Abstract
Aperparalines produced by Aspergillus japonicus JV-23 induce paralysis in silkworm (Bombyx mori) larvae, but the target underlying insect toxicity remains unknown. In the present study, we have investigated the actions of asperparaline A on ligand-gated ion channels expressed in cultured larval brain neurons of the silkworm using patch-clamp electrophysiology. Bath-application of asperparaline A (10 μM) had no effect on the membrane current, but when delivered for 1 min prior to co-application with 10 μM acetylcholine (ACh), it blocked completely the ACh-induced current that was sensitive to mecamylamine, a nicotinic acetylcholine receptor (nAChR)-selective antagonist. In contrast, 10 μM asperparaline A was ineffective on the γ-aminobutyric acid- and L-glutamate-induced responses of the Bombyx larval neurons. The fungal alkaloid showed no-use dependency in blocking the ACh-induced response with distinct affinity for the peak and slowly-desensitizing current amplitudes of the response to 10 μM ACh in terms of IC50 values of 20.2 and 39.6 nM, respectively. Asperparaline A (100 nM) reduced the maximum neuron response to ACh with a minimal shift in EC50, suggesting that the alkaloid is non-competitive with ACh. In contrast to showing marked blocking action on the insect nAChRs, it exhibited only a weak blocking action on chicken α3β4, α4β2 and α7 nAChRs expressed in Xenopus laevis oocytes, suggesting a high selectivity for insect over certain vertebrate nAChRs.

Introduction
Aperparalines are alkaloids produced by Aspergillus japonicus JV-23 when grown on “okara” media (soybean residue resulting from tofu manufacturing). They are known to paralyze silkworm (Bombyx mori) larvae when administered orally using artificial diets [1]. Aperparalines A, B and C possess unique 3-spiro-succinimide and cyclopent[ff]indolizine moieties along with a 4-methylamide bridge [2] (Fig. 1). The unique structures of aperparalines have prompted challenges for total synthesis [3], but their targets and selectivity have not yet been elucidated.

It is presumed that the likely target of asperparaline A is the nervous system or neuromuscular junction, since the compound induces paralysis in the silkworm larvae. By applying whole-cell patch-clamp electrophysiology to larval neurons of B. mori, we were able to record the neurotransmitter-evoked responses of native ligand-gated ion channels and study the actions of asperparaline A. Having detected a blocking action on nicotinic acetylcholine receptors (nAChRs), we also investigated the actions of asperparaline A on vertebrate (avian) α3β4, α4β2 and α7 nAChRs expressed in Xenopus laevis oocytes using two-electrode voltage-clamp electrophysiology. We found that the fungal metabolite specifically and non-competitively blocked the ACh-induced response of the native nAChRs in the insect neurons, but hardly affected receptors for γ-aminobutyric acid (GABA) and L-glutamate. Much weaker blocking actions of asperparaline A were observed on 3 classes (α3β4, α4β2 and α7) of vertebrate (avian) nAChRs, suggesting selectivity for invertebrate nAChRs.

Materials and Methods
Approval of this study and animal treatment
This study using living modified organisms (LMO) has been approved by the committee of Kinki University for the experiments involving the production of LMOs (ID number: KDas-16-015). We used an anesthetic intracaine to reduce the pain of female frogs (Xenopus laevis) as much as possible when we
removed oocytes from the frogs by referring to the U.K. Animals (Scientific Procedures) Act, 1986.

B. mori neurons

Heads were dissected from last instar larvae of B. mori and placed in a Ca²⁺-free physiological saline solution of the following composition: 135 mM NaCl, 3 mM KCl, 4 mM MgCl₂, 10 mM glucose and 10 mM HEPES (pH 7.3, adjusted with NaOH) supplemented with 50 units ml⁻¹ penicillin and 50 μg ml⁻¹ streptomycin. The brains were isolated and desheathed using fine forceps and then treated with 1.0 mg ml⁻¹ collagenase (Type IA, Sigma-Aldrich Japan, Tokyo, Japan) dissolved in the Ca²⁺-free saline for 30–40 min at room temperature. After washing with the Ca²⁺-free saline, the brains were transferred to a Ca²⁺-free supplemented incubation saline of the following composition: 135 mM NaCl, 3 mM KCl, 4 mM MgCl₂, 5 mM CaCl₂, 10 mM glucose, 10 mM trehalose and 10 mM HEPES (pH 7.3, adjusted with NaOH) supplemented with 10% fetal bovine serum and 50 units ml⁻¹ penicillin and 50 μg ml⁻¹ streptomycin. The neurons were dissociated by gentle pipetting using a 1,000 μl micropipette tip, and the resultant cell suspension was placed onto poly-D-lysine (Sigma-Aldrich Japan, Tokyo, Japan)-coated coverslips which were placed in a 35-mm diameter culture dish and left for 60 min. The B. mori neurons were then incubated at 25°C for 18–36 h before electrophysiology. All salines used in the cell culture were filter sterilized.

Whole-cell patch-clamp electrophysiology

The whole-cell patch-clamp electrophysiology [4] was conducted at 20–23°C. The recording electrodes (patch pipette) were prepared from glass capillaries (PG150T-10, Harvard Apparatus, Holliston, MA, USA) using a PE-83 puller (Narishige, Tokyo, Japan). The patch pipette was filled with an internal solution (100 mM KCl, 1 mM CaCl₂, 4 mM MgCl₂, 20 mM sodium pyruvate, 10 mM EGTA and 10 mM HEPES (pH 7.3, adjusted with Tris)). Only pipettes having a resistance of 5–6 MΩ when filled with the internal solution were used for experiments. Coverslips with neurons attached were carefully transferred to the recording chamber (RC-16, Warner Instruments, Hamden, CT, USA) and superfused continuously at 5 ml min⁻¹ with a physiological saline (135 mM NaCl, 3 mM KCl, 5 mM CaCl₂, 4 mM MgCl₂, 10 mM glucose and 10 mM HEPES (pH 7.3, adjusted with NaOH)). The membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) and low-pass filtered at 10 kHz using a four pole-Bessel filter. Data were stored on a personal computer, for subsequent analysis, using a Digidata 1320A data acquisition system (Molecular Devices, Sunnyvale, CA, USA). The holding membrane potential of the neuronal membrane was −60 mV. The current-clamp method that keeps the membrane current at zero was also used to examine the effect of asperparaline A on the resting membrane potential of the neuron. ACh, L-glutamate and GABA were applied to the B. mori neurons using a U-tube; fipronil, mecamylamine and asperparaline A were applied by either U-tube or bath-application.

Expression of vertebrate nicotinic acetylcholine receptors in X. laevis oocytes

Oocytes at stage V or VI of development were removed from female X. laevis under anesthetic in 1.5 g l⁻¹ tricaine [5,6,7]. Oocytes were then treated for 30–40 min at room temperature with 2.0 mg ml⁻¹ collagenase (Type IA, Sigma-Aldrich Japan, Tokyo, Japan) dissolved in the Ca²⁺-free standard oocyte saline (SOS) of the following composition: 100 mM NaCl, 2 mM KCl, 1.8 mM CaCl₂, 1 mM MgCl₂ and 5 mM HEPES 5.0 (pH 7.6). After washing in Ca²⁺-free SOS to remove collagenase, the follicle cell layer was manually removed using forceps, and followed with the nuclear injection of 20 nl cDNAs of the chicken nAChR subunits (α2, α4, α7, β2 and β3) in the pcDNA3.1 (+) expression vector in distilled water (final concentration of each cDNA: 0.1 ng ml⁻¹). For α3β4 and α3β2, 1:1 mixtures of the α and the non-α (β2 and β3) cDNA solution were injected into oocytes. The injected oocytes were incubated at 18°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. Electrophysiology was conducted 3–5 days after nuclear injection of cDNAs.

Two-electrode voltage-clamp (TEVC) electrophysiology

TEVC electrophysiology was performed at room temperature (18–23°C). The X. laevis oocytes were secured in a Perspex recording chamber that was continuously perfused with SOS (7–10 ml min⁻¹) as previously described [7,8]. Membrane currents were recorded using a GENECLAMP 300B amplifier (Molecular Devices, Sunnyvale, CA, USA) at a holding potential of −100 mV. The electrodes were filled with 2 M KCl and had a resistance of 1–5 MΩ when measured in SOS. Signals were digitized using a Digidata 1200 data acquisition system (Molecular Devices) and recorded using Clampex 9.0 (Molecular Devices). Agonists were dissolved in SOS and were applied to oocytes for 3–5 s, with an interval of 1–5 min between applications, to ensure a full recovery from desensitization. Asperparaline A (10 μM) was bath-applied to oocytes for 1 min and then co-applied with ACh.

Analysis of electrophysiological data

The membrane current data were analyzed using Clampfit 9.2 (Molecular Devices, Sunnyvale, CA, USA). The concentration-inhibition curves for asperparaline A were fitted with the following equation, using Prism 4.03 (GraphPad Software, CA, USA):

\[
Y = \frac{I_{\text{max}}}{1 + 10^{(\log \text{IC}_{50} - \log C)/n_H}}
\]

where \(Y\) is the normalized response, \(I_{\text{max}}\) is the normalized maximum response, \(\text{IC}_{50}\) (M) is the half maximal inhibitory concentration, \([A]\) is the logarithm of the concentration of asperparaline A (M) and \(n_H\) is the Hill coefficient. On the other hand, the concentration-response curves for ACh were fitted with
Figure 2. Acetylcholine (ACh)-induced currents (A), the effects of blockers (mecamylamine and fipronil) on the ACh- (B), \(\gamma\)-aminobutyric acid (GABA) (C)- and L-glutamate (D)-induced currents and the actions of asperparaline A on the resting-state (E) and neurotransmitter-evoked currents (F–H) in the silkworm (Bombyx mori) larval neurons. The holding potential was \(-60\) mV. ACh (10 \(\mu\)M), L-glutamate (30 \(\mu\)M) and GABA (30 \(\mu\)M) was applied for 2 s using the U-tube, whereas mecamylamine and fipronil were bath-applied for 1 min prior to co-application with the agonists. In (E), asperparaline A was applied alone at 1 \(\mu\)M for 2 s using the U-tube, whereas in (F–H), it was bath-applied for 1 min prior to co-application with neurotransmitters ACh (F), GABA (G) and L-glutamate (H). Note that both peak and slowly desensitizing current amplitudes of the ACh-evoked response were blocked reversibly, selectively and almost completely by 1 \(\mu\)M asperparaline A (F).

doi:10.1371/journal.pone.0018354.g002
the following equation:

\[ Y = \frac{\text{Imax}}{1 + 10^{\text{logEC}_{50}/\text{A}]}^{\text{nH}}} \]  

(2)

where EC_{50} (M) is the half maximal effective concentration.

**Chemicals**

Fipronil and mecamylamine hydrochloride were purchased from Sigma-Aldrich Japan (Tokyo, Japan). Asperparaline A was obtained by purifying the okara broth of *A. japonicus* JV-23 as previously reported [1,2]. Stock solutions of fipronil, mecamylamine and asperparaline A were prepared in DMSO at a concentration of 10–100 mM and stored at −20°C until use. These stock solutions were diluted with the physiological saline described below. The final concentration (v/v) of DMSO in test solutions was 0.1% or lower, which had no adverse effect on the cellular response under investigation. Test solutions of ACh, L-glutamate and GABA were prepared by directly dissolving the stock solutions in saline immediately prior to experiments.

**Results**

**Membrane currents induced by three neurotransmitters in *B. mori* larval brain neurons and actions of asperparaline A on the membrane currents**

Application of ACh (10 μM) resulted in a rapid inward current at a holding potential of −60 mV with fast and slow desensitizing phases. The ACh-induced currents were stably recorded using intracellular (pipette) and extracellular (bath) solutions for 15 min
or longer (Fig. 2A). The entire current was completely blocked by bath-applied 100 μM mecamylamine (n = 4, Fig. 2B), a non-competitive antagonist of nAChRs. Both GABA- and L-glutamate-induced currents at the same holding potential were attenuated by bath-applied 10 μM fipronil, a phenylpyrazole insecticide known to block the chloride channels of GABA- and L-glutamate-gated chloride channels in insects (Fig. 2C (n = 4), D (n = 5)) [9,10].

To examine if asperparaline A activates any of ligand-gated ion channels expressed in the silkworm neurons, it was applied alone to the neurons at 10 μM. Asperparaline A had no effect on the membrane current amplitude to clamp the membrane potential of the B. mori larval neurons at -260 mV (n = 4, Fig. 2E). In addition, the compound was also ineffective on the resting membrane potential of the neuron when tested under the current clamp condition (n = 5, data not shown). Hence, it was bath-applied for 1 min, prior to co-application for 2 s with ACh (10 μM), GABA (30 μM) and L-glutamate (30 μM) (These neurotransmitter concentrations are close to EC50), to explore any possible antagonist actions on any ligand-gated ion channels present on the neurons. Asperparaline A markedly and reversibly blocked the ACh-induced current when applied at 1 μM (Fig. 2F). However, the alkaloid barely affected the peak current amplitude of the GABA (n = 5, Fig. 2G)- and L-glutamate (n = 5, Fig. 2H)-evoked responses.

Effects of repeated application of ACh and pre-application on the blocking action of asperparaline A

To examine whether the blocking action of asperparaline A was use-dependent, asperparaline A was continuously bath-applied at 30 nM, during which ACh was also applied at 10 μM for 2 s every minute. In such experiments, the blocking action was not accelerated by repeated ACh-application over a 10 min period (n = 4, Fig. 3A, B).

Figure 4. Effects of pre-application on the antagonist action of asperparaline A. (A) Asperparaline A was co-applied at 30 nM with 10 μM ACh for 2 s without pre-application, or applied for 1, 2 and 5 min prior to co-application with 10 μM ACh. (B) The antagonist action of asperparaline A with and without pre-application for 1, 2 and 5 min. Each bar graph represents the mean ± standard error of the mean (n=4) of the peak current amplitude of the ACh-induced response normalized by that taken before the application of asperparaline A. The pre-application of asperparaline A significantly enhanced the antagonist action (p<0.05, One-way ANOVA, Tukey’s test), but there were no significant differences in the blocking action between 1, 2, and 5 min pre-applications.

doi:10.1371/journal.pone.0018354.g004

Figure 5. Concentration-inhibition curves for asperparaline A in terms of attenuation of the responses to ACh of the silkworm larval neurons. (A) The ACh-induced responses recorded before and after bath-application of asperparaline A for 1 min prior to co-application with 10 μM ACh. The peak and slowly desensitizing currents are indicated by “a” and “b”, respectively. (B) Concentration-inhibition curves for asperparaline A. Data were normalized to the maximum response to ACh (10 μM). Each plot represents the mean ± the standard error of the mean of 4 experiments. The concentration-inhibition curves were obtained by fitting the data to Eq. (1) (see Materials and Methods). The pIC50 (=-log(1/IC50)) values for the peak and slowly desensitizing currents were 7.69±0.02 (n = 4, IC50 = 20.2 nM) and 7.40±0.04 (n = 4, IC50 = 39.6 nM), respectively. These two values are significantly different (p<0.05, t-test).

doi:10.1371/journal.pone.0018354.g005

Effects of repeated application of ACh and pre-application on the blocking action of asperparaline A

To examine whether the blocking action of asperparaline A was use-dependent, asperparaline A was continuously bath-applied at 30 nM, during which ACh was also applied at 10 μM for 2 s every minute. In such experiments, the blocking action was not accelerated by repeated ACh-application over a 10 min period (n = 4, Fig. 3A, B).
Amplitudes were determined to be 7.69 ± 0.04 of asperparaline A for the peak and slowly desensitizing current of paraline A, 4.94 ± 0.04 (n = 7, EC50 = 11.4 μM) and 7.40 ± 0.04 (n = 7, IC50 = 39.6 μM), respectively. No significant shift in EC50 was observed by the application of asperparaline A.

The antagonist potency of asperparaline A observed without pre-application was significantly lower than when pre-applied (n = 4, p < 0.05, one-way ANOVA, Tukey’s test, Fig. 4A, B). Thus, the effects of three different pre-application times (1, 2 and 5 min) on the blocking action were examined. No significant difference in the blocking action was observed between the pre-application times tested (n = 4, Fig. 4A, B).

Mode of blocking action of asperparaline A on B. mori nicotinic acetylcholine receptors

It has been shown that a neonicotinoid insecticide imidacloprid differentially modulated two phases (desensitizing and non-desensitizing) of the ACh-induced currents in the American cockroach neurons [11]. Hence we examined whether asperparaline A differentially blocks the peak and slowly desensitizing currents. Using the 1 min pre-application protocol, the pIC50 (= log1/IC50) values determined in the presence and absence of asperparaline A were 4.98 ± 0.10 (n = 4, EC50 = 10.5 μM) and 4.94 ± 0.04 (n = 7, EC50 = 11.4 μM), respectively. No significant shift in EC50 was observed by the application of asperparaline A.

Actions of asperparaline A on vertebrate nicotinic acetylcholine receptors expressed in X. laevis oocytes

Asperparaline A was tested on the chicken α3β4, α4β2 and α7 nAChRs expressed in X. laevis oocytes (Fig. 7). When tested alone, the alkaloid showed no agonist action on these three nAChRs, at concentrations up to 10 μM (data not shown). Thus it was bath-applied at 10 μM for 1 min prior to co-application with 100 μM ACh. It reduced the peak current amplitude of the ACh-induced response of α3β4 nAChR by 33.4 ± 3.3% (n = 3, Fig. 7A), while barely influencing the amplitudes of the responses to ACh of the α4β2 (n = 4, Fig. 7B) and α7 (n = 3, Fig. 7C) nAChRs.

Discussion

Since the discovery of asperparaline A in 1997, its target has remained unknown. Here we have for the first time tested asperparaline A on ligand-gated ion channels present on the silkworm larval neurons using patch-clamp electrophysiology. Asperparaline A was found to selectivity reduce the ACh-induced currents (Fig. 2F) that were also blocked by mecamylamine (Fig. 2B). In addition, it barely affected the GABA (Fig. 2G) and L-glutamate (Fig. 2H)-induced currents, indicating a specific antagonist action on nAChRs present in the neuron. In insects, however, cation-permeable, ionotropic glutamate receptors mediate fast-acting neuromuscular transmission and are targeted by several venoms [12]. As such, tests of asperparaline A on this type of ligand-gated ion channels are of importance to ensure that the toxicity of this compound to the silkworm larvae is the result of the selective antagonist action on nAChRs.

Asperparaline A was not an open channel blocker of the nAChRs because there was no evidence of use-dependency in the blocking action (Fig. 3). The ACh-induced currents consisted of fast and slow desensitizing phases (Figs. 2–5), which may reflect the presence of several receptor subtypes as reported for other insect neurons [11]. The peak and slowly-desensitizing ACh-induced currents showed different asperparaline-sensitivity (Fig. 5). Given that the isoforms of all the silkworm nAChR subunits resulting from splicing and RNA editing have been elucidated [13], it will be of interest in future to examine the affinity of asperparaline A for nAChR subtypes. Nonetheless, it is at present difficult to express functional and robust nAChRs consisting of only insect receptor subunits including those of the silkworm in heterologous cells, which should be resolved primarily.

We examined the effects of asperparaline A on the concentration-response curve for ACh. The alkaloid (100 nM) reduced the normalized maximum response to ACh, while scarcely influencing EC50 (Fig. 6), suggesting that ACh and asperparaline A do not compete for the same binding site at nAChRs.

To investigate whether asperparaline A is a selective antagonist of insect nAChRs, or equally effective on vertebrate nicotinic AChRs, its actions on the chicken α3β4, α4β2 and α7 nAChRs expressed in X. laevis oocytes were investigated using two-electrode voltage-clamp electrophysiology. Although α3β4 nAChR showed higher asperparaline A-sensitivity than others, the blocking effect was only 33.4% of the control response at 10 μM, a concentration about 250–500-fold higher than the IC50 for the B. mori nAChRs (Fig. 7). Moreover, the blocking action on α4β2 and α7 was very weak at this concentration, suggesting a high selectivity for insect over certain vertebrate (avian) nAChRs. We cannot of course rule out that other vertebrate nAChRs may show higher sensitivity to this alkaloid than α3β4, α4β2 and α7 [14].

Figure 6. Effects of asperparaline A on the concentration-response curve for ACh in the silkworm larval neurons. The ACh-induced responses were measured at various concentrations in the presence and absence of 100 nM asperparaline A. The concentration-response curves were obtained by fitting the data to Eq. (2) (see Materials and Methods). The pEC50 (=log1(EC50)) values determined in the presence and absence of asperparaline A were 4.98 ± 0.10 (n = 4, EC50 = 10.5 μM) and 4.94 ± 0.04 (n = 7, EC50 = 11.4 μM), respectively. No significant shift in EC50 was observed by the application of asperparaline A.

doi:10.1371/journal.pone.0018354.g006

Asperparaline A, a new antagonist of insect nAChRs
In conclusion, this is the first study to have shown that asperparaline A from *A. japonicus* JV-23 targets the nAChRs among the ligand-gated ion channels expressed by *B. mori* neurons, offering an explanation, at least in part, for the paralysis exhibited by silkworm larvae exposed to this compound. The asperparaline A acts on native *B. mori* nAChRs as a non-competitive antagonist, and is highly selective to insect (silkworm), over vertebrate (chicken), nAChRs. Future research should focus on elucidation of the mechanism of the selectivity, which may pave a new way for novel pest control chemicals.

**Author Contributions**

Conceived and designed the experiments: KM HH. Performed the experiments: KH SK SF KM. Analyzed the data: KH SK KM. Contributed reagents/materials/analysis tools: HH. Wrote the paper: KH HH KM.

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