Effects of Celangulin IV and V From Celastrus angulatus Maxim on Na⁺/K⁺-ATPase Activities of the Oriental Armyworm (Lepidoptera: Noctuidae)

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Abstract

Na⁺/K⁺-ATPase (sodium pump) is an important target for the development of botanical pesticide as it is responsible for transforming chemical energy in ATP to osmotic work and maintaining electrochemical Na⁺ and K⁺ gradients across the cell membrane of most animal cells. Celangulin IV (C-IV) and V (C-V), which are isolated from the root bark of Celastrus angulatus, are the major active ingredients of this insecticidal plant. The activities of C-IV and C-V on Na⁺/K⁺-ATPase were investigated by ultramicro measuring method to evaluate the effects of C-IV and C-V on Na⁺/K⁺-ATPase activities of the brain from the fifth Mythimna separata larvae and to discuss the insecticidal mechanism of C-IV and C-V. Results indicate that inhibitory activities of Na⁺/K⁺-ATPase by C-IV and C-V possess an obvious concentration-dependent in vitro. Compared with C-IV, the inhibition of C-V on Na⁺/K⁺-ATPase was not striking. In vivo, at a concentration of 25 mg/liter, the inhibition ratio of C-IV on Na⁺/K⁺-ATPase activity from the brain in narcosis and recovery period was more remarkable than that of C-V. Furthermore, the insects were fed with different mixture ratios of C-IV and C-V. The inhibition extent of Na⁺/K⁺-ATPase activity was corresponded with the dose of C-IV. However, C-V had no notable effects. This finding may mean that the mechanism of action of C-IV and C-V on Na⁺/K⁺-ATPase were different. Na⁺/K⁺-ATPase may be an action target of C-IV and C-V.

Key words: Celangulin, Mythimna separata, Na⁺/K⁺-ATPase, target
gradients across the plasma membrane (Natacica and Gadsby 2014). In insect neurons, the role of the Na\(^+/K^+\)-ATPase is crucial for basic metabolic requirements and for the specialized functions of nerve impulse transmission (Vacek et al. 2013, Shattock et al. 2015). The establishment of the resting membrane potential, the uptake of neurotransmitters and the efflux of calcium are coupled to its activity (Haruo and Masahiro 2013). When the Na\(^+/K^+\)-ATPase activity is inhibited, Na\(^+\) ion cannot be extruded out of the plasma membrane and the intracellular Na\(^+\) ion concentration would increase, which can change the transmembrane potential, result in a long excitation time of the nerve cell membrane. This phenomenon leads to the disorder of function of nerve system and causes the death of insects (Petschenka et al. 2012, Ismail Seham and Shaker 2014).

In this study, the effects on Na\(^+/K^+\)-ATPase activities of the larval brain from the oriental armyworm, Mythimna separata were determined using methodology (Ilenchuk and Davey 1982) in order to determine whether Na\(^+/K^+\)-ATPase is a putative target of C-IV and C-V. The results will establish a foundation for further research on the mechanisms of action and target localization of Celangulin compounds in agricultural pests.

**Materials and Methods**

**Compounds**

C-IV and C-V were isolated from the root bark of C. angulatus by the Institute of Pesticide Science, Northwest Agriculture and Forestry University (NWAFU, Yangling, China). As shown in Fig. 1 (Wu et al. 1994), chemical structures of C-IV and C-V were elucidated as 1β, 2β, 6α, 8β-tetraacetoxy-9β-benzoyloxy-12-isobutenyloxy-4α-hydroxy-β-dihydroagarofuran and 2β, 8α-diacetoxy-9β-benzoyloxy-1β, 12-diisobutenyloxy-4α, and 6α-dihydroxy-β-dihydroagarofuran. The structure of the compounds was confirmed via nuclear magnetic resonance and mass spectra analysis and its purity was over 95% according to HPLC analysis. All reagents were of analytical grade unless specified otherwise.

**Insects**

The 1-d-old fifth instar larvae of the oriental armyworm, M. separata, were provided by the Institute of Pesticide Science, NWAFU (Yangling, China). The colony of M. separata was kept in the laboratory condition for 18 yr at 25°C, 70% relative humidity, and a photoperiod of 16:8 (L:D) h.

**Preparation of Na\(^+/K^+\)-ATPase**

To determine Na\(^+/K^+\)-ATPase activities in the brain tissue of M. separata, homogenates of whole heads were used. The heads of fifth instar larvae were cut, pooled, immersed in an ice-cold dissection solution (1.4 mol/liter sucrose, 0.1 mol/liter Hepes-Tris, pH 7.0, 2.5 mmol/liter dithiothreitol, 1 mmol/liter phenylmethanesulfonyl fluoride) and weighed. The brain tissues were homogenized in 10 vols of Tris–HCl buffer (pH 7.0), and centrifuged at 4°C at 2,500 × g for 10 min; the supernatant was centrifuged at 30,000 × g for 30 min using a high-speed refrigerated centrifuge (Thermo Scientific, Beijing, China). The supernatant was discarded, and the sediment containing the Na\(^+/K^+\)-ATPase was resuspended with 70 vol of Tris–HCl buffer (pH 7.0). Fifty insects were used for each experiment. The final preparation of Na\(^+/K^+\)-ATPase was kept frozen at −20°C until used for the enzymatic assays.

**Activities Assay of Na\(^+/K^+\)-ATPase**

In vitro, the ATPase activity was determined by an ultramicro-measuring method based on the determination of inorganic phosphate content (Feng 1981, Korpeka and Tähti 1988, Phillips and Hayes 1989, Vaalavirta and Tähti 1995). Three groups of reaction systems were carried out. The following are the reaction systems with 100 µl reaction mixture: 50 mmol/liter Tris–HCl (pH 7.0), 5 mmol/liter MgCl\(_2\) and 6–10 µg protein of enzyme suspension. Therefore, reaction 1 contained 150 mmol/liter NaCl and 20 mmol/liter KCl; reaction 2 contained 0.5 mmol/liter Ouabain; reaction 3, only control. The reaction was carried out in a 37°C water bath. The reaction mixtures were mixed and incubated for 5 min; ATP (0.5 mmol/liter) was added, and the mixture were incubated for 5 min once again. The reaction was terminated by ice-cold trichloroacetic acid (15% w/v). Then, 50µl of the reagent (1% NH\(_4\)MoO\(_4\)·4H\(_2\)O = 0.5 NH\(_2\)SO\(_4\) solution) and 100 µl of 1% ascorbic acid were added. After a 25-min reaction time, the absorbance of the samples was read at 625 nm with MULTISKAN MK3 (Thermo Co.). The experiments were replicated three times. The enzyme activities were expressed as micromoles of inorganic phosphate formed per hour per mg of protein (1 mmol Pi/h/mg protein). Na\(^+/K^+\)-ATPase activity is defined as the difference between total ATPase and Mg\(^2+\)-dependent ATPase activities. The test substances C-IV and C-V dissolved in acetone were added to the above reaction systems (C-IV and C-V final concentration: 0, 6.25, 12.5, 25, 50, 100, 200, 400, or 800 mg/liter) and were incubated for 5 min; ATP was then added the reaction systems (0.5 mmol/liter); then the systems were incubated again for 5 min. The results normalized, and the ATPase activities of treated samples were given as percentages of the activities of control samples.

In vivo, the leaf discs of known areas were treated with known amounts of the test samples (w/v 2% mixtures of C-IV:C-V=1:3, 1:1, or 3:1) dissolved in acetone. Each testing larvae was fed with a fresh wheat leaf disc. The Na\(^+/K^+\)-ATPase activities were determined in different intoxicating period of C-IV and C-V. In addition, after feeding the leaf discs, the Na\(^+/K^+\)-ATPase activities in different time of C-IV, C-V, and the mixtures of C-IV:C-V=1:3, 1:1, or 3:1, respectively, were determined. The preparation of Na\(^+/K^+\)-ATPase and the determination of the Na\(^+/K^+\)-ATPase activities were similar to that of in vitro.

**Protein Content Determination**

The estimation of protein content was done following the method of Bradford (1976) using a protein determination kit (Solarbio, Beijing, China). Absorbance was converted to protein concentration by analysis against a simultaneously determined standard curve of bovine serum albumin.

**Statistics Analysis Methods**

The determinations were made in triplicates at each dose level and the independent measurements were repeated 10 times. SPSS 19.0
software was used for analysis of photometric data (SPSS Inc., Chicago, IL). Significant differences were analyzed by independent sample t-test, and were compared with the control. The means were considered significantly different at $P \leq 0.05$.

Results

Effects of C-IV and C-V on Na\(^+/K^+\)–ATPase Activity In Vitro

In vitro, the inhibition activities of ATPase by C-IV and C-V had an obvious concentration dependence (Fig. 2). At the concentration of 25 mg/liter, C-IV decreased the activity of Na\(^+/K^+\)–ATPase from the brain by 7.59%, and at 400 mg/liter by 40.36%. In comparison with C-IV, the inhibition of C-V on Na\(^+/K^+\)–ATPase was not striking. The inhibition percentage of activity on the brain Na\(^+/K^+\)–ATPase were 3.60% at 400 mg/liter and 15.74% only at 25 mg/liter. The results showed that the inhibition of C-IV was more significant than that of C-V.

Effects of Na\(^+/K^+\)–ATPase Activity After Feeding Celangulin IV and V In Vivo

In vivo, the inhibition percentage of C-IV on the Na\(^+/K^+\)–ATPase activity from the brain in narcosis and recovery period of insect treated with 25 mg/liter concentration were 32.39 and 37.73%, respectively. However, the activity inhibition percentage of C-V on Na\(^+/K^+\)–ATPase from the brain in excitation, convulsion, and dehydration periods were 10.57, 18.70, and 17.53%, respectively. In comparison with C-IV, the inhibition of C-V on Na\(^+/K^+\)–ATPase was not striking. All of these can facilitate the understanding of the mechanisms of Na\(^+/K^+\)–ATPase in insects after C-IV and C-V treatment.

Effects on Na\(^+/K^+\)–ATPase Activities After Feeding Mixtures of Celangulin IV and V

Figure 3 shows the effects on Na\(^+/K^+\)–ATPase activities when the testing insects were fed with different mixture ratios of C-IV and C-V. The inhibition of the lower ratio of C-IV (C-IV:C-V = 1:3) in the mixture on Na\(^+/K^+\)–ATPase from the brain was the same with the C-V, and their highest inhibition percentages were 26.84%, and 22.52% for C-V. However, the effects of the higher ratio of C-IV (C-IV:C-V = 3:1) was similar as the C-IV; thus, its highest inhibition ratios were 34.84% after 8 h in the brain, and 42.47% for C-IV. Moreover, when the testing insects were fed with different ratio mixtures of C-IV and C-V, the inhibition extent of Na\(^+/K^+\)–ATPase was not striking. All of these instructed that the action mechanism of Celangulin compounds against insects can inhibit the activities of Na\(^+/K^+\)–ATPase. Moreover, when the testing insects were fed with different ratio mixtures of C-IV and C-V, the inhibition extent of Na\(^+/K^+\)–ATPase activity was also corresponded with the dose of C-IV, whereas C-V had no notable effects.

Discussion

Na\(^+/K^+\)–ATPase plays an important role in maintaining transmembrane potential and conducting nerve impulse in eukaryotes (Oliveira et al. 2015). Many research showed that high-concentration pyrethroid has significant inhibition on the insect Na\(^+/K^+\)–ATPase, and some scholars think it is an important target of pyrethroid insecticides against pest insect (Ning and Shang 1998, He et al. 1999). Na\(^+/K^+\)–ATPase is also the receptor for cardiac glycosides, including ouabain and digoxin, which can specifically inhibit the enzyme by binding to the extracellular surface of the enzyme (Blanco and Mercer 1998). All of these can facilitate the understanding of the mechanisms of Na\(^+/K^+\)–ATPase in insects after C-IV and C-V treatment.

This study demonstrated that C-IV and C-V have inhibiting effects on insect brain Na\(^+/K^+\)–ATPase both in vitro and in vivo, and inhibitory activities of Na\(^+/K^+\)–ATPase in vitro manifested dependence in concentration. In comparison with C-IV, the inhibition of C-V on Na\(^+/K^+\)–ATPase was not striking. All of these instructed that the action mechanism of Celangulin compounds against insects can inhibit the activities of Na\(^+/K^+\)–ATPase. Moreover, when the testing insects were fed with different ratio mixtures of C-IV and C-V, the inhibition extent of Na\(^+/K^+\)–ATPase activity was also corresponded with the dose of C-IV. However, C-V had no notable effects suggesting that Na\(^+/K^+\)–ATPase might be a target of C-IV and C-V.

The poison process of pesticides on the insect is relatively complex and death is often a combination of a variety of effects. The role of Na\(^+/K^+\)–ATPase as one of the targets after pesticides have effects on the insects has been confirmed. Previous studies have shown that Na\(^+/K^+\)–ATPase is one of the important targets of Terpinen-4-ol, which has a strong inhibition on Na\(^+/K^+\)–ATPase in brain synaptosomes in vitro (Ma et al. 2004).
Deoxypodophyllotoxin exhibited toxicity against the third-instar larvae of *Pieris rapae*, indicating that Na\(^+\)/K\(^-\) ATPases may be an important target of deoxypodophyllotoxin (Zhang et al. 2007). Hu et al. (2008) showed that Na\(^+\)/K\(^-\) ATPase in the central nervous system was inhibited by wilfordine and wilforine, and the inhibition rate was dependent on the concentration. Therefore, Na\(^+\)/K\(^-\) ATPase was an acting target of wilfordine and wilforine against some larvae of insects.

The effects of C-IV and C-V on Na\(^+\)/K\(^-\) ATPase might be different. In vivo, C-IV and C-V had some inhibiting effect on the insect brain Na\(^+\)/K\(^-\) ATPase at the concentration of 25 mg/liter. However, in vitro, no any effect was observed under this concentration. Compared with the control group, C-IV and C-V showed an inhibitory effect on Na\(^+\)/K\(^-\) ATPase at the concentration of 100 and 200 mg/liter, respectively, and showed a concentration effect. This finding suggested that inhibition effect in vitro did not play a direct effect on Na\(^+\)/K\(^-\) ATPase and only an effect of backwardness. Therefore, C-IV and C-V resulted in reverse nerve poisoning symptoms (i.e., narcosis and excitation).

In conclusion, C-IV and C-V can inhibit insect brain Na\(^+\)/K\(^-\) ATPase of different levels, and Na\(^+\)/K\(^-\) ATPase is speculated to be an acting target of C-IV and C-V. However, the specific mechanism of action still needs to be validated prospectively in further studies. This study successfully applied an ultramicro-measuring approach in understanding the regulation of Na\(^+\)/K\(^-\) ATPase on *M. separata* larvae brain by C-IV or C-V treatment, yielding informative data and providing novel perspectives for action mechanism of Celangulin compounds against pest insects.

C-IV and C-V can inhibit insect brain Na\(^+\)/K\(^-\) ATPase of different levels, however, the effects of C-IV and C-V on Na\(^+\)/K\(^-\) ATPase were obviously different. In addition, the inhibition effect in vivo does not directly affect Na\(^+\)/K\(^-\) ATPase, and may be an effect of backwardness. Furthermore, we speculate that the Na\(^+\)/K\(^-\) ATP might be one action target of C-IV and C-V. The results in this research can provide basic guidance information for further deeper research on the specific action mechanisms of Celangulin compounds.

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### Table 1. Effects on Na\(^+\)/K\(^-\) ATPase activities of the brain of *M. separata* larvae after feeding Celangulin IV and V

| Treatment | Symptom                | Activity (mean±S.E.) (nmolPi/min/mg) | Inhibition percentage (%) |
|-----------|------------------------|--------------------------------------|--------------------------|
| IV        | Narcosis               | 24.44 ± 0.27                         | 32.39                    |
|           | Recovery               | 22.51 ± 0.60                         | 37.73                    |
| V         | Excitation             | 32.33 ± 0.74                         | 10.57                    |
|           | Convulsion             | 29.39 ± 0.36                         | 18.70                    |
|           | Dehydration            | 30.24 ± 0.35                         | 17.53                    |
| CK        |                        | 36.15 ± 0.07                         | –                        |

### Fig. 3. Effects on Na\(^+\)/K\(^-\) ATPase activities of the brain from *M. separata* larvae after feeding mixtures of Celangulin IV and V at different ratios.
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