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Original Article

Quantitative relationship between insecticidal activity and Ca^{2+} pump stimulation by flubendiamide and its related compounds

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Flubendiamide is the first benzenedicarboxamide compound developed as an insecticide with potent activity against lepidopterous pests. It has already been clarified that insecticidal activity of the compound is mediated through modulation of the ryanodine-sensitive Ca^{2+} release channel, (ryanodine receptors; RyRs). The present study showed close correlation between insecticidal activities and stimulating effects on the Ca^{2+} pump, a functional co-operative element with RyR activity, and implied the involvement of Ca^{2+} pump stimulation in insecticidal activity. This result suggested that Ca^{2+} pump activity could be a convenient indicator for determining RyR activity.

Keywords: flubendiamide, Ca^{2+} pump, insecticide, benzenedicarboxamide

Introduction

The ryanodine-sensitive Ca^{2+} release channel, (ryanodine receptors; RyRs), is known as a crucial element for intracellular Ca^{2+} mobilization.[1)] Cytoplasmic Ca^{2+} released through activated RyRs triggers the expression of versatile functions of excitable cells. In most animals, muscle contraction is one of the fundamental functions evoked by the increase of cytoplasmic Ca^{2+} following RyR activation. In addition to its structural and pharmacological identity, the mediation of RyRs in muscle contraction has also been established for insect RyRs.[2–5)] Because of their vital role in muscle contraction, several researchers view the insect RyRs as a promising target site for potent insecticides.[6–9)]

Flubendiamide, a new benzenedicarboxamide compound (Table 1), is the first synthetic insecticide that controls lepidopterous pests through functional modulation of the RyR.[10–13)] Our previous study clarified that flubendiamide stabilized the insect RyR in an open state, evoking massive Ca^{2+} release from intracellular Ca^{2+} stores.[10)] Further study elucidated that specific interaction between the compound and insect RyR induced marked stimulation of the Ca^{2+} pump to resequester released Ca^{2+} into intracellular Ca^{2+} stores.[11)] Ca^{2+} pump stimulation should reflect specific characteristics of the functional modulation by flubendiamide, since the intensity of Ca^{2+} pump stimulation was evidently prominent compared to conventional RyR modulators, ryanodine and caffeine.[10)]

The insecticidal activity of flubendiamide is accompanied by characteristic symptoms with high selectivity to lepidopterous insects.[11)] The compound caused several symptoms such as gradual contraction, thickening and shortening of the insect body without convulsions immediately after treatment, which can be clearly distinguished from the symptoms caused by conventional insecticides.[12)] The characteristic symptoms caused by the compound were directly attributable to sustained muscle contraction evoked by the functional modulation of RyR; however, toxicological significance of Ca^{2+} pump stimulation has not been sufficiently clarified. To clarify the relationship between Ca^{2+} pump stimulation and insecticidal activity, the quantitative aspect of Ca^{2+} pump stimulations by flubendiamide and its related compounds was examined in this study.

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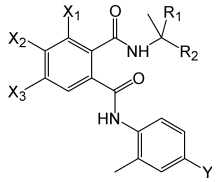
Table 1. Ca^{2+} pump stimulation and insecticidal activity of flubendiamide and its related compounds[a)]



Compound	X_1	X_2	X_3	Y	R_1	R_2	Ca^{2+} pump stimulation (μM)		Insecticidal activity (ppm)
							EC_{50}	95% confidence interval	LC_{50}
1[b)]	I	H	H	$CF(CF_3)_2$	CH_3	$CH_2SO_2CH_3$	0.01[c)]	0.008–0.014	0.03–0.1[d)]
2	I	H	H	$CF(CF_3)_2$	CH_3	CH_2SCH_3	0.01	0.005–0.03	0.03–0.1
3	I	H	H	$CF(CF_3)_2$	H	CH_3	0.03	0.02–0.04	0.1–0.3[d)]
4	I	H	H	CF_2CF_3	H	CH_3	0.04	0.03–0.05	0.3
5	I	H	H	OCF_3	H	CH_3	0.09	0.07–0.13	1–3[d)]
6	I	H	H	$OCHF_2$	H	CH_3	0.44	0.37–0.57	1–3
7	Br	H	H	Cl	H	CH_3	0.42	0.3–0.6	10[d)]
8	H	H	H	Cl	H	CH_3	11.6	7.8–13.9	10–100[d)]
9	I	Cl	Cl	$CF(CF_3)_2$	CH_3	CH_2SCH_3	>30	N.A.	>10

[a)] EC_{50} value for Ca^{2+} pump stimulation was determined based on the results shown in Fig. 2. LC_{50} value was determined against *S. litura* by the leaf dipping method. [b)] Flubendiamide. [c)] Data from ref. 11. [d)] Data from ref. 12.

Materials and Methods

1. *Chemicals and reagents*

All compounds listed in Table 1 were synthesized at the Research Center, Nihon Nohyaku Co., Ltd. (Osaka, Japan). Chemical structures and the purity of tested compounds were checked by ^{1}H-NMR spectroscopy. The purity of tested compounds was above 95%. The compounds were dissolved into dimethyl sulfoxide prior to the measurement of Ca^{2+} pump activity (below 0.5% final concentration). Ryanodine, ATP/Tris, thapsigargin, A23187 and alamethicin were purchased from Sigma Aldrich (St. Louis, MO, USA). All other chemicals used in this study were the highest available grade.

2. *Insects*

The strain of *Spodoptera litura* (Lepidoptera: Noctuidae) used in this study has been maintained at Research Center, Nihon Nohyaku Co., Ltd. for 15 years. Larvae of *S. litura* were reared on an artificial diet of Insecta LFS, purchased from Nosan Corporation (Yokohama, Japan). The colony was maintained at 25°C under a 16 hr-light/8-hr dark photoperiod, with constant access to the larval diet.

3. *Preparation of muscle membranes*

To assess the effect on Ca^{2+} pump activity, we employed a membrane preparation from insect muscle containing sarcoplasmic membrane, which retains functional cooperativity between Ca^{2+} pumps and RyRs,[11)] which enabled us to investigate RyR mediated effects of the compounds on the Ca^{2+} pump. The membrane preparation of *S. litura* was prepared according to the previously described procedure.[11)] Briefly, sixth-instar larvae of *S. litura* were dissected and longitudinal ventral muscle tissues were collected, followed by homogenization on ice in nine volumes (based on tissue wet weight) of the homogenization solution [250 mM sucrose, 5 mM dithiothreitol (DTT), 10 mM tris(hydroxymethyl)aminomethane (Tris)–HCl, pH 7.4] with a Teflon-pestle homogenizer. The supernatants obtained by centrifugation at 9,000 g_{max} (7,000 g_{avg}) for 30 min at 4°C were filtered through Cell Strainer® (BD Falcon, Bedford, MA, USA), followed by centrifugation at 140,000 g_{max}, (104,000 g_{avg}) for 1 h at 4°C. The resulting pellets were re-suspended in four volumes (based on tissue wet weight) of homogenization solution without DTT, followed by re-centrifugation under the conditions described above. The resulting pellets were suspended in a half volume of the homogenization solution without DTT. The suspensions of the obtained membrane preparation were stored at −80°C until use.

4. *Measurements of Ca^{2+} pump activity*

The membrane preparation used in this study contained Ca^{2+}-, Na^+/K^+- and Mg^{2+}-dependent ATPase activity (basal activity). Ca^{2+} dependent ATPase activity (Ca^{2+} pump) was specifically determined by subtracting basal activity from ATPase activity in the presence of Ca^{2+} and Mg^{2+}, as described below. Membrane preparations (20 μg protein) were sus-

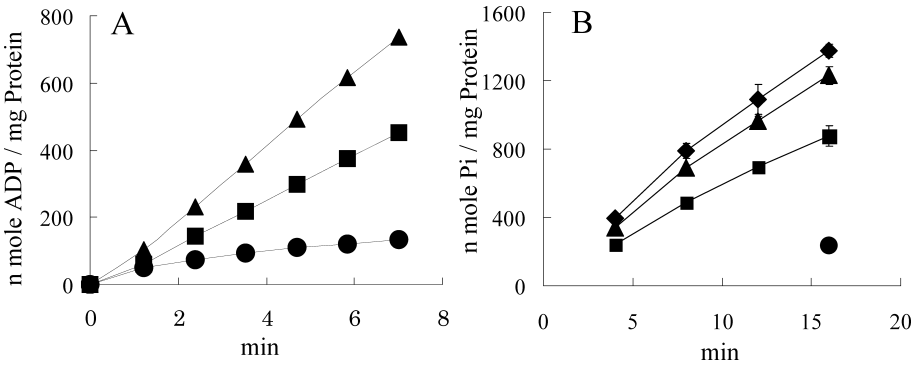


Fig. 1. Effects of compound **2** on Ca^{2+} ATPase activity of *S. litura* membrane preparation. A: ADP production was monitored in the presence of 1.0 μM compound **2** (▲), or absence of the compound (■). Basic activity was determined in the presence of 0.8 mM EGTA (●). Data shows the average of two independent assays. B: liberated inorganic phosphate was determined in the presence of 1.0 μM (◆) or 0.1 μM (▲) compound (**2**) or in the absence of the compound (■). Basic activity was determined as described above (●). Error bar shows standard deviation of quadruplicate samples.

pended in 100 μl of Ca^{2+} pump assay solution (100 mM KCl, 0.05 mM $CaCl_2$, 6 mM $MgCl_2$, 50 mM Tris/3-morpholinopropanesulfonic acid, pH 7.4). After 30-min preincubation at 25°C, reactions were initiated by the addition of ATP/Tris. Unless otherwise stated, the added ATP/Tris concentration was fixed at 1 mM. To determine basal activity, 0.8 mM *O*,*O*′-bis(2-aminoethyl)ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) was added to the assay solution. Inorganic phosphate (Pi) liberated during the reaction was colorimetrically determined according to the previously described method.[14] EC_{50} values for each compound were calculated by curve fitting the data to the sigmoid equation with nonlinear least-square regression using SAS system software (SAS Institute Inc. Japan).

In addition, ATPase activity was evaluated using the ATP regeneration system according to reported method[15] with minor modification. Briefly, 40 μg membrane preparation was suspended in 2 mL assay solution containing the coupling mixture (100 mM KCl, 6 mM $MgCl_2$, 0.05 mM $CaCl_2$, 50 mM Tris/HCl, pH 7.4, 0.2 mM β-nicotinamide adenine dinucleotide (β-NADH), 2 mM phosphoenolpyruvate, 4.3 unit/mL pyruvate kinase, 6.3 unit/mL lactate dehydrogenase (LDH)). The reactions were performed at 25°C with continuous stirring of the reaction mixture. Consumption of β-NADH was monitored by measuring absorbance at 340 nm with a double-beam spectrophotometer (U-3000, Hitachi, Tokyo, Japan).

5. Determination of insecticidal activity

Insecticidal activities of benzenedicarboxamides against a lepidopterous insect, *S. litura*, were determined by the leaf dipping method, as previously described.[12] Briefly, insects were released onto cabbage leaf disks which had been dipped into aqueous suspension containing graded concentrations of the compounds. Insecticidal activities were visually inspected between 4 and 7 days after insect release. LC_{50} values are shown as the range between immediately higher and lower concentrations exerting 50% lethal effect.

Results

1. Ca^{2+} pump stimulation

We reported earlier that flubendiamide specifically stimulates the Ca^{2+} pump as a consequence of stabilization of the insect RyR in an open state, whereas the compound did not stimulate Mg^{2+}- and Na^+/K^+-dependent ATPase activity.[11] In this study, the stimulating effects of a flubendiamide-related compound were confirmed by the ADP recycling system, in which hydrolyzed ADP was continuously converted to ATP by LDH and pyruvate kinase. As shown in Fig. 1, the compound (**2**) with comparable insecticidal activity evidently stimulated ATPase activity. The stimulating effect on the Ca^{2+} pump was represented by the constant increase of catalytic cycles of Ca^{2+} pump activity. The increase rate in the presence of supramaximal concentration of the compound was 162% toward control activity.

The stimulating effect was also confirmed by determination of liberated Pi, which was produced through hydrolysis of ATP. The compound increased inorganic phosphate production to 157% of the control, consistent with the result from the ADP recycling system described above.

2. Correlation between stimulation and insecticidal activity

To clarify the relationship between the stimulating effect on the Ca^{2+} pump and insecticidal activity, the effects of benzenedicarboxamides listed in Table 1 on the Ca^{2+} pump were examined. Flubendiamide and its related compounds **1**–**8** showed insecticidal activities accompanied by the characteristic symptoms consistent with the result reported previously.[12] All examined compounds possessing insecticidal activity evidently stimulated the Ca^{2+} pump (Fig. 2). EC_{50} values of Ca^{2+} pump stimulation were nicely correlated with the LC_{50} value for insecticidal activity against *S. litura* (Fig. 3 and Table 1).

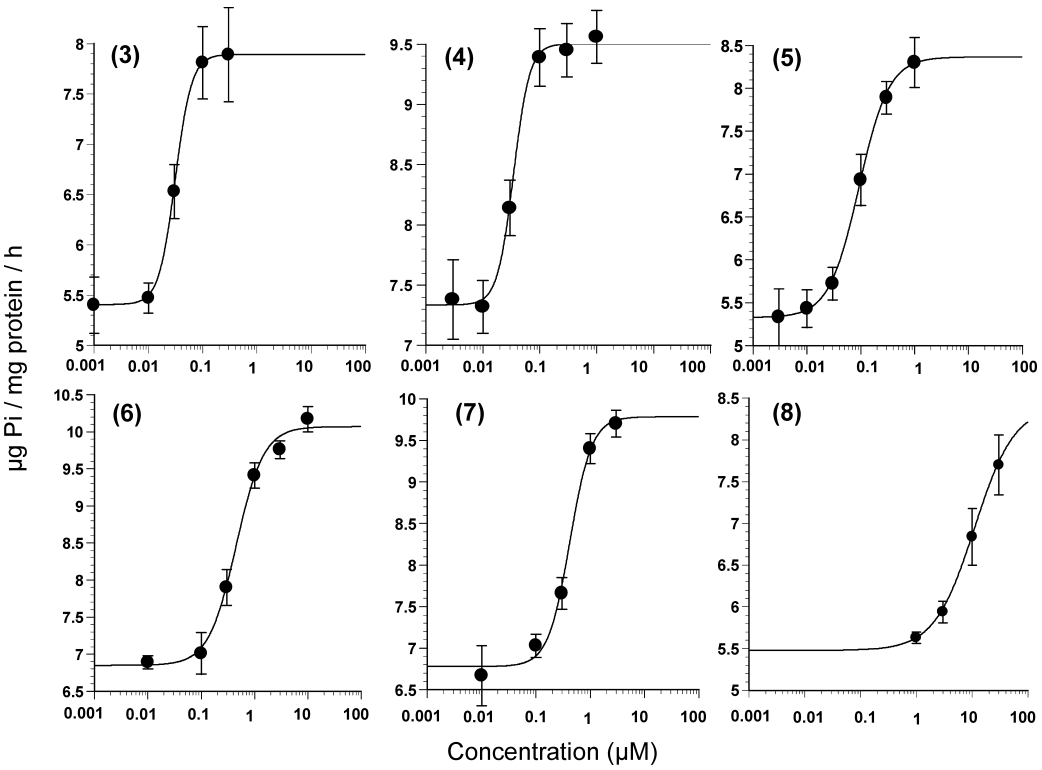


Fig. 2. Effects of benzenedicarboxamide compounds on Ca^{2+} ATPase activity of *S. litura* membrane preparation. Assays were performed in Ca^{2+} ATPase assay solution containing 50 μM free Ca^{2+}. Activity was determined by Pi production over 20 min. Data are the mean±S.D. of quadruplicate samples. The number in parentheses shows the tested benzenedicarboxamides as shown in Table 1. Response curve was fitted to the sigmoid equation with the nonlinear least square regression method.

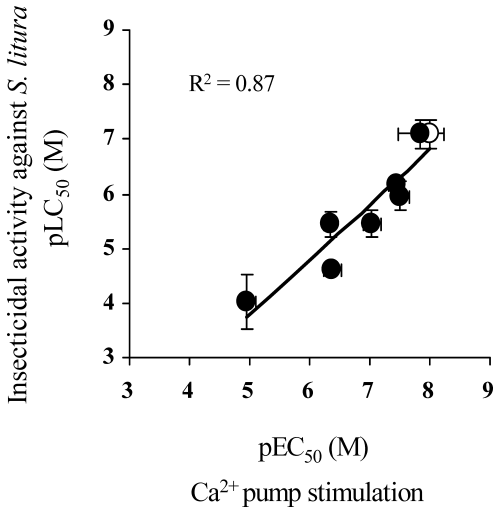


Fig. 3. Correlation between Ca^{2+} pump stimulation and insecticidal activity against *S. litura*. LC_{50} values were determined by the leaf dipping method as described in the text. The values were expressed as the negative of the common logarithm (p). X and Y error bars mean 95% confident interval of EC_{50} and approximate range of LC_{50}, respectively.

In addition, compound **9** without apparent insecticidal activity failed to affect Ca^{2+} pump activity.

3. *Effect of ryanodine on Ca^{2+} pump stimulation of flubendiamide*

The mediation of RyR in the Ca^{2+} pump stimulation of flubendiamide was examined using ryanodine, a specific

Table 2. The effects of ionophores, a specific inhibitor and a calcium chelator on ATPase activity from *S.litura*

Treatment		Average (%)[a)]	S.D.
A23187	0.4 μM	157.9	8.4
	2.0	357.5	3.4
Alamethicin	50 μg/mL	107.7	1.6
	500	102.7	3.2
Thapsigargin	0.1 μM	67.8	3.7
	1.0	31.7	0.9
	10	31.0	2.4
EGTA	0.8 mM	33.6	4.1
Control		100	3.0

[a)] Values are expressed as percentiles toward the control value (3.9 μmole Pi/mg protein/h).

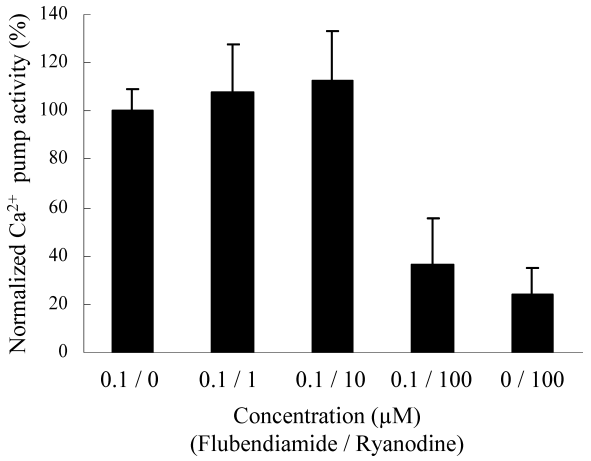


Fig. 4. Effect of ryanodine on Ca^{2+} pump stimulation by flubendiamide. Ca^{2+} pump activities stimulated by 0.1 μM flubendiamide were determined in the presence of ryanodine. Vertical axis represents the Ca^{2+} pump stimulation rate normalized to activity in the presence of 0.1 μM flubendiamide. Data are the mean±S.D. of triplicate samples.

modulator of RyRs. As shown in Fig. 4, Ca^{2+} pump activity stimulated by supramaximal concentration of flubendiamide was significantly suppressed to 36% of control activity in the presence of 100 μM ryanodine. Treating ryanodine alone at the same concentration slightly stimulated Ca^{2+} pump activity in membrane preparation. Suppression of Ca^{2+} pump activity at higher concentrations of ryanodine supported the mediation of RyR in Ca^{2+} pump stimulation.

In addition, the effect of Ca^{2+} permeability on catalytic cycle of Ca^{2+} ATPase activity was examined on the membrane preparation from a lepidopterous insect using a Ca^{2+} ionophore, A23187 as shown in Table 2. The increase of Ca^{2+} permeability in the presence of A23187 clearly facilitated Ca^{2+} ATPase activity, whereas the increased permeability of monovalent cations caused by alamethicin, a peptide known as a channel forming-ionophore, failed to affect Ca^{2+} ATPase activity. This Ca^{2+} ATPase activity was suppressed by a specific Ca^{2+} pump inhibitor, thapsigargin, suggesting that Ca^{2+} ATPase activity reflects enhanced transmembrane Ca^{2+} permeability.

Discussion

We earlier showed that marked stimulation of the Ca^{2+} pump by flubendiamide employing the measurement of liberated Pi, since Ca^{2+} transport by the Ca^{2+} pump is stoichiometrically coupled to the hydrolysis rate of ATP (ATPase activity).[11] For further investigation of the effect on the Ca^{2+} pump, the present study employed the ADP recycling system, an alternative method for measuring of ATPase activity, in addition to Pi measurement (Fig. 1). In both assays, compound (**2**), which is as insecticidal as flubendiamide, showed comparable stimulation of Ca^{2+} pump activity. The evidence further confirmed the stimulating effect of the compound on the Ca^{2+} pump. These results indicated a stimulating effect of the compound on the Ca^{2+} pump, reflecting continuous Ca^{2+} release through activated RyRs. In addition, this stimulating effect can be suppressed by ryanodine, a specific modulator of RyRs (Fig. 4). It is broadly accepted that ryanodine stabilizes RyRs at a subconductance state at which Ca^{2+} flow through RyRs is markedly attenuated due to partial occupation of the channel pore by bound ryanodine.[16] In particular, a higher concentration of ryanodine (~100 μM) is known to induce persistent inactivation of a RyR;[17] therefore, the suppression of the stimulating effect on the Ca^{2+} pump by 100 μM ryanodine was considered to be the result of decreased Ca^{2+} flow through the RyR. This evidence further supported the mediation of the RyR in Ca^{2+} pump stimulation by flubendiamide.

Despite the confirmed evident effect on the Ca^{2+} pump, the toxicological roles of Ca^{2+} pump stimulation have not been sufficiently clarified. In the present study, the relationship between Ca^{2+} pump stimulation and insecticidal activity was quantitatively investigated. As shown in Fig.3 and Table 1, the stimulating effect on the Ca^{2+} pump by flubendiamide and its related compounds was correlated to insecticidal activities. This evidence strongly suggested an intrinsic relationship between insecticidal activity and stimulating effect on the Ca^{2+} pump. Although the details of the direct cause of insect death treated by the compounds have not been clarified, it is assumed that starvation under long-lasting paralysis due to uncontrolled muscle contraction could have a lethal effect on the insect.[12] Stimulation of the Ca^{2+} pump by flubendiamide could result in the acceleration of ATP consumption by catalytic cycles of the Ca^{2+} pump; thus, it is speculated that insect death could be facilitated by the rapid depletion of ATP in treated insects.

The characteristic symptoms caused by the compound could be attributable to continuous muscle contraction induced by released Ca^{2+} through the RyR. In insects, functionally distinguishable muscles, known as synchronous and asynchronous muscle, have been identified.[18] It is known that lepidopterous insects including *S. litura* have synchronous muscle, in which contraction is precisely controlled by sarcoplasmic Ca^{2+} concentration, whereas in asynchronous muscle, sarcoplasmic Ca^{2+} plays a restricted role in muscle contraction.[19–20] Thus the strong synchrony between sarcoplasmic Ca^{2+} concentration and muscle contraction in lepidopterous insects could express the characteristic symptoms even with a slight increase of sarcoplasmic Ca^{2+} concentration as a result of rapid sequestration of the stimulated Ca^{2+} pump.

Ca^{2+} pump activity in the membrane preparation used in this study selectively responded to increase of Ca^{2+} permeability (Table 2), which indicated that the stimulation of Ca^{2+} pump activity in the presence of benzenedicarboxamides reflected the increase of Ca^{2+} flow through the RyR. Generally, the intrinsic activity of RyRs has been directly measured using the lipid bi-layer method, in which the open probability

of the channel was determined by measuring transmembrane conductance through RyRs incorporated into the lipid bilayer. This method, however, seems to be inconvenient due to the need for high content RyRs in test preparations. On the other hand, intracellular Ca^{2+} kinetics is recognized as an important indicator of channel functionality, which can be determined by Ca^{2+} imaging or Ca^{2+} release assay. The results of this study suggest that the Ca^{2+} pump assay is one method to determine RyR activities, which precisely reflect Ca^{2+} release through RyRs.

The importance of insect RyRs as target molecules for developing a new insecticide has further increased, since anthranilic diamide derivatives were developed as a new insecticidal compound with a specific effect on insect RyRs.[21–23] The present study demonstrated that Ca^{2+} pump stimulation, a result of disrupting intracellular calcium kinetics caused by functional modulation of the RyR, is closely correlated to insecticidal activities of the compounds. The results of this study can provide technical bases for a new assay method to screen insecticides targeting intracellular Ca^{2+} kinetics, a new promising target for selective insecticides.

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