IJP: Drugs and Drug Resistance 8 (2018) 350–360

Contents lists available at ScienceDirect

IJP: Drugs and Drug Resistance

journal homepage: www.elsevier.com/locate/ijpddr

The fungal alkaloid Okaramine-B activates an L-glutamate-gated chloride channel from *Ixodes scapularis*, a tick vector of Lyme disease

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ARTICLE INFO

Keywords:
Glutamate-gated chloride channel
Tick *Ixodes scapularis*
Ivermectin
Okaramine B
Acaricide

ABSTRACT

A novel L-glutamate-gated anion channel (IscaGluCl1) has been cloned from the black-legged tick, *Ixodes scapularis*, which transmits multiple pathogens including the agents of Lyme disease and human granulocytic anaplasmosis. When mRNA encoding IscaGluCl1 was expressed in *Xenopus laevis* oocytes, we detected robust 50–400 nA currents in response to 100 μM L-glutamate. Responses to L-glutamate were concentration-dependent (pEC_{50} 3.64 ± 0.11). Ibotenate was a partial agonist on IscaGluCl1. We detected no response to 100 μM aspartate, quisqualate, kainate, AMPA or NMDA. Ivermectin at 1 μM activated IscaGluCl1, whereas picrotoxinin (pIC_{50} 6.20 ± 0.04) and the phenylpyrazole fipronil (pIC_{50} 6.90 ± 0.04) showed concentration-dependent block of the L-glutamate response. The indole alkaloid okaramine B, isolated from fermentation products of *Penicillium simplicissimum* (strain AK40) grown on okara pulp, activated IscaGluCl1 in a concentration-dependent manner (pEC_{50} 5.43 ± 0.43) and may serve as a candidate lead compound for the development of new acaricides.

1. Introduction

Ticks are major ectoparasites of livestock and are also vectors of human and animal diseases worldwide (Jongejan and Uilenberg, 2004). They transmit a greater diversity of infectious agents than any other group of blood-feeding arthropods (Gulia-Nuss et al., 2016), including the Lyme disease spirochaete, *Borrelia burgdorferi* (Burgdorfer, 1984), and many other human and animal pathogens. At present, only a limited number of chemicals are available for their control (Woods and Williams, 2007; Van Leeuwen et al., 2015). Improved understanding of the molecular targets of tick control chemicals (acaricides) will enhance our ability to tackle tick-borne livestock diseases, with important implications for veterinary medicine. L-glutamate-gated chloride channels (GluCls), which belong to the di-cysteine loop-containing superfamily of ligand-gated ion channels (Cys-loop LGICs), are present in invertebrates but not vertebrates and are therefore suitable targets for antiparasitic drugs, most of which show good host-tolerance (Raymond-Delpech et al., 2005; Wolstenholme, 2012). For example, GluCls are activated by the endectocide ivermectin (22, 23-dihydro-avermectin B1a), a macrocyclic lactone isolated from the actinomycete, *Streptomyces avermitilis*, which controls both nematode endoparasites and ectoparasites such as ticks (Rugg et al., 2005). Ivermectin also targets GABA-gated chloride channels (Duce and Scott, 1985; Sattelle, 1990). First introduced in 1981, by the second half of that decade ivermectin had become the world's biggest-selling animal health product (Omura and Crump, 2014).

Other chemotypes targeting arthropod Cys-loop LGICs include the phenylpyrazole, fipronil (Cole et al., 1993; Davey et al., 1998; Denny, 2001; Zheng et al., 2003; Raymond-Delpech et al., 2005), and the isoxazolines, a group including fluralaner, afoxalaner and sarolaner (Ozoe et al., 2010; García-Reynaga et al., 2013; Gassel et al., 2014; Shoop et al., 2014; McTier et al., 2016), which block GABA-gated chloride channels and GluCls. Fluralaner is effective against multiple life stages of ticks of the Ixodidae and Argasidae families (Gassel et al., 2014;

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https://doi.org/10.1016/j.ijpddr.2018.06.001
Received 2 October 2017; Received in revised form 31 May 2018; Accepted 3 June 2018
Available online 04 June 2018

Rohdich et al., 2014; Wengenmayer et al., 2014; Williams et al., 2015). Okaramines are indole alkaloids isolated from fermentation products of *Penicillium simplicissimum* (strain AK40) grown on the okara pulp resulting from Soybean cake production. They are toxic to larvae of the silkworm, *Bombyx mori* (Bm) (Hayashi et al., 1989) and show strong selectivity for these lepidopteran BmGluCls (Furutani et al., 2014b). For example, they activate BmGluCls but not the silkworm GABA receptor (BmRDL). They are also ineffective on both human GABA-gated chloride channels (type A GABA receptors) and glycine-gated chloride channels (GlyCls) (Furutani et al., 2014b). Furthermore, their insecticidal profile agrees well with their LD_{50} profile on lepidopteran larvae (Furutani et al., 2017). To our knowledge, okaramine B has not been tested on tick GluCls.

Many invertebrate genomes have now been sequenced providing access to GluCls from many pests and parasites (Wolstenholme, 2012). Completion of the genomes of the medically important tick, *Ixodes scapularis* (Gulia-Nuss et al., 2016), and the agricultural pest, the two-spotted spider mite, *Tetranychus urticae* (Grbić et al., 2011), indicates that the acarine GluCl family may be quite diverse. We recently cloned and heterologously expressed in *Xenopus laevis* oocytes a member of this family from *I. scapularis* (IscaGluCl1) which formed a presumed homomeric functional GluCl responding to L-glutamate but none of the other neurotransmitters (GABA, 5-HT, ACh, dopamine, tyramine and histamine) known to activate particular invertebrate ligand-gated anion channels (Gulia-Nuss et al., 2016). This expressed GluCl was also unresponsive to glycine, which together with GABA (Olsen et al., 1999) is an important inhibitory neurotransmitter in mammalian brain.

Here we describe aspects of the pharmacology of IscaGluCl1, including the actions of ibotenate, picrotoxinin, fipronil, ivermectin and the novel indole-alkaloid, okaramine B, which activates the receptor. Okaramine B may therefore serve as a candidate lead not only for the development of novel insecticides (Furutani et al., 2014b, 2017), but also for the development of novel acaricides.

2. Materials and methods

2.1. Cloning of an Ixodes scapularis GluCl, IscaGluCl1

Unfed adult male and female *Ixodes scapularis* ticks (Wikel strain) (stored in RNAlater®) were kindly supplied by Professor Daniel Sonenshine. A mixed population of adults (ranging from 2 to 3 unfed adult ticks — mixed sex for each extraction) were stored in TRIzol® and homogenised using a Vibration Mixer Mill Retsch MM300, and total RNA was extracted according to the manufacturer's protocol. Tick (*I. scapularis*) cDNA was prepared using oligo dT(15) (Promega) and MMLV-RT RNaseH- (Promega). A partial predicted *I. scapularis* GluCl gene was identified from Vectorbase (ISCW022629). The full-length gene was obtained using degenerate primers based on the previously identified RsGluCl1 sequence (ACX33155 and US patent 7202054). The full length sequence was deposited in NCBI under accession number KR107244. The complete coding sequence of IscaGluCl1 was cloned into the p-GEM-T-Easy vector (Promega), and transcribed using SP6 Message Machine kit (Ambion) after linearisation with ApaI prior to oocyte injection.

2.2. Chemicals

L-Glutamate, D-glutamate, ivermectin and picrotoxinin (PTX) were obtained from Sigma-Aldrich (UK). Fipronil was a gift from Dr. Lance Hammerland (Merial Ltd). Kainic acid (referred to as *kainate* throughout this paper), N-methyl-D-aspartic acid (NMDA), quisqualic acid (referred to as *quisqualate* throughout this paper), L-aspartatic acid (referred to as *aspartate* throughout this paper), α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA) were obtained from Tocris (UK), whereas ibotenic acid (referred to as ibotenate throughout this paper) was obtained from Wako Pure Chemical Industries (Osaka, Japan). Okaramine B was isolated from fermentation products of *P. simplicissimum* according to the original paper (Hayashi et al., 1989).

2.3. Electrophysiology on IscaGluCl1 expressed in Xenopus laevis oocytes

Ovaries were removed from adult female *Xenopus laevis* under anaesthetic (1.5 g L tricaine) according to the UK Animals (Scientific Procedures) Act 1986. Isolated oocytes were defolliculated manually following a 30 min incubation with collagenase type 1 A (2 mg ml) (Sigma) and each oocyte was injected with 50 ng of cRNA encoding IscaGluCl1. Oocytes prepared in this way were maintained in standard oocyte saline (SOS) at 16 °C (Buckingham et al., 2006). Membrane currents were recorded 24–48 h post-injection using standard two-electrode voltage clamp methods, with oocytes voltage-clamped at E_h −80 mV (Buckingham et al., 2006) unless otherwise stated. Data were only collected from oocytes which yielded stable responses to at least three control doses of 100 μM L-glutamate applied at 3 min intervals.

Agonist actions of test compounds were examined by challenging the oocyte with increasing concentrations of agonist for 5 s at a flow-rate of 7–10 ml min with at least 3 min between challenges to minimise the effects of desensitisation. Peak amplitudes of responses were normalised to the response to 1 mM L-glutamate. To evaluate allosteric or antagonist actions, test compounds were first applied alone for 1 min and then co-applied with agonists. In this case, peak amplitudes of observed responses were normalised to the response to 100 μM L-glutamate. For studies on the blocking actions of picrotoxinin and fipronil, only a single concentration of compound was tested on an individual oocyte. Picrotoxinin, ivermectin, fipronil and okaramine B were first dissolved in dimethylsulphoxide (DMSO) and then diluted in SOS to the required concentrations. Care was taken that the final concentration of DMSO did not exceed 1% (v/v) to prevent any impact of DMSO on electrophysiological recordings.

2.4. Data analysis

Data are presented as mean ± SEM of 2–6 independent experiments. Data were normalised to the peak amplitude evoked by either 1 mM or 100 μM L-glutamate as indicated in the previous section and analysed using GraphPad Prism version 5.0 (GraphPad Software Inc., USA). To calculate concentration-response relationships, normalised data were fitted to the following equation:

$$Y = I_{min} + \frac{I_{max} - I_{min}}{1 + 10^{(\log EC_{50} - X) n_H}} \quad (1)$$

where Y is the normalised response amplitude, I_{max} and I_{min} are the maximum and minimum normalised responses respectively, EC_{50} is the concentration giving half the maximum normalised response, X is log [Agonist/Antagonist (M)] and nH is the Hill coefficient. To obtain the concentration-inhibition relationship, the response after co-application of agonist and antagonist was normalised to the control response to 100 μM L-glutamate and analysed to obtain the pIC_{50} value, using above equation but in this case the pEC_{50} was replaced with pIC_{50} and I_{max} was constrained to be 1. Statistical tests were performed for comparison of pIC_{50} values using *t*-test with a significance level of $P < 0.05$.

3. Results

3.1. Sequence of IscaGluCl1

The full-length IscaGluCl1 DNA (1350 nucleotides) encodes a 449 amino acid protein which shows the characteristic features of a Cys-loop LGIC subunit including: a large extracellular N-terminal domain, a dicysteine loop (Cys-loop) with cysteines separated by 13 residues, 4 transmembrane (TM) regions and a large intracellular TM3-TM4 loop (Fig. 1). A second N-terminal loop, characteristic of ligand-gated anion