Introduction

Intracellular calcium is widely accepted as a pivotal regulator of cell functions. Diverse components are involved in the precise and dynamic control of intracellular calcium homeostasis. It is also recognized that functional modulations of components such as Ca\(^{2+}\)/H\(^{+}\) pumps or Ca\(^{2+}\)/H\(^{+}\) release channels have significant physiological impact on cell functions through intracellular calcium kinetics. Because of such physiological importance, several researchers have pointed out that the components involved in intracellular calcium homeostasis should be promising targets for insecticides. In fact, extracts from a tropical shrub, *Ryania speciosa*, which affect calcium release channels, have been applied for pest control in the United States (EP A registration was withdrawn in 1997); however, synthetic organic compounds affecting intracellular Ca\(^{2+}\) have never been commercially developed as pesticides. Flubendiamide is a new insecticide with potent activity against lepidopterous pests. The present study demonstrated that the compound possessed insecticidal activity through a specific effect on intracellular Ca\(^{2+}\) kinetics, which was signified by pronounced Ca\(^{2+}\) pump stimulation. Further study clarified that the compound intrinsically activated the ryanodine-sensitive calcium release channel (ryanodine receptors, RyRs). The results of this study indicated that insect RyR is a promising target molecule for a new insecticide with high selectivity and low toxicity to mammals.

Keywords: flubendiamide, Ca\(^{2+}\) pump, insecticide, benzendicarboxamide.

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1. Characteristic symptoms in insects

Insects treated with flubendiamide expressed characteristic symptoms, including thickening and shorting of the body, vomiting and defecation. Identical symptoms were also observed in insects treated with ryanodine, a specific modulator of RyRs. Insects treated with these compounds could respond to contact stimuli but failed to behave integrally, which resulted in rapid cessation of feeding behavior. The appearance of treated insects seemed to reflect the specific effect of the compounds, since symptoms caused by the compound were clearly distinguishable from conventional insecticides.

2. Specific stimulation of Ca\(^{2+}\) pump activity

To assess the effect on intracellular Ca\(^{2+}\) kinetics, we determined the activity of the Ca\(^{2+}\) pump, a vital component in intracellular Ca\(^{2+}\) homeostasis, by measuring of liberated inorganic phosphate, since Ca\(^{2+}\) transport was stoichiometrically coupled to catalytic cycles of Ca\(^{2+}\) ATPase activity. Flubendiamide specifically stimulated the Ca\(^{2+}\) pump in a concentration-dependent manner (EC\(_{50}\)=10 nM). The potency of flubendiamide was evidently pronounced in comparison with the effect of the known RyR modulators, ryanodine and caffeine. Further analysis demonstrated that insecticidal activities of flubendiamide and its related compounds were quantitatively correlated to Ca\(^{2+}\) pump stimulation. The results suggested the insecticidal activity of flubendiamide was mediated by a specific effect on intracellular calcium kinetics.
3. Effect on intracellular calcium kinetics
In addition to the \( \text{Ca}^{2+} \) pump, \( \text{Ca}^{2+} \) release channels govern intracellular calcium kinetics, which mediated calcium release from intracellular calcium stores. To evaluate the effects of the compound on \( \text{Ca}^{2+} \) release, a \( \text{Ca}^{2+} \) release assay was conducted using a membrane preparation from skeletal muscles of *Spodoptera litura*. Treatment of flubendiamide after active loading of \( \text{Ca}^{2+} \) into the membrane preparation induced remarkable calcium release from the membrane preparation. Interestingly, this \( \text{Ca}^{2+} \) release was observable only in the presence of a \( \text{Ca}^{2+} \) pump inhibitor, thapsigargin, indicating that the released \( \text{Ca}^{2+} \) might be rapidly re-sequestered by the stimulated \( \text{Ca}^{2+} \) pump.

4. Specific interaction between flubendiamide and insect RyR
If insect RyR is involved in the expression of characteristic symptoms through uncontrolled calcium release, conformational change of the RyR should be induced in the presence of flubendiamide. The binding assay using \( ^{3}\text{H}-\text{ryanodine} \) is a convenient technique to detect the conformational change induced by specific modulators of RyRs. This assay is based on allosteric modulation of ligand bindings by compounds, which potentiate the binding affinity of \( ^{3}\text{H}-\text{ryanodine} \) concurrently with channel activity. In this assay, flubendiamide evidently increased the binding affinity of \( ^{3}\text{H}-\text{ryanodine} \) to insect RyR, indicating that the compound specifically activated insect RyR.

For further elucidation of the specific interaction between flubendiamide and insect RyR, a binding assay using \( ^{3}\text{H}-\text{flubendiamide} \) was performed and indicated a single binding isotherm of \( ^{3}\text{H}-\text{flubendiamide} \) binding \( (K_{d}=7 \text{ nM}) \), which was consistent with the EC\(_{50} \) value under \( \text{Ca}^{2+} \) pump stimulation. The binding affinities of related compounds of flubendiamide, which were estimated by IC\(_{50} \) values in competitive inhibition, were closely correlated to LC\(_{50} \) values against *S. litura*. The evidence indicated that the affinity to the \( ^{3}\text{H}-\text{flubendiamide} \) binding site determined the intrinsic activity of the compounds.

5. Selectivity in the functional modulation of RyRs
According to the toxicological profile of ryanodine, uncontrolled RyR opening seems to lead to serious toxicity to mammals, with similar symptoms observed in insects. The non-selective toxicity of ryanodine is mediated by a high affinity-binding domain conserved in mammalian homologous RyR, whereas flubendiamide showed no acute toxicity in rats, consistent with the evidence that \( ^{3}\text{H}-\text{flubendiamide} \) failed to bind to the rabbit membrane preparation. This evidence inferred that the high affinity-binding domain of flubendiamide was involved in selective toxicity between insects and mammals.

The results of this study indicated that insect RyRs could be a promising target molecule for new insecticide with high selectivity and low toxicity to mammals.