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The pharmacological profile of ELIC, a prokaryotic GABA-gated receptor

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ABSTRACT

The *Erwinia* ligand-gated ion channel (ELIC) is a bacterial homologue of vertebrate Cys-loop ligand-gated ion channels. It is activated by GABA, and this property, combined with its structural similarity to $GABA_A$ and other Cys-loop receptors, makes it potentially an excellent model to probe their structure and function. Here we characterise the pharmacological profile of ELIC, examining the effects of compounds that could activate or inhibit the receptor. We confirm that a range of amino acids and classic $GABA_A$ receptor agonists do not elicit responses in ELIC, and we show the receptor can be at least partially activated by 5-aminovaleric acid and γ-hydroxybutyric acid, which are weak agonists. A range of $GABA_A$ receptor non-competitive antagonists inhibit GABA-elicited ELIC responses including α-endosulfan (IC_{50} = 17 μM), dieldrin (IC_{50} = 66 μM), and picrotoxinin (IC_{50} = 96 μM) which were the most potent. Docking suggested possible interactions at the 2′ and 6′ pore-lining residues, and mutagenesis of these residues supports this hypothesis for α-endosulfan. A selection of compounds that act at Cys-loop and other receptors also showed some efficacy at blocking ELIC responses, but most were of low potency (IC_{50} > 100 μM). Overall our data show that a number of compounds can inhibit ELIC, but it has limited pharmacological similarity to GLIC and to Cys-loop receptors.

1. Introduction

The Cys-loop family of ligand-gated ion channels are membrane proteins responsible for fast excitatory and inhibitory synaptic neurotransmission in the central and peripheral nervous systems. Members of this family share a common quaternary structure of five subunits that can be homomeric or heteromeric. Each of the subunits has three distinct regions that are known as the extracellular, transmembrane and intracellular domains. The N-terminal extracellular domain contains the neurotransmitter binding sites, which are located at subunit interfaces. They are created by the convergence of three amino acid loops (loops A–C) from the principal subunit and three β-sheets (loops D–F) from the adjacent complementary subunit (Brejc et al., 2001; Unwin, 2005). The transmembrane domain consists of 4 transmembrane α-helices from each subunit (M1–M4) that span the membrane, with the M2 helices surrounding the central ion pore. The intracellular domain is largely unstructured, and is responsible for receptor trafficking, regulation by intracellular modulators, and has a role in channel conductance (Hales et al., 2006; Deeb et al., 2007; Carland et al., 2009).

One of the major problems in understanding the mechanisms of action of this family of channels is the paucity of high resolution structures. Nevertheless the identification of prokaryotic Cys-loop receptor homologues has significantly improved our understanding of many structural details (Tasneem et al., 2005). An X-ray crystal structure of a Cys-loop receptor homologue from *Erwinia chrysanthemi* (*Erwinia* ligand-gated ion channel or ELIC) was solved in 2008, and one from *Gloeobacter violaceous* (*Gloeobacter* ligand-gated ion channel, or GLIC) in 2009 (Hilf and Dutzler, 2008, 2009; Bocquet et al., 2009). These prokaryotic receptors share many of their structural features with Cys-loop receptors, although they do not possess an N-terminal α-helix, an intracellular domain, or the disulphide bonded loop that gives the eukaryotic family its name. The crystallisation conditions of these proteins (ELIC unliganded; GLIC at high pH) led to the proposal that ELIC is in a closed conformation, while GLIC is in an open conformation, although recent work suggests that the structure of GLIC may represent a desensitized state (Parikh et al., 2011). GLIC is activated by protons and ELIC is activated by a range of small amine molecules, including GABA (Ulens et al., 2011; Zimmermann and Dutzler, 2011). The

Abbreviations: nACh, nicotinic acetylcholine; AChBP, acetylcholine binding protein; GABA, γ-aminobutyric acid; ELIC, *Erwinia* ligand-gated ion channel; GLIC, *Gloeobacter* ligand-gated ion channel; 5-AV, 5-aminovaleric acid; GHB, gamma-hydroxybutyric acid; PXN, picrotoxinin; ACh, acetylcholine; 5-HT, 5-hydroxytryptamine.

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potency of GABA on ELIC is low compared to its eukaryotic counterparts, but work on bacterial receptors in other systems (e.g. Singh et al., 2007; Zhou et al., 2007), suggest that even if the potencies are not in the same range, their mechanism of action at homologous proteins are similar, making ELIC an attractive model system to understand the molecular mechanisms of Cys-loop receptors. Although ELIC shows low sequence similarity with Cys-loop receptors overall, it shows high sequence homology (>60%) in the M2 region (Fig. 1). The pharmacology of ELIC, however, has still not been comprehensively explored. Here we report the effects of a range of compounds that could potentially activate or inhibit the receptor.

2. Materials and methods

2.1. Cell culture and oocyte Maintenance

Xenopus laevis oocyte-positive females were purchased from NASCO (Fort Atkinson, Wisconsin, USA) and maintained according to standard methods. Harvested stage V–VI *Xenopus* oocytes were washed in four changes of ND96 (96 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 5 mM HEPES, pH 7.5), de-folliculated in 1.5 mg ml^{-1} collagenase Type 1A for approximately 2 h, washed again in four changes of ND96 and stored in ND96 containing 2.5 mM sodium pyruvate, 50 mM gentamycin, 0.7 mM theophylline.

2.2. Receptor expression

The ELIC sequence (Genbank accession number POC7B7) was purchased from Genscript as a synthetic gene with optimized codon usage for expression in *Escherichia coli*. For electrophysiological recordings from *Xenopus* oocytes, the mature sequence of ELIC (residue numbers 8-322) was cloned into pGEMHE with the signal sequence (MRCSPGGVWLALAASLLHVSLQ) of the human α7 nAChr receptor (Liman et al., 1992). cRNA was *in vitro* transcribed from linearised pGEMHE cDNA template using the mMessage mMachine T7 Transcription kit (Ambion, Austin, Texas, USA). Stage V and VI oocytes were injected with 20 ng cRNA, and currents recorded 1–3 days post-injection.

2.3. Electrophysiology

Using two-electrode voltage-clamp, *Xenopus* oocytes were clamped at −60 mV using an OC-725 amplifier (Warner Instruments, Connecticut, USA), Digidata 1322A and the Strathclyde Electrophysiology Software Package (Department of Physiology and Pharmacology, University of Strathclyde, UK). Currents were recorded at 5 kHz and filtered at a frequency of 1 kHz. Micro-electrodes were fabricated from borosilicate glass (GC120TF-10, Harvard Apparatus, Edenbridge, Kent, UK) using a one stage horizontal pull (P-87, Sutter Instrument Company, California, USA) and filled with 3 M KCl. Pipette resistances ranged from 1.0 to 2.0 MΩ. Oocytes were perfused with ND96 at a constant rate of 12 ml min^{-1}. Drug application was via a simple gravity fed system calibrated to run at the same rate. Inhibition by test compounds was measured at the GABA EC_{50} (1.6 mM).

Analysis and curve fitting was performed using Prism v4.03 (GraphPad Software, San Diego, California, USA). Concentration–response data for each oocyte were normalised to the maximum current for that oocyte. The mean and S.E.M. for a series of oocytes were plotted against agonist or antagonist concentration and iteratively fitted to the following equation:

$$I_A = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{n_H(\log A_{50} - \log A)}} \quad (1)$$

where A is the concentration of ligand present; I_A is the current in the presence of ligand concentration A; I_{min} is the current when $A = 0$; I_{max} is the current when $A = \infty$, A_{50} is the concentration of A which evokes a current equal to $(I_{max} + I_{min})/2$; and n_H is the Hill coefficient. The relative current amplitudes (R_{max}) were expressed as the maximal current amplitude evoked by the test compound divided by the maximal current amplitude evoked by GABA.

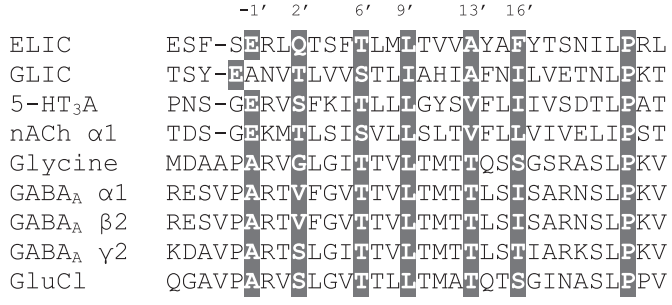

Fig. 1. An alignment of channel-lining residues for a range of eukaryotic Cys-loop receptors and prokaryotic homologues. As is common for these receptors, a prime notation is used to facilitate comparison between different subunits, with 0′ being the conserved charged residue at the start of M2. Grey indicates residue conservation. Accession numbers are: ELIC P0C7B7, GLIC Q7NDN8, 5-HT_3 P46098, nACh α1 P02708, Gly P23415, $GABA_A$ α1 P14867, $GABA_A$ β2 P47870, $GABA_A$ γ2 P18507, GluCl Q94900.

2.4. Docking

Docking was performed using an ELIC crystal structure (pdbid: 2VL0) downloaded from the RCSB Protein Data Bank. A three-dimensional structure of β-endosulfan was extracted from the Cambridge Structural Database (Ref. code: β-Endosulfan = ENSULF). β-Endosulfan was converted into the α conformer and the protonated form constructed in Chem3D Ultra 7.0 and energy-minimized using the MM2 force field.

Docking of the protonated ligand into ELIC was carried out using GOLD 3.0 (The Cambridge Crystallographic Data Centre, Cambridge, UK). The binding site was constrained as a docking sphere with a 20 Å radius surrounding the C_α of the 6′ residues in chains A and C. These amino acids were chosen based on the binding locations of ligands in eukaryotic Cys-loop receptors, but the docking sphere covered the full length of the transmembrane region of the channel. Ten genetic algorithm runs were performed on each docking exercise using default parameters. The structures were visualised using PyMOL v 1.3 and ViewerLite v 5.0.

3. Results

3.1. ELIC agonists

Application of GABA produced large, reversible inward currents (Fig. 2). These will be predominantly Na^+ currents, given the composition of our buffers and the fact that ELIC is cation-selective (Zimmermann and Dutzler, 2011). Plotting current amplitude against a range of GABA concentrations yielded an EC_{50} of 1.6 mM ($pEC_{50} = 2.78 \pm 0.04$, $n = 6$) and Hill slope of 2.1 ± 0.6. At 1 mM, the amino acid Ala, Arg, Asn, Asp, Cys, Gln, Glu, His, Ile, Leu, Lys, Met, Phe, Pro, Ser, Thr, Trp, Tyr, Val) had no effect on ELIC. At 10 mM several native Cys-loop receptor ligands (ACh, Gly and 5-HT) also yielded no ELIC responses (Table 1).

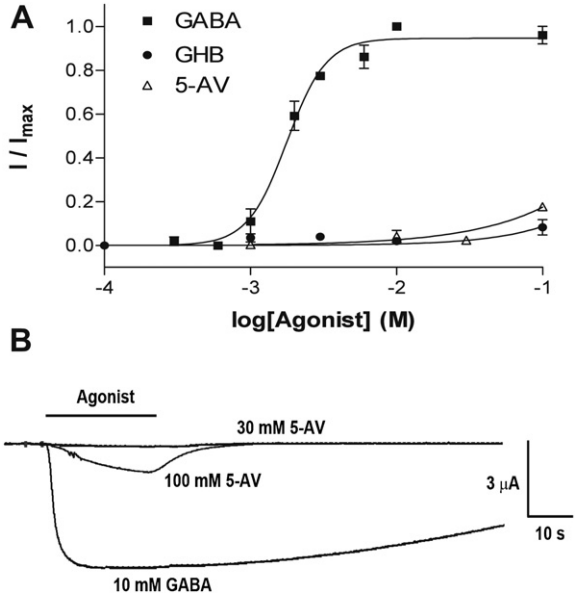

Fig. 2. GABA and 5-AV agonist concentration–response curves (A) and example responses (B). The black bar is the application of agonist. Data = mean ± SEM, $n \geq 4$.