NeuroToxicology 32 (2011) 123–129

Contents lists available at ScienceDirect

NeuroToxicology

Three austin family compounds from *Penicillium brasilianum* exhibit selective blocking action on cockroach nicotinic acetylcholine receptors

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ARTICLE INFO

Article history:
Received 2 March 2010
Accepted 14 October 2010
Available online 30 October 2010

Keywords:
Austin
Dehydroaustin
Acetoxydehydroaustin
Periplaneta americana
Nicotinic acetylcholine receptor
Whole-cell patch-clamp electrophysiology

ABSTRACT

Austin (AT) and its derivatives (dehydroaustin (DAT) and acetoxydehydroaustin (ADAT)) produced by *Penicillium brasilianum* MG-11 exhibit toxicity to insects, yet their targets are unknown. Here, we used whole-cell patch-clamp electrophysiology to investigate the action of AT family compounds on cockroach acetylcholine (ACh), γ-aminobutyric acid (GABA) and L-glutamate receptors expressed in the American cockroach (*Periplaneta americana*) neuron. U-tube application of AT or its derivatives did not induce any current amplitudes, suggesting that they did not act as agonist of these three receptors. In the second step of experiments, they were bath-applied for 1 min before co-application with the corresponding ligand. We found that AT and its derivatives had no effect on GABA and L-glutamate-induced currents, whereas they significantly reduced ACh- and epibatidine-induced currents, showing that these compounds acted as selective antagonists of nicotinic acetylcholine receptors (nAChRs) expressed in the cockroach neuron. Of the compounds, DAT showed the highest blocking potency for nAChRs, differentially attenuating the peak and slowly desensitizing current amplitude of ACh-induced responses with pIC_{50} (= $-\log IC_{50}$ (M)) values of 6.11 and 5.91, respectively. DAT reduced the maximum normalized response to ACh without a significant shift in EC_{50}, suggesting that the blocking action is not competitive with ACh.

1. Introduction

Nature provides a rich source of leads for pest control. We have been exploring the potential use of fungi as one insecticide source because few pest control chemicals have been developed from this source. A feeding assay was employed to test fungal products for toxicity in the silkworm *Bombyx mori*. One outcome of this screen was the isolation of austin (AT), dehydroaustin (DAT), and acetoxydehydroaustin (ADAT) (Fig. 1) from the fermentation products 'okara', of a waste byproduct of the tofu manufacturing process, with *Penicillium brasilianum* strain MG-11 (Hayashi et al., 1994). Although none of these compounds affected the larval motility when tested alone, they enhanced convulsion induced by another fungal product verruculogen (Hayashi et al., 1991).

Fourteen years later, the toxicity to insects of some austin family compounds were reinvestigated by Geris et al. (2008). These authors found a direct insecticidal action of DAT and ADAT (Fig. 1) against the dengue fever vector *Aedes aegypti*. Hence, we tested AT, DAT and ADAT on male adult American cockroaches by injection and noted that injected cockroaches became paralyzed. The paralysis of cockroaches was observed within 1 h after injection (Kataoka et al., unpublished).

Ionotropic receptors in the nervous system and at neuromuscular junctions are the target of several chemicals that result in paralysis, including insecticides (Narahashi, 2002). Therefore, to elucidate the mechanism for the paralysis of cockroaches induced by AT and its derivatives (AT, DAT and ADAT), we have investigated the action of these three compounds on neuronal ligand-gated ion channels present on cockroach neurons using whole-cell patch-clamp electrophysiology. Here, we report for the first time that the AT-family compounds act selectively as antagonists on neuronal nicotinic acetylcholine receptors.

2. Materials and methods

2.1. Cells preparation

Cockroach (*Periplaneta americana*) neurons from the sixth abdominal ganglion were prepared as described in previous reports (Ihara et al., 2005, 2006, 2007). The terminal abdominal

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doi:10.1016/j.neuro.2010.10.003

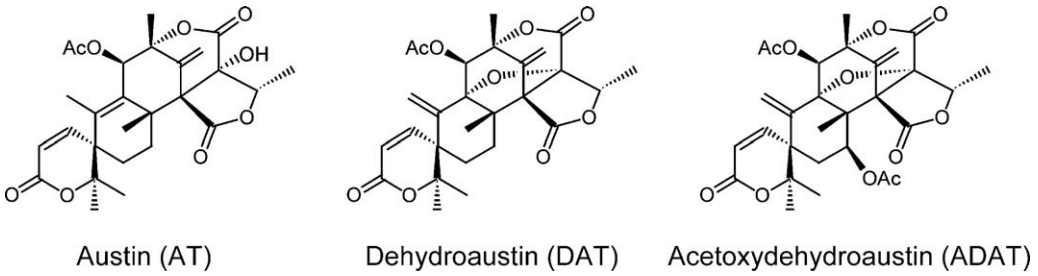

Fig. 1. Chemical structure of austin (AT), dehydroaustin (DAT), and acetoxydehydroaustin (ADAT) produced by the fungal species *Penicillium brasilianum*.

ganglia (TAGs) were taken from adult male American cockroaches and desheathed in Ca^{2+}-free solution with the following composition (in mM): NaCl 200, KCl 3.1, $MgCl_2$ 4.0, and HEPES 10 (pH 7.4) supplemented with 50 U/ml penicillin and 50 μg/ml streptomycin. Isolated TAGs were then treated with 0.5 mg/ml collagenase (Type IA, Sigma) for 30 min at 22–25 °C. After washing with Ca^{2+}-free solution, the enzyme-treated TAGs were transferred to a cockroach culture medium containing (in mM): NaCl 200, KCl 3.1, $MgCl_2$ 4.0, $CaCl_2$ 5.0, glucose 10, trehalose 10 and HEPES 10 (pH 7.4 adjusted with Tris), supplemented with 10% fetal bovine serum, 50 U/ml penicillin and 50 μg/ml streptomycin, and neurons were dissociated by gentle trituration using the disposable tip of a 1000-μl pipette. The dissociated neurons were placed on a poly-D-lysine coated coverslip for 30 min, and were incubated for 16–24 h in the culture medium at 20–23 °C prior to the electrophysiological experiments.

2.2. Patch-clamp recordings

The cockroach neurons on the coverslip were superfused at a constant flow of 2 ml/min with cockroach extracellular solution of the following composition (in mM): NaCl 200, KCl 3.1, $MgCl_2$ 4.0, $CaCl_2$ 5.0, and HEPES 10, pH 7.4 adjusted with NaOH. Recording (patch) electrodes were prepared using PG150T-10 glass capillaries (Harvard Apparatus, Holliston, MA, USA) and filled with cockroach intracellular solution containing (in mM): KCl 170, $MgCl_2$ 4.0, $CaCl_2$ 1.0, EGTA 10, HEPES 20 and sodium pyruvate 20 (pH 7.4 adjusted with Tris). Only recording electrodes showing a resistance of 4–5 MΩ were used for experiments. Membrane currents were recorded using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA, USA) at a membrane potential of −60 mV. Tests were begun 10 min after establishing whole-cell configuration. Neurotransmitters and the fungal products were applied to the neurons using a U-tube (Ihara et al., 2005) when testing their agonist actions. The fungal products were bath-applied prior to U-tube co-application with neurotransmitters when testing for antagonist (blocking) actions. Each neuron studied was tested with only one concentration of the test compound to avoid the cumulative effects caused by repeated application of compounds. The current data was digitized and stored on a personal computer via a Digidata 1320A digitizer (Molecular Devices) for subsequent analysis using pClamp 9.2 software (Molecular Devices).

2.3. Stock solutions

Solutions of neurotransmitters (acetylcholine (ACh), γ-aminobutyric acid (GABA), and L-glutamate), all purchased from Sigma Aldrich Japan (Tokyo, Japan), were prepared in the extracellular solution immediately before the experiments. AT, DAT and ADAT were isolated from the okara medium after culturing with the *P. brasilianum* strain MG-11 as reported previously (Hayashi et al., 1994). Stock solutions of these compounds were made in dimethyl sulfoxide (DMSO) and test solutions were prepared by diluting each stock solution with the extracellular solution. The final DMSO concentration was 0.1% (v/v) or lower and DMSO at this concentration range had no effect on the membrane currents.

2.4. Data analysis

Concentration–inhibition data was fitted by nonlinear regression analysis using "Prism" software ver. 4.03 (GraphPad Software, CA, USA) according to Eq. (1) as reported previously (Shimomura et al., 2002, 2006).

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

where Y is the normalized response of a compound applied at logarithmic concentration [A]. I_{max} and I_{min} are the maximum and minimum normalized responses, respectively, EC_{50} is the concentration giving half the maximum normalized response and n_H is the Hill coefficient. To determine a time constant for the desensitization of the response to ACh from the peak to the end of ACh application, changes in the current amplitude from the peak to the end of compound application were fitted by Eq. (2) using pClamp 9.2 software (Molecular Devices).

$$Y = A \exp\left(\frac{-t}{\tau}\right) + C \quad (2)$$

where Y is the current amplitude of the response to ACh at time t, τ is the time constant and C is the current amplitude at infinite time (at steady state). A is a constant that can be determined by the peak current amplitude of the response to ACh (time is zero) and the constant C.

For all data, mean ± standard error of the mean was calculated to be given in Section 3. One-way ANOVA (Tukey's test) and the t-test were used to compare mean values between three and two groups, respectively.

2.5. Toxicity to American cockroaches

In vivo toxicity of AT, DAT and ADAT to male adult American cockroaches was investigated by injection. DMSO solutions of the compounds were injected into the cockroach abdomen and the cockroach symptoms were observed at 1 h after injection. Ten cockroaches were used for each assay and each assay was repeated four times. DMSO (<10 μl) alone had no effect on the motility of the insects. EC_{50} paralyzing 50% of injected cockroaches was determined by the non-linear regression analysis (Eq. (1)) for each compound.

3. Results

AT, DAT and ADAT did not affect the membrane currents of cockroach neurons when tested alone at 10 μM, the maximum