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Pharmacological activity of C10-substituted analogs of the high-affinity kainate receptor agonist dysiherbaine

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ABSTRACT

Kainate receptor antagonists have potential as therapeutic agents in a number of neuropathologies. Synthetic modification of the convulsant marine toxin neodysiherbaine A (NDH) previously yielded molecules with a diverse set of pharmacological actions on kainate receptors. Here we characterize three new synthetic analogs of NDH that contain substituents at the C10 position in the pyran ring of the marine toxin. The analogs exhibited high-affinity binding to the GluK1 (GluR5) subunit and lower affinity binding to GluK2 (GluR6) and GluK3 (GluR7) subunits in radioligand displacement assays with recombinant kainate and AMPA receptors. As well, the natural toxin NDH exhibited ~100-fold selectivity for GluK2 over GluK3 subunits, which was attributable to the C8 hydroxyl group in NDH. We used molecular dynamic simulations to determine the specific interactions between NDH and residues within the ligand-binding domains of these two kainate receptor subunits that contribute to the divergent apparent affinities for the compound. These data demonstrate that interactions with the GluK1 subunit are preserved in analogs with substitutions at C10 in NDH and further reveal the determinants of selectivity and pharmacological activity of molecules acting on kainate receptor subunits, which could aid in design of additional compounds that target these receptors.

1. Introduction

Understanding the diverse functional roles played by kainate receptors in the mammalian central nervous system will require development of new pharmacological agents. Most existing kainate receptor agonists and antagonists act either non-selectively or preferentially on receptors containing the GluK1 (formerly GluR5) subunit (Jane et al., 2008), and some selective antagonists show therapeutic potential in pre-clinical animal models of pain and other neuropathologies (Jane et al., 2008). Further insight into the molecular interactions between selective ligands and residues within the receptor subunit ligand-binding domains (LBDs) could guide future synthetic efforts at generation of molecules selective for the other kainate receptor subunits, GluK2 (GluR6), GluK3 (GluR7), GluK4 (KA1), and GluK5 (KA2).

The natural marine excitotoxins dysiherbaine (DH) and neodysiherbaine A (NDH) serve as useful scaffolds to explore such molecular interactions. Both DH and NDH were isolated from the marine sponge *Lendenfeldia chodrodes* and are potent convulsants with a high apparent binding affinity for GluK1 receptors (Sakai et al., 1997, 2001a). They contain a shared structural template, consisting of a functionalized perhydrofuranopyran bicyclic ring containing a backbone of L-glutamate. Two functional groups at the C8 and C9 ring positions are important for activity and selectivity at (*S*)-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) and kainate receptors (Lash et al., 2008; Sanders et al., 2005). Removal of these substituents profoundly alters pharmacological activity, converting high-affinity agonism into a selective antagonism of GluK1 receptors (Sanders et al., 2005). Epimerization of chiral centers within the glutamate backbone of NDH also produced an antagonist, 2,4-epi-NDH, with selectivity for both GluK1 and GluK2 receptors (Lash et al., 2008). Thus, the unique structures of DH and NDH serve as useful lead compounds from which to develop additional analogs with distinct pharmacological profiles.

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Here we describe the pharmacological properties of a new set of NDH analogs containing substitutions at the C10 ring position, a site on the template molecule that has not been explored to date (Sasaki et al., 2006). As with other related molecules, the C10 analogs preferentially bound GluK1 subunits with high-affinity relative to other kainate receptor subunits. Intriguing differences in affinity for GluK2 and GluK3 predominantly arose in part from the functional group at the C8 position, which led us to discover that the parent compound, NDH, itself exhibited a striking divergence in affinity for these two receptor subunits. Non-conserved residues and subtle rearrangement of hydrogen bonds within the ligand binding domains of GluK2 and GluK3 could account for the subunit selectivity of NDH. These studies offer new insight into the determinants of ligand specificity within different kainate receptor subunits.

2. Materials and methods

2.1. Molecular biology and nomenclature

Site-directed mutagenesis of GluK2 and GluK3 cDNAs was performed using the QuikChange site mutagenesis protocol (Stratagene, La Jolla, CA). All mutant cDNAs were sequenced in full at the Northwestern University Genomics Core Facility. Kainate receptor splice isoforms used in this study were rat GluK1-2a, GluK2a, and GluK3a (GluR5-2a, GluR6a and GluR7a). AMPA receptor subunits were rat GluA1 (flip) and GluA2 (flip). Amino acids are referred to using single-letter codes in the physiology and binding assays for the sake of brevity, whereas the three-letter code was used when referring to the modeling results, to be consistent with prior studies.

2.2. Cell culture and electrophysiology

HEK-293-T/17 cells were maintained and transfected as described previously (Lash et al., 2008). Cells were transfected with receptor cDNAs (0.05–0.2 μg) in combination with enhanced green fluorescent protein (eGFP) using the TransIT-LT1 transfection reagent (Mirus, Madison, WI) according to the manufacturer's protocol. External recording solution contained 150 mM NaCl, 2.8 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, and 10 mM HEPES, at pH 7.3. Thick-walled borosilicate glass electrodes (Warner Instruments, Hamden, CT) were pulled to a final resistance of 1.5–2.5 MΩ and were filled with internal solution containing the following: 110 mM CsF, 30 mM CsCl, 4 mM NaCl, 0.5 mM $CaCl_2$, 10 mM HEPES, and 5 mM EGTA, at pH 7.3. Drugs were applied rapidly through a three-barreled glass flow pipe mounted on a piezo-bimorph. Glutamate-evoked currents from transfected cells lifted into the laminar solution flow had a 10–90% rise-time of 0.8–1.5 ms. Whole-cell voltage-clamp recordings were carried out using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA). Data were analyzed with Origin 7.5 (OriginLab Corp., Northampton, MA), and Prism 4 (GraphPad Software Inc., La Jolla, CA); inhibition-response curves were plotted and fit with a one-site competition curve constrained to fixed minima (0) and maxima (100). Time constants of recovery were determined by an exponential fit of recovery data.

2.3. Modeling and molecular dynamics simulations

2.3.1. Ligand structures

The 3D structures of ligands were built with SYBYL 7.3 (Tripos, Inc., St Louis, MO) and geometry-optimized quantum mechanically with GAUSSIAN03 (Gaussian Inc., Wallingford, CT) at the HF/6-31+G* level with the continuum water (PCM) model. The atom-centered point charges were created from the electrostatic potentials (GAUSSIAN03; HF/6-31+G*) using RESP methodology (Bayly et al., 1993; Cieplak et al., 1995; Cornell et al., 1993). The charges of chemically comparable atoms in the ligands were set to identical values.

2.3.2. Protein structures

The 3D dimer structures of the GluK1-ligand binding core (LBC) (PDB-code: 1YCJ) (Naur et al., 2005) and the GluK2-LBC (PDB-code: 1S7Y) (Mayer, 2005) in complex with L-glutamate were obtained from the PDB. The closed GluK3-LBC monomer structure was built based on the alignment of the B chain of 1YCJ and the human GluK3 sequence (GRIK3) (Puranam et al., 1993) using MALIGN in the BODIL modeling environment (Lehtonen et al., 2004) and NEST in JACKAL 1.5 (accessible online).

2.3.3. Ligand docking

The ligands were docked flexibly into the GluK1-LBC with GOLD3.1.1 (Jones et al., 1995, 1997) into a predefined search area of a 15 Å radius sphere centered at the O^{OH}-atom of Tyr489 (GluK1 numbering used) in the ligand-binding site, otherwise default values were used. The ligand poses for MD simulations were selected based on two criteria: (a) a fitness value given by GOLD, and (b) similarity of the conformation to that observed for DH in the crystal structure of GluK1–DH complex (Frydenvang et al., 2009). The docked ligands were included in GluK2- and GluK3-LBCs by superpositioning the ligand-GluK1 docking results using BODIL to assure the same initial conformation. To accommodate 8-desmethyl-10-HM-DH into the GluK2-LBC, the side chain of Asn721 was adjusted by using the side chain rotamer library (Lovell et al., 2000) incorporated within BODIL. Side chain adjustments of Glu738 and Asn721 were also necessary for 8-desmethyl-10-HM-DH positioning into the GluK3-LBC.

2.3.4. Starting structures for molecular dynamics simulations

After docking of ligands and prior to the molecular dynamics (MD) simulation, water molecules located in a 1.4 Å radii around the ligands were removed from the crystal structures. Protonation of histidines were determined based on the possible hydrogen bonds with adjacent residues and water molecules in the initial protein structure. The Gly545-Thr546 artificial linker used in the crystallization process to connect the S1 and S2 segments of the LBC was removed from all chains to allow free domain movement. TLEAP in ANTECHAMBER-1.27 (Wang et al., 2006) was used to (a) set the force field parameters for the protein (parm99) and ligands (gaff), (b) add hydrogen atoms, (c) neutralize the LBC–ligand complex with chloride or sodium ions, (d) solvate the system with a rectangular box of transferable intermolecular potential three-point water molecules (TIP3P) 13 Å in all directions, and (e) to add disulphide bridges.

2.3.5. Molecular dynamics simulations

The constructed LBC–ligand complexes were used as starting structures for the MD simulations. The two-step energy minimization, MD equilibration, and free MD simulation were run with NAMD 2.6 (Phillips et al., 2005). Firstly, the water molecules, counter-ions, and amino acid side chains were minimized with the conjugate gradient algorithm (15,000 steps) as the C^{α}-atoms were restrained into their initial positions with the harmonic force of 5 kcal mol^{-1} $Å^{-2}$. Secondly, the whole complex was minimized without constraints (15,000 steps) to assure complete equilibration of the system. The MD equilibration was done in constant temperature with restrained C^{α}-atoms (180,000 steps). The production simulation was performed without constraints in constant temperature and pressure for 14 ns for all ligands in complex with the LBC. All ligand–LBC complexes were simulated once.

The simulated complexes were held at constant temperature of 300 K with Langevin dynamics for all non-hydrogen atoms, using a Langevin damping coefficient of 5 ps^{-1}. A constant pressure of 1 atm was upheld by a Nosé–Hoover Langevin piston with an oscillation timescale of 200 fs and a damping timescale of 100 fs. An integration time step of 2 fs was used under a multiple time stepping scheme (Schlick et al., 1999). The bonded and short-range interactions were calculated every time step and long-range electrostatic interactions every third step. A cutoff value of 12 Å was used for the van der Waals and short-range electrostatic interactions. The MD equilibration and constraint-free simulation were conducted under the periodic boundary conditions with the full-system, and the long-range electrostatics were counted with the particle-mesh Ewald (PME) method (Darden et al., 1993; Toukmaji et al., 2000). The bonds involving hydrogen atoms were restrained by the SHAKE algorithm.

Snapshot structures were extracted from the MD trajectories at 360 ps intervals with PTRAJ 6.5 (available online); the structures at the ~14 ns time point were utilized to represent the protein–ligand complexes.

2.4. Radioligand binding

Membrane preparations from HEK-293-T/17 cells were prepared and used in radioligand binding assays as described previously (Sanders et al., 2005). Unlabeled analogs were used to displace [^{3}H]kainate (10–20 nM, PerkinElmer Life and Analytical Sciences, Boston, MA) or [^{3}H]AMPA (20 nM, PerkinElmer Life and Analytical Sciences, Boston, MA) from kainate and AMPA receptors, respectively. The GluK4 subunit was not included in the analysis because we were unable to establish conditions that yielded specific binding to the radioligand. Nonspecific binding was determined in the presence of 1 mM glutamate. After 1 h incubation at 4 °C, samples were harvested by rapid filtration onto Whatman GF/C membranes. Upon addition of scintillation fluid, membranes were incubated for 1 h at room temperature. A Beckman LS5000TD scintillation counter was used for quantification (Beckman Coulter Inc., Fullerton, CA). Data were plotted and fit with a one-site competition curve with fixed minima and maxima using Prism 4 (GraphPad Software, Inc). K_is were calculated with the Cheng–Prusoff equation using IC_{50} values derived from the fitted data and the radioligand K_d values. Saturation experiments with varying concentrations of [^{3}H]kainate (1–600 nM) were performed under the same conditions as above. These data were plotted and fit with a one-site binding hyperbola curve using Prism 4 (GraphPad Software, Inc) to determine the radioligand K_d values for receptors and mutants as follows: GluK2, 13 nM; GluK2 (Y443F), 30.9 nM; GluK2 (A518T), 5.0 nM; GluK2 (Y443F/A518T), 7.6 nM; GluK3, 53.8 nM; GluK3 (F446Y), 65.8 nM; GluK3 (T520A), 186 nM; GluK3 (F446Y/T520A), 121 nM.