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SCIENCE DIRECT
Neuropharmacology 50 (2006) 174–181
NEURO PHARMACOLOGY
www.elsevier.com/locate/neuropharm

Accessibility to residues in transmembrane segment four of the glycine receptor

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Received 14 April 2005; received in revised form 23 August 2005; accepted 24 August 2005

Abstract

Glycine receptors (GlyRs) are members of the ligand-gated ion channel superfamily. Each subunit has four transmembrane segments (TM1–TM4). Several studies suggest that amino acids in all four TMs face into a water-filled, alcohol and anesthetic binding cavity in the extracellular portion of the transmembrane domain. TM4 should contribute a ''wall'' to this cavity, but the residues involved are unknown. Here, we determined the ability of an alcohol analog, propyl methanethiosulfonate (propyl MTS), to covalently react with twelve GlyR TM4 positions (I401–I412) after mutating the original amino acids to cysteines. Reactivity of a cysteine with propyl MTS implies that the cysteine is exposed to water. W407C, I409C, Y410C, and K411C showed altered receptor function following reaction with propyl MTS in the presence or absence of glycine. The cysteine mutations alone eliminated the effects of ethanol for I409C, Y410C, and K411C, and reduced the effects of octanol for I409C and isoflurane for K411C. The ability of propyl MTS to reduce isoflurane and chloroform potentiation was examined in the reactive mutants. Potentiation by isoflurane was significantly reduced for I409C after reaction. These data demonstrate water-accessibility of specific TM4 positions in the GlyR and suggest involvement of these residues with alcohol and anesthetic action.

Keywords: Glycine receptor; Alcohols; Anesthetics; Binding site; *Xenopus laevis* oocytes; Methanethiosulfonate

1. Introduction

Glycine receptors (GlyRs) are members of the cys-loop superfamily of ligand-gated ion channels. Each subunit of the pentameric receptor has four transmembrane segments (TM1–TM4). GlyRs are targets of alcohols and volatile anesthetics, and their function is potentiated by these drugs. Three amino acids were identified as critical for alcohol and/or volatile anesthetic action on GlyRs (and the homologous $GABA_A$ receptor): I229 (in TM1), S267 (in TM2) and A288 (in TM3) (Greenblatt and Meng, 1999; Jenkins et al., 2001; Mihic et al., 1997; Ueno et al., 2000; Wick et al., 1998; Yamakura et al., 1999; Ye et al., 1998). All three sites are located in the extracellular portion of the transmembrane domain. These three amino acids are believed to face into a water-filled, drug-binding cavity at the center of the transmembrane domain of each receptor subunit. This model is supported by mutagenesis, substituted cysteine accessibility method (SCAM), and electrophysiological studies, as well as biochemical crosslinking between S267C and A288C, and molecular modeling data (Jenkins et al., 2001; Jung et al., 2005; Lobo et al., 2004a, b; Mascia et al., 2000; Mihic et al., 1997; Wick et al., 1998; Williams and Akabas, 1999; Yamakura et al., 2001). We hypothesize that the drug-binding cavity is bound by amino acids contributed by all four TMs, including TM4.

Abbreviations: GlyR, glycine receptor; WT, wild type; TM, transmembrane segment; MTS, methanethiosulfonate; GABA, γ-aminobutyric acid; EC, effective concentration; SCAM, substituted cysteine accessibility method; nAChR, nicotinic acetylcholine receptor; MBS, modified Barth's solution; DMSO, dimethyl sulfoxide.

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0028-3908/$ - see front matter © 2005 Elsevier Ltd. All rights reserved.
doi:10.1016/j.neuropharm.2005.08.017

While amino acids on TM1, TM2, and TM3 have been the subject of study in the GlyR, the orientation of amino acids involved in alcohol and anesthetic action suggest that residues in the extracellular portion of TM4 also contribute to form a wall of the drug-binding cavity. In the recently published 4 Å nicotinic acetylcholine receptor structure, the mostly lipid-facing TM4 forms a left-handed, alpha helical bundle with the other three transmembrane segments, and has probable contacts with TM1 and TM3 (Miyazawa et al., 2003; Unwin, 2005). Experimental evidence from mutagenesis in the $GABA_A$ receptor suggest that positions in TM4 are important for anesthetic action (Jenkins et al., 2002). When 12 positions in the extracellular portion of TM4 were mutated to the bulky amino acid tryptophan in the α1 subunit of the $GABA_A$ receptor, a number of positions were found to either increase or decrease the effects of the volatile anesthetics: isoflurane, halothane, and chloroform (Jenkins et al., 2002). Presently, it is unknown which amino acids in TM4 of the glycine receptor are contributing to the drug-binding pocket. Homology modeling of the GlyR implicated that the amino acids in the extracellular portion of TM4, Y406 to Y410, are the most likely participants in drug binding (Bertaccini et al., 2005).

Here, we tested 12 single cysteine mutants (I401C-I412C) in TM4 of the GlyR α1 subunit for their accessibility to the sulfhydryl-specific reagent propyl methanethiosulfonate (MTS) using the substituted cysteine accessibility method (SCAM; Karlin and Akabas, 1998). These amino acids were selected for mutation because they are on the extracellular side of the transmembrane domain, and we hypothesize that amino acids in this region could be facing into the water-filled, drug-binding pocket. Reaction with MTS reagents requires that the sulfhydryl side chain of the substituted cysteine is ionized, and ionization occurs predominantly in the presence of water (Karlin and Akabas, 1998). Because alcohols and anesthetics are proposed to bind in a water-filled cavity at the center of the transmembrane domain, SCAM results would provide information about the structure of TM4 and indicate possible candidate positions for drug action. As in our previous studies, we used an alkyl MTS reagent (rather than more conventionally used sulfhydryl-specific reagents), because when bound to a cysteine an alkyl thiol is structurally similar to an alcohol or anesthetic molecule bound covalently to the protein (Lobo et al., 2004a; Mascia et al., 2000). Here, we tested for reactivity under conditions where the channel was either closed or open (in the absence or presence of glycine) using two-electrode voltage clamping in *Xenopus* oocytes.

Covalent reaction with propyl MTS results in altered channel function, and we found that four positions covalently reacted with propyl MTS (W407C, I409C, Y410C, and K411C), indicating that these positions are water-accessible. To our knowledge, this study is the first to determine water-accessibility to residues in TM4 for any ligand-gated ion channel. We tested whether these four mutants were responsive to alcohols (ethanol and octanol) and tested two anesthetics (isoflurane and chloroform) both before and after reaction with propyl MTS. These experiments tested the effect of the single cysteine substitutions on drug modulation and tested whether reaction with propyl MTS could occlude potentiation by anesthetic molecules, one criterion previously proposed for an alcohol/anesthetic binding site (Mascia et al., 2000). Additionally, following reaction of I409C with propyl MTS, potentiation of the glycine response by isoflurane was significantly reduced. These data suggest that there are water-accessible sites in TM4 of the glycine receptor and that these positions are important for alcohol and anesthetic action.

2. Methods

2.1. Mutagenesis and expression of human GlyR α1 subunit cDNA

Site-directed mutagenesis in the human glycine receptor α1 subunit was performed on cDNA subcloned into the pBK-CMV vector using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Point mutations were verified by partial sequencing of the sense and antisense strands. *Xenopus laevis* oocytes were isolated and injected (1 ng per 30 nl) with either human glycine receptor α1 wild type cDNA or with the following α1 mutants: I401C, F402C, N403C, M404C, F405C, Y406C, W407C, I408C, I409C, Y410C, K411C, or I412C. The cDNAs were injected into the nucleus using a microdispenser (Drummond Scientific, Broomwall, PA) (Colman, 1984). Injected oocytes were singly stored in incubation media and stored at 15 °C. Incubation media is composed of sterile modified Barth's solution [MBS; 88 mM NaCl, 1 mM KCl, 10 mM HEPES, 0.82 mM $MgSO_4$, 2.4 mM $NaHCO_3$, 0.91 mM $CaCl_2$, and 0.33 mM $Ca(NO_3)_2$, adjusted to pH 7.5] and supplemented with 10 mg/l streptomycin, 10,000 units/l penicillin, 50 mg/l gentamicin, 90 mg/l theophylline, and 220 mg/l pyruvate. Electrophysiological reagent chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Extraction of oocytes from *Xenopus laevis* frogs was in accordance with the National Institutes of Health guide for the care and use of laboratory animals, as previously described (Beckstead et al., 2000). When expressed in *Xenopus laevis* oocytes, GlyR α1 subunits assemble homomerically, forming functioning receptors with properties similar to those of native receptors (Taleb and Betz, 1994).

2.2. Electrophysiology

Electrophysiological measurements were made in oocytes 1–10 days after injection. Oocytes were placed in a rectangular chamber (approximately 100 μl volume) and perfused (2.0 ml/min) with MBS, in the presence or in the absence of drugs, via a roller pump (Cole-Parmer Instruments Co., Chicago, IL) through 18-gauge polyethylene tubing (Becton Dickinson, Sparks, MD). Oocytes were impaled in the animal pole with two glass electrodes filled with 3 M KCl and clamped at −70 mV using a Warner Instruments OC725C (Hamden, CT) oocyte clamp. Currents were continuously plotted using a chart recorder (Cole-Parmer Instrument Co, Chicago, IL).

Glycine (Biorad, Hercules, CA) was dissolved in MBS. Concentrations of 100 μM glycine and higher were applied for 20 s, and lower concentrations were applied to oocytes for 30 s to reach a peak response. Isoflurane was purchased from Ohmeda Caribe Inc. (Liberty Corner, NJ), ethanol was purchased from AAPER Alcohol and Chemicals Co. (Shelbyville, KY), and octanol and chloroform were purchased from Sigma Chemical Co. (St. Louis, MO).

Glycine concentration response curves were determined for the WT and each of the 12 TM4 mutants. The maximal glycine response was elicited at 1 mM glycine for all mutants except Y410C, which required 5 mM glycine for the concentration response curve to plateau. Concentrations of glycine ranging from 10 μM to 5 mM were applied to each oocyte to measure the response curves. For each mutant, the data were fitted for individual oocytes with nonlinear regression curve fitting, and the mean values of the EC_{50} and Hill coefficients were calculated. These values, as well as the average maximal response to glycine, were compared to those obtained for the WT receptors by one-way ANOVA.