Bromo-eudistomin D, a Novel Inducer of Calcium Release from Fragmented Sarcoplasmic Reticulum That Causes Contractions of Skinned Muscle Fibers*

Yoichi Nakamura†, Jun'ichi Kobayashi, Jeremy Gilmore‡, Mark Mascal§, Kenneth L. Rinehart, Jr.∥, Hideshi Nakamura, and Yasushi Ohizumi¶

From the Mitsubishi-Kasei Institute of Life Sciences, Minamiooya, Machida, Tokyo 194, Japan and §Roger Adams Laboratory, University of Illinois at Urbana-Champaign, Urbana, Illinois 61801

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Bromo-eudistomin D induced a contraction of the chemically skinned fibers from skeletal muscle at concentrations of 10 μM or more. This contractile response to bromo-eudistomin D was completely blocked by 10 mM procaine. The extravascular Ca2+ concentrations to bromo-eudistomin D was completely blocked by 10 mM procaine. However, these are not satisfactory drugs for use in the identification or the purification of the molecules operating in Ca2+ release, because of their low affinity. Ruthenium red blocks Ca2+ release at a concentration as low as 0.1 μM (6). However, a labeled derivative of this compound is unavailable.

Bromo-eudistomin D is a monobrominated derivative of eudistomin D, a marine natural product with antiviral activity which has been isolated from the Caribbean tunicate Elasmostoma ollaceum (7). In the present paper, we show that bromo-eudistomin D induces a contraction of chemically skinned fibers and a release of Ca2+ from a heavy fraction of the fragmented SR (HSR) at a concentration as low as 1 μM.

Bromo-eudistomin D might become a powerful tool for investigation of the mechanism of Ca2+ release from the SR.

EXPERIMENTAL PROCEDURES

Materials—Bromo-eudistomin D was synthesized by bromination of 6-methoxy-7H-pyrido[3,4-b]indole (8), with bromine in acetic acid, followed by demethylation with BB₃. The structure of bromo-eudistomin D was confirmed by 1H and 13C nuclear magnetic resonance and fast atom bombardment mass spectra as shown in Fig. 1. 6-Hydroxy-β-carboline was prepared by demethylation of 6-methoxy-7H-pyrido[3,4-b]indole described above. β-Carboline was synthesized from tryptamine and glyoxylic acid. The details of the synthetic procedures will be reported elsewhere. Eudistomins A, D, G-J, M-O, and Q were isolated from extracts of E. ollaceum (7). These compounds were dissolved in ethanol or dimethyl sulfoxide prior to application.

The HSR was prepared by the method of Kim et al. (9). The homogenate of rabbit white muscle in 5 volumes of 5 mM Tris-maleate (pH 7.0) was fractionated by differential centrifugations, in which vesicles remaining in the supernatant after centrifugation at 5,000 × g for 5 min were pelleted at 12,000 × g for 30 min. The fraction was washed with buffer A containing 0.1 M KCl and 5 mM Tris-maleate at 4 °C. The purified Ca2+-ATPase of SR (10), myosin (11), and creatine kinase (12) were also prepared from rabbit skeletal muscle. Na+K+-ATPase from porcine cerebral cortex was purchased from Sigma.

CaCl₂ was purchased from New England Nuclear, procaine HCl from Sigma, caffeine and ruthenium red from Wako Pure Chemical Industries, and saponin from ICN Pharmaceuticals.

Tension Measurement of Skinned Fibers—The isometric tension of chemically skinned fibers isolated from psoas muscle of guinea pigs was measured by the method of Endo and Kitaizawa (13). A small bundle (~0.1 mm in diameter, ~3 mm in length) of 4-6 fibers was set

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† Present address: Laboratory of Clinical Investigation, Miyazaki Medical College Hospital, Kiyotake, Miyazaki 889-16, Japan.

‡ To whom reprint requests should be addressed: Mitsubishi-Kasei Institute of Life Sciences, 11 Minamiooya, Machida-shi, Tokyo 194, Japan.

§ The abbreviations used are: SR, sarcoplasmic reticulum; HSR, heavy fraction of fragmented SR; EGTA, ethylene bis(oxyethyl)enitrilo)tetraacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid.
on the needles of a strain gauge (T7-8-240, Toyo Baldwin, Japan). The bundle was treated with 50 μg/ml saponin in a relaxation solution containing 2 mM EGTA, 4 mM ATP, 90 mM KMeSO₄, 5 mM MgSO₄, and 50 mM MOPS (pH 7.0) for 30 min in a 5-ml Petri dish with a magnetic stirrer at 20 °C. After flushing out saponin with the relaxation solution, 2 mM CaCl₂ was added (~14 μM free Ca²⁺) to check the tension development (usually 30–60 mg). The medium was changed to the relaxation solution and then to a solution containing the same reagents as the relaxation solution without EGTA. After 15 to 30 min, spontaneous contractions occurred repeatedly at constant intervals of 4 to 5 min.

**Measurements by Ca²⁺ Electrode**—The extravesicular Ca²⁺ concentration was monitored with a Ca²⁺ electrode prepared by the method of Tsien and Rink (14) with modifications. We used Pipetman polyethylene tips (C20, Gilson), instead of siliconized micro-glass pipettes. A small amount of the Ca²⁺ sensor, a mixture of 5 μl of Ca²⁺-cocktail (Fluka 21048) and 0.7 mg of polyvinyl chloride in 35 μl of tetrahydrofuran, was attached at the tip (~0.3 mm in diameter) of the polyethylene tips that had been filled with an internal solution containing 5 mM CaCl₂, 5 mM EGTA, 5 mM MgCl₂, 0.1 M KCl, and 50 mM MOPS (pH 7.0). The reference electrode of a glass pipette was filled with 1.5% agar in the internal solution without CaCl₂ and EGTA. The electrodes were connected to a high impedance pH meter (Schott CG-822) with platinum terminals and dipped in a 1-ml sample kept in a thermostated bath with a magnetic stirrer. This Ca²⁺ electrode showed Nernstian response (slope: 27–29 mV/pCa unit) in the calibration solutions containing Ca²⁺-EGTA between pH 4.5 and 7.0.

**RESULTS AND DISCUSSION**

The sarcoplasmic reticulum (SR) in skinned muscle fibers is known to be morphologically intact although connections between the transverse tubular systems and SR are disrupted. Endo et al. (4) proposed the "Ca-induced Ca release" hypothesis from the finding that skinned fibers showed spontaneous and oscillatory contractions in solutions containing submicromolar Ca²⁺, which was attained with low concentration of EGTA. The contraction of skinned fibers is an adequate system to survey the effects of various drugs on Ca²⁺ release from the SR, possibly occurring through Ca²⁺ channels in SR membranes.

The skinned fibers showed spontaneous contractions repeatedly at constant intervals of 4 to 5 min (Fig. 2). The addition of 10 μM bromo-eudistomin D just after relaxation induced a tension spike of the same or larger size as the spontaneous contractions. This contraction was followed by frequent and disordered contractions. Similar contractions were observed by the addition of 0.4 mM caffeine. In the presence of 10 mM procaine, tension did not develop following the addition of 20 μM bromo-eudistomin D or 1 mM caffeine (data not shown). These facts suggest that bromo-eudistomin D activates Ca²⁺ channels in a manner similar to caffeine which is known to be an inducer of Ca²⁺ release from the SR.

We examined the effect of bromo-eudistomin D on SR by monitoring directly extravesicular concentrations of Ca²⁺ of HSR with a Ca²⁺ electrode (Fig. 3). The reaction medium contained 1.75 mg/ml HSR, 50 μM CaCl₂ (added), ATP-regenerating system, i.e. 10 mM creatine phosphate and 0.1 mg/ml creatine kinase, and 0.4 mM (α-ε) or 4 mM (d and ε) MgCl₂ (see "Experimental Procedures" for details). The semilogarithmic graph (Fig. 3) demonstrates that, upon the addition of 0.5 mM ATP, free Ca²⁺ concentrations decreased rapidly due to the formation of Ca-ATP complexes and further decreased gradually due to Ca²⁺ uptake by the HSR. Thus Ca²⁺ uptake phase was almost linear in an antilogarithm plot (not shown). Ca²⁺ uptake was slowed when the concentration of Ca²⁺ was reduced to submicromolar levels. The addition of 10 μM bromo-eudistomin D to the Ca²⁺-filled HSR induced a rapid Ca²⁺ release followed by a Ca²⁺ reuptake. The rate of Ca²⁺ reuptake was almost the same as that before the addition of bromo-eudistomin D. The transient Ca²⁺ release was observed repeatedly upon addition of 10 μM bromo-eudistomin D. A similar Ca²⁺ release was induced by the addition of 2 mM caffeine (Fig. 3b). Ca²⁺ release by 20 μM bromo-eudistomin D was completely blocked after the addition of 2 μM ruthenium red (Fig. 3c). In the presence of 4 mM MgCl₂, the Ca²⁺ uptake rate increased significantly but no Ca²⁺ release was induced by 20 μM bromo-eudistomin D or 4 mM caffeine (Fig. 3, d and e). These results suggest that bromo-eudistomin...
**Fig. 3.** Ca\(^{2+}\) release induced by bromo-eudistomin D from HSR loaded with Ca\(^{2+}\) by the ATP-dependent Ca\(^{2+}\) pump. The extravesicular concentration of Ca\(^{2+}\) was monitored with a Ca\(^{2+}\) electrode in the presence of 1.75 mg/ml HSR, 0.5 mM ATP, 50 \(\mu\)M CaCl\(_2\) (added), 0.4 mM (a–c), or 4 mM (d and e) MgCl\(_2\), 90 mM KCl, 10 mM creatine phosphate (CP), 0.1 mg/ml creatine kinase (CK), and 42 mM MOPS at pH 7.0 and 30 °C. At the beginning of each experiment 10 \(\mu\)M CaCl\(_2\) was added stepwise 5 times as the internal standard (see “Experimental Procedures”). In b–e the traces are those only after ATP addition. At the time shown by arrows, 10 \(\mu\)M bromo-eudistomin D (a), 2 mM caffeine (Caff) (b), 2 \(\mu\)M ruthenium red (RR), and 20 \(\mu\)M bromo-eudistomin D (c), 20 \(\mu\)M bromo-eudistomin D (d), and 4 mM caffeine (e) were added. BED, bromo-eudistomin D.

**Fig. 4.** Enhancement of \(^{45}\)Ca\(^{2+}\) efflux by bromo-eudistomin D from HSR passively loaded with \(^{45}\)Ca\(^{2+}\). The time course of the decrease in the \(^{45}\)Ca content in the vesicles was measured after 100-fold dilution of 20 mg/ml HSR preloaded with 5 mM \(^{45}\)CaCl\(_2\) into a medium containing various concentrations of bromo-eudistomin D with 1% ethanol (A) or caffeine (B), and 0.4 mM CaCl\(_2\), 1.9 mM EGTA, 90 mM KCl, and 50 mM MOPS at pH 7.0 and 0 °C. The concentrations of bromo-eudistomin D added in A were 0 (C), 0.3 (O), 1 (A), 3 (A), 10 (E), and 30 (B) \(\mu\)M and caffeine in B were 0 (C), 0.1 (O), 0.3 (A), 1 (A), 3 (C), and 10 (B) mM. BED, bromo-eudistomin D.

D induces Ca\(^{2+}\) release through specific Ca\(^{2+}\) channels, which are blocked by ruthenium red and high concentrations of Mg\(^{2+}\). It is also implied that bromo-eudistomin D-sensitive channels are the same as or similar to channels activated by caffeine, although the affinity of the channels for bromo-eudistomin D is much higher than that for caffeine.

The mechanism which is responsible for the reuptake of Ca\(^{2+}\) after its release is not clear. However, two possibilities are present: 1) the activated channels close after a certain period and the released Ca\(^{2+}\) is reincorporated into vesicles or 2) the activated channels are kept open and the released Ca\(^{2+}\) is taken up into vesicles which have no Ca\(^{2+}\) channels. Ca\(^{2+}\) release from the SR was induced by the addition of A-23187, a Ca\(^{2+}\) ionophore, whereas Ca\(^{2+}\) reuptake was not observed (data not shown). The presence of the Ca\(^{2+}\) reuptake phase, thus, excludes the possibility that bromo-eudistomin D acts on the vesicles as a Ca\(^{2+}\) ionophore.

We measured the concentration-dependent effect of bromo-eudistomin D and caffeine on Ca\(^{2+}\) efflux from HSR under the conditions in which the Ca\(^{2+}\) pump did not work. The suspension of HSR preloaded passively with 5 mM \(^{45}\)CaCl\(_2\) was transferred into 100 volumes of a reaction medium containing various concentrations of bromo-eudistomin D or caffeine, 0.4 mM CaCl\(_2\), and 1.9 mM EGTA at pH 7.0 and 0 °C. The amount of \(^{45}\)Ca remaining in the vesicles decreased and reached a constant basal level of 9.5 ± 1.0 nmol/mg 20 min after the dilution irrespective of drug concentrations. The time courses of the change in \(^{45}\)Ca content of the HSR were plotted logarithmically and the plots were linear as shown in Fig. 4. The rate of Ca\(^{2+}\) efflux was markedly accelerated from 0.11 to 0.75 min\(^{-1}\) by 10 \(\mu\)M bromo-eudistomin D. Caffeine also increased the rate of Ca\(^{2+}\) efflux, but required much higher concentrations in the range of several hundred micromolar. In the absence of drugs, the rate of Ca\(^{2+}\) efflux was slightly higher in A than in B, indicating that 1% ethanol (in A) raised the efflux rate. The effect of ethanol was reported by Ohnishi et al. (17). Hill plots (not shown) of the apparent rate constants, which were determined from the slopes in Fig. 4, showed \(n_h = 1.9\) and \(K = 1.5\) \(\mu\)M for bromo-eudistomin D and \(n_h = 1.9\) and \(K = 0.62\) mM for caffeine. \(^{45}\)Ca\(^{2+}\) efflux, accelerated by 3 \(\mu\)M bromo-eudistomin D, was completely blocked in the presence of 2 \(\mu\)M ruthenium red or 0.5 mM MgCl\(_2\) (data not shown).

We also examined the effect on Ca\(^{2+}\) release of 12 compounds related to bromo-eudistomin D, eudistomin A, D, G–J, M–O, and Q (7) as well as \(ß\)-carboline and 6-hydroxy-\(ß\)-carboline using a Ca\(^{2+}\) electrode. Among them, only three compounds, eudistomin A, D, and J, stimulated Ca\(^{2+}\) release...
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from the HSR, although severalfold higher concentrations than bromo-eudistomin D were required (data not shown). These results suggest that 5-Br, 6-OH, and 7-Br in the benzenoid ring are important for the activity.

In addition, the effects of bromo-eudistomin D were examined on other enzymatic reactions which play important roles in muscle contraction mechanisms. Bromo-eudistomin D (up to 30 \(\mu\)M) had no effect on the myosin ATPase activity in the presence of 5 mM EDTA and 0.5 mM KCl. It also had no effect on the activity of the Ca\(^{2+}\)-ATPase purified from the SR or on the Na\(^+\),K\(^+\)-ATPase.

The present study thus indicates that a low concentration of bromo-eudistomin D induces contractions of skinned muscle fibers by triggering Ca\(^{2+}\) release from intact SR through Ca\(^{2+}\) channels without affecting other muscular enzymes. Isolation of BED-sensitive components in HSR by using bromo-eudistomin D as the reporter group is currently in progress in our laboratory.

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