Running title: Mode of action of okaramines on *Bombyx mori* glutamate-gated chloride channels (GluCls).

Okaramine insecticidal alkaloids show similar activity on both exon 3c and exon 3b variants of glutamate-gated chloride channels of the larval silkworm, *Bombyx mori*

Research Article for publication in a special issue of Neurotoxicology

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Abstract

The okaramine indole alkaloids were recently shown to be more selective than ivermectin in activating the glutamate-gated chloride channels of the silkworm larvae of *Bombyx mori* (BmGluCls). Those studies were carried out using the exon 3b variant as a representative of BmGluCls. However, it remains unknown whether okaramines are similarly effective on other silkworm GluCl variants and whether they share the same binding site as ivermectin on GluCls. To begin to address these questions, we examined the potency of four okaramines on the exon 3c variant of BmGluCls by two-electrode voltage clamp voltage recordings of glutamate-induced chloride currents. The potency of okaramines in activating the exon 3c BmGluCl agreed well with findings on the exon 3b BmGluCl and insecticidal potency. Okaramine B (10 μM) reduced the maximum binding (B_{max}) but not the dissociation constant (K_D) of [^{3}H]ivermectin in studies on plasma membrane fractions of HEK293 cells expressing the exon 3c variant. These findings indicate that activation of GluCls is important in the insecticidal actions of okaramines.

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Keywords: Okaramines, ivermectin, glutamate-gated chloride channels, silkworm (*Bombyx mori*), voltage-clamp electrophysiology, binding assay.

Abbreviations: BmGluCl, *Bombyx mori* glutamate-gated chloride channel.

Introduction

Okaramines (Fig. 1) are prenylated indole alkaloids produced by the molds *Penicillium simplicissimum* AK-40 and *Aspergillus aculeatus*. The first two compounds, okaramines A and B, were isolated as insecticidal compounds from the okara medium fermented by *P. simplicissimum* and tested by oral application to larvae of the silkworm *Bombyx mori* (Hayashi et al., 1989). Thereafter, a quest for other okaramines from these fungal metabolites led to the discovery of 16 more compounds (Hayashi, 2015). Of all the okaramine family, okaramine B was the most potent in insect toxicity tests and its potent action was shown to be dependent on the presence of azocine and azetidine rings (Hayashi, 2015, Shiono et al., 2000a).

Okaramines paralyze silkworm larvae within a few hours following their application by injection, indicating a possible action on the nervous system (Furutani et al., 2014b). We have employed the whole-cell patch-clamp technique to investigate their actions and found that okaramines induced chloride currents in the silkworm larval neurons. We went on to test these alkaloids on a *Bombyx* γ-aminobutyric acid (GABA)-gated chloride channel (BmRDL), which is a homologue of the exon 3b/exon 6d variant of the *Drosophila* RDL GABA receptor (ffrench-Constant and Rocheleau, 1993), as well as the exon 3b variant of glutamate-gated chloride channels (GluCls) (Furutani et al., 2014a). Neither agonist, nor antagonist actions of okaramines were observed on expressed BmRDL (Furutani et al., 2014b). By contrast, the *Bombyx mori* GluCl (BmGluCl) was activated in a concentration-dependent manner by okaramines with their relative potency showing correlations with their insecticidal actions on silkworm larvae (Furutani et al., 2014b). A similar order of potency was observed in GluCl studies on native *Bombyx mori* neurons, pointing to GluCl as a primary target of

okaramines (Furutani et al., 2014b). However, we did not test the okaramines on the exon 3c variant of BmGluCl although it is expressed at a level similar to that of the 3b variant in the brain and 3rd thoracic ganglion of *Bombyx mori* (Furutani et al., 2014a). Thus, it is important to address whether okaramines act on the exon 3c variant and the exon 3b variant with similar potency, thereby implicating both variants in the insecticidal activity of these natural products on the silkworm larvae.

Like okaramines, the macrolide compound ivermectin also modulates GluCls, inducing persistent chloride currents when measured by voltage clamp electrophysiology (Furutani et al., 2014a, Raymond and Sattelle, 2002, Wolstenholme and Kaplan, 2012, Wolstenholme and Rogers, 2005). Co-crystallisation studies show that ivermectin interacts with the allosteric site formed by transmembrane regions (TMs) and a TM2-TM3 linker in the *Caenorhabditis elegans* GluCl (Hibbs and Gouaux, 2011). It remains to be determined if okaramines interact with GluCl in a manner similar to that of ivermectin.

To begin to address the roles of exon 3 variants and whether okaramines and ivermectin act in the same way, we have investigated 1) the actions of four okaramines (A, B, 4',5'-dihydrookaramine B (Okaramine B-H_2) and Q) on the exon 3c variant of BmGluCls expressed in *Xenopus* oocytes by using two-electrode voltage clamp electrophysiology, and 2) the effects of okaramine B on the binding of [^{3}H] ivermectin to membrane preparations including the exon 3c variant.

Materials and Methods.

Chemicals.

Okaramines A, B and Q were obtained from the okara media fermented by *P.*

simplicissimum AK-40 (Hayashi et al., 1989, Shiono et al., 2000b), while okaramine B-H_2 was prepared by partial reduction of okaramine B (Shiono et al., 2000a). Ivermectin and L-glutamate were purchased from Sigma Aldrich (St. Louis, MO, USA), while [^{3}H]ivermectin (50 Ci/mmol) was purchased from American Radiolabeled Chemicals, Inc. (St. Louis, MO, USA).

cRNA preparation.

cDNAs of exon 3b (Accession number; KC342244) and 3c (Accession number: KC342245) variants of BmGluCls cloned into the pcDNA3.1 (+) vector (Thermo Fisher Scientific, Waltham, MA USA) were used for preparation of cRNAs. The cRNA was prepared by *in vitro* transcription using the mMESSAGE mMACHINE T7 ULTRA kit (Life Technologies, CA, USA) from the linearized cDNA according to the kit manual. cRNA was dissolved in RNase-free water at 1 mg ml^{-1} and stored at -80ºC until use.

Functional expression of BmGluCl variants in Xenopus laevis oocytes.

Excised varies of *Xenopus laevis* were treated with 2.0 mg ml^{-1} collagenase (Type IA, Sigma-Aldrich) in the Ca^{2+}-free standard oocyte saline (SOS) containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$ and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) 5.0 (pH 7.6 adjusted with NaOH), and then transferred into SOS (100 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$ and 5 mM HEPES (pH 7.6)) (Ihara et al., 2003, Matsuda et al., 1998, Shimomura et al., 2002, Shimomura et al., 2006). Each oocyte was injected with 50 nl of the cRNA solution and incubated for 1 day at 16°C in SOS supplemented with penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (20 μg ml^{-1}) and 2.5

mM sodium pyruvate prior to conducting electrophysiology experiments.

Voltage-clamp electrophysiology

The two-electrode voltage-clamp electrophysiology was conducted at 18 – 23°C. The *Xenopus* oocytes were perfused with SOS at a flow rate of 7 – 10 ml min^{-1} (Ihara et al., 2003, Shimomura et al., 2002, Shimomura et al., 2006). Membrane currents were recorded using a Geneclamp 500B amplifier (Molecular Devices, Sunnyvale, CA, USA) at -80 mV. The electrodes were filled with 2 M KCl and had a resistance of 1 – 5 MΩ. Signals were digitized by a Digidata 1200 (Molecular Devices) and recorded by Clampex 8 (Molecular Devices). L-Glutamate was directly dissolved in SOS, whereas okaramine solution was prepared by diluting the 10 mM stock solution in dimethyl sulfoxide with SOS. Test solutions were applied to oocytes for 3 – 5 s with a 3–5 min interval (Furutani et al., 2014a, Furutani et al., 2014b).

Data analysis

Peak current amplitude of each okaramine response was measured by Clampfit 9 (Molecular Devices) and normalized to that of the L-glutamate response at 100 μM, a submaximal concentration where no GluCl desensitization was observed. The concentration-response curves for the okaramine-induced response of BmGluCl were fitted according to the following equation using Prism 4 (GraphPad Software, CA, USA):

$$Y = \frac{Imax}{1+10^{(\log EC_{50}-[A])n_H}}$$

where Y is the normalized response to okaramines at a concentration of A, I_{max} is the maximal normalized response, EC_{50} (M) is the half maximal effective concentration,

[A] is the logarithm of the concentration and n_H is the Hill coefficient.

Binding assay

Binding experiments using [^{3}H]ivermectin were conducted as previously described (Furutani et al., 2014a). The HEK293 cells were transfected with the BmGluCl exon 3b or exon 3c variant cDNA in the pcDNA 3.1 (+) vector and collected 48 h after transfection. Cells were homogenized in 50 mM HEPES buffer (pH 7.4) supplemented with cOmplete protease inhibitor cocktail (F. Hoffmann-La Roche, Basel, Switzerland) and homogenates were centrifuged at 1,000 x g for 10 min. The supernatants were centrifuged at 25,000 x g for 30 min and resulting pellets were resuspended in 50 mM HEPES buffer (pH 7.4) for binding assays. Aliquots of such membrane preparations containing10 μg protein were incubated with 1 nM [^{3}H]ivermectin in the absence and presence of test ligands, including okaramines, in 500 μL of 50 mM HEPES buffer (pH 7.4) containing 0.02% Triton X-100 at 22°C for 60 min. Candidate ligands were incubated for 10 min prior to application of [^{3}H]ivermectin. Also, concentration-[^{3}H]ivermectin binding relations for the membrane preparations containing the exon 3c variant were measured in the absence and presence of 10 μM okaramine B. Reactions were terminated by filtration through GF/B glass fiber filters (GE Healthcare Life Sciences, Uppsala, Sweden) using the M-24 cell harvester (Brandel, MD, USA). The filters were washed with cold distilled water containing 0.25% Triton X-100. The filter radioactivity was measured using the LSC-5100 liquid scintillation counter (Hitachi (formerly Aloka), Tokyo, Japan). In all experiments, specific binding fractions were obtained by subtracting a non-specific binding observed in the presence of 1 μM cold ivermectin from the total [^{3}H]ivermectin binding.

Results

1. Effects of exon 3 variation on GluCl activating potency of okaramines.

Okaramines A, B, B-H_2 and Q induced inward currents (Fig. 2A) in *Xenopus* oocytes expressing exon 3c variant of BmGluCl. The peak current amplitude of each response was normalized to that induced by 100 μM L-glutamate and plotted against okaramine concentrations (Fig. 2B). The concentration-normalized okaramine-induced response relationships were fitted by non-linear regression to determine pEC_{50} (= -log EC_{50} (M)) values (Table 1). The higher the pEC_{50} value, the higher is the GluCl-activating potency, and thus a rank of the GluCl-activating potency was okaramine B > B-H_2 > A > Q.

The pEC_{50} values of okaramines on exon 3c showed a good correlation with those reported on exon 3b with a correlation coefficient r^2 of 0.90 (Fig. 3A). Also, the exon 3c activating potency also correlated with the insecticidal potency on *Bombyx* larvae in pLD_{50} with a correlation coefficient r^2 of 0.95 (Fig. 3B).

2. Effects of okaramine B and ivermectin on [^{3}H]ivermectin binding to exon 3c variant of GluCl expressed in HEK293 cells

To examine whether okaramine B interacts with the ivermectin binding site in GluCl, we investigated the effects of 10 μM okaramine B on total binding of 1 nM [^{3}H]ivermectin on the membrane fractions prepared from the HEK293 cells expressing the exon 3b and exon 3c variants. [^{3}H]Ivermectin showed a significant binding activity on membrane preparations from HEK293 cells expressing exon 3c as well as exon 3b as previously observed (Fig. 4A). Unlabeled ivermectin (1 μM) markedly reduced the total 1 nM [^{3}H] ivermectin binding to both variants, leaving a non-specific binding fraction

(Fig. 4A). On the other hand, 10 μM okaramine B reduced the total 1 nM [^{3}H]ivermectin binding to exon 3b and 3c by 10% and 16%, respectively (Fig. 4A).

[^{3}H]Ivermectin binding to the exon 3c variant was investigated in the presence and absence of 10 μM okaramine B (n = 3, Fig. 4B). Okaramine B significantly reduced the specific maximum binding (Bmax) of [^{3}H]ivermectin from 8.68 ± 0.19 pmol/mg protein to 7.34 ± 0.29 pmol/mg protein (t-test, $p < 0.05$), whereas it had no significant effect on the dissociation constant K_D (- okaramine B, 0.390 ± 0.026 nM: +10 μM okaramine B, 0.282 ± 0.037 nM) (Fig. 4B).

Discussion

We have previously investigated the effects of okaramines on the exon 3b variant in a study of the mode of insecticidal action of okaramines in *Bombyx mori* larvae. However, the exon 3c variant also expressed abundantly in the silkworm brain and 3rd thoracic ganglion (Furutani et al., 2014a), and forms a robust functional GluCl when expressed in *Xenopus* oocyte and HEK293 cells. Thus, it was important to test the same set of okaramines on the exon 3c variant to better understand the mode of action of these natural products.

Replacing exon 3b by 3c had a minimum effect on the GluCl activating potency of the okaramines measured by EC_{50} values (Figure 3). This implies that residues that differ in 3c and 3b (Furutani et al., 2014a) are not part of the okaramine binding site in GluCl. The okaramine potency on exon 3c showed a correlation with the insecticidal potency (Fig. 3B), suggesting that both exon 3b and 3c variants play important roles in determining the insecticidal action on *Bombyx mori* larvae.

The presence of 10 μM okaramine B had a significant but low impact on the

[^{3}H]ivermectin binding to the exon 3c variant (Fig. 4A, B), whereas its effect on K_D was minimal (Fig. 4B), pointing to a binding site for okaramines on GluCls distinct from that of ivermectin. However, appropriate caution is needed in the interpretation of the binding data because the experimental conditions were best tuned for detecting specific binding of [^{3}H]ivermectin. Thus, the effects of mutations at the ivermectin binding site on the GluCl activating action of okaramines should be explored to test further the hypothesis that okaramine binds to a novel site on GluCls. In addition to such binding site discussion on GluCl, more studies are required to examine if okaramines have some effects on other ion channels that are targeted by pesticides.

In conclusion, we have shown that okaramines act on the exon 3c variant of *Bombyx* GluCl expressed in *Xenopus* oocytes in a manner similar to their actions on the exon 3b variant. Hence both variants appear to contribute to the insecticidal actions of these molecules. We also demonstrated that okaramine B does not displace [^{3}H]ivermectin binding to GluCls. It is conceivable that okaramines interact with a distinct site from ivermectin but further studies are needed to test this hypothesis. Okaramines are more selective than ivermectin on GluCls. It is therefore of importance to clarify the mechanism of selectivity and to test them on a broader range of pesticide targets. They may serve as new leads, which could be useful in the design of novel, safer compounds for the control of pest insect species. Understanding the basis of selective toxicity was a field pioneered by Professor Toshio Narahashi (Narahashi et al., 2007, Narahashi et al., 2010).

Funding

This study was supported by KAKENHI from Japan Society for the Promotion of Science [grant numbers 21310147 and 26292031]; Strategic Project to Support the Formation of Research Bases at Private Universities: Matching Fund Subsidy from Ministry of Education, Culture, Sports, Science and Technology [grant number S1101035]. Support from The Division of Medicine, University College London is gratefully acknowledged.

Dedication

We dedicate this article to the memory of Professor Toshio Narahashi (1927-2013), a world leader in Neuropharmacology and Neurotoxicology.

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Table 1. GluCl-activating potency of okaramines A, B, B-H_2 and Q

Compounds	Exon 3c[a]	Exon 3b[b]	Insecticidal potency[b]
	pEC_{50}	pEC_{50}	pLD_{50}
Okaramine A	5.32 ± 0.14	5.51	9.29
Okaramine B	6.18 ± 0.14	6.11	10.81
Okaramine B-H_2	5.43 ± 0.30	5.64	9.85
Okaramine Q	4.93 ± 0.13	4.81	8.40

a. Each data represents mean ± standard error of the mean (n = 4).

b. Data are from Furutani et al., 2014b.

Figure legends

Figure 1. Structures of okaramines A, B, 4'5'-dihydrookaramine B (okaramine B-H_2) and okaramine Q tested in this study.

Figure 2. Okaramine-induced currents in *Xenopus laevis* oocytes expressing the exon 3c variant of BmGluCl (A) and concentration-response curves for 4 okaramines on GluCls (B). In B, the data are expressed as normalized responses relative to the peak current amplitude of the response to 100 μM L-glutamate. Each plot represents mean ± standard error of the mean (n = 4).

Figure 3. Correlation of BmGluCl exon 3c variant activating potency with BmGluCl exon 3b variant activating potency (A) and insecticidal potency (B). Data for the potency on exon 3b variant and the silkworm larvae are taken from (Furutani et al., 2014b).

Figure 4. The effects of okaramine B and ivermectin on [^{3}H]ivermectin binding to membrane fractions from HEK293 cell expressing BmGluCls. Each bar graph represents mean ± standard error of the mean (n = 3). A, Effects of 10 μM ivermectin and 10 μM okaramine B on total binding of 1 nM [^{3}H]ivermectin. B, Relationships between concentration and specific binding of [^{3}H]ivermectin to membrane fractions prepared from HEK293 cells expressing the exon 3c variant of BmGluCl in the absence and presence of 10 μM okarmine B. Each bar in A and plot in B represents the mean ± standard error of the mean (n = 3). Okaramine B significantly reduced Bmax (t-test, $p < 0.05$), while hardly affecting K_D of [^{3}H]ivermectin (See text for values).