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Properties of amentoflavone, a potent caffeine-like Ca^{2+} releaser in skeletal muscle sarcoplasmic reticulum

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Abstract

Amentoflavone, like caffeine, caused a concentration-dependent increase in Ca^{2+} release from the heavy fraction of fragmented sarcoplasmic reticulum of rabbit skeletal muscle. The Ca^{2+}-releasing activity of amentoflavone was approximately 20 times more potent than that of caffeine. The bell-shaped profile of Ca^{2+} dependence for amentoflavone was almost the same as that for caffeine. Typical blockers of Ca^{2+}-induced Ca^{2+} release channels, such as Mg^{2+}, procaine and ruthenium red, inhibited markedly amentoflavone- and caffeine-induced $^{45}Ca^{2+}$ release. The maximum $^{45}Ca^{2+}$ release in response to amentoflavone was not changed by caffeine, but was further increased by adenosine-5′-(β,γ-methylene) triphosphate. This compound enhanced [^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum with a decrease in K_D but without a change in B_{max}. These results suggest that amentoflavone, which does not contain a nitrogen atom, probably binds to the caffeine-binding site in Ca^{2+} channels and thus potentiates Ca^{2+}-induced Ca^{2+} release from the heavy fraction of fragmented sarcoplasmic reticulum.

Keywords: Amentoflavone; Ca^{2+}-induced Ca^{2+} release; Caffeine-binding site; Ca^{2+} release channel; Ryanodine receptor; Sarcoplasmic reticulum

1. Introduction

The ryanodine receptor, which functions as a Ca^{2+} release channel in the sarcoplasmic reticulum, is postulated to play a key role in excitation–contraction coupling in skeletal muscle (McPherson and Campbell, 1993; Coronado et al., 1994; Sutko and Airey, 1996). Several natural products, such as imperatoxins (Valdivia et al., 1992) and bastadins (Mack et al., 1994), have been shown to modulate the Ca^{2+} release mediated by the ryanodine receptor in sarcoplasmic reticulum. In the course of our survey of natural compounds with Ca^{2+}-releasing activity in the heavy fraction of fragmented sarcoplasmic reticulum, we have found that 9-methyl-7-bromoeudistomin D (MBED) is useful as chemical probe to elucidate the molecular mechanisms of Ca^{2+} release (Seino et al., 1991). We have recently isolated amentoflavone (Fig. 1), a biflavonoid from *Nandina domestica* (Berberidaceae), and have found that this compound potently induces Ca^{2+} release from the heavy fraction of fragmented sarcoplasmic reticulum. Amentoflavone has been isolated from various species of Hypericum and is reported to have anti-human immunodeficiency virus (HIV) activity (Lin et al., 1997) and nonenzymic lipid peroxidation inhibitory activity (Mora et al., 1990). However, the detailed pharmacological properties of this compound have not yet been studied. Here we demonstrate for the first time the detailed pharmacological study of Ca^{2+} release from the heavy fraction of fragmented sarcoplasmic reticulum induced by amentoflavone.

2. Materials and methods

2.1. Materials

Amentoflavone was isolated from the dried fruits of *N. domestica*. Briefly, the dried fruits of *N. domestica* (3 kg), purchased from Uchida-Wakanyaku, Japan, were extracted

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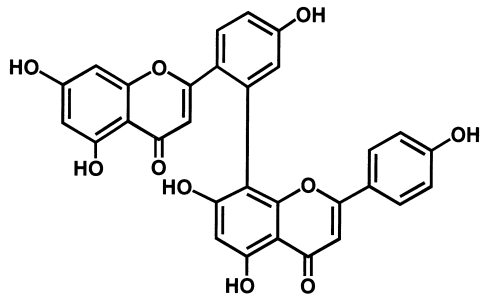

Fig. 1. Chemical structure of amentoflavone.

with hot MeOH. The MeOH extract was concentrated under reduced pressure, and the viscous concentrate was partitioned between *n*-BuOH and H_2O. The *n*-BuOH-soluble fraction was chromatographed on silica-gel with a stepwise gradient of $CHCl_3$–MeOH as eluant to give six fractions. Fraction III was repeatedly subjected to silica-gel column chromatography with $CHCl_3$–MeOH–H_2O (80:10:1; 70:10:1) as eluant, resulting in the isolation of amentoflavone (524 mg). The substances we used were purchased from the sources indicated: ryanodine (S.B. Penick, New York, NY), procaine (Sigma, St. Louis, MO), $^{45}CaCl_2$ (0.7 Ci/mmol) (NEN Life Science Products) and [^{3}H]ryanodine (60 Ci/mmol) (NEN Life Science Products). All other chemicals were of analytical grade.

2.2. Preparation of the sarcoplasmic reticulum vesicles from skeletal muscle

The heavy fraction of fragmented sarcoplasmic reticulum enriched in Ca^{2+}-induced Ca^{2+} release activity was prepared from rabbit skeletal muscle as previously reported (Seino et al., 1991) with a slight modification. White muscle was homogenized four times with a National MX-915C mixer in 5 vols. of 5 mM Tris–maleate (pH 7.0) for 30 s with 30-s intervals. The homogenate was centrifuged at $5000 \times g$ for 15 min. The supernatant was filtered through cheesecloth, and the filtrate was centrifuged again at $12{,}000 \times g$ for 30 min. The pellets were suspended in a solution containing 90 mM KCl and 5 mM Tris–maleate (pH 7.0) and centrifuged at $70{,}000 \times g$ for 40 min. The pellets were suspended in a solution containing 90 mM KCl, 5 mM Tris–maleate (pH 7.0) and 0.3 M sucrose. The obtained heavy fraction of fragmented sarcoplasmic reticulum was stored in the same solution at −80°C until use. The protein concentration was determined by the method of Bradford (1976) with bovine serum albumin as a standard. The animals used in this study were treated in accordance with the principles and guidelines of Tohoku University Council on Animal Care.

2.3. Ca^{2+} electrode experiments

The extravesicular Ca^{2+} concentration was monitored with a Ca^{2+} electrode at 30°C (Nakamura et al., 1986; Seino et al., 1991). The Ca^{2+} electrode showed a Nernstian response (slope, 27–29 mV/ − log [Ca^{2+}] unit) in the calibration solutions containing Ca^{2+}–EGTA at Ca^{2+} concentrations between 0.3 μM and 1 mM. The time for 90% response was approximately 0.6 s when the Ca^{2+} concentration was reduced from 100 to 1 μM. The assay solution (final volume, 1 ml) contained 0.05 mM $CaCl_2$, 90 mM KCl, 0.5 mM $MgCl_2$, 50 mM 3-morpholinopropanesulfonic acid (MOPS)–Tris (pH 7.0), 0.75 mg/ml of the heavy fraction of fragmented sarcoplasmic reticulum, 5 mM creatine phosphate, 0.1 mg/ml of creatine kinase and 0.5 mM ATP. The Ca^{2+} uptake reaction was started by the simultaneous addition of creatine kinase and ATP.

2.4. $^{45}Ca^{2+}$ release experiments

$^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum vesicles passively preloaded with $^{45}Ca^{2+}$ was measured at 0°C as described previously (Nakamura et al., 1986) with a slight modification. After a 12-h pre-incubation of 20 mg/ml of the heavy fraction of fragmented sarcoplasmic reticulum with 5 mM $^{45}CaCl_2$ in a solution containing 90 mM KCl and 50 mM MOPS–Tris (pH 7.0) at 0°C, the suspension was diluted with 100 vols. of an ice-cold reaction medium containing 0.4 mM $CaCl_2$ with various concentrations of EGTA, 90 mM KCl and 50 mM MOPS–Tris (pH 7.0). For measurement of the amount of $^{45}Ca^{2+}$ in the heavy fraction of fragmented sarcoplasmic reticulum at time 0, the suspension was diluted with the reaction medium containing 5 mM $LaCl_3$. At an appropriate time 5 mM $LaCl_3$ was added to stop $^{45}Ca^{2+}$ release. The reaction mixture was then filtered through a Millipore filter (HAMP type, 0.45 μm pore size) which was then washed with 5 ml of a solution containing 5 mM $LaCl_3$, 5 mM $MgCl_2$, 90 mM KCl and 50 mM MOPS–Tris (pH 7.0). The amount of $^{45}Ca^{2+}$ remaining in the heavy fraction of fragmented sarcoplasmic reticulum vesicles was measured by counting the radioactivity on the washed filters.

The free Ca^{2+} concentration was maintained by using Ca^{2+}–EGTA buffer (0.5 mM $CaCl_2$ plus various concentrations of EGTA) and was estimated by using a microcomputer program that took into account the binding constant of Ca^{2+}–EGTA, pH and the concentration of K^+, Mg^{2+} and nucleotides (Sillen and Martell, 1964, 1971).

2.5. Binding assays

[^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum was examined as described previously (Furukawa et al., 1994) with a slight modification. The heavy fraction of fragmented sarcoplasmic reticulum (100 μg/ml) was incubated with 2.5 nM [^{3}H]ryanodine at 37°C for 2 h in a solution containing 0.3 M sucrose, 0.3 M KCl, 100 μM $CaCl_2$ and 20 mM Tris–HCl

(pH 7.4). The amount of [^{3}H]ryanodine bound was determined by filtration of the reaction mixture through Whatman filters (GF/B). Nonspecific binding was determined in the presence of 10 μM unlabeled ryanodine.

2.6. Statistical analysis

The data are expressed as means ± S.E.M. Statistical comparisons were made by using Student's *t*-test for unpaired data. $P < 0.05$ was considered significant.

3. Results

3.1. Ca^{2+} release experiments with a Ca^{2+} electrode

The effect of amentoflavone on the Ca^{2+}-mobilizing activity of fragmented sarcoplasmic reticulum can be visualized clearly by monitoring the extravesicular Ca^{2+} concentration of the heavy fraction of fragmented sarcoplasmic reticulum directly with a sensitive Ca^{2+} electrode. Fig. 2 demonstrates that, upon the addition of 0.5 mM ATP, the free Ca^{2+} concentration decreased promptly due to the formation of Ca^{2+}–ATP complexes and further decreased gradually because of Ca^{2+} uptake by the heavy fraction of fragmented sarcoplasmic reticulum. When the concentration of Ca^{2+} was reduced to a submicromolar level, the Ca^{2+} uptake slowed down. At this point, the addition of 1 mM caffeine or 10 μM amentoflavone to the Ca^{2+}-filled heavy fraction of fragmented sarcoplasmic reticulum induced a prompt release of Ca^{2+} followed by a rapid re-uptake of Ca^{2+} (Fig. 2A and B). The Ca^{2+}-releasing action of amentoflavone (10 μM) was abolished by pretreatment with ruthenium red (2 μM), $MgCl_2$ (5 mM) or ryanodine (10 μM) (Fig. 2C–E).

3.2. $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum

Another more quantitative way of examining Ca^{2+}-releasing activity involves the measurement of $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum vesicles under conditions in which the Ca^{2+} pump does not work. The $^{45}Ca^{2+}$ release was significantly increased by amentoflavone and caffeine in a time-dependent manner (0 to 2 min) and reached a plateau 2 min after application of amentoflavone and caffeine. The concentration–response curve of amentoflavone or caffeine for causing $^{45}Ca^{2+}$ release is shown in Fig. 3A. $^{45}Ca^{2+}$ release was increased by amentoflavone (the EC_{50} value of 67 μM) and caffeine (the EC_{50} value of 1.2 mM) in a concentration-dependent manner. Fig. 3B shows the effect of amentoflavone and caffeine on $^{45}Ca^{2+}$ release over a wide range of free Ca^{2+} concentrations. $^{45}Ca^{2+}$ release was increased by increasing the free Ca^{2+} concentration from 10 nM to 1 μM (the EC_{50} value of 0.12 μM). There was no significant effect of amentoflavone and caffeine on the maximum $^{45}Ca^{2+}$ release at a Ca^{2+} concentration of 1 μM. However, at Ca^{2+} concentrations of 0.3 μM or lower, the concentration–response curve for $^{45}Ca^{2+}$ re-

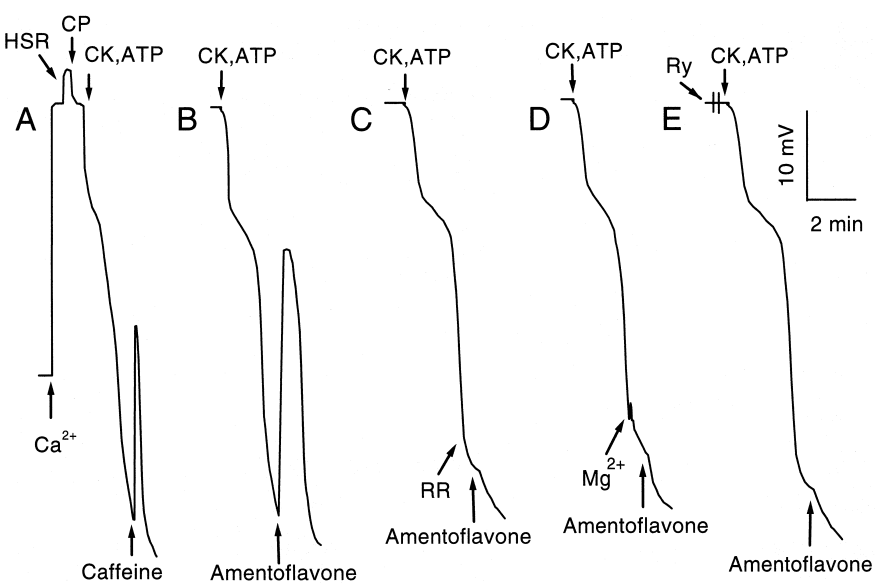

Fig. 2. Ca^{2+} release induced by amentoflavone from the heavy fraction of fragmented sarcoplasmic reticulum in the presence or absence of Mg^{2+}, ruthenium red (RR) and ryanodine (Ry). The assay solution (final volume, 1 ml) contained 0.05 mM $CaCl_2$, 90 mM KCl, 0.5 mM $MgCl_2$, 50 mM MOPS–Tris (pH 7.0), 0.75 mg/ml of the heavy fraction of fragmented sarcoplasmic reticulum, 5 mM creatine phosphate (CP), 0.1 mg/ml of creatine kinase (CK) and 0.5 mM ATP. Vertical calibration bars indicate the response to a voltage change (10 mV) corresponding to $0.3 - \log[Ca^{2+}]$ unit. In panels B to E, the traces are those after the addition of ATP. (A) 1 mM caffeine; (B) 10 μM amentoflavone; (C) 2 μM RR + 10 μM amentoflavone; (D) 5 mM Mg^{2+} + 10 μM amentoflavone; (E) 10 μM Ry + 10 μM amentoflavone. Ryanodine treatment was performed for 3 min just before the addition of ATP.

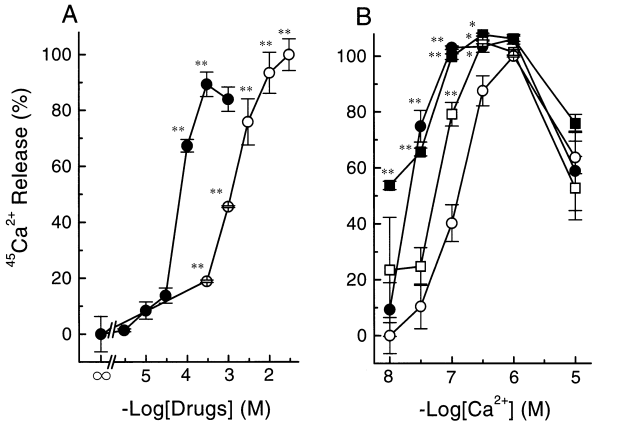

Fig. 3. Stimulatory effects of amentoflavone and caffeine on $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum. (A) Concentration-dependent acceleration of $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum by amentoflavone and caffeine. $^{45}Ca^{2+}$ release was measured at a Ca^{2+} concentration of 0.1 μM. The amount of $^{45}Ca^{2+}$ released was calculated from the decrease in the $^{45}Ca^{2+}$ content of heavy sarcoplasmic reticulum vesicles in 1 min after dilution. Each value was obtained by subtracting the amount of $^{45}Ca^{2+}$ release measured in the absence of amentoflavone from that measured in its presence. Caffeine (○); amentoflavone (●). (B) Effects of free Ca^{2+} concentration on the $^{45}Ca^{2+}$ release from the heavy fraction of fragmented skeletal muscle sarcoplasmic reticulum in the absence or presence of amentoflavone and caffeine. Control (○); 1 mM caffeine (●); 30 μM amentoflavone (□); 300 μM amentoflavone (■). $^{45}Ca^{2+}$ release is expressed as a percentage of the maximum release (100%) in the absence of amentoflavone and caffeine at a Ca^{2+} concentration of 1 μM. Values are means ± S.E.M. ($n = 3$). Statistically significant difference from the control $^{*}P < 0.05$, $^{**}P < 0.01$.

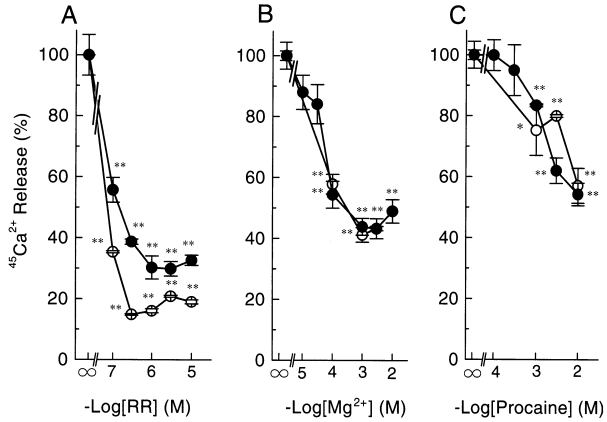

Fig. 4. Effects of inhibitors of Ca^{2+}-induced Ca^{2+} release channels such as RR (A), Mg^{2+} (B) and procaine (C) on amentoflavone- and caffeine-induced $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum. Data are expressed as the difference between $^{45}Ca^{2+}$ release in the presence and in the absence of amentoflavone and caffeine. Values are means ± S.E.M. ($n = 3$). Caffeine (○); amentoflavone (●). Statistically significant difference from the control (100%) $^{*}P < 0.05$, $^{**}P < 0.01$.

lease plotted against the external Ca^{2+} concentration was shifted to the left in a parallel manner by amentoflavone and caffeine. After treatment with amentoflavone (30 μM) and caffeine (1 mM), the EC_{50} value of Ca^{2+} was decreased to 21 and 53 nM, respectively. The effects of typical inhibitors of Ca^{2+}-induced Ca^{2+} release channels on amentoflavone- and caffeine-induced $^{45}Ca^{2+}$ release are shown in Fig. 4. $^{45}Ca^{2+}$ release induced by 100 μM amentoflavone and 1 mM caffeine was inhibited by ruthenium red (RR) (Fig. 4A), Mg^{2+} (Fig. 4B) and procaine (Fig. 4C) in a concentration-dependent manner. The interrelations among the Ca^{2+}-releasing activities of amentoflavone, caffeine and adenosine-5′-(β,γ-methylene) triphosphate (AMP-PCP) were examined by measuring $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum. AMP-PCP was used as an unhydrolyzable adenine nucleotide analogue. Amentoflavone caused a maximum increase in $^{45}Ca^{2+}$ release at 300 μM (Fig. 3A). The additional application of AMP-PCP (300 μM) further increased the maximum $^{45}Ca^{2+}$ release induced by 300 μM amentoflavone (data not shown), indicating that the Ca^{2+}-releasing effects of amentoflavone and AMP-PCP were additive. In contrast, 30 mM caffeine did not alter the maximum response to 300 μM amentoflavone (data not shown), suggesting that amentoflavone and caffeine share the same binding site in the heavy fraction of fragmented sarcoplasmic reticulum vesicles.

3.3. [^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum

[^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum was examined in the presence or absence of amentoflavone. Amentoflavone increased [^{3}H]ryanodine binding by approximately 40% in a concentration-dependent manner (data not shown). The

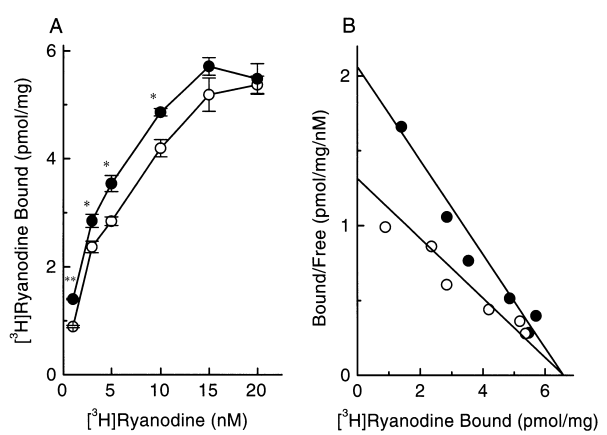

Fig. 5. A typical saturation curve (A) and its corresponding Scatchard (B) plot of [^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum in the presence or absence of 100 μM amentoflavone. The heavy fraction of fragmented sarcoplasmic reticulum (100 μg/ml) was incubated with 1–20 nM [^{3}H]ryanodine for 2 h at 37°C in the presence or absence of 100 μM amentoflavone. Control (○); 100 μM amentoflavone (●). Values are means ± S.E.M. ($n = 3$). Statistically significant difference from the control $^{*}P < 0.05$, $^{**}P < 0.01$.

degree of enhancement by amentoflavone was comparable to that of caffeine (by 25%) and MBED (by 20%) (Seino et al., 1991). The amentoflavone (30 and 300 μM)-induced increase in [^{3}H]ryanodine binding was further enhanced by AMP-PCP (100 μM) by 10% and 30%, respectively (data not shown). Fig. 5 shows the saturation curve (A) and the corresponding Scatchard (B) of [^{3}H]ryanodine binding in the presence or absence of amentoflavone. Amentoflavone decreased the K_D value from 5.26 to 3.34 without affecting the B_{max} value.

4. Discussion

The ryanodine receptor, which is known as a Ca^{2+}-induced Ca^{2+} release channel, may be the machinery of excitation–contraction coupling in skeletal muscle (Ford and Podolsky, 1970; Endo et al., 1970; Endo, 1977). Ryanodine was reported to selectively bind to its receptor in an open state (McPherson and Campbell, 1993). The Ca^{2+} channel has been purified using [^{3}H]ryanodine as a specific ligand (Inui et al., 1987; Hymel et al., 1988; Wagenknecht et al., 1989). Not only ryanodine but also a variety of natural products such as imperatoxin (Valdivia et al., 1992) and MBED (Seino et al., 1991) have attracted the attention of pharmacologists, physiologists and biochemists, because they act on their specific binding sites in the ryanodine receptor with high affinity.

In our present experiments, amentoflavone and caffeine induced a rapid release of Ca^{2+} from the heavy fraction of fragmented sarcoplasmic reticulum followed by the re-uptake of Ca^{2+}. Thus, the Ca^{2+} released from the heavy fraction of fragmented sarcoplasmic reticulum can be taken up by a Ca^{2+} pump using an ATP-regenerating system even in the presence of amentoflavone and caffeine. The reason for the transient nature of Ca^{2+} release is not clear, but a possible explanation is that the activated channels are closed by a negative feedback mechanism or a spontaneous closing mechanism (Kim et al., 1983) and that released Ca^{2+} is rapidly re-incorporated via the Ca^{2+} pump into the heavy fraction of fragmented sarcoplasmic reticulum vesicles. The Ca^{2+}-mobilizing effect of amentoflavone was inhibited by typical blockers of Ca^{2+}-induced Ca^{2+} release channels such as Mg^{2+} and ruthenium red. Treatment with a high concentration of ryanodine blocked the Ca^{2+} release induced by amentoflavone. Both amentoflavone and caffeine caused an increase in $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum under conditions in which the Ca^{2+} pump did not work. The potency of the $^{45}Ca^{2+}$-releasing action of amentoflavone was greater than that of caffeine. The Ca^{2+} dependence of amentoflavone-induced $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum had a bell-shaped profile similar to that of caffeine. The $^{45}Ca^{2+}$ release induced by amentoflavone and caffeine was significantly inhibited by typical blockers of Ca^{2+}-induced Ca^{2+} release channels including procaine. These results suggest that amentoflavone, like caffeine, induced Ca^{2+} release by affecting Ca^{2+}-induced Ca^{2+} release channels in the heavy fraction of fragmented sarcoplasmic reticulum.

The Ca^{2+}-induced Ca^{2+} release channel is modulated by a variety of agents such as Mg^{2+}, caffeine, adenine nucleotides, local anesthetics and ryanodine. Four functional domains comprise the Ca^{2+}-ryanodine receptor complex: (1) a regulatory domain responsible for gating the Ca^{2+} channel which binds Ca^{2+} (and Mg^{2+}) and contains critical sulfhydryl moieties; (2) an alkaloid binding domain in close proximity to the channel which binds ryanodine only in its Ca^{2+}-activated state and rapidly closes upon complex formation; (3) a domain which binds caffeine and directly influences the sensitivity of the Ca^{2+} regulatory site to Ca^{2+}; and (4) a domain which binds adenine nucleotides and influences the gating efficiency or lifetime of the open channel (Endo et al., 1981; Martonosi, 1984; Pessah et al., 1987). The maximum $^{45}Ca^{2+}$ release from the heavy fraction of fragmented sarcoplasmic reticulum induced by caffeine was increased further by AMP-PCP (Seino et al., 1991). In the present experiments, the interrelation between the stimulatory effects of amentoflavone and each channel effector was examined. The maximum $^{45}Ca^{2+}$ release in response to amentoflavone was further increased by AMP-PCP, whereas it was not changed by caffeine. These observations suggest that the stimulatory action of amentoflavone on the heavy fraction of fragmented sarcoplasmic reticulum may be mediated by an interaction of amentoflavone with the binding site of caffeine in the channel protein whereas there is no such interaction with that of AMP-PCP.

[^{3}H]ryanodine binding is potentiated by channel activators, such as caffeine and adenine nucleotides but is decreased by channel inhibitors (Su and Chang, 1995; Ohkura et al., 1996). [^{3}H]ryanodine binding experiments are useful for studying the functional state of the channel (Coronado et al., 1994). Amentoflavone, like caffeine, markedly enhanced [^{3}H]ryanodine binding to the heavy fraction of fragmented sarcoplasmic reticulum. Scatchard analysis of [^{3}H]ryanodine binding revealed that amentoflavone decreased the K_D without affecting the B_{max}. These results suggest that amentoflavone associates with the ryanodine receptor to facilitate channel opening.

Generally, potent Ca^{2+} releasers possess at least one nitrogen atom in their structure, which suggests that this atom is essential for the Ca^{2+}-releasing activity in the heavy fraction of fragmented sarcoplasmic reticulum. We have succeeded in finding amentoflavone, a novel compound with Ca^{2+}-releasing activity which does not contain a nitrogen atom.

In conclusion, these results suggest that the pharmacological properties of amentoflavone are indistinguishable from those of caffeine, except for its 30-fold higher potency. This compound may serve as a useful pharmaco-

logical tool for clarifying the regulatory mechanism of Ca^{2+}-induced Ca^{2+} release in the heavy fraction of fragmented skeletal muscle sarcoplasmic reticulum.

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