Functional insight into development of positive allosteric modulators of AMPA receptors

Autumn M. Weeks [a], Jonathan E. Harms [a], Kathryn M. Partin [a, *], Morris Benveniste [b]

[a] Department of Biomedical Sciences, Colorado State University, Fort Collins, CO 80523-1617, USA
[b] Neuroscience Institute, Morehouse School of Medicine, 720 Westview Drive SW, Atlanta, GA 30310-1495, USA

ARTICLE INFO

ABSTRACT

Positive allosteric modulators of α-amino-3-hydroxy-5-methyl-isoxazole-propionic acid (AMPA) ionotropic glutamate receptors facilitate synaptic plasticity and contribute essentially to learning and memory, properties which make AMPA receptors targets for drug discovery and development. One region at which several different classes of positive allosteric modulators bind lies at the dimer interface between the ligand-binding core of the second, membrane-proximal, extracellular domain of AMPA receptors. This solvent-accessible binding pocket has been the target of drug discovery efforts, leading to the recent delineation of five "subsites" which differentially allow access to modulator moieties, and for which distinct modulator affinities and apparent efficacies are attributed. Here we use the voltage-clamp technique in conjunction with rapid drug application to study the effects of mutants lining subsites "A" and "B" of the allosteric modulator pocket to assess affinity and efficacy of allosteric modulation by cyclothiazide, CX614, CMPDA and CMPDB. A novel analysis of the decay of current produced by the onset of desensitization has allowed us to estimate both affinity and efficacy from single concentrations of modulator. Such an approach may be useful for effective high throughput screening of new target compounds.

© 2014 Elsevier Ltd. All rights reserved.

1. Introduction

Glutamate receptors are responsible for the majority of excitatory transmission throughout the CNS. The AMPA-subtype glutamate receptors (GluA) are ligand-gated ion channels composed of four transmembrane subunits (GluA 1–4); (Boulter et al., 1992; Mansour et al., 2001; Rosenmund et al., 1998). Each subunit has an alternatively-spliced "flip/flop" region between the M3 and M4 domains that has a major influence on receptor gating kinetics (Koike et al., 2000; Partin et al., 1996; Sommer et al., 1990; Sun et al., 2002). GluA receptors initiate rapid depolarization of excitatory post-synaptic potentials (EPSPs) and serve as a trigger for induction of long-term potentiation of synaptic strength (LTP), believed to be

the cellular basis for learning and memory (Malinow et al., 2000). GluA receptors mediate changes in synaptic strength through changes in their copy number and subunit composition in the postsynaptic membrane (Malenka, 2003) and through post-translational modification (Anggano and Huganir, 2012). Subunit composition of GluA receptors varies by brain region throughout the CNS (Traynelis et al., 2010; Collingridge et al., 2009) and are implicated in and shown to be valuable therapeutic targets for a wide range of disease states, including Alzheimer's, Parkinson's, ADHD, autism, Schizophrenia, and stroke (Black, 2005; Menniti et al., 2013; Morrow et al., 2006; Ward et al., 2010). However, because GluA receptors are both ubiquitous and diverse, there is a pointed need for more selective drug design to match this diversity and better target these diseases (Schwenk et al., 2012).

Positive allosteric AMPA receptor modulators prolong the probability GluA receptors remain in the open state, thereby, prolonging EPSPs and increasing the likelihood of inducing LTP (Lynch et al., 2011; Staubli et al., 1994a,b). Like the GluA receptors they regulate, AMPA receptor modulators need to be diverse to selectively target specific GluA receptor subclasses. Known positive allosteric AMPA receptor modulators have been chemically classified as benzothiazides, benzamides, benzoxainones, and

biarylsulfonamides, but have varying degrees of specificity, particularly splice-isoform selectivity for flip or flop GluA variants (e.g. CTZ and CX614) (Fernandez et al., 2006; Jin et al., 2005; Krintel et al., 2012, 2013; Arai and Kessler, 2007; Harms et al., 2013; Mueller et al., 2011; Sun et al., 2002; Timm et al., 2011). Although this variation exists for modulators, finer selectivity is required for significant therapeutic value (Ward et al., 2010).

Experimentally, major differences in kinetics exist for the different GluA receptor isoforms. When exposed to brief pulses of agonist, channels will open and then close upon agonist removal (deactivation). However, when subjected to prolonged agonist exposure, channels will open briefly then close even with agonist bound (desensitization). Agonist dissociation is then required for recovery from desensitization. At present, AMPA receptor modulators have been shown to increase both deactivation and desensitization rates. A common concern about modulators that slow the onset of receptor desensitization is that substantially increased desensitization rates may result in excitotoxicity, making the drug, therapeutically, much less attractive (Moudy et al., 1994). Some discovery approaches have focused on the possibility of teasing apart the biophysical process of deactivation from that of desensitization. However, in no case has a positive allosteric modulator of deactivation been identified which does not also modulate desensitization (Ahmed et al., 2010; Arai et al., 2000; Mitchell and Fleck, 2007; Quirk and Nisenbaum, 2002; Sun et al., 2002; Timm et al., 2011).

The co-crystal structure of GluA2 ligand binding domain solved with several classes of modulator provides the opportunity to understand how GluA2 receptors interact with their modulators. These studies have revealed a hydrophilic modulator binding pocket which consists of 5 subsites that have been proposed to differentially affect receptor modulation and function (Ptak et al., 2009). Previously, we compared the structure and function of two novel positive allosteric modulators, CMPDA (phenyl-1,4-bis-alkylsulfonamide) and CMPDB (phenyl-1,4-bis-carboxythiophene) to cyclothiazide (CTZ) and the AMPAkine CX614 (2H,3H,6aH-pyr-rolidino(2,1-3′,2′)1,3-oxazino(6′,5′-5,4)benzo(e)1,4-dioxan-10-one) on WT GluA2 receptors (Timm et al., 2011). Here we extend that analysis using point mutations of subsites A and B of GluA2 receptors. Using patch-clamp electrophysiology, rapid-perfusion of modulators, and a novel method of data analysis, we examine the effects of several classes of positive allosteric modulator on GluA2 receptors with point mutations within the modulator-binding pocket (subsites A, B and B′). Computer simulations also provide a proof of concept study for our novel analysis which may serve as a beneficial tool for the future of characterizing and categorizing novel GluA receptor modulators.

2. Materials and methods

2.1. Positive allosteric modulators

Four positive allosteric modulators were used for this study: cyclothiazide (CTZ; Tocris Bioscience; Bristol, UK); CX614 (kindly provided by Cortex Pharmaceuticals, Inc; Irvine, CA); and two compounds developed by Lilly Research Laboratories (Timm et al., 2011), bis-Alkylsulfonamide 506091 (referred to in this study as CMPDA) and bis-Carboxythiophene 2152080 (referred to in this study as CMPDB).

2.2. AMPA receptor cDNA plasmids

cDNAs were a gift of Dr. Peter Seeburg (University of Heidelberg, Germany). The flip and flop isoforms of rat GluA2 receptors were expressed in pRK5, a CMV expression vector, fused in-frame with yellow fluorescent protein (YFP). "WT" GluA2 cDNA was produced from the cDNA plasmids into which a pore mutation ($R_{607}Q$) was made, using site-directed mutagenesis (QuikChange II XL Site-Directed Mutagenesis Kit, Stratagene; La Jolla, CA). Mutant receptors express easily-measured currents in HEK293 cells, while receptors with an arginine at position 607 express currents that are too small to measure with conventional patch-clamp electrophysiology (Hume et al., 1991; Verdoorn et al., 1991).

2.3. HEK293 cell culture and transient transfection

Human embryonic kidney 293 (HEK293) cells (CRL 1573; American Type Culture Collection; Manassas, VA) were cultured in 35 mm polystyrene dishes (Becton–Dickinson and Company; Lincoln Park, NJ) up through cell passage P45. Cells were transiently transfected using FuGene 6 reagent (Roche Diagnostic Corp., Indianapolis, IN) with GluA2 (i, flip o, flop) cDNA and enhanced yellow fluorescent protein (EYFP) cDNA (1 and 0.2 μg/35 mm dish, respectively). Media was changed 4–5 h post-transfection and NBQX was added to a final concentration of 10–20 μM in some experiments in order to prevent cyto-toxicity, but no NBQX was present when currents were measured.

2.4. Patch-clamp electrophysiology

Currents were recorded 1–2 days after transfection from cells expressing fluorescence arising from the YFP fusion protein. Outside-out membrane patches were held under voltage-clamp at −60 mV using an Axopatch 200B amplifier (Molecular Devices; Union City, CA). Synapse software (version 3.6d; Synergy Research, Inc.; Silver Springs, MD) controlled data acquisition and movement of a two-barrel flowpipe perfusion system driven by a piezo-electric device (Burleigh Instruments; Fishers, NY). Micropipettes (TW150F; 2–5 MΩ; World Precision Instruments; Sarasota, FL) contained the following intracellular solution (in mM): 135 CsCl, 10 CsF, 10 HEPES, 5 Cs₄BAPTA, 1 MgCl₂, and 0.5 CaCl₂, pH 7.2. Patches were perfused at 0.2 mL/min with solutions emitted from a two-barrel flowpipe made with theta tubing (BT150-10; Sutter Instruments; Novato, CA). One barrel contained control solution (in mM): 145 NaCl, 5.4 KCl, 5 HEPES, 1 MgCl₂, 1.8 CaCl₂, with 0.01 mg/mL phenol red, pH 7.3. The other barrel contained L-glutamate (10 mM) dissolved in the control solution. For drug studies, each barrel additionally contained either 100 μM cyclothiazide (CTZ; Tocris Bioscience; Bristol, UK), 100 μM CX614 (Cortex Pharmaceuticals, Inc; Irvine, CA), or 10 μM CMPDA or CMPDB (Eli Lilly & Company; Indianapolis, IN). CTZ and CX614 were dissolved in 1% DMSO. After attaining whole-cell voltage clamp, outside-out patches were pulled from cells, raised off the dish and positioned near the interface between the glutamate-free and glutamate-containing solutions of the flowpipe. Agonist applications were achieved by stepping into and out of the glutamate containing solution for either 1 or 500 ms to test for deactivation and desensitization, respectively. Because of extremely slow washout of modulator from cells and from tubing, only one modulator was used per cell, and control glutamate applications were applied first. Solution exchange was measured for each patch by measurement of junction potentials after obliterating the patch at the end of the experiment. Solution exchange times were approximately 500 μs. Responses were digitized at 20 kHz, and stored on a PowerPC Macintosh computer (Apple, Inc.; Cupertino, CA) using an ITC-16 interface (InstruTech, Port Washington, NY) connected through a USB-16 adapter (HEKA Instruments Inc.; Bellmore, NY).

2.5. Data analysis

Current traces and graphs were plotted using KaleidaGraph 3.6 (Synergy Software; Reading, PA). Traces were averaged (2–25 traces per sweep) and I_{Peak} was calculated using an average of three points. I_{SS}/I_{Peak} was calculated using an averaged steady state (SS) current. A single exponential was initially fit to the trace, but because most modulated responses were best fit with the sum of two exponentials, all data were fit with two exponentials and a weighted average was used for statistical comparisons of deactivation and desensitization kinetics.

Additional analyses were run on a Macintosh MacBook Pro computer using Igor Pro 6.2 to determine the time to 95% decay of the peak current to the steady state current in glutamate alone. I_A, I_B and I_C were then calculated, where I_A is defined as the peak current response to glutamate and modulator, I_B is the current amplitude for glutamate and modulator measured at the predetermined time of 95% decay in the absence of modulator, and I_C is the averaged steady-state current in glutamate and modulator (see Fig. 3).

2.6. Kinetic simulations

Simulations of currents under voltage clamp to an AMPA receptor model were performed using code originally written by John Clements (Benveniste et al., 1990), revised and converted by M.B. to an Igor Pro XOP. Receptor state occupancies at each time point were determined numerically. The change in each state occupancy was calculated according to first order reaction rate kinetics for transitions into and out of each state, as detailed in Benveniste et al. (1990). This was done iteratively at least 20 times per time point. Simulations, fitting and related analyses were run on a Macintosh MacBook Pro computer utilizing Igor Pro 6.2.

2.7. Visualization of protein structures

Protein structures were visualized using The PyMol Molecular Graphics System (version 1.3, Schrödinger, LLC) in cartoon representation, using PDB files 2AL4 for CX614 (Jin et al., 2005); 3H6T for cyclothiazide (Hald et al., 2009); 3RN8 and 3RNN for CMPD A and B, respectively (Timm et al., 2011), obtained from the RCSB Protein Data Bank.