Flubendiamide, a Novel Ca\textsuperscript{2+} Channel Modulator, Reveals Evidence for Functional Cooperation between Ca\textsuperscript{2+} Pumps and Ca\textsuperscript{2+} Release

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ABSTRACT
Flubendiamide, developed by Nihon Nohyaku Co., Ltd. (Tokyo, Japan), is a novel activator of ryanodine-sensitive calcium release channels (ryanodine receptors; RyRs), and is known to stabilize insect RyRs in an open state in a species-specific manner and to desensitize the calcium dependence of channel activity. In this study, using flubendiamide as an experimental tool, we examined an impact of functional modulation of RyR on Ca\textsuperscript{2+} pump. Strikingly, flubendiamide induced a 4-fold stimulation of the Ca\textsuperscript{2+} pump activity (EC\textsubscript{50} = 11 nM) of an insect that resquesters Ca\textsuperscript{2+} to intracellular stores, a greater increase than with the classical RyR modulators ryanodine and caffeine. This prominent stimulation, which implies tight functional coupling of Ca\textsuperscript{2+} release with Ca\textsuperscript{2+} pump, resulted in a marginal net increase in the extravesicular calcium concentration despite robust Ca\textsuperscript{2+} release from the intracellular stores by flubendiamide. Further analysis suggested that luminal Ca\textsuperscript{2+} is an important mediator for the functional coordination of RyRs and Ca\textsuperscript{2+} pumps. However, kinetic factors for Ca\textsuperscript{2+} pumps, including ATP and cytoplasmic Ca\textsuperscript{2+}, failed to affect the Ca\textsuperscript{2+} pump stimulation by flubendiamide. We therefore conclude that the stimulation of Ca\textsuperscript{2+} pump by flubendiamide is mediated by the decrease in luminal calcium, which may induce calcium dissociation from the luminal Ca\textsuperscript{2+} binding site on the Ca\textsuperscript{2+} pump. This mechanism should play an essential role in precise control of intracellular Ca\textsuperscript{2+} homeostasis.

Pharmacological probes have gained widespread acceptance as reagents to reveal the functional consequences of intracellular calcium regulation. Many exogenous effectors modulate intracellular calcium homeostasis through effects on ryanodine-sensitive calcium release channels (ryanodine receptors; RyRs). However, their actions on RyRs are often complex, not completely clarified, or both (Zucchi and Roncai-Testoni, 1997). This complexity is thought to be attributable to calcium dependence of the functional modulation, leading to apparently antithetical effects on the RyR. Such complexity is a serious obstacle to understanding the sequential effects of RyR activity in fluctuating calcium conditions.

Flubendiamide, a new phthalic diamide compound (Fig. 1), stabilizes insect RyR to an open state, evoking massive calcium release from intracellular stores (Ebbinghaus-Kintscher et al., 2006). The compound possesses distinct pharmacological characteristics, mediated by a binding site different from that of ryanodine, a widely used probe for the RyR. Interestingly, flubendiamide achieved maximum activation of the RyR in the presence of a broad range of calcium concentrations, including in low calcium conditions. This characteristic might contribute to revealing the relationships between functionally linked components by minimizing the complexity in functional modulation of the RyR. Thus, this compound is expected to provide a new experimental device for exploring the impacts of RyR activity.

The action of flubendiamide is highly specific to insect RyRs and results in selective toxicity in restricted taxa, including Lepidoptera (Tohnishi et al., 2005). The characteristic symptoms caused by continuous muscle contractions through straightforward activation of the insect RyR suggest a pivotal role for the RyR in insect muscle contraction. The physiological importance of RyR in insect muscle implies that basic understanding of excitation-contraction coupling, which has been postulated from evidence accumulated in comprehensive investigations (Lockyer et al., 1998; Quinn et al., 1998; Takekura and Franzini-Armstrong, 2002; Vázquez-Martínez et al., 2003), is broadly deducible in the animal kingdom, including the Insecta. However, distinct pharma-

ABBREVIATIONS: RyR, ryanodine receptor; cADPR, cyclic ADP ribose; SR, sarcoplasmic reticulum; diBr-BAPTA, 1,2-bis(2-amino-5-bromophenoxy)ethane-N,N',N''-tetraacetic acid; DTT, dithiothreitol; MOPS, 3-(N-morpholino)propanesulfonic acid; A23187, calcimycin.

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ological characteristics are seen in the functional properties of the insect RyR, despite its essential similarity to mammalian isoforms (Lehmburg and Casida, 1994; Puente et al., 2000; Scott-Ward et al., 2001). We therefore believe that the insect muscle is a unique model for understanding the physiological impact of altered RyR activity.

The Ca\(^{2+}\) released via the RyR during excitatory states is eventually returned to intracellular stores by the Ca\(^{2+}\) pump. Functional interference with the Ca\(^{2+}\) pump by the RyR is implied in the recent controversy around studies of cADPR (Lukyanenko et al., 2001a). cADPR was first reported as an endogenous RyR activator, but subsequent studies suggested RyR activation by cADPR was a result of Ca\(^{2+}\) pump activation mediated by increased luminal calcium.

In this study, we used a unique approach to elucidate the physiological impact of RyR activity using this new probe in a distinct RyR isoform derived from an insect. In particular, we focused our attention on SR Ca\(^{2+}\) pump activity, a functionally complementary factor of the RyR. The results presented here demonstrate an intriguing relationship between the Ca\(^{2+}\) pump and RyR activation by flubendiamide, mediated by luminal Ca\(^{2+}\).

Materials and Methods

Chemicals and Reagents. Flubendiamide (Fig. 1) was synthesized at Nihon Nohyaku Co., Ltd. The compound was dissolved in dimethyl sulfoxide for all assays (below 0.5% final concentration). [\(^3\)H]Ryanodine (56 Ci/mmol) was purchased from PerkinElmer Life and Analytical Sciences (Boston, MA). Ryanodine, ATP/Tris, thapsigargin, A23187 (calcimycin), and caffeine were purchased from Sigma-Aldrich (St. Louis, MO). DiBr-BAPTA and Rhod-2 were purchased from Invitrogen (Carlsbad, CA). All other chemicals were the best available grade.

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

Preparation of Muscle Membranes. Sixth instars of S. litura were dissected under a stereoscopic microscope, and longitudinal ventral muscle tissues were collected. Collected tissues were homogenized on ice in 9 volumes (based on tissue wet weight) of the homogenization solution (250 mM sucrose, 5 mM DTT, and 10 mM Tris/HCl, pH 7.4) with a Teflon-pestle homogenizer. The homogenization solution (250 mM sucrose, 5 mM DTT, and 10 mM Tris/HCl, pH 7.4) was maintained at 25°C under a 16-h light/8-h dark photoperiod, with constant access to the larval diet.

Fig. 1. Flubendiamide.
Ca\(^{2+}\) might be rapidly resequestered by the Ca\(^{2+}\) pump, which is tightly coupled to RyR activity as described in the next section. The calcium release induced by flubendiamide in the presence of 3 mM Mg\(^{2+}\) was suppressed by increasing the Mg\(^{2+}\) concentration to 10 mM. This inhibition by Mg\(^{2+}\), an endogenous inhibitor of RyR (Scott-Ward et al., 2001), supports the view that the RyR mediates the calcium release by flubendiamide. A calcium ionophore, A23187, released a large fraction of the residual Ca\(^{2+}\) after the completion of calcium release by flubendiamide, which confirmed that P\(_i\) precipitation in the vesicle did not have a limiting effect on calcium release.

The specific Ca\(^{2+}\) pump inhibitor thapsigargin itself induced moderate calcium release, which was not sensitive to Mg\(^{2+}\). Berman (2000) reported that thapsigargin could release Ca\(^{2+}\) bound in ATPase molecules in calcium-limited states. According to the Mg\(^{2+}\) sensitivity, calcium release induced by flubendiamide was characteristically distinguishable from that caused by thapsigargin.

**Specific Stimulation of the Ca\(^{2+}\) Pump by Flubendiamide.** The effects of flubendiamide on calcium transport by the Ca\(^{2+}\) pump were evaluated by measuring Ca\(^{2+}\)-ATPase activity, indicative of the catalytic cycles of the Ca\(^{2+}\) pump, because calcium transport is stoichiometrically coupled to hydrolysis of ATP. As shown in Fig. 3, flubendiamide in the nanomolar range specifically stimulated Ca\(^{2+}\)-ATPase in a concentration-dependent manner (EC\(_{50}\) = 11 nM). The maximum velocity of Ca\(^{2+}\)-ATPase was 160% of control activity at supramaximal concentrations of flubendiamide. The potency of flubendiamide was evidently pronounced in comparison with the effects of the known RyR modulators ryanodine and caffeine (Fig. 3). Contrary to the definitive stimulation of the Ca\(^{2+}\)-ATPase, flubendiamide exerted no stimulation on Mg\(^{2+}\)- and Na\(^+\)/K\(^+\)-dependent ATPase activity, indicating specificity for Ca\(^{2+}\)-ATPase. Identical experiments using an ATP-regenerating system reproduced these results (data not shown). The ADP concentration is known to be a kinetic factor that can suppress the catalytic cycles of the Ca\(^{2+}\) pump by accumulation of ADP-bound phosphoenzymes (Inesi and de Meis, 1989). This can be avoided in the ATP-regenerating system, because the ADP formed is efficiently converted to ATP. Hence, the result obtained with the ATP-regenerating system proved that flubendiamide stimulated the Ca\(^{2+}\) pump by a mechanism other than enhancement of ADP dissociation from phosphoenzymes.

**Evidence Suggesting the Importance of Luminal Ca\(^{2+}\) in Ca\(^{2+}\) Pump Stimulation by Flubendiamide.** Ca\(^{2+}\) within intracellular stores (luminal Ca\(^{2+}\)) is regarded as an essential factor in the kinetic regulation of the Ca\(^{2+}\) pump. In the presence of sufficient extravesicular Ca\(^{2+}\), a decrease in luminal calcium induced acceleration of the Ca\(^{2+}\) pump activity because of facilitation of calcium dissociation from a low-affinity calcium binding site (luminal site). To investigate the possible involvement of luminal calcium in Ca\(^{2+}\) pump stimulation, the luminal Ca\(^{2+}\) concentration was indirectly manipulated by a Ca\(^{2+}\) ionophore and a calcium chelator (Table 1). As shown in Table 1, A23187 induced a 5-fold increase in Ca\(^{2+}\) pump activity. This effect of A23187 was attributable to a decrease in luminal calcium, because A23187 reduced 60Ca\(^{2+}\) accumulation in the membrane preparations to 24.6% of control (T. Masaki, unpublished data) as a result of an increase in calcium permeability (Scarpa et al., 1972). Under this condition, Ca\(^{2+}\) pump stimulation by flubendiamide was mostly eliminated, suggesting the involvement of luminal Ca\(^{2+}\) in the Ca\(^{2+}\) pump stimulation by flubendiamide. The stimulatory effect of flubendiamide on Ca\(^{2+}\) pump was also diminished in the calcium buffers containing calcium chelators with high and low calcium affinity (Table 1). The low-affinity calcium chelator diBr-BAPTA (K\(_d\) = 3.7 \mu M) evidently accelerated the catalytic cycles of Ca\(^{2+}\) pump, as in the case with A23187. The result also implies importance of luminal calcium, because the low affinity of this chelator could not interrupt the calcium association with high-affinity binding sites (cytoplasmic site) on Ca\(^{2+}\)-ATPase. Thus, the acceleration of Ca\(^{2+}\)-ATPase suggests only calcium dissociation from luminal site was facilitated in the presence of

![Fig. 2. Calcium release from the insect membrane preparation induced by flubendiamide.](image)

To load calcium into the vesicles, 1 mM ATP was added at time 0. A, extravesicular Ca\(^{2+}\) was monitored in the presence of 3 mM Mg\(^{2+}\). Flubendiamide was added after calcium sequestration was completed. Residual Ca\(^{2+}\) in the vesicles was released by the addition of 2 \mu M A23187. B, extravesicular Ca\(^{2+}\) was monitored in the presence of 3 mM (solid line) or 10 mM (dashed line) Mg\(^{2+}\). After calcium sequestration had been completed, the Ca\(^{2+}\) pump was inhibited by addition of 0.4 \mu M thapsigargin; thereafter, flubendiamide was added. Residual Ca\(^{2+}\) in the vesicles was released by 2 \mu M A23187.

![Fig. 3. Effect of flubendiamide (○), ryanodine (▲) and caffeine (×) on the Ca\(^{2+}\)-ATPase activity of the membrane preparation. Assays were performed in Ca\(^{2+}\)-ATPase assay solution containing 50 \mu M free Ca\(^{2+}\). The activity was determined by P\(_i\) production over 20 min at 25 °C. Data represents the mean ± S.D. of quadruplicate samples. The ordinate axis shows ATPase activity in the presence of flubendiamide normalized to control activity.](image)
diBr-BAPTA. Conversely, high calcium affinity of EGTA ($K_d = 0.1 \mu M$) disturbed calcium binding to both of the cytoplasmic and luminal site of the molecule, which suppressed wholesale catalytic cycles of the Ca$^{2+}$ pump (Table 1). The results demonstrate that affinities for calcium are essential for apparent effect on Ca$^{2+}$ pump activity of the chelators. Simultaneously, the elimination of effect of flubendiamide on Ca$^{2+}$ pump with A23187 or the chelators inferred flubendiamide has no direct effect on the Ca$^{2+}$ pump.

In addition, the maximum velocity of the flubendiamide-stimulated Ca$^{2+}$-ATPase activity in the presence of 50 \mu M Ca$^{2+}$ was notably augmented under low calcium conditions (1 \mu M of free Ca$^{2+}$, as imposed by a Ca$^{2+}$-EGTA buffer; Fig. 4), whereas apparent flubendiamide affinity based on the Ca$^{2+}$ concentration range, Ca$^{2+}$ ($K_m$) was altered due to a change of the Ca$^{2+}$ pump stimulation by flubendiamide. It was also indicated that the extravesicular calcium concentration failed to affect the affinity of flubendiamide for the molecule.

**Effect of ATP and Ca$^{2+}$ Concentrations on Ca$^{2+}$ Pump Stimulation by Flubendiamide.** The catalytic cycles of the Ca$^{2+}$ pump are also regulated by cytoplasmic Ca$^{2+}$ concentrations through cytoplasmic calcium binding site on the molecule. Flubendiamide failed to affect the apparent calcium affinity of the cytoplasmic calcium binding site, because the $K_m$ values of Ca$^{2+}$ (0.3 \mu M) for Ca$^{2+}$-ATPase activity were not altered by the presence of a supramaximal concentration (100 nM) of the compound (Fig. 5A). The $K_m$ values of Ca$^{2+}$ were determined at calcium concentrations around the reported $K_d$ value for the cytoplasmic calcium binding site (1 \mu M; de Meis and Vianna, 1979). In this calcium concentration range, Ca$^{2+}$ pump stimulation by flubendiamide was characterized by increases in the $V_{max}$ of the activity.

**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Ca$^{2+}$-ATPase Activity (\mu mol Pi/mg protein/h)</th>
<th>B/A</th>
</tr>
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<tr>
<td>A23187</td>
<td>Control 0.1 \mu M Flubendiamide</td>
<td>%</td>
</tr>
<tr>
<td>0.0 \mu M</td>
<td>2.9 ± 0.1</td>
<td>155</td>
</tr>
<tr>
<td>0.4 \mu M</td>
<td>5.2 ± 0.3</td>
<td>119</td>
</tr>
<tr>
<td>2.0 \mu M</td>
<td>13.1 ± 0.1</td>
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<tr>
<td>diBr-BAPTA</td>
<td>0.08 mM 0.9 ± 0.2</td>
<td>217</td>
</tr>
<tr>
<td>0.15 mM</td>
<td>0.9 ± 0.2</td>
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</tr>
<tr>
<td>0.30 mM</td>
<td>1.6 ± 0.1</td>
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</tr>
<tr>
<td>0.60 mM</td>
<td>2.2 ± 0.0</td>
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<tr>
<td>1.20 mM</td>
<td>3.6 ± 0.1</td>
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<tr>
<td>6.00 mM</td>
<td>14.9 ± 0.3</td>
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EGTA

<table>
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<tr>
<th>Treatment</th>
<th>Ca$^{2+}$-ATPase Activity (\mu mol Pi/mg protein/h)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 mM</td>
<td>2.8 ± 0.1</td>
<td>294</td>
</tr>
<tr>
<td>1.2 mM</td>
<td>2.7 ± 0.2</td>
<td>194</td>
</tr>
<tr>
<td>1.6 mM</td>
<td>3.0 ± 0.2</td>
<td>112</td>
</tr>
<tr>
<td>4.0 mM</td>
<td>0.6 ± 0.1</td>
<td>106</td>
</tr>
</tbody>
</table>

**ATP** is another kinetic factor for the catalytic cycles of Ca$^{2+}$ pump. Ca$^{2+}$ pump stimulation by flubendiamide was also unaffected in the presence of graded concentrations of ATP (Fig. 5B). ATP concentrations in the millimolar range enhance Ca$^{2+}$-ATPase activity, which is thought to be mediated by a regulatory binding site on Ca$^{2+}$-ATPase (Ogawa, 1972; Nakamura et al., 2002). Therefore, these results infer that flubendiamide could not enhance the Ca$^{2+}$-ATPase activity by affecting the cytoplasmic calcium binding site or the low-affinity ATP binding site.

**Effects of Flubendiamide on $[^3H]$Ryanodine Binding to the Membrane Preparations.** Our previous report indicates that the RyR is the primary target of flubendiamide; therefore, the stimulation of the Ca$^{2+}$ pump observed in this study should be triggered by activation of insect RyR. To confirm the involvement of RyR, conformational changes of the RyR in the membrane preparation was examined by $[^3H]$ryanodine binding. As shown in Fig. 6, the binding isotherm indicates a single binding site for $[^3H]$ryanodine in the membrane preparation. The $K_d$ and $B_{max}$ values for ryanodine binding were 15.4 ± 5.5 nM and 0.54 ± 0.06 pmol/mg, respectively, in optimal binding solution containing 1 mM Ca$^{2+}$. In the presence of a supramaximal concentration (1 \mu M) of flubendiamide, the binding affinity of the $[^3H]$ryanodine was significantly increased without a evident effect on the $B_{max}$ value. This result indicates that flubendiamide shifts the RyR conformation to the open state in this membrane preparation from *S. litura*.

Numerous endogenous modulators such as Ca$^{2+}$, Mg$^{2+}$, or ATP affect the activity of RyR. The effect of those modulators on RyR was evaluated by comparing the $EC_{50}$ values of flubendiamide for potentiation of $[^3H]$ryanodine binding (Table 2). The $EC_{50}$ value of flubendiamide in the presence of 1 mM Ca$^{2+}$ was 0.12 nM, which was invariable even after the addition of 1 mM ATP or 6 mM Mg$^{2+}$, whereas addition of 1 mM EGTA, which excludes free Ca$^{2+}$ from the assay solution, drastically increased the $EC_{50}$ value to 26 nM. It is therefore indicated that the efficacy of flubendiamide binding is altered due to a change of the Ca$^{2+}$ concentration. The effective concentration of flubendiamide for the Ca$^{2+}$ pump ($EC_{50} = 11$ nM) was in a similar range to the $EC_{50}$ values for RyR, determined in the various solutions listed in Table 2. Indeed, the $EC_{50}$ for RyR obtained in the Ca$^{2+}$-ATPase assay solution (2.5 nM) was rather close to the $EC_{50}$ value for the Ca$^{2+}$ pump. The consistency of the effects of the $EC_{50}$ values for both two components suggests that the Ca$^{2+}$ pump stim-
ulation by flubendiamide is accompanied by RyR activation. This evidence further supports coordination between the RyR and the Ca\(^{2+}\) pump.

**Discussion**

Flubendiamide and its analogous compounds modify intracellular calcium kinetics in cultured insect neurons. This effect is mediated by a specific interaction with the insect RyR (Ebbinghaus-Kintscher et al., 2006). The results of this study revealed Ca\(^{2+}\) release from muscle membrane preparations from an insect, *S. litura*, as a consequence of the functional modulation of the RyR by flubendiamide. Interestingly, Ca\(^{2+}\) pump inhibition was necessary for flubendiamide to produce an observable increase in extravesicular calcium (Fig. 2), despite the robust calcium release induced by flubendiamide. This obscuring of the observable calcium release should be achieved by rapid calcium sequestration by the Ca\(^{2+}\)/H\(^{+}\) pump, which was concurrently stimulated by flubendiamide (Fig. 3). The stimulation of the Ca\(^{2+}\) pump by flubendiamide was clearly pronounced compared with the effects of the classical RyR modulators ryanodine and caffeine, which are known to induce calcium release even in the absence of the Ca\(^{2+}\) pump inhibitor (Palade, 1987). Furthermore, Ca\(^{2+}\) pump stimulation by flubendiamide seemed to be linked with RyR activation, because the EC\(_{50}\) values of flubendiamide for these two components were comparable (Fig. 3; Table 2). This evidence strongly suggests tight functional coupling between the Ca\(^{2+}\) pump and the RyR.

With respect to the underlying mechanism of the functional coordination between the RyR and the Ca\(^{2+}\) pump, we focused on the possibility of luminal calcium as the mediator, an important regulator for both components (Ikemoto and Yamamoto, 2000; Lukyanenko et al., 2001b). In membrane preparations forming sealed vesicles, millimolar concentrations of luminal Ca\(^{2+}\) are quickly achieved, and thereby the catalytic cycles of the Ca\(^{2+}\) pump and calcium transport are impeded (Inesi and de Meis, 1989). This high luminal Ca\(^{2+}\) concentration is efficiently reduced in the presence of the calcium ionophore A23187, which results in acceleration of Ca\(^{2+}\) pump activity (Scarpa et al., 1972). In the presence of A23187, the stimulatory effect of flubendiamide on the Ca\(^{2+}\)

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**Fig. 5.** Ca\(^{2+}\)-ATPase stimulation by flubendiamide at various concentrations of Ca\(^{2+}\) (A) and ATP (B). A, assays were performed in Ca\(^{2+}\)-ATPase assay solution containing 2.6 to 2300 nM free Ca\(^{2+}\). Activities were determined by Pi production over 20 min at 25°C in the presence (●) or absence (○) of flubendiamide (100 nM). Free Ca\(^{2+}\) concentrations imposed by the presence of 0.5 mM EGTA were calculated using the free software program WINMAXC (http://www.stanford.edu/~capton/maxc.html). B, assays were performed in Ca\(^{2+}\)-ATPase assay solution containing 50 μM Ca\(^{2+}\) and 0.03 to 3 mM ATP/Tris.

**Fig. 6.** Effect of flubendiamide on[^H]ryanodine binding to the insect membrane preparation. Saturation kinetics (A) and Scatchard transformation (B) of[^H]ryanodine binding in the presence (●; \(K_d = 3.1 \pm 0.1\) nM; \(B_{\text{max}} = 0.54 \pm 0.057\) pmol/mg of protein) and absence (○; \(K_d = 15 \pm 0.06\) nM; \(B_{\text{max}} = 0.47 \pm 0.06\) pmol/mg of protein) of flubendiamide (1 μM). Binding was equilibrated in binding solution containing 1 mM calcium. Results are expressed as the means of three preparations.
pump activity was evidently eliminated, which was considered to be due to reduction of the luminal calcium concentration, because the effect of A23187 was accompanied by acceleration of the Ca\(^{2+}\) pump (Table 1).

Furthermore, the importance of luminal Ca\(^{2+}\) was also indicated by the amplification of flubendiamide-stimulated Ca\(^{2+}\) pump activity with an increased transmembrane Ca\(^{2+}\) gradient (Fig. 4). At low extravesicular calcium concentrations, Ca\(^{2+}\) pump stimulation by flubendiamide was enhanced. This might be due to an increased flow rate of Ca\(^{2+}\) through the activated RyR with the steeper transmembrane calcium gradient.

The catalytic cycles of the Ca\(^{2+}\) pump are also regulated by cytoplasmic Ca\(^{2+}\), which accelerates the catalytic cycles of the Ca\(^{2+}\) pump via cytoplasmic calcium binding site on the molecule. Flubendiamide failed to increase the apparent calcium affinity for cytoplasmic binding site (Fig. 5A). In addition, flubendiamide stimulated the Ca\(^{2+}\) pump even under buffered calcium conditions in which released calcium was rapidly clamped by the calcium chelator (Fig. 4); therefore, released Ca\(^{2+}\) could not participate in the stimulation of the Ca\(^{2+}\) pump by flubendiamide. Hence, we conclude that luminal Ca\(^{2+}\) is the pivotal mediator of the coordination between the Ca\(^{2+}\) pump and the RyR.

The luminal Ca\(^{2+}\) affects the catalytic cycles of the Ca\(^{2+}\) pump via the luminal Ca\(^{2+}\) binding site on the molecule, which has a low calcium affinity with a high dissociation constant of around 1 mM (de Meis and Vianna, 1979; Ikemoto, 1982; MacLennan et al., 1997). The decrease in luminal Ca\(^{2+}\) is considered to increase calcium dissociation rates from luminal binding sites and subsequently enhances the Ca\(^{2+}\) pump activity based on the unifying model of calcium transport by the Ca\(^{2+}\) pump. Similar to the case of A23187, a Ca\(^{2+}\) buffer composed of diBr-BAPTA accelerated the catalytic cycles of Ca\(^{2+}\) pump activity (Table 1). This result is attributable to enhancement of the calcium dissociation rate from the luminal binding sites, because the chelator has a higher affinity for Ca\(^{2+}\) (K\(_d\) = 3.6 \(\mu\)M; Tsien, 1980) compared with the reported affinity of the luminal calcium binding sites (K\(_d\) = 1 mM; de Meis and Vianna, 1979). Conversely, the affinity of diBr-BAPTA for Ca\(^{2+}\) is lower compared with the cytoplasmic binding sites on the Ca\(^{2+}\) pump (K\(_d\) = 0.3 \(\mu\)M; Fig. 5A), which indicates that the calcium association to cytoplasmic binding site cannot be interrupted by the chelator. Therefore, enhancement of the calcium dissociation rate from the luminal binding sites by diBr-BAPTA can be directly reflected in acceleration of the Ca\(^{2+}\) pump. Hence, the elimination of Ca\(^{2+}\) pump stimulation by flubendiamide in the presence of diBr-BAPTA further supports the mediation by luminal Ca\(^{2+}\) from the view of a subsequent increase of calcium dissociation rate from luminal binding site. However, the precise mechanism for the acceleration of the Ca\(^{2+}\) pump in the presence of diBr-BAPTA remains to be clarified.

The importance of luminal calcium in the coordination between calcium release and resequestration in the mammalian SR has been previously reported by Ikemoto and Yamamoto (2000). Their experiments, however, used polylysine as an inducer of calcium release, which could interact with components of the membrane (de Kruijff and Cullis, 1980). Therefore, this study first elucidated the coordination between calcium release and resequestration using a specific probe for the RyR. Luminal Ca\(^{2+}\) is increasingly recognized as a modulator of RyRs, especially cardiac RyRs (Lukyanenko et al., 2001b), which can also modulate the sensitivity for exogenous modulators (Dettborn and Palade, 1997). As for the Ca\(^{2+}\) pump, the luminal calcium binding site contributes to preventing overloading by suppressing calcium uptake. Based on the increasing background information described above, several researchers have indicated the importance of luminal Ca\(^{2+}\) homeostasis from the view of optimization of cytoplasmic calcium regulation (Fabio, 1992; Ikemoto and Yamamoto, 2000; Lukyanenko et al., 2001b). The mediation of luminal calcium with cooperation between the Ca\(^{2+}\) pump and the RyR in the insect suggests that the physiological importance of luminal calcium is ubiquitous over long evolutionary distances.

It seems that flubendiamide has no direct effect on Ca\(^{2+}\) pump, although flubendiamide binding to the isolated Ca\(^{2+}\)-ATPase has not been investigated, because Ca\(^{2+}\)-ATPase activity in light SR from rabbit skeletal muscles was not affected by the compound (T. Masaki, unpublished data). The elimination of effect of flubendiamide on Ca\(^{2+}\) pump with A23187 or the chelators (Table 1) should provide additional evidence for this speculation. It should also be noted that intracellular calcium in Chinese hamster ovary cells expressing native Ca\(^{2+}\)-ATPase exhibited no fluctuations by the treatment of flubendiamide (Ebbinghaus-Kintscher et al., 2006). Hence, the stimulation of the Ca\(^{2+}\) pump observed in this study should be triggered by activation of insect RyR.

In this study, we demonstrated that the selective and straightforward RyR activation by flubendiamide enables us to elucidate sequential effects on the Ca\(^{2+}\) pump. In addition, insect RyR isoforms have distinct characteristics, such as lower calcium sensitivity than mammalian isoforms (Scott-Ward et al., 2001), which can be exactly discriminated using flubendiamide. Thus, this compound should provide a promising probe for understanding the diversity of functional regulations of the RyR. Moreover, precise investigations using the compound can provide pertinent information for discovering unexplored target site for insecticides. The practical

### Table 2

<table>
<thead>
<tr>
<th>Solution</th>
<th>Ca(^{2+})</th>
<th>Additives</th>
<th>EC(_{50})</th>
<th>B(_{max})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Binding</td>
<td>1</td>
<td>ATP (1 mM)</td>
<td>0.12</td>
<td>0.35</td>
</tr>
<tr>
<td>Binding</td>
<td>1</td>
<td>Mg(^{2+}) (6 mM)</td>
<td>0.14</td>
<td>0.41</td>
</tr>
<tr>
<td>Binding</td>
<td>1</td>
<td>EGTA (1 mM)</td>
<td>0.12</td>
<td>0.39</td>
</tr>
<tr>
<td>Ca(^{2+})-ATPase assay</td>
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<td>ATP (1 mM), Mg(^{2+}) (6 mM)</td>
<td>2.5</td>
<td>0.14</td>
</tr>
</tbody>
</table>

Characteristics of the potentiation of \([^{3}H]\)ryanodine binding by flubendiamide

The effects of the compound on the binding of 5 nM \([^{3}H]\)ryanodine were determined in the solutions with endogenous modulators for RyR. Binding solution contained 1 M KCl, 0.3 M sucrose, and 10 mM Tris/HCl, pH 7.4. Ca\(^{2+}\)-ATPase assay solution contained 100 mM KCl, 6 mM Mg\(^{2+}\), 0.05 mM CaCl\(_2\), 1 mM ATP, and 50 mM Tris/MOPS, pH 7.4.

The practical...
flubendiamide binding domain on RyR and its functional role, including interactions with accessory factors, remain to be determined for further clarification of the mode of action of flubendiamide.

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