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Identification and characterization of heptapeptide modulators of the glycine receptor

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ABSTRACT

The glycine receptor is a member of the Cys-loop receptor superfamily of ligand-gated ion channels and is implicated as a possible therapeutic target for the treatment of diseases such as alcoholism and inflammatory pain. In humans, four glycine receptor subtypes (α1, α2, α3, and β) co-assemble to form pentameric channel proteins as either α homomers or αβ heteromers. To date, few agents have been identified that can selectively modulate the glycine receptor, especially those possessing subtype specificity. We used a cell-based method of phage display panning, coupled with two-electrode voltage-clamp electrophysiology in *Xenopus laevis* oocytes, to identify novel heptapeptide modulators of the α1β glycine receptor. This involved a panning procedure in which the phage library initially underwent subtractive panning against Human Embryonic Kidney (HEK) 293 cells expressing alternative glycine receptor subtypes before panning the remaining library over HEK 293 cells expressing the target, the α1β glycine receptor. Peptides were identified that act with selectivity on α1β and α3β, compared to α2β, glycine receptors. In addition, peptide activity at the glycine receptor decreased when zinc was chelated by tricine, similar to previous observations of a decrease in ethanol's enhancing actions at the receptor in the absence of zinc. Comparisons of the amino acid sequences of heptapeptides capable of potentiating glycine receptor function revealed several consensus sequences that may be predictive of a peptide's enhancing ability.

1. Introduction

The Cys-loop receptor superfamily constitutes a major class of ligand-gated ion channels involved in fast inhibitory and excitatory neurotransmission throughout the central nervous system and periphery. Members include excitatory cation-selective channels such as the nicotinic acetylcholine receptor and the serotonin type 3 receptor, as well as inhibitory anion-selective channels such as the γ-aminobutyric acid type A and glycine receptors (Thompson et al., 2010). Due to their involvement in a variety of central nervous system disorders, several members of this receptor superfamily serve as targets for compounds in clinical use, as well as investigational agents (Dineley et al., 2015; Nys et al., 2013). In particular, the glycine receptor has been identified as a potential target for a variety of therapeutic applications, including the treatment of inflammatory pain and alcoholism (Foster et al., 2015; Lynch and Callister, 2006; Molander et al., 2005; Molander and Söderpalm, 2005a).

Although glycine receptors predominate in the brain stem and spinal cord, they are also expressed in higher brain regions such as the nucleus accumbens, frontal cortex, and hippocampus (Jonsson et al., 2012, 2009; Molander and Söderpalm, 2005b). A variety of drugs of abuse, including alcohol, inhalants and volatile anesthetics enhance glycine receptor function at concentrations that are achieved *in vivo* (Lynch, 2004; Molander et al., 2005; Xiong et al., 2009). Individual receptor subunits of this Cys-loop superfamily form pentamers around central ion-conducting pores. In humans, there are 4 glycine receptor subunits (α1, α2, α3, and β) that can form α-homomeric or αβ-heteromeric channels. Enhanced activation of these channels through positive allosteric modulation effectively treats inflammatory pain in rodents and may aid in the treatment of addictions such as alcoholism, through modulation of dopamine release (Molander et al., 2005; Molander and Söderpalm, 2005a, 2005b; Xiong et al., 2009). However, while α3-containing receptors are thought to predominate in glycine receptor-mediated analgesia, it is not entirely clear which subtypes are responsible for the behavioral and dopamine-modulating effects of alcohol. While α1− and α2−containing receptors

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seem to be the most likely *in vivo* targets of alcohol (Blednov et al., 2015), there would be considerable utility in the development of glycine receptor modulators with subunit selectivity, to definitively determine the relative contributions of each particular subtype to alcohol's pharmacological effects. Our previous work validated the use of phage display for the discovery of novel peptide modulators of the glycine receptor (Tipps et al., 2010). In the present report, we expand on these studies by identifying and characterizing the action of peptides with selectivity for α1β and α3β over α2β−containing glycine receptors and identify several possible amino acid consensus sequences within the heptapeptides. Interestingly, the actions of these peptides appear to be zinc-dependent. This zinc dependence was also previously shown to affect alcohol modulation of the glycine receptor (McCracken et al., 2010), suggesting that the presence of zinc may be necessary for efficient modulation of glycine receptor activity by allosteric modulators.

2. Materials and methods

2.1. Reagents

Unless stated otherwise, all chemicals were obtained from Sigma-Aldrich (St. Louis, MO). Peptides were synthesized as HCl salts at 98% purity by Peptide 2.0 Inc. (Chantilly, VA). Peptides were received as a lyophilized powder and suspended at a concentration of 10 mM in ultrapure H_2O, based on the theoretical peptide molecular weight and the dry weight of lyophilized peptide material. Suspended peptides were stored as single-use aliquots at −20 °C for up to 4 weeks before use.

2.2. Human Embryonic Kidney (HEK) cell culture and expression of glycine receptors

HEK 293 cells were obtained from the American Type Culture Collection and grown according to standard procedures (Freshney, 2002). Briefly, cells were cultured at 37 °C and 5% CO_2 in Gibco® Dulbecco's modified Eagle's medium with L-glutamine, sodium pyruvate, and 10% fetal bovine serum (Thermo-Fisher Scientific, Waltham, MA). Cells were split every 5 days with Gibco® trypsin-EDTA (Thermo-Fisher Scientific) up to 25 times, after which new aliquots of early-passage cells were started. Cells were transfected with 4 μg of glycine receptor α1β, α2β, or α3β cDNA (1:20 α: β ratio) in modified pBK-cytomegalovirus vectors (Mihic et al., 1997) using PolyFect reagent (Qiagen, Valencia, CA). All cells were incubated for at least 48 h before use in panning.

2.3. Phage display

The Ph.D.−7 phage library was purchased from New England Biolabs (Ipswich, MA) and the panning procedures followed were the same as described in Tipps et al. (2010), except as noted below. The phage display procedure consisted of two separate panning series, D7.1 and D7.2. Each series was identical except for the subtractive panning step against negative selection cells. Subtractive panning for series D7.1 consisted of washing the phage library over negative selection HEK 293 cells expressing α2β glycine receptors while D7.2 consisted of a pair of washes of library over negative selection HEK 293 cells expressing α2β, followed by HEK 293 cells expressing α3β glycine receptors.

Amplified phage were tittered and diluted in phosphate-buffered saline containing 1.5% bovine serum albumin and 0.1% Tween to ~10^{11} PFU/ml for each round of panning. After four rounds of panning, individual plaques from the final round were isolated and incubated overnight in LB broth containing a 1:100 dilution of an overnight culture of *E. coli* ER2738, at 37 °C with gentle agitation. Phage DNA was isolated from overnight cultures using the S. N. A. P. MiniPrep kit (Thermo-Fisher Scientific) and sequenced using a −96gIII sequencing primer in order to identify the peptides displayed on the *N*-terminal portions of the pIII coat protein, presumably responsible for glycine receptor binding.

2.4. Oocyte isolation and cDNA Injection

Xenopus laevis were obtained from Nasco (Fort Atkinson, WI) and housed at a 19 °C on a 12 h light/dark cycle. A portion of the ovaries was removed surgically, in accordance with the Association for Assessment and Accreditation of Laboratory Animal Care regulations, and placed in isolation media (108 mM NaCl, 1 mM EDTA, 2 mM KCl, and 10 mM HEPES). Forceps were used to manually remove the thecal and epithelial layers from stage V and VI oocytes before removal of the follicular layer by a 10 min incubation in 0.5 mg/ml type 1 A collagenase in buffer containing 83 mM NaCl, 2 mM $MgCl_2$ and 5 mM HEPES. A 32.2 nl sample of 50 ng/μl α1β, α2β, or α3β (at a 1:20 α:β ratio) cDNA, in a modified pBK-cytomegalovirus vector (Mihic et al., 1997), was injected into the animal pole of each oocyte using a micropipette (10–15 μm tip size) attached to an electronically active microdispenser. Oocytes were stored individually at room temperature in 96-well plates containing modified Barth's saline (MBS) [89 mM NaCl, 1 mM KCl, 2.4 mM $NaHCO_3$, 10 mM HEPES, 0.82 mM $MgSO_4$•7 h_2O, 0.33 mM $Ca(NO_3)_2$, 0.91 mM $CaCl_2$, pH 7.5] supplemented with 2 mM sodium pyruvate, 0.5 mM theophylline, 10 U/ml penicillin, 10 mg/l streptomycin and 50 mg/l gentamicin, and sterilized by passage through a 0.22 μM filter.

2.5. Two-electrode voltage-clamp electrophysiology

Electrophysiological recordings were conducted 1–5 days post cDNA injection. Oocytes were placed in a 100 μl bath with the animal poles facing upwards and impaled with two high-resistance (0.5–10 MΩ) glass electrodes filled with 3 M KCl. Oocytes were perfused with MBS at a rate of 2 ml/min through 18-gauge polyethylene tubing using a Masterflex peristaltic pump (Cole Parmer Instrument Co., Vernon Hills, IL) and voltage-clamped at −70 mV using an OC-725C oocyte clamp (Warner Instruments, Hamden, CT). All solutions were prepared in MBS or MBS +2.5 mM tricine. Peptides were diluted in MBS or MBS +2.5 mM tricine to final working concentrations from frozen 10 mM stocks. When maximally-effective concentrations of agonists were applied, applications lasted for 15 s and were followed by 10–15 min washouts with MBS to allow for receptor re-sensitization. For experiments using submaximal concentrations of glycine, concentrations that yielded 5-10% of the maximally-effective glycine response (EC_{5-10}) were applied for 45 s followed by 3–5 min washouts with MBS to allow for receptor re-sensitization. Peptides were co-applied with agonist following a 30 s pre-incubation of peptide alone. Data were acquired at a rate of 1 kHz using a Powerlab 4/30 digitizer with LabChart version 7 software (ADInstruments, Bella Vista, NSW, Australia).

2.6. Determination of zinc concentration in peptide stocks

Zinc concentrations in peptide stocks were determined using a quadrupole-based Agilent 7500ce inductively-coupled plasma mass spectrometer (ICP-MS) at the Jackson School of Geosciences Isotope Geochemistry Facility at the University of Texas at Austin. 10 mM stocks of peptide were diluted 10-fold in 2% HNO_3 before analysis. Electrophysiological and ICP-MS data were collected in parallel from the same batch of each peptide.