer consists of one NR1 and one NR2 subunit, the arrangement of the dimer of dimers is not known, i.e. are the NR1 subunits adjacent, or across in the dimer of dimers. Using a modified NMDA receptor with specific donor:-acceptor fluorophores at the N-terminus, and at various sites on the domain 1 of the ligand binding domain we have measured the distances between the NR1 subunits, between the NR2 subunits, and between NR1 and NR2 subunits, using luminescence resonance energy transfer. Using these distance constraints we show that the NR1-NR1 subunits and the NR2-NR2 subunits are adjacent to each other in forming the dimer of dimers.

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Activation Mechanism of Native NMDA Receptors in Cultured Rat Neurons in Culture

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The activation mechanisms of recombinant NMDA receptor isoforms have been established in sufficient detail to account for their responses at both single-channel and macroscopic levels. Still, the reaction mechanism of native receptors remains uncertain due to indetermination of isoforms expressed, regulatory mechanisms and difficulties in regulating channel number. To investigate the activation mechanism of native receptors we recorded single-channel currents from cultured neurons dissociated from rat cerebral cortex and hippocampus. In several cell-attached patches, the currents originated from only one channel which remained active for tens of minutes. Activity recorded from neurons maintained for less than 19 days in vitro (DIV) resembled those of recombinant NR1/NR2B isoforms (means, s.d.): Po = 0.12 ± 0.06, MOT = 3.2 ± 0.5 ms (n = 8; 783,147 events). In contrast, in all one-channel records obtained from 21 - 35 DIV neurons we observed kinetics similar to those of recombinant NR1/NR2A receptors (means, s.d.): Po = 0.33 ± 0.10 and MOT = 5.6 ± 0.9 ms, (n = 7; 890,060 events). Importantly, for both types of activity we routinely identified clusters of activity characterized by three distinct open durations consistent with modal behavior. These data support previous reports indicating that dissociated neurons in culture recapitulate the developmental pattern of isoform observed in the intact animal and show for the first time that, like recombinant receptors in heterologous cells, receptors native to rat cortical neurons can adopt modal kinetics.

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Kinetic Effects of Perturbations in the Ligand Binding Domain of NMDA Receptors

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NMDA receptors become active only after both glycine and glutamate bind to their cognate ligand binding domain (LBD) on NR1 and NR2 subunits, respectively. It has been proposed that the conformational changes that lead to channel opening are initiated by agonist-induced closure of the LBDs and that the stability of the closed structure correlates with the agonist's efficacy. To probe this hypothesis we characterized kinetic changes in receptor gating arising from complementary perturbations at the NR2-LBD: a) NR2-specific partial agonists, L-homocysteate or SYM2081, each reported to have ~80% efficacy; and b) NR2-LBD mutations (K487C, N687C) which lock the LBD in a closed-cleft conformation. We recorded steady-state single-channel currents from cell-attached patches containing only one receptor at saturating agonist concentrations. Kinetic analyses of these data indicated that neither perturbation affected the core gating mechanism of the channel which consisted of five closed and two open states, including desensitization. Open durations were only minimally affected with most of the kinetic effects observed resulting from changes in the duration of closures. Two closed time components were significantly changed compared to Control (tau_2, 1.7 ± 0.1 ms, tau_3 = 4.6 ± 0.2 ms, n = 5). These were increased by partial agonists to 3.5 ± 0.3 ms and 9.1 ± 0.8 ms, respectively (n = 10, $p<0.05$) and decreased for the locked-LBD mutant to 0.9 ± 0.1 ms and to 3.2 ± 0.2 ms (n = 4, $p<0.05$). These results are consistent with the hypothesis that the stability of the closed NR2-LBD conformation correlates with channel gating efficacy. Further, the data show that distinct local perturbations at the NR2-LBD affect gating through the same mechanism observed as changes in the duration of two specific closed time components.

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Diversity of NR1/NR2B Receptor Gating Kinetics

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NMDA receptors are heteromeric glutamate-activated ion channels composed of NR1- and NR2-subunits. Controlled expression of four NR2-isoforms

(A - D) results in receptors with distinct gating properties, contributing to the diversity of excitatory post-synaptic currents. Additionally, the NR1/NR2A-isoform can itself respond with distinct kinetics due to modal gating. To investigate whether the NR1/NR2B-isoform also gates with modal kinetics, we recorded steady-state single-channel activity from cell-attached patches of HEK293-cells transfected with NR1 and NR2B, in the continuous presence of saturating agonists. Single-channel records (n=37) revealed a variety of gating patterns illustrated by a 25-fold range in measured equilibrium open probability: range, 0.02 - 0.49; P_o (mean ± s.e.m.) = 0.20 ± 0.02. This diversity reflects mainly differences in mean closure durations per file: range, 6 - 200 ms; mean closed time = 43 ± 8 ms; with less spread for mean open durations: range, 1.8 - 10.5 ms; mean open time = 4.8 ± 0.3 ms. Kinetic analyses revealed that each record had 2-4 open and at least 5 closed components in the respective interval duration distributions. As with NR1/2A-receptors, in all NR1/2B-records we observed sporadic gating changes due to sudden changes in mean open durations, indicative of modal behavior. We identified three gating regimes, each having at least two open time components: a ubiquitous brief component (0.27 ± 0.01 ms) and at least one of three longer components (tau-L = 2.5 ± 0.1 ms; tau-M = 5.0 ± 0.2 ms; or tau-H = 10.0 ± 1.0 ms). In contrast to NR1/2A-channel behavior where modal gating allowed characterization of all observed channels, for the NR1/2B-receptor we also observed gating patterns which differ in mean duration of closures. These data and analyses reveal the variety of mechanisms generating the previously observed diversity of macroscopic NR1/2B-responses.

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Effect of Protons on the NR1/NR2A NMDA Receptor Kinetics

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NMDA receptors are glutamate-activated ion-channels that mediate fast excitatory transmission, synaptic plasticity and excitotoxicity. They assemble as heterotetramers of two NR1 and two NR2 subunits. Multiple isoforms with distinct kinetics, pharmacology and physiologic roles are differentially expressed in the central nervous system. Of these the NR1/NR2A and NR1/NR2B isoforms are most abundant. They are both inhibited by physiological proton concentrations but so far, the kinetic mechanism of proton inhibition has been characterized only for the NR1/NR2B isoform. To determine the mechanism of proton inhibition of NR1/NR2A receptors we recorded single-channel currents from cell-attached patches of HEK 293 cells transfected with NR1-1a, NR2A and GFP. The patch pipette contained saturating concentrations of glutamate and glycine and several proton concentrations in the range: pH 6.5 to 8.5. These records confirmed that protons do not change the channel's conductance and act solely by decreasing channel open probability (IC_{50} = 7.3). Kinetic analyses of our single-channel data showed that with increasing proton concentrations (pH 8, n = 5 vs. pH 6.5, n = 4) the mean channel open time decreased (7 ± 1.3 ms to 1.7 ± 3 ms) and the mean channel closed time increased (12 ± 0.1 ms to 94 ± 16 ms). To identify the rate constants affected by proton-binding we used best fit kinetic models to our single channel data. Results showed that similar to the mechanism previously reported for NR1-1a/NR2B receptors, protons inhibit NR1-1a/2A receptors by increasing the stability of two pre-open conformations. The rate constants we report here will help understand the role of protons in regulating synaptic transmission, plasticity and neuroprotection.

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A LRET Based Method To Studying Intersubunit Conformational Changes In The Ligand Binding Domain Of A Functional AMPA Receptor

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Ionotropic glutamate receptors are the main excitatory neurotransmitter receptors in the mammalian central nervous system. During activation of the receptor, an agonist binds to an extracellular domain initiating a sequence of conformational changes leading to the opening of a cation-selective channel, which subsequently closes during desensitization. Structures of the isolated ligand binding domain of the AMPA subtype of the receptor have provided the first clues of the structural movements within the ligand binding domain; however, these structures lack the crucial functional portion of the protein, the transmembrane segments. Additionally, these limited structures do not reveal the structural changes associated with desensitization, unless artificially decoupled with a disulfide bond. In order to determine how the agonist controls receptor activation and desensitization, it is necessary to investigate the changes in the ligand binding domain in the presence of the transmembrane segments. We have modified a functional AMPA receptor (ΔN*-AMPA) to serve as an LRET based probe that allows us to measure distance changes of the ligand binding domain in the presence of the transmembrane segments. This receptor has been modified such that fluorophores can be introduced at defined sites to serve as a readout of intersubunit distance measurements associated with the apo, activated, and desensitized state. These investigations suggest that the apo state in the presence of the transmembrane segments is decoupled, and during activation, the interface is coupled due to the driving force of cleft closure, thereby stabilizing the open channel, and then the interface decouples thus leading to desensitization.

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Partial Agonism And Lobe Orientation In The Glutamate Receptor, Glur2

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Ionotropic glutamate receptors (iGluRs) mediate the majority of excitatory synaptic transmission in the vertebrate CNS. iGluRs are ligand-gated ion channels and their complete structure is unknown; however, studies on the soluble constructs of ligand binding cores (S1S2) have provided considerable insight into structure, function and dynamics. A number of X-ray structures of these constructs bound to various ligands have been determined, all showing a bilobal structure open to different degrees depending on the bound ligand. Some structures of GluR2 S1S2 suggested a direct correlation between the degree of lobe closure and the efficacy of channel opening. However, significantly different structures were obtained in several cases for the same ligand at different crystallization conditions. The measurement by NMR spectroscopy of residual dipolar couplings (RDCs) in partially aligned proteins provides a means of orienting protein domains in solution. We used this method to determine the domain orientation of GluR2 S1S2 bound to partial agonists and an antagonist. The precision required and the limited stability of S1S2 made it necessary for us to develop a somewhat novel approach. The main limitation for achievable precision is the presence of ''structural noise'' in X-ray structures. We refined several structures using NH RDCs measured in 5 alignment media for S1S2 bound to glutamate. These structures were then shown to exhibit reduced structural noise when used with RDCs measured with other ligands. This allowed us to calculate the difference in the lobe orientation between glutamate and any other ligand with high precision. The results indicate that the degree of lobe closure is not necessarily correlated to the efficacy of a ligand, and that in some cases, the lobe orientation is likely to be highly dynamic.

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Microsecond-to-second Timescale Motions In The Ligand Binding Domain Of Glutamate Receptor 2

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Within the central nervous system, AMPA-type glutamate receptors mediate fast synaptic transmission and deactivate on the millisecond timescale. In this study, we characterize backbone amide nuclear spin dynamics associated with conformational and hydrogen exchange events in the ligand binding domain of glutamate receptor 2 and obtain a novel view of the backbone motions occurring over five orders of magnitude of timescale, spanning microsecond-to-second timescale motions. Most notably, we find that hydrogen exchange rates of particular residues in the ligand binding site provide important clues about the sequence of events leading to ligand detachment from the ligand binding domain. These results thus provide insights into the mechanism of receptor deactivation.

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Functional Characteristics of iGluR3 AMPA Receptor-Channels in Cell Attached Recordings

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Ionotropic glutamate receptors (iGluR's) are ligand gated ion channels that mediate most of the fast excitatory neurotransmission in the CNS. Aberrant function of glutamate neurotransmission can lead to epilepsy and other neurodegenerative disorders. The extracellular ligand binding domain is a bilobal structure that binds an agonist and induces channel activation. Data from single channel recordings from homomeric AMPA receptor subtype (GluR3) in cell-attached patches were analyzed using QuB software to examine preliminary kinetic models of agonist dependent channel activity. Cell attached recordings were performed with both full and partial agonists on stably transfected HEK 293 cells. Amplitude analysis uncovered three conductance states, 15 pS, 27 pS, and 40 pS, in the presence of the full agonist, glutamate, as well as the partial agonists, fluorowillardiine, chlorowillardiine and nitrowillardiine. Different modes of activation ranging from low to high open probability exist for this channel. In the presence of the full agonist, glutamate, during a high mode of activation, the channel