3663-Pos

Molecular Dynamics Simulations of the GABA Type A Receptor

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The type A GABA-receptors (GABARs) are chloride ion channels found in the brain and are the major inhibitory neurotransmitter receptors. Upon binding of an agonist, the GABAR opens and increases the intraneuronal concentration of chloride ions, thus hyperpolarizing the cell and inhibiting the transmission of the nerve action potential. GABARs can also be modulated by a variety of pharmacologically important drugs, such as diazepam and also other regularly abused drugs such as ethanol. Here, we present homology models (based upon the 4 Å resolution EM acetylcholine receptor structure) and simulations of the most abundant GABAR subtype ($\gamma 2\alpha 1\beta 2$), and also the alcohol-sensitive subtype ($\delta\alpha 6\beta 3$). As well as GABA itself, a variety of ligands were docked to the proteins to assess the potential binding sites, and also the affect of ligand binding on the properties of the structure. Through the modeling and simulation of the two different receptor subtypes, and comparison of the ligand effects upon each one, we are able to gain greater insight into the specific residues important in binding, and also details of the different behavior of the subtypes. This work performed under the auspices of the U.S. Department of Energy by Lawrence Livermore National Laboratory under Contract DE-AC52-07NA27344. Release number LLNL-ABS-417208.

3664-Pos

An Electrophysiology Assay for the Fast Characterization of $GABA_A$ Ion Channel Modulators

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The $GABA_A$ receptors belong to a family of ligand-gated ion channels mediating fast synaptic transmission. They are drawing great attention in the pharmaceutical industry due to their potential role in the development of new therapeutics affecting anxiety, sleep disorders, and muscle relaxation. However, ligand-gated ion channel screening has been hampered by the lack of suitable high throughput electrophysiology platforms. The existing electrophysiology screening platform (IonWorks) is unable to apply ligands rapidly during current recording, which can be a major drawback for inactivating channels. While some studies have shown that it is possible to record the GABA tail current and use that information during a screen, such methods have an inherently lower signal to noise ratio and cannot be used on faster desensitizers.

Here we present the use of a novel electrophysiology screening platform integrating a microfluidics network for the study of $GABA_A$ receptor pharmacology. This platform features fast (<100ms) solution exchange coupled with simultaneous data recording. A novel assay could monitor GABA response in real time, and obtain a 3 point EC_{50} dose curve within 1 minute.

The $GABA_A$ $\alpha 1\beta 3\gamma 2$ expressing HEK cells from Millipore were used for this study. The channel was targeted with agonists, including GABA and muscimol, inhibitors (picrotoxin, bicuculline, and gabazine), and positive modulators, including diazepam, zolpidem and chlordiazepoxide. The positive modulators produced concentration dependent augmentation of the GABA EC_{20} response. The pharmacology data determined using this method was consistent with the literature values obtained using other platforms. Statistical data for inter and intra-plate reproducibility, current stability, and Z-values, is used to validate this approach.

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Characterization of the Etomidate Binding Site in Expressed $\alpha 1\beta 3$ $GABA_AR$ by Photoaffinity Labeling

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A major site of general anesthetic action is the inhibitory γ-aminobutyric acid type A receptor ($GABA_AR$), which responds to various anesthetics by an enhanced GABA response. Using a photoreactive analog of the general anesthetic etomidate ([^{3}H]azietomidate), the etomidate binding site in the bovine brain $GABA_AR$ was identified by the pharmacologically specific photolabeling of two amino acids in the transmembrane domain, βMet-286 in the M3 helix and αMet-236 ($\alpha 1$ numbering) in the M1 helix, which both project into the interface between the β and α subunits (Li et al, J. Neurosci., 2006 26:11599-605). The $GABA_AR$ preparation used in that study was heterogeneous, as it was isolated on a benzodiazepine affinity column from a detergent extract of brain membranes. To examine anesthetic interactions with $GABA_AR$ of known subunit composition, we expressed $\alpha 1\beta 3$ $GABA_AR$ in HEK cells with an N-terminal FLAG epitope tag on $\alpha 1$ and purified hundreds of picomoles of receptor by detergent solubilization, affinity chromatography, and reconstitution into lipid. When this $GABA_AR$ was photolabeled with [^{3}H]azietomidate, the ^{3}H incorporation was enhanced by GABA and inhibited by etomidate, as determined by SDS-PAGE. From a preparative scale [^{3}H]azietomidate photolabeling of $GABA_AR$ (350 pmol of muscimol binding sites), potential photoincorporation in each of the 4 transmembrane helices from both the $\alpha 1$ and β3 subunits was examined by Edman degradation. In the $\alpha 1\beta 3$ $GABA_AR$, [^{3}H]azietomidate photolabeled the two residues at the β-α interface identified previously, as well as three additional residues, two within the β3M3 helix and the third in the $\alpha 1$M3 helix. To determine if these new labeled residues contribute to an etomidate binding site or represent lipid-exposed labeling, we are currently characterizing the etomidate inhibition of [^{3}H]azietomidate photolabeling at the level of individual amino acids.

3666-Pos

Effect of GABA Concentration on Current-Voltage Properties of the GABA(A) Receptor

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The γ-aminobutyric acid type A ($GABA_A$) receptor functions as a chemical to voltage transducer in the central nervous system, converting neurotransmitter signals to changes in postsynaptic membrane potential. The kinetics of GABAergic currents are known to be affected by membrane potential, but the dependence on neurotransmitter concentration has not been fully explored. The present study investigates the current-voltage (IV) relationship of $\alpha 1\beta 2\gamma 2$s $GABA_A$ receptors across a range of GABA concentrations using whole-cell patch clamp of transfected HEK293 cells. We have determined and validated a voltage ramp protocol verses a more traditional stepwise procedure, in order to rapidly quantify rectification, hysteresis and the reversal potential at GABA concentrations ranging from 0.3 to 1000 μM. Results show that the rectification and hysteresis of the IV curves are dependent on GABA concentration, whereas the reversal potential is independent. Specifically, measures of rectification and hysteresis segregated into two distinct populations, at low (0.3-10 μM) and high (30-1000 μM) GABA concentrations. We provide evidence that the current-voltage properties of $GABA_A$ receptors are concentration dependent and, consequently, that the GABA concentration must be taken into context when designing experiments and interpreting results. Finally, these results have implications for a better understanding of synaptic responses where receptor location plays a role in the level of GABA exposure.

3667-Pos

Molecular Determinants in Glycine and GABA-A Receptors that Sense Intracellular Chloride Concentration

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GABA-A and glycine receptors are ligand-gated ion channels belonging to the Cys-loop superfamily. They are permeable to anions and mediate fast synaptic inhibition in the central nervous system. We recently showed that intracellular chloride can affect the time course of deactivation of these channels (1). The aim of the present work was to identify the residues that are involved in ''sensing'' the intracellular chloride concentration.

We used the fast concentration jump technique to apply brief pulses (1-3 ms) of saturating glycine or GABA to outside-out patches from transiently-transfected HEK cells expressing alpha1 or alpha1 beta glycine receptors or alpha1 beta2 gamma2L GABA-A receptors. The effect of intracellular chloride could be mediated either by residues in intracellular loops, such as M1-M2 and M3-M4, or by the residues lining the channel itself. We used a combination of deletion and Ala/Cys-scanning mutagenesis approaches to address this question.

In other nicotinic channels, the long M3-M4 intracellular loop can be replaced by the equivalent domain (7 amino acids) of the orthologous prokaryotic channel from *Gloeobacter violaceus* without losing channel function (2). We found that chimeric constructs of glycine and GABA-A receptors containing the prokaryotic M3-M4 domain retained modulation by intracellular chloride, suggesting that the major cytoplasmic domain is unlikely to mediate this effect. The results of mutagenesis of residues facing the ion permeation pathway on the contrary strongly suggests that chloride ions affect channel kinetic by binding to a site along the pore; we have identified conserved residues that reduce the differences between the deactivation rates measured at different chloride concentrations.

(1) Pitt, Sivilotti and Beato (2008) J.Neurosci 28, 11454-11467
(2) Jansen, Bali and Akabas (2008) J.Gen.Physiol. 131, 137-146

3668-Pos

Microsecond Simulations Show that Ethanol Binds between Subunits and Stabilizes the Open Form of a Glycine Receptor Model

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Cys-loop receptors are a superfamily of ligand gated ion channels comprising such receptors as acetylcholine, serotonin, Glycine and GABA. Despite the low level of sequence conservation all these receptors are thought to share common structural characteristics. Numerous experiments describe in detail the pore channel and the extracellular domain. However due to the low sequence conservation between members of Cys-loop family, and the lack of any high resolution structures of these receptors in the trans-membrane domain (TMD), little information is available concerning the potential allosteric binding site. This potentiating binding site is of extreme importance for a pharmacological perspective, as it is the target of anesthetic an alcohol molecules.

Recently the crystal structure of the open form of Gloeobacter violaceus pentameric ligand-gated ion channel (GLIC) has been solved [1]. As Glycine receptors (GlyR) are the closest receptors in terms of sequence, to GLIC, we decided to create a homology model of the human homomeric alpha-1 GlyR. Furthermore we chose the well-known ethanol molecule as a ligand target for studying the potentiating effect of such molecules on Cys-loop receptors [2]. In total we present 2 microseconds of molecular dynamics simulations, the longest simulation ever presented for Cys-loop receptors. Our simulations show a spontaneous binding of ethanol in cavities of the TMD. These cavities are located between subunits of GlyR TMD, and involve several residues previously identified by mutations as crucial for the potentiating effect of GlyR by ethanol. We also show that ethanol is stabilizing the open form of the GlyR, which could explain the effect of allosteric ligand on Cys-loop receptors.

[1] Bocquet, N, et al. Nature (2009): (457)111-114.
[2] Harris, RA, Trudell, JR and Mihic, SJ. Sci Signal. (2008): 1(28):re7.

3669-Pos

The Activation Mechanism of the Rat Homomeric alpha2 Glycine Receptor

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The alpha2 glycine receptor subunit, abundant in embryonic neurons, is replaced by alpha1 in the adult nervous system. Glycine-activated single-channel currents were recorded in the cell-attached configuration at +100 mV pipette holding potential from alpha2 homomeric receptors expressed in HEK293. At all glycine concentrations (0.02 - 10 mM), openings were grouped in long (> 300 ms) bursts/clusters with high open probability (0.99). The mean duration of individual apparent openings was c. 30 ms. Shut-time intervals within groups of openings were dominated by short shuttings of 5-10 μs. The properties of these groups of openings appeared to have an unusually steep concentration dependence and it is unclear whether they represent receptor activations (i.e. bursts) or clusters. Several mechanisms were fitted by maximising the likelihood of the entire sequence of open and shut times, with exact allowance for missed events (program HJCFIT, ref.1). Several records obtained at different glycine concentrations were fitted simultaneously. Good fits were obtained with several qualitatively different mechanisms incorporating 2 or 3 binding sites. In fits with the flip mechanism (2), we found that the closing rate constant of alpha2 glycine receptors is slow (about 500 s-1) and thus the efficacy for the final opening step in the activation is >10 fold higher than that of alpha1 beta channels, the receptor in adult synapses. On the other hand, flipping efficacy and binding affinity were lower for alpha2 than for alpha1 beta channels. These differences confirm that the alpha2 glycine receptor properties make it less suitable than alpha1 beta channels to mediate fast synaptic transmission (3).

1. Colquhoun et al. (2003) J Physiol 547, 699-728.
2. Burzomato et al. (2004) J Neurosci 24, 10924-10940.
3. Mangin et al. (2003) J Physiol 553, 369-386.

3670-Pos

Transient Currents from Glycine Receptors Depend on Intracellular Chloride

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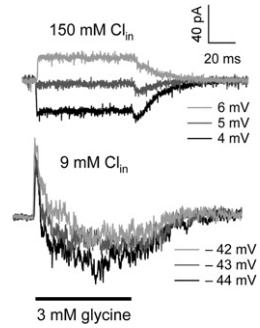
We transfected HEK-293 cells with cDNA for homomeric human α1 glycine receptors. 2-3 days after transfection, we recorded currents and used a submillisecond perfusion system to apply glycine to outside-out patches. The extracellular solution contained 150 mM chloride but the intracellular solution contained either 150 mM chloride (symmetric) or 9 mM chloride and 127 mM acetate (''physiological''). We clamped at a range of potentials near the current reversal potential for each condition. With symmetric chloride, currents were nearly constant during the 70 ms application of 3 mM glycine. With ''physiological'' chloride, currents reversed direction from inward to outward on the 5 ms time scale during agonist application. Similar biphasic currents were seen with gluconate as the substitute intracellular anion. Biphasic currents were observed at external pH values from 6.4-8.0, with HEPES, TRIS or PIPES as external buffer and with external choline replacing sodium. Activation of GlyR channels is dependent on Cl_{in} (J Neurosci 28:11454, 2008) but this cannot explain the observed reversal of currents. We are considering that local changes in Cl_{in} can cause this effect or that time-dependent changes in pore selectivity are possible.



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3671-Pos

A Novel Mechanism for the Inhibitory Action of Hydrocortisone at 5-HT$_3$A Receptor

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The 5-HT$_3$A receptor is a member of the Cys-loop family of ligand-gated ion channels. Due to its low conductance, analysis of this receptor has been restricted to the macroscopic level. We introduced mutations in the 5-HT3A subunit to obtain a high-conductance form so that single channels can be detected. We studied the actions of the neuroactive steroid, hydrocortisone (HC), in the high-conductance form of the 5-HT$_3$A receptor. Channel activity elicited by 1 μM 5-HT appears as opening events of 4.6 ± 0.4 pA (−70 mV) forming bursts, which coalesce into long clusters. A minor population of lower amplitude events (~2.8 pA) is observed, which corresponds to 0-10 % of the total events in all recordings. HC produces a concentration-dependent reduction in the duration of bursts and of the slowest open component (from ~100 ms in the control to ~3.6 ms at 400 μM HC), which can be explained on the basis of a slow block mechanism. Interestingly, amplitude histograms reveal a concentration-dependent increase in the relative area of the low-amplitude component without changes in its mean value. At 400 μM HC, the low-amplitude events correspond to 40 % of the total events. This channel population shows an amplitude of 2.8 ± 0.5 pA (−70 mV). Macroscopic currents elicited by 5-HT in the presence of HC show reduced peak currents (~50% at 400 μM HC) and increased decay rates compared to those recorded in the absence of the steroid. Taken together, our results reveal that hydrocortisone negatively modulates 5-HT$_3$A receptors and show a novel mechanism which involves the stabilization of a subconductance state.

3672-Pos

Analysis of CorA-Catalyzed Mg^{2+} Selective Currents in Xenopus Oocytes

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In prokaryotes, Magnesium homeostasis is tightly controlled through the workings of three distinct transport systems, with CorA being the primary source of Mg^{2+} uptake. The recent structures of CorA from the thermophilic eubacteria *Thermotoga maritima* (TmCorA) have provided an excellent model for a molecular understanding of Mg^{2+} transport. Yet, a detailed characterization of CorA function has been conspicuously missing so far, as only *in-vivo* bulk assays have been available to evaluate Mg^{2+} translocation leaving unanswered fundamental questions related to CorA ion selectivity and specificity, gating mechanism and regulation. Here we have developed a system for high-level expression of TmCorA in *Xenopus* oocytes that allows for an accurate functional readout of Mg^{2+} transport through several electrophysiological techniques. Mg^{2+} currents from TmCorA in the order of 1 to 10 μA were routinely obtained with the cut-open voltage clamp technique and two electrodes Voltage clamp. We demonstrate that CorA act as a strong inward rectifier, an observation in agreement with its physiological role as the primary source of Mg^{2+} uptake. As expected, Mg^{2+} currents are blocked by Co(III) hexamine, a structural analog of a hydrated Mg^{2+}. CorA selectivity towards Magnesium *vs.* other divalent and monovalents was characterized using a perfusion TEV setup. The divalent selectivity series for CorA was determined as $Co^{2+} >= Mg^{2+} > Ni^{2+} > Zn^{2+} >>> Ca^{2+} = Ba^{2+}$. Macroscopic CorA currents record from oocytes macropatches show that Mg^{2+}-binding to the cytoplasm domain act as a gating factor. Single channel transitions are still below our detection capabilities and efforts are ongoing to characterize the conductance of TmCorA through noise analysis. The ability to evaluate CorA function by means of electrophysiological methods will allow for a detailed analysis of structure/function relationships in CorA.