Running title: Effect of an L319F mutation on okaramine actions on GluCl

An L319F mutation in transmembrane region 3 (TM3) selectively reduces sensitivity to okaramine B of the *Bombyx mori* L-glutamate-gated chloride channel

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Abbreviations. *Bombyx mori* L-Glutamate-gated chloride channel, BmGluCl; *Bombyx mori* γ-aminobutyric acid-gated chloride channel, BmGABACl; third transmembrane (TM3)

Abstract

Okaramines produced by *Penicillium simplicissimum* AK-40 activate L-glutamate-gated chloride channels (GluCls) and thus paralyze insects. However, the okaramine binding site on insect GluCls is poorly understood. Sequence alignment shows that the equivalent of residue Leucine319 of the okaramine B sensitive *Bombyx mori* GluCl is a phenylalanine in the okaramine B insensitive *B. mori* γ-aminobutyric acid-gated chloride channel (BmGABACl) of the same species. This residue is located in the third transmembrane (TM3) region, a location which in a nematode GluCl is close to the ivermectin binding site. The *B. mori* GluCl containing the L319F mutation retained its sensitivity to L-glutamate, but responses to ivermectin were reduced and those to okaramine B were completely blocked.

Key words

Okaramine, L-glutamate-gated chloride channel, ion channel, ivermectin. *Bombyx mori.*

Introduction

Fungi produce diverse secondary metabolites in response to environmental changes [1, 2]. An intriguing example is the production by fungi of insect regulating metabolites in the soybean pulp “okara”. In 1989, *Penicillium simplicissimum* AK-40 cultured on okara was found to produce the indole alkaloids okaramines A and B (Fig. 1A), compounds showing insecticidal activity on the silkworm larvae of *Bombyx mori* [3]. Both okaramines possess a novel skeleton featuring the eight-membered azocine ring, and the pioneering studies inspired the total synthesis of okaramine N by Baran *et al*. at Harvard University [4]. Further exploration of insect-active fungal metabolites led to the isolation of insecticidal meroterpenoids, cyclic peptides as well as the other alkaloids [5].

Although successive discoveries of insect-modulating fungal metabolites were reported, their sites of action remained elusive, triggering investigations on possible targets. When tested on silkworm larvae, uncoordinated motility was rapidly observed, followed by paralysis or death, indicating that such compounds may modulate neural transmission [5]. Whole-cell patch-clamp electrophysiology was employed to show that whereas asperparaline A [6] and chrodrimanins [7] blocked the acetylcholine- and γ-aminobutyric acid (GABA)-induced currents, respectively, okaramines induced chloride currents in neurons [8]. In particular, okaramines activated *B. mori* L-glutamate-gated chloride channels (BmGluCls), while having no effect on *B. mori* GABA-gated chloride channels (BmGABACls). The order of their GluCl activating potency (okaramine B > 4,5’-dihydrookaramine B > okaramine A > okaramine Q) correlated with their insecticidal potency, thereby supporting the view that the primary target of

okaramines is GluCl [8].

To explore further the site of action of okaramine B, we tested its capacity to displace the specific binding of [^{3}H]ivermectin to membranes prepared from HEK293 cells expressing the exon 3c variant of BmGluCl. Okaramine B displaced [^{3}H]ivermectin binding in a non-competitive fashion in that Bmax was reduced whereas Kd was unaffected, pointing to the possibility of a distinct site of binding from that of ivermectin [9]. However, this hypothesis requires further testing, because both classes of compounds activate GluCl, inducing persistent chloride currents in oocytes expressing BmGluCl.

In the crystal structure of the *C. elegans* GluCl α-subunit in complex with ivermectin [10], glycine342 and methionine345 in the third transmembrane region (TM3) (Fig. 1B) are located closest to ivermectin (Fig. 1C). Of these two amino acids, the glycine342 in the *C. elegans* GluCl is preserved in insect GluCls and GABACls, whereas the equivalent of methionine345 is leucine319 in the okaramine B-sensitive BmGluCl and phenylalanine319 in the okaramine B-insensitive *B. mori* GABA-gated chloride channel (BmGABACl) (Fig. 1B). Therefore, we investigated the effects of the L319F mutation in BmGluCl on the GluCl-activating action of okaramine B.

Materials and Methods

Chemicals

Okaramine B was isolated from the *P. simplicissimum* AK-40 metabolites generated when the fungus was grown on okara [3]. Ivermectin and sodium L-glutamate were purchased from Merck/Sigma-Aldrich (St. Louis, MO, USA) and Wako Pure Chemical Industries (Osaka, Japan), respectively.

cRNA preparation and injection to *Xenopus* oocytes

The exon 3c variant of BmGluCl (accession number; KC342245) [11] was employed to investigate the actions of compounds in terms of inducing inward currents in oocytes expressing the BmGluCls, since the current amplitude of its response to L-glutamate and ivermectin is largest among all the splice variants [11]. The cDNA was cloned into the pcDNA3.1 (+) vector (Thermo Fisher Scientific) and the nucleotide was mutated by polymerase chain reaction. Wild type and mutant cDNAs were linearized by cutting the 3' end with BamHI and cRNA was prepared with the linearized cDNA using the mMESSAGE mMACHINE Ultra T7 kit (Thermo Fisher Scientific) as previously described [11].

Oocytes were isolated from female *Xenopus laevis* anesthetized according to the according to the U.K. Animals Act, 1986 and the follicle membranes were removed from oocytes after collagenase treatment [12, 13, 14]. Each oocyte was injected with 50 ng of cRNA and incubated in Standard Oocyte Saline (SOS) containing 100 mM NaCl, 2 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, and 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (pH 7.6) supplemented with antibiotics [penicillin (100 units ml^{-1}), streptomycin (100 μg ml^{-1}), gentamycin (20

μg ml^{-1})] and 2.5 mM sodium pyruvate for 24 h prior to recording GluCl response by two-electrode voltage-clamp electrophysiology.

Two-electrode voltage-clamp electrophysiology

Oocytes were perfused extracellularly at a flow rate of 7-10 ml min^{-1} [12, 13, 14]. The transmembrane currents of oocytes were recorded using 3MKCl-filled glass microelectrodes (Resistance 0.5–5 MΩ) coupled to a GeneClamp 500B amplifier using Clampex 8 software (Molecular Devices, Sunnyvale, CA, USA). The currents were digitized using a Digidata1200 A/D converter (Molecular Devices) and analyzed using Clampfit 9 software (Molecular Devices). Oocytes were clamped at a holding potential (Eh) = -80 mV. The peak current amplitude of the response to each ligand was normalized to the peak current amplitude of the response to 100 μM L-glutamate. The concentration-normalized response data were fitted by non-linear regression with Prism 5 (GraphPad, CA, USA) according to the equation (1).

$$Y = Imax/(1+10^{(\log EC_{50}-X)n_H}) \quad (1)$$

In equation (1), X is log[ligand concentration (M)], Imax is maximal normalized response, EC_{50} is half-maximal concentration and n_H is the Hill coefficient. The Imax and pEC_{50} values for each ligand were obtained by repeated experiments (n = 4).

Results

L-glutamate concentration-response curves for wild type BmGluCl and the L319F mutant

When bath-applied alone, L-glutamate resulted in concentration-dependent, inward currents at Eh –80 mV in oocytes expressing either wild type or L319F mutant BmGluCl (Fig. 2). In the case of wild type BmGluCl, the concentration-response curve for L-glutamate reached a maximum at 100 μM with a pEC value of 3.61 ± 0.20 (Fig. 2B, Table 1). The L319F mutation did not significantly affect the pEC_{50} value of L-glutamate (3.54 ± 0.07) (Table 1).

Actions of ivermectin on wild type BmGluCl and the L319F mutant

It is well documented that ivermectin primarily targets GluCls, although it has some modulatory actions on GABACls and related ligand-gated ion channels [15]. Ivermectin induced inward currents in *Xenopus* oocytes expressing wild type and L319F mutant BmGluCl, with rise and decay times slower than those observed for L-glutamate-induced currents (Fig. 3A). At micromolar concentrations, such ivermectin actions were irreversible and thus a single oocyte was used to record only one ivermectin-induced response at each concentration. Using this protocol, the pEC_{50} and Imax values of ivermectin for the wild type GluCl were determined to be 6.23 ± 0.09 and 0.24 ± 0.02, respectively (Fig. 3B). The L319F mutation slowed desensitization of the ivermectin-induced response (Fig. 3A) and shifted both pEC_{50} and Imax values to 5.11 ± 0.71 and 0.08 ± 0.05, respectively (Fig. 3B) (Table 1).

Actions of okaramine B on wild type BmGluCl and the L319F mutant

Okaramine B was bath-applied to oocytes expressing the wild type and L319F mutant BmGluCl; it activated the wild type BmGluCl, inducing the inward currents which were concentration-dependent (Fig. 4A, B) with pEC_{50} and Imax values of 6.25 ± 0.10 and 0.19 ± 0.01, respectively (Table 1). However, the compound was completely ineffective when tested on oocytes expressing the L319F mutant (Fig. 4A, B) at concentrations from 10 nM to100 μM. Thus, a pEC_{50} value could not be determined.

Effect of co-application of okaramine B on the L-glutamate-induced response of the L319F mutant of BmGluCl.

As shown in Fig. 4, okaramine B was incapable of activating the L319F mutant of BmGluCl. However, it could potentially act as antagonist or allosteric modulator on the mutant. Hence, okaramine B was applied at 10 μM for 1 min and then co-applied with 100 μM L-glutamate. This resulted in a 31% reduction of the peak current amplitude of the L-glutamate response (Figure 5, n = 4).

Discussion

In this work, we have for the first time investigated the effects of the L319F mutation in TM3 of larval silkworm (*B. mori*) GluCl on the receptor's sensitivity to L-glutamate, okaramine B and ivermectin. Based on structural studies on the *C. elegans* GluCl with ivermectin bound [10], the residue equivalent to the one investigated here is likely also involved in the formation of the allosteric site. Thus, not surprisingly perhaps, the L319F mutation had no significant effect on

the concentration-response curve of L-glutamate. The *C. elegans* studies of GluCl with L-glutamate bound showed binding of the natural ligand with the orthosteric site [10]. In contrast, the L319F mutation reduced the response to both ivermectin and okaramine B. The more profound effect was observed on the actions of okaramine B, the response to which was obliterated over the concentration range 10 nM to 100 µM. The response to ivermectin was considerably reduced but concentration-dependent responses were still detected in the range 300 nM – 30 µM. One plausible interpretation of this result is that okaramine B and ivermectin partly share a common allosteric binding site on BmGluCl but that bound okaramine B is located closer to leucine319 than is the case for bound ivermectin. Our previous observation that okaramine B displaced the binding of [^{3}H] ivermectin to the membrane fractions containing BmGluCl in a non-competitive fashion [9] may be reconciled if the binding of okaramine B to one of five allosteric sites on BmGluCl attenuates the binding of ivermectin to the remaining four allosteric sites. To examine whether this is the case, it will be necessary to solve the X-ray crystal structure of an insect GluCl in complex with okaramine B.

The complete block of an activating response to okaramine B in the L319F mutant allowed us to address any possible modification (enhancement or block) by okaramine B of the response to L-glutamate. We detected a small but significant antagonist action in the L319F mutant. This suggests that whereas in the wild type BmGluCl occupation of the okaramine B binding site allows unimpaired coupling between the orthosteric binding site movement and channel opening, in the L319F mutant okaramine B can still bind but coupling between

orthosteric site movement and channel opening in response to L-glutamate is attenuated. It should also be noted that the reduction by okaramine B of the peak response to L-glutamate of the L319F mutant of BmGluCl was only 31%, a not strong effect.

Since the L319F mutation hardly affects the response to L-glutamate, this may facilitate the generation of viable GluCl mutants of insects that are okaramine B- and ivermectin-resistant. Such mutants would be valuable in counter screens of small molecule mimics of okaramine B in the search for new insecticide leads. Our findings also suggest that it will be important to remain alert for the detection of resistance due to mutations in the equivalent residue to *B. mori* leucine319 in pest lepidoptera and other insect and acarine species exposed to abamectin and ivermectin. We have in the past pointed to the possibility of resistance emerging from mutations in loop D of the nicotinic acetylcholine receptor, which impact on neonicotinoids but not acetylcholine [13] and later such mutations were found in field populations [16].

In conclusion, we have shown that a novel L319F mutation in TM3 of BmGluCl reduced okaramine B sensitivity more profoundly than its impact on ivermectin sensitivity, indicative of okaramine B interactions with GluCl in the vicinity of leicine319. In spite of these differences in their actions and the non-competitive actions of okaramine B in the displacement of [^{3}H]ivermectin binding to the membrane fractions prepared from H293 cells expressing BmGluCl, further studies, in particular structural studies, will be needed to determine whether okaramine B interacts at a site distinct from that of ivermectin on BmGluCl.

Disclosure statement

No potential conflict of interest was reported by the authors.

Author contribution

Kazuhiko Matsuda conceived and designed the study and wrote the manuscript; Shogo Furutani performed the experiments and wrote the manuscript; Daiki Okuhara and Anju Hashimoto performed the experiments; Makoto Ihara performed the data analysis and wrote the manuscript; Kenji Kai and Hideo Hayashi provided okaramine B and wrote manuscript; David B. Sattelle discussed the results and commented on the manuscript. All the authors approved the final version of the manuscript.

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Table 1. pEC_{50} and Imax values[1] for L-glutamate, ivermectin and okaramine B actions on wild type and L319F mutant BmGluCl of the larval silkworm *Bombyx mori* expressed in *Xenopus laevis* oocytes

Compounds	Wild type		L319F Mutant	
	pEC50	Imax	pEC50	Imax
L-Glutamate	3.61 ± 0.20	1.37 ± 0.12	3.54 ± 0.07	1.13 ± 0.05
Ivermectin	6.23 ± 0.09	0.24 ± 0.02	5.11 ± 0.71	0.08 ± 0.05
Okaramine B	6.25 ± 0.10	0.19 ± 0.01	ND[2]	ND

[1] Data are represented as mean ± standard error of the mean (n = 4).

[2]Could not be determined because okaramine B was inactive in the range 10-100 μM when tested on oocytes expressing the L319F BmGluCl mutant.

Figure legends

Figure 1. Structures of ivermectin B1a and okaramine B (A) and the alignment of amino acid sequences of *C. elegans* and insect GluCls and insect GABA-gated chloride channels (GABACls) (B). Accession numbers of protein sequences compared: *C. elegans* GluCl, AAA50785.1; *B. mori* GluCl, BAD16657.1; *Drosophila melanogaster* GluCl, AAG40735.1; *M. domestica* GluCl AGW27406.1; *Anopheles gambiae* GluCl, AGS43092.1; *B. mori* GABACl, BAN92442.1; *D. melanogaster* GABACl, AAA28556.1; *M. domestica* GABACl, BAD16658.2; *A. gambiae* GABACl, APA16887.1. Amino acid sequences are numbered according to the *Bombyx* GluCl when counted from the start methionine1. The position of leucine319 of BmGluCl (=methionine345 of *C. elegans* GluCl) is indicated by an arrow. (C) The crystal structure of the *C. elegans* GluCl with ivermectin bound (3RHW). The figures were generated using PyMOL software. Methionine345 corresponding to leucine319 of BmGluCl and phenylalanine319 of BmGABACl is indicated by space filling model where carbons, oxygen, nitrogen and sulfur are colored white, red, blue and yellow, respectively. Ivermectin is shown as a wire model where carbons and oxygens are colored green and red, respectively.

Figure 2. Effects of the BmGluCl L319F mutation on the response to L-glutamate. (A) Inward currents induced by L-glutamate in *Xenopus laevis* oocytes expressing the wild type and L319F mutant GluCl. (B) Concentration-response curve of L-glutamate for the wild type and L319F mutant GluCl. Data are represented as mean ± standard error (n = 4).

Figure 3. Effects of the BmGluCl L319F mutation on the response to ivermectin. (A) Inward currents evoked by ivermectin in *Xenopus laevis* oocytes expressing the wild type and L319F mutant of BmGluCl. The application of ivermectin is indicated by horizontal lines. (B) Concentration-response curve of ivermectin for the wild type and L319F mutant BmGluCl. Data are represented as mean ± standard error (n = 4).

Figure 4. Effects of the BmGluCl L319F mutation on the response to okaramine B. (A) Inward currents evoked by okaramine B in *Xenopus laevis* oocytes expressing the wild type and L319F mutant BmGluCl. Application of okaramine B is indicated by horizontal lines. (B) Concentration-response curve of okaramine B for the wild type and L319F mutant BmGluCl. Data are represented as mean ± standard error (n = 4).

Figure 5. Effects of co-application of okaramine B on the response to L-glutamate of BmGluCl expressed in *Xenopus laevis* oocytes. (A) After recording a control response to 100 μM L-glutamate, 10 μM okaramine B was bath-applied at 10μM for 1 min and then co-applied with 100 μM L-glutamate for 2 s to an oocyte expressing BmGluCl. After washing for 3 min with SOS, 100 μM L-glutamate was applied again. Application of L-glutamate and okaramine B are indicated by solid and hashed horizontal lines, respectively. (B) Effects of 10 μM okaramine B on the peak amplitude of the response to 100 μM L-glutamate of BmGluCl. The data represent mean ± standard error of the mean (n = 4). *, Difference is statistically significant by paired t test ($P<0.05$).