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Short communication

# Structure–activity relationship of gramine derivatives in $Ca^{2+}$ release from sarcoplasmic reticulum

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**Abstract**

5,6-Dibromo-1,2-dimethylgramine evoked $Ca^{2+}$ release from skeletal muscle sarcoplasmic reticulum through ryanodine receptors in a concentration-dependent manner with an $EC_{50}$ of 22.2 μM. Since the $EC_{50}$ of caffeine was 0.885 mM, 5,6-dibromo-1,2-dimethylgramine was 40 times more sensitive than caffeine. Among 14 gramine derivatives having different substituents at N-1, C-2, C-5 or C-6 of the indole skeleton, we found that five derivatives were effective. Study of the structure–activity relationship for $Ca^{2+}$ release indicated that 1-methylation and/or both 5- and 6-bromination are important for $Ca^{2+}$ release. Thus, gramine derivatives are useful tools for the investigation of $Ca^{2+}$ release from sarcoplasmic reticulum. 

*Keywords:* $Ca^{2+}$ release; Sarcoplasmic reticulum; Ryanodine receptor; Gramine derivatives; Caffeine

## 1. Introduction

$Ca^{2+}$ has an important role as an intracellular second messenger in diverse cellular functions (Berridge, 1993). In excitation–contraction coupling of skeletal muscle, depolarization of the transverse tubule leads to $Ca^{2+}$ release from the sarcoplasmic reticulum, resulting in muscle contraction (Schneider and Chandler, 1973; Rios and Burm, 1987; Numa et al. 1990). The ryanodine receptor in sarcoplasmic reticulum is responsible for $Ca^{2+}$ release from the $Ca^{2+}$ store as it is a $Ca^{2+}$ release channel (Block et al., 1988; McPherson and Campbell, 1993; Coronado et al., 1994). It is well known that caffeine or adenine nucleotide activates the ryanodine receptor. Although some accessory proteins have been shown to modulate the channel activity of the ryanodine receptor (Mackrill, 1999), the molecular mechanisms involved in the regulation of the channel are still to be resolved. Caffeine is a typical $Ca^{2+}$ releaser acting on the ryanodine receptor, but relatively high concentrations are necessary to cause $Ca^{2+}$ release. Thus, another $Ca^{2+}$ releaser in addition to caffeine will be extremely useful to characterize ryanodine receptor function.

It has been shown that 2,5,6-tribromo-1-methylgramine is the most potent anti-fouling agent against barnacles (Kon-ya et al., 1994). In order to characterize the nature of the compound, we examined the effects of the related gramine derivatives on animal tissues or cells. In the course of the study, we found that the gramine derivatives released $Ca^{2+}$ from skeletal muscle sarcoplasmic reticulum. Thus, the present study dealt with the structure–activity relationship of gramine derivatives for causing $Ca^{2+}$ release from skeletal muscle sarcoplasmic reticulum.

## 2. Materials and methods

### 2.1. Materials

Chemicals were obtained from the following source: ryanodine was purchased from S.B. Penick (New York, NY), and aprotinin and benzamidine hydrochloride were from Sigma (St. Louis, MO). [$^3$H]Ryanodine (2.62 kBq/pmol) was from DuPont/New England Nuclear (Boston, MA). Gramine derivatives were supplied by Marine

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Biotechnology Institute (Shimizu, Japan). All other chemicals were purchased from Wako (Osaka, Japan).

### 2.2. Preparation of sarcoplasmic reticulum vesicles

Sarcoplasmic reticulum vesicles were prepared from rabbit skeletal muscle, according to the method described by Kim et al. (1983) in the presence of protease inhibitors: aprotinin (76.8 μM) and benzamidine (0.83 mM). Rabbit white muscle was homogenized in five volumes of 5 mM Tris–maleate (pH 7.0) and centrifuged at $5000 \times g$ for 15 min. The supernatant was centrifuged at $12{,}000 \times g$ for 30 min. The pellet was suspended in 5 mM Tris–maleate (pH 7.0) containing 90 mM KCl and centrifuged at $70{,}000 \times g$ for 40 min. The pellet (sarcoplasmic reticulum) was resuspended and stored at −80°C until use. The protein concentration of sarcoplasmic reticulum was determined by the method previously described (Bradford, 1976) using bovine serum albumin as a standard.

### 2.3. Measurement of $Ca^{2+}$ concentration using $Ca^{2+}$ electrode

The concentration of extravesicular $Ca^{2+}$ in the sarcoplasmic reticulum suspension was measured at 30°C with a $Ca^{2+}$ electrode as described previously (Seino et al., 1991). The $Ca^{2+}$ electrode showed a Nernstian response (slope, 27–29 mV/pCa unit) in the calibration buffer containing $Ca^{2+}$-EGTA. The assay solution (final volume, 1 ml) contained 100 μM $CaCl_2$, 90 mM KCl, 0.5 mM $MgCl_2$, 50 mM 3-(*N*-morpholino)propanesulfonic acid (MOPS)–Tris (pH 7.0), 0.75 mg/ml of sarcoplasmic reticulum, 10 mM creatine phosphate, 0.1 mg/ml creatine kinase and 0.5 mM ATP. The $Ca^{2+}$ release activity of a gramine derivative was estimated from the peak $Ca^{2+}$ concentrations after the addition of drug. Caffeine was used as a positive control to evoke $Ca^{2+}$ release.

### 2.4. [$^3$H]Ryanodine binding

The sarcoplasmic reticulum vesicles (0.2 mg/ml) were incubated with 1.0 nM [$^3$H]ryanodine for 60 min in a binding buffer of 0.3 M sucrose, 0.35 M KCl, 100 μM $CaCl_2$, 20 mM Tris–HCl, pH 7.4. The binding was measured by filtering aliquots of the sample through Whatman GF/B filters. The non-specific binding was determined in the presence of 10 μM ryanodine.

## 3. Results

The $Ca^{2+}$-releasing activities of gramine derivatives were compared with that of caffeine, using a $Ca^{2+}$ electrode. 5,6-Dibromo-1,2-dimethylgramine (GD[N]) as well as caffeine caused a transient $Ca^{2+}$ release from sarcoplasmic reticulum (Fig. 1A). This response was potently inhibited by pretreatment with ryanodine or ruthenium red. GD[N] increased [$^3$H]ryanodine binding to a similar extent to caffeine, indicating the mediation of ryanodine receptor in the $Ca^{2+}$ release. The $Ca^{2+}$-releasing activities of 14 gramine derivatives were examined using a $Ca^{2+}$ electrode and are summarized in Fig. 2 together with their chemical structures. Derivatives GD[F], GD[H], GD[L], GD[M] and GD[N] evoked $Ca^{2+}$ release from sarcoplasmic reticulum in a concentration-dependent manner (Fig. 1C) with $EC_{50}$ values of 65.7, 39.8, 70.8, 20.0 and 22.2 μM, respectively (Fig. 2B). These active derivatives were much more potent than caffeine, as determined by comparing their $EC_{50}$ values. GD[M] showed a relatively small maximum response (intrinsic activity), compared with that of caffeine (Fig. 2B). The active derivatives have common substituents, bromines, at C-5 and C-6 of the indole skeleton. GD[K] or GD[L], in which the methyl group at N-1 was eliminated from the structure of GD[M] or GD[N], evoked

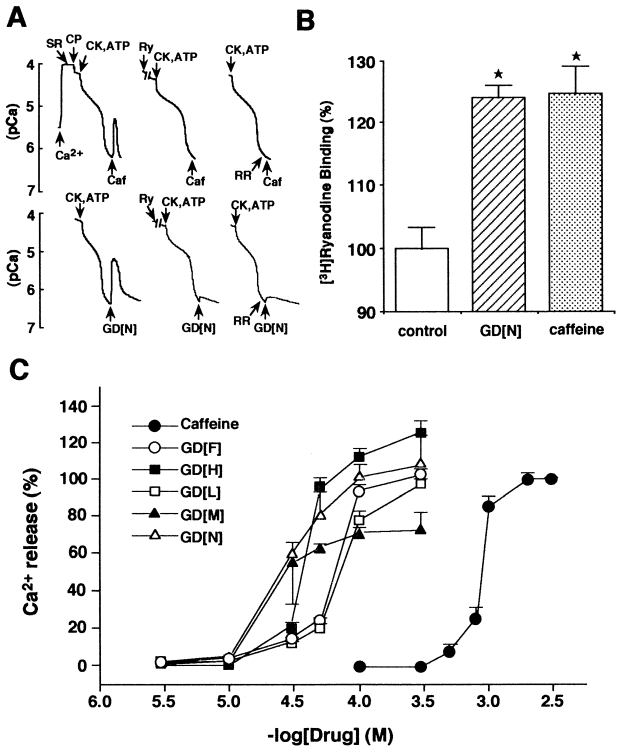
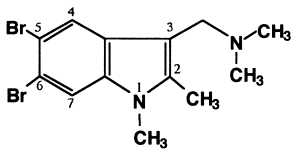
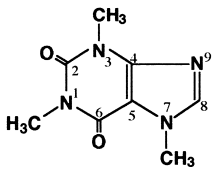



Fig. 1. Effects of gramine derivatives on $Ca^{2+}$ release and [$^3$H]ryanodine binding in rabbit sarcoplasmic reticulum. (A) Effects of ryanodine (Ry, 10 μM) and ruthenium red (RR, 2 μM) on $Ca^{2+}$ release by 5,6-dibromo-1,2-dimethylgramine (GD[N], 30 μM) and caffeine (Caf, 1 mM) in $Ca^{2+}$ electrode experiments. Sarcoplasmic reticulum started $Ca^{2+}$ uptake after addition of creatine kinase (CK) and ATP after creatine phosphate (CP). (B) Effects of 5,6-dibromo-1,2-dimethylgramine (GD[N], 30 μM) and caffeine (1 mM) on [$^3$H]ryanodine binding. The results are expressed as % increase from basal binding. Each column represents the mean ± S.E.M. of three determinations. ★ Significant difference from control ($P < 0.05$). (C) Concentration–response curves for five active gramine derivatives and caffeine for causing $Ca^{2+}$ release in $Ca^{2+}$ electrode experiments. Results are shown as percentage of caffeine (2 mM)-induced $Ca^{2+}$ release. Each point represents the mean ± S.E.M. of three to eight determinations.

**A**



5,6-Dibromo-1,2-dimethylgramine (GD[N])



caffeine

**B**

| Gramine derivative | Position of indole 1 | 2 | 5 | 6 | $EC_{50}$ | Intrinsic activity |
|---|---|---|---|---|---|---|
| GD[A] | | | Br | | > 100 μM | – |
| GD[B] | | | | Br | > 100 μM | – |
| GD[C] | $CH_3$ | | Br | | > 100 μM | – |
| GD[D] | $CH_3$ | | | Br | > 100 μM | – |
| GD[E] | | Br | | Br | > 100 μM | – |
| GD[F] | | | Br | Br | 65.7 μM | 1.03 |
| GD[G] | $CH_3$ | Br | | Br | > 100 μM | – |
| GD[H] | $CH_3$ | | Br | Br | 39.8 μM | 1.13 |
| GD[I ] | $CH_3$ | $CH_3$ | Br | | > 100 μM | – |
| GD[J] | $CH_3$ | $CH_3$ | | Br | > 100 μM | – |
| GD[K] | | Br | Br | Br | > 100 μM | – |
| GD[L] | | $CH_3$ | Br | Br | 70.8 μM | 0.97 |
| GD[M] | $CH_3$ | Br | Br | Br | 20.0 μM | 0.71 |
| GD[N] | $CH_3$ | $CH_3$ | Br | Br | 22.2 μM | 1.01 |
| caffeine | – | – | – | – | 885 μM | 1.0 |

Fig. 2. Chemical structure and $Ca^{2+}$-releasing activity of gramine derivatives and caffeine. (A) Chemical structure of gramine derivatives and caffeine. (B) $EC_{50}$ values and intrinsic activities of 14 different gramine derivatives. Intrinsic activity was determined as the maximum response relative to that of caffeine (1.0).

no or slight $Ca^{2+}$ release (Fig. 2). Furthermore, GD[D] or GD[J], in which the bromine at C-5 was eliminated from the structure of GD[H] or GD[N], evoked no or only slight $Ca^{2+}$ release. Similarly, GD[C] or GD[I], in which the bromine at C-6 was eliminated from the structure of GD[H] or GD[N], evoked no $Ca^{2+}$ release. However, the $Ca^{2+}$-releasing activities were not changed by methylation or bromination at C-2 of the indole skeleton.

## 4. Discussion

In the present study, we show for the first time that some gramine derivatives cause $Ca^{2+}$ release from skeletal muscle sarcoplasmic reticulum through ryanodine receptors. Among 14 gramine derivatives having different substituents at N-1, C-2, C-5 or C-6 of the indole skeleton, we found five potent derivatives, which released $Ca^{2+}$ in lower concentrations than caffeine did.

Comparison of the $Ca^{2+}$-releasing activities of the pairs of derivatives of GD[K] and GD[M] or GD[L] and GD[N] indicated that the methyl group at N-1 of the indole skeleton is important for the ability of gramine derivatives to evoke $Ca^{2+}$ release. Furthermore, comparison of some pairs of derivatives such as GD[D] and GD[H], GD[J] and GD[N], GD[C] and GD[H] or GD[I] and GD[N] showed that bromination of both C-5 and C-6 of the indole skeleton is essential for $Ca^{2+}$ release. Since substitution in the chemical structure at C-2 resulted in a small change in $Ca^{2+}$-releasing activity, it seems likely that there is no or little relationship between the substituent at C-2 and $Ca^{2+}$-releasing activity.

There are several studies concerning the relationships between chemical structure and the $Ca^{2+}$-releasing activity of drugs that affect the function of the ryanodine receptor. Rousseau et al. (1988) have reported the structure–activity relationship for caffeine, a $Ca^{2+}$ releaser that acts through the ryanodine receptor. They showed that the methyl groups at N-1, N-3 and N-7 were essential for its $Ca^{2+}$-releasing activity. The methyl group at N-1 of gramine derivatives may correspond with that at N-7 of caffeine. Kobayashi et al. (1989) reported that modulation of the chemical structure of eudistomin derivatives having a carboline skeleton resulted in a change in their $Ca^{2+}$-releasing activity. Further study demonstrated that 9-methyl-7-bromoeudistomin D was the most potent $Ca^{2+}$ releaser (Seino et al., 1991), and 4,6-dibromo-3-hydroxycarbazole was a potent inhibitor of $Ca^{2+}$-induced $Ca^{2+}$ release from sarcoplasmic reticulum (Takahashi et al., 1995). Thus, the present study together with the above-mentioned studies about the structure–activity relationship provides information about the underlying molecular site of action of the $Ca^{2+}$-releasing drug.

In conclusion, we found that 5,6-dibromo-1,2-dimethylgramine and its related gramine derivatives cause $Ca^{2+}$ release from the sarcoplasmic reticulum through the ryanodine receptor. Study of the structure–activity relationship of gramine derivatives revealed that bromination both at C-5 and C-6, and methylation at N-1 of the indole structure are important for activity. These gramine derivatives are useful pharmacological tools for investigating the regulation of $Ca^{2+}$ release via the ryanodine receptor.

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