Eudistomin D and Penaresin Derivatives as Modulators of Ryanodine Receptor Channels and Sarcoplasmic Reticulum Ca^{2+} ATPase in Striated Muscle[§]

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ABSTRACT

Eudistomin D (EuD) and penaresin (Pen) derivatives are bioactive alkaloids from marine sponges found to induce Ca^{2+} release from striated muscle sarcoplasmic reticulum (SR). Although these alkaloids are believed to affect ryanodine receptor (RyR) gating in a “caffeine-like” manner, no single-channel study confirmed this assumption. Here, EuD and MBED (9-methyl-7-bromoeudistomin D) were contrasted against caffeine on their ability to modulate the SR Ca^{2+} loading/leak from cardiac and skeletal muscle SR microsomes as well as the function of RyRs in planar bilayers. The effects of these alkaloids on [^3H]ryanodine binding and SR Ca^{2+} ATPase (SERCA) activity were also tested. MBED (1–5 μM) fully mimicked maximal activating effects of caffeine (20 mM) on SR Ca^{2+} leak. At the single-channel level, MBED mimicked the

agonistic action of caffeine on cardiac RyR gating (i.e., stabilized long openings characteristic of “high-open-probability” mode). EuD was a partial agonist at the maximal doses tested. The tested Pen derivatives displayed mild to no agonism on RyRs, SR Ca^{2+} leak, or [^3H]ryanodine binding studies. Unlike caffeine, EuD and some Pen derivatives significantly inhibited SERCA at concentrations required to modulate RyRs. Instead, MBED’s affinity for RyRs ($\text{EC}_{50} \sim 0.5 \mu\text{M}$) was much larger than for SERCA ($\text{IC}_{50} > 285 \mu\text{M}$). In conclusion, MBED is a potent RyR agonist and, potentially, a better choice than caffeine for microsomal and cell studies due to its reported lack of effects on adenosine receptors and phosphodiesterases. As a high-affinity caffeine-like probe, MBED could also help identify the caffeine-binding site in RyRs.

Introduction

Ryanodine receptors [RyRs; skeletal muscle RyRs (RyR1) and cardiac RyRs (RyR2)]/ Ca^{2+} release channels are crucial for excitation-contraction coupling (Sitsapasan and Williams, 1998; Fill and Copello, 2002; Meissner, 2004; Fleischer, 2008; Lanner et al., 2010). After isolation and reconstitution in artificial bilayers, RyRs display complex single-channel kinetics, which include modal gating (Zahradnikova and Zahradnik, 1995; Rosales et al., 2004). RyRs also exhibit functional heterogeneity in their response to physiologic modulators (cytosolic Ca^{2+} , Mg^{2+} , and ATP). This heterogeneity results from the fact that some channels can dwell more often in high-open-probability (P_o) mode, characterized by long openings and short closures,

while others display frequent low- P_o mode, distinguished by frequent and brief flickering openings (Copello et al., 1997, 2002). RyRs dwelling in the high- P_o mode were found to display increased sensitivity to physiologic agonists, lower sensitivity to blockers, and increased ability to associate with neighboring channels for coupled gating (Gyorke and Fill, 1993; Zahradnikova et al., 1999; Copello et al., 2002; Diaz-Sylvester et al., 2011; Porta et al., 2012). Thus, the domains in the RyR molecule involved in the stabilization of high- and low- P_o modes have a huge significance for the synchronous gating of the channels. Unfortunately, the topography of these domains remains largely unknown.

Of the many pharmacologic agents that modulate RyRs (Conley and Brammar, 1996; West and Williams, 2007), the diagnostic ligand caffeine particularly raised our interest due to its ability to target domains that stabilize RyRs in high- P_o mode (Rousseau and Meissner, 1989; Sitsapasan and Williams, 1990; Porta et al., 2011). However, caffeine and other xanthines are inadequate for binding studies or molecular labeling due to their relatively low affinity for RyRs (Liu and Meissner, 1997).

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ABBREVIATIONS: APIII, antipyrilazo III; BAPTA, 1,2-bis(o-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid; [Ca^{2+}]_{cyt}, cytosolic Ca^{2+} concentration; CPZ, cyclopiazonic acid; EuD, eudistomin D; MBED, 9-methyl-7-bromoeudistomin D; MPenM, *N*-methyl-penaresin methyl ester; MPenNO, *N*-methyl-penaresin nitro derivative; n_H , Hill coefficient; Pen, penaresin; PenCN, penaresin dicyano derivative; PenM, penaresin methylester; P_o , open probability; RyR, ryanodine receptor; RyR1, skeletal muscle ryanodine receptor; RyR2, cardiac ryanodine receptor; SERCA, sarcoplasmic reticulum Ca^{2+} ATPase; SR, sarcoplasmic reticulum; TC, terminal cisternae.

In addition, caffeine, as well as other xanthines, has other cellular targets, including adenosine receptors and phosphodiesterases, which can also affect intracellular Ca^{2+} signaling (Zahradnik and Palade, 1993; Francis et al., 2011; Müller and Jacobson, 2011; Riksen et al., 2011).

Among other bioactive agents isolated from marine sponges, the indole alkaloids bromoeudistomin D and penaresin (Pen) were found to induce Ca^{2+} release from skeletal muscle and heart sarcoplasmic reticulum (SR) (Nakamura et al., 1986; Kobayashi et al., 1990). Subsequently, MBED (9-methyl-7-bromoeudistomin D) was reported to increase the binding of ryanodine and to be an even more powerful Ca^{2+} releaser from cardiac and skeletal muscle SR (Seino et al., 1991; Seino-Umeda et al., 1998). However, no studies to date have examined the nature of the direct effects of penaresins and eudistomins (especially MBED) on RyRs at the single-channel level. Consequently, the goal of this study was to contrast various penaresin and eudistomin derivatives against caffeine in their ability to modulate Ca^{2+} loading and release, as well as the activity of the SR Ca^{2+} ATPase (SERCA) in SR microsomes. We also studied the direct effects of these agents on single-channel function of skeletal muscle and cardiac RyRs reconstituted into lipid bilayers. Our results suggest that only one of these compounds (MBED) would act as a highly potent and specific “caffeine-like” agonist of RyR channels. The other tested agents show either weaker agonism or complex effects, such as inhibition of SERCA.

Materials and Methods

Cardiac and Skeletal Muscle SR Microsomes. All procedures involving animals were designed to minimize pain and suffering and conformed to the guidelines of the National Institutes of Health. Southern Illinois University School of Medicine animal research procedures have Association for Assessment and Accreditation of Laboratory Animal Care accreditation and Public Health Service assurances numbers 000551 and A3209-01, respectively. The Laboratory Animal Care and Use Committee of Southern Illinois University School of Medicine reviewed and approved the protocols for animal use in our laboratory (196-05-021 and 196-11-010).

R2 and R4 fractions of skeletal muscle SR microsomes were isolated from predominantly fast-twitch skeletal muscle from adult New Zealand white rabbits, as previously described (Saito et al., 1984; Chu et al., 1988). The R4 fraction of SR is highly enriched in terminal cisternae (TC) of SR, which consists of the junctional face membrane, where RyR1 localizes; and the calcium pump membrane, where the Ca^{2+} ATPase protein (SERCA 1a) localizes (Saito et al., 1984; Fleischer, 2008). Thus, the R4 fractions contain both RyR1 and SERCA 1a, and, therefore, they were used to test RyR1 function in planar bilayers, as well as RyR1 and SERCA 1a function in microsome assays (leak and loading, respectively). The R2 fraction of SR is enriched in longitudinal tubules of SR, which contain a high density of SERCA 1a and essentially no RyR1 (Chu et al., 1988; Fleischer, 2008). The R2 microsomes were used to characterize SERCA 1a using the ATPase assay.

Enriched porcine cardiac SR microsomes were prepared following protocols developed for dog heart SR microsomes (Chamberlain et al., 1983). All preparations were stored in liquid nitrogen. Every month, SR microsomes were separated into aliquots to be used in experiments. Aliquots of 15 μl (bilayers) or 100 μl (loading/release/ATPase assays) at a concentration of 5–15 mg protein/ml in 5 mM imidazole-Cl and 290 mM sucrose (pH ~7) were quickly frozen and stored at -80°C . For experiments, aliquots were quickly thawed in water, kept on ice, and used within 5 hours.

Measurements of Ca^{2+} Loading/Leak by SR Microsomes. Ca^{2+} uptake by cardiac SR microsomes or R4 fractions of skeletal muscle TC

microsomes was measured with a spectrophotometer (Cory 50; Varian, Walnut Creek, CA) using the Ca^{2+} -sensitive dye antipyrilazo III (APIII), as previously described (Chamberlain et al., 1984a; Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). Briefly, Ca^{2+} uptake was initiated by adding 40 nM CaCl_2 to 40–100 μg SR membranes resuspended in 1 ml of buffer [in mM: KH_2PO_4 , 100; MgCl_2 , 5 (free Mg^{2+} , ~0.3 mM); ATP, 5; and APIII, 0.2; pH 7.0]. The rate of Ca^{2+} uptake by the skeletal muscle (R4 fractions) and cardiac microsomes was measured in the absence (control) or presence of the RyR blocker ruthenium red (5 μM), and the effect of penaresins and eudistomins (preincubated for 5 minutes) was measured as changes in the absorbance (710–790 nm) of APIII. Initial rate of uptake, in micromoles of Ca^{2+} per milligram of protein per minute, was estimated as previously described (Neumann et al., 2011). The rate of Ca^{2+} leak from skeletal muscle R4 fractions preloaded with Ca^{2+} (three pulses of 40 μM Ca^{2+}) was measured after addition of cyclopiazonic acid (CPZ; 20 μM), which inhibits SERCA, plus 1 μl dimethylsulfoxide (control) or penaresins/eudistomins in dimethylsulfoxide. Leak experiments were carried out in the absence or presence of ruthenium red (5 μM).

Measurements of ATPase Activity in SR Microsomes. The effects of penaresins and eudistomins (preincubated for 5 minutes) were studied at 50 μM Ca^{2+} and 300 nM Ca^{2+} to measure the effects of the drugs on SERCA ATPase activated at maximal (V_{max}) and half-maximal rate, respectively. The methods for measuring ATPase activity have been previously described (Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). Briefly, skeletal muscle SR fractions (10–40 μg) enriched in longitudinal tubules (R2 fractions) were incubated with buffer containing (in mM) KCl, 140; MgCl_2 , 5; HEPES, 5; phosphoenolpyruvate, 2; CaCl_2 , 0.3; and variable amounts of BAPTA [1,2-bis(*o*-aminophenoxy)ethane-*N,N,N',N'*-tetraacetic acid] for free Ca^{2+} levels of ~50 and 0.3 μM . The solution also contained pyruvate kinase (8.4 units/ml) and lactate dehydrogenase (12 units/ml). pH was adjusted to 7.0 with KOH. The reaction starts by adding ATP (1 mM), which is hydrolyzed to ADP by the ATPases. ADP is regenerated to ATP by reactions that consume one molecule of NADH (oxidation to NAD^+) per ATP hydrolyzed (Chu et al., 1988). The rate of ATP hydrolysis was estimated from the decrease in absorbance at 340 nm as previously described (Neumann and Copello, 2011; Neumann et al., 2011). In skeletal muscle R2 fractions, ~99% of the ATPase activity is inhibited by CPZ (a SERCA blocker). In heart microsomes, there are significant non-SERCA components of ATPase activity, which required addition of sodium azide (1 mM) and ouabain (100 μM) for inhibition. In the presence of these agents, 70–80% of the total ATPase activity is inhibited by CPZ.

Measurements of [^3H]Ryanodine Binding in SR Microsomes. [^3H]Ryanodine binding was measured under control conditions and in the presence of caffeine or alkaloids (eudistomin and penaresin derivatives) following protocols previously described (Barg et al., 1997; Neumann and Copello, 2011) with minor modifications. Briefly, aliquots of 100 μg of SR microsomes were incubated for 2.5 hours (in 1.5-ml polyethylene vials) with 0.6 ml of solution containing (in mM) KCl, 130; K-phosphate, 20 (pH ~7.0); EGTA, 1; hydroxyethyl ethylenediaminetriacetic acid (HEDTA), 1; and CaCl_2 levels to buffer free [Ca^{2+}] at 1 μM . The solution also contained 100 nM [^3H]ryanodine (5000 cpm/pMole; PerkinElmer, Inc., Boston, MA) for measurements of total SR [^3H]ryanodine binding. Cold ryanodine (30 μM) was also added to the solution for measuring nonspecific binding. After incubation, SR microsomes containing bound [^3H]ryanodine were pelleted by centrifugation (15,000g) at 4°C for 40 minutes using an 18.1 Beckman rotor and a Beckman J2-21M/E centrifuge (Beckman Coulter, Brea, CA). The supernatant was removed from the vial by suction and the pellet washed twice with 1.5 ml of iced ryanodine-free buffer. Then pellets were incubated 24 hours with 50 μl of BTS-450 tissue solubilizer (Beckman Coulter) with occasional agitation/vortexing. Subsequently, 1.5 ml of liquid scintillation fluid was added to the vials, mixed, and left to rest for 48 hours (which secures the extinction of bioluminescence generated by the reaction of tissue

solubilizer and scintillation fluid). Finally, the samples were counted twice for 10 minutes in a liquid scintillation counter.

RyR Channel Measurements in Planar Lipid Bilayers. Planar lipid bilayers made of 50% phosphatidylethanolamine, 40% phosphatidylserine, and 10% phosphatidylcholine (total, 50 mg/ml) (Avanti Polar Lipids, Alabaster, AL) were formed on ~100- μ m-diameter circular holes in Teflon septa, separating two 1.2-ml compartments (Copello et al., 1997). The *trans* bilayer solution containing 250 mM HEPES and 50 mM $\text{Ca}(\text{OH})_2$ (pH 7.4) was clamped at 0 mV with an Axopatch 200B (Axon Instruments, Foster City, CA). The *cis* compartment (ground) contained 250 mM HEPES and 120 mM Tris (pH 7.4). Subsequent addition, while stirring, of 500–1000 mM CsCl, 1 mM CaCl_2 , and SR microsomes (5–15 μ g) to the *cis* solution allowed for reconstitution of RyRs with their cytosolic surface facing the *cis* chamber (Copello et al., 1997). Excess CsCl and Ca^{2+} in the *cis* chamber was removed by superfusion (5 minutes at 4 ml/min with HEPES-Tris solution). As previously described (Copello et al., 1997; Porta et al., 2012), BAPTA (1 mM) and dibromo-BAPTA (1 mM) was used to buffer the free $[\text{Ca}^{2+}]$ on the cytosolic surface of the channel ($[\text{Ca}^{2+}]_{\text{cyt}}$).

Channel recordings (4- to 8-minute durations in each condition) were filtered through a low-pass Bessel filter at 1 kHz, digitized at 20 kHz with a 12-bit analog-to-digital converter, and stored on an optical disk for computer analysis, using pClamp9 software (Axon Instruments). Measurements of open times, closed times, and open probabilities were determined by half-amplitude threshold analysis of single-channel recordings as done before (Copello et al., 1997). For simplicity of the analysis, exponential fitting of the dwell-time histograms was performed considering that all open and closed time distributions included only two components, which is an approximation (Sitsapasan and Williams, 1994; Copello et al., 1997; Rosales et al., 2004).

Drugs and Chemicals. CaCl_2 standard for calibration was from World Precision Instruments, Inc. (Sarasota, FL). Phospholipids (phosphatidylethanolamine, phosphatidylserine, and phosphatidylcholine) were obtained from Avanti. The agents tested here included eudistomin D (EuD), a compound originally isolated from *Eudistoma olivaceum* (Kobayashi et al., 1984), and its synthetic derivative MBED, which may be a potent RyR agonist (Seino et al., 1991). We also tested Pen, an agent originally isolated from *Penares* sp. that has also been reported to increase SR Ca^{2+} release (Kobayashi et al., 1990). Additionally, we studied the natural Pen derivative penaresin methylester (PenM), originally isolated from *Iotrochota* sp. (Dellar et al., 1981); and three synthetic Pen derivatives: penaresin dicyano derivative (PenCN), *N*-methyl-penaresin methyl ester (MPenM), and *N*-methyl-penaresin nitro derivative (MPenNO). All Pen and EuD derivatives were a gift from Dr. M. Große-Bley, Bayer AG Central Research, Leverkusen, Germany. The structures of these agents are shown in Supplemental Fig. 1.

Statistical Analysis. Data are presented as means $\pm$ S.E.M. of *n* measurements. Statistical comparisons between groups were performed with a paired *t* test. Differences were considered statistically significant at *P* < 0.05, and figures indicate *P* values.

Results

Single-Channel Studies and [^3H]Ryanodine Binding Measurements Indicate That MBED Is a Potent Caffeine-Like Agonist of Cardiac RyR2. The agonistic effects of eudistomin and penaresin derivatives on single RyR2 receptors from pig heart were studied after reconstitution of the channels from SR membranes into planar lipid bilayers. In all experiments, the pH was 7.4. In most cases, the bilayer membrane was clamped at 0 mV, and all experiments were carried out using 50 mM Ca^{2+} (in the *trans* chamber) as current carrier.

Initially, all agents were tested in the presence of Mg-ATP and cytosolic $[\text{Ca}^{2+}]$ at ~1 μ M. Under these conditions, RyR2 receptors have low-to-moderate activity (P_o , 0.03–0.2) but are strongly activated by 10 mM caffeine (reaching P_o > 0.95).

This would allow us to recognize even minor activating effects of high doses of agents that act as caffeine. Figure 1A shows the activating effect of EuD on single RyR2. Notice that the P_o levels reached in the presence of EuD are not as high as those observed after addition of caffeine. Figure 1, B and C, shows examples of single RyR2 exposed to Pen and its derivative MPenNO, which produced minor activation and no effect, respectively. The results of equivalent sets of single-channel experiments testing several EuD and Pen derivatives are summarized in Fig. 1D. MBED was the only agent that matched the agonistic action of caffeine. Under these experimental conditions, EuD induced activation, but subsequent addition of caffeine significantly increased P_o . Pen and MPenM also displayed some partial agonistic action, while PenCN, MPen, and MPenNO did not produce any effect. Not shown are studies of RyR2 current amplitude versus voltage that indicated that these agents neither significantly affected the channel's slope conductance nor induced any substates. In all cases, the effects of all agents were reversible. EuD and Pen derivatives were added to the cytosolic (*cis*) solution in most experiments for technical reasons. The multiple superfusions required to add/remove these drugs and other compounds can only be done safely (i.e., without disrupting our bilayers) on the cytosolic surface of the channel. Notice, however, that MBED and caffeine were also effective when added to the *trans* side/luminal surface of the RyR2 channels (data not shown).

To complement the experiments performed on single channels, we also tested the effects of EuD and Pen derivatives on [^3H]ryanodine binding to cardiac SR microsomes incubated with 1 μ M Ca^{2+} (in the absence of Mg-ATP). The values obtained in this test should reflect the effect of these drugs on RyR2 activity, as [^3H]ryanodine only binds to open RyRs (Ogawa, 1994). The results shown in Fig. 1E indicate that MBED (fully) and EuD (partially) mimicked the activating effects of caffeine on [^3H]ryanodine binding to SR microsomes. Pen and its derivatives were found ineffective with this test.

The results shown in Fig. 1 suggest that, among all the tested agents, MBED was the only compound that had agonistic effects matching those of caffeine. Thus, we focused on determining if this agent affects RyR2 channel gating in the same way that caffeine does. As shown in Fig. 2A, RyR2 channels bathed with 100 nM cytosolic Ca^{2+} have very low activity in the absence of agonists. An increase to high- P_o values was observed upon addition of either MBED or caffeine. However, the EC_{50} of MBED was 683 nM, while much higher doses of caffeine were required to produce the same degree of activation (EC_{50} ~3.7 mM). RyR2 activation by MBED and caffeine had Hill coefficients (n_H) around 2 (1.8 and 2.1, respectively), suggesting cooperativity between multiple sites. Figure 2B shows that, in the absence of agonists (open circles), RyR2 channels have very low activity at resting cytosolic Ca^{2+} levels (100 nM), and they activate with increasing micromolar $[\text{Ca}^{2+}]_{\text{cyt}}$ (EC_{50} ~3 μ M $[\text{Ca}^{2+}]_{\text{cyt}}$), as previously reported (Copello et al., 1997, 2002; Diaz-Sylvester et al., 2011; Porta et al., 2011). In the presence of 1 μ M MBED or 10 mM caffeine, the $[\text{Ca}^{2+}]_{\text{cyt}}$ required to activate RyR2 was much lower (EC_{50} s were ~81 and 93 nM, respectively). Moreover, the maximal P_o values reached in the presence of caffeine or MBED (~0.98 and ~0.96, respectively) were higher than that observed under control conditions (0.83; for details see Fig. 2B legend).

As shown in the recordings of Fig. 2B, RyR2 can reach high- P_o values with 10–100 μ M $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of other

Fig. 1. Effect of eudistomins and penaresins on cardiac RyR2 single-channel activity and $[^3H]$ ryanodine binding to cardiac SR microsomes. (A–C) Single-channel recordings of pig cardiac RyR2 at membrane voltage of 0 mV. Luminal solution contained (in mM) $Ca(OH)_2$, 50; and HEPES, 250 (pH 7.4). Cytosolic solution contained (in mM) Tris, 100; and HEPES, 250 (pH 7.4). Channel openings are depicted as positive deflections in the current. Top panels show 2-second segments of channel activity and P_o values estimated from 4-minute recordings under control conditions ($[Ca^{2+}]_{cyt} \sim 1 \mu M$ in the presence of 5 mM total ATP and 1 mM free Mg^{2+}). RyR2 recordings performed after addition of EuD (A), Pen (B), or MPenNO (C) are shown in the middle panels. As a positive control, 10 mM caffeine was added at the end of each experiment (bottom panels). (D) Mean $P_o \pm$ S.E.M. of RyR2 channels under control conditions (open bars), after subsequent addition of either 10 μM MBED or 200 μM other agents (EuD, Pen, PenCN, MPenM, MPenNO, PenM) (gray bars), and finally after addition of 10 mM caffeine (black bars). Each drug was tested on a minimum of five different channels. (E) Mean $\pm$ S.E.M. ($n = 5$) of specific $[^3H]$ ryanodine binding to cardiac SR microsomes before (control) and after the addition of the indicated drugs. With exception of MBED (10 μM) and caffeine (20 mM), all other agents were used at 200 μM . * $P < 0.05$ versus Control.

agonists. Figure 2C (top panel) shows that the gating of Ca^{2+} -activated RyR2 includes abundant brief events (closures or openings), which are an identifying mark for these channels (Fill and Copello, 2002). As shown in Fig. 2C (middle and bottom panels), in the presence of 5 μM MBED or 10 mM caffeine, RyR2 reached full activation at 100 nM $[Ca^{2+}]_{cyt}$, but brief events were scarce or not observed. Typical open and closed time distribution histograms and average values are shown in Fig. 2D and in its legend, respectively. Time constants (τ) for Ca^{2+} -activated and caffeine/MBED-activated RyR2 channels were obtained by fitting the logarithmic dwell-time distributions (open or closed time distribution histograms) with two components (τ_{o1} , τ_{o2} for openings; τ_{c1} , τ_{c2} for closures), which is a practical simplification of a more complex gating behavior (Sitsapasan and Williams, 1994; Fill and Copello, 2002). Open times of Ca^{2+} -activated channels distributed with $\tau_{o1} = 3.4$ milliseconds; and $\tau_{o2} = 42.2$ milliseconds. As evidenced from the channel recordings and dwell open time distribution (Fig. 2, C and D), significantly longer open events were observed in caffeine-activated ($\tau_{o1} = 18$ milliseconds; and $\tau_{o2} = 321$ milliseconds) or MBED-activated channels ($\tau_{o1} = 11$ milliseconds; and $\tau_{o2} = 336$ milliseconds). Most closed times of Ca^{2+} -activated channels were quite brief (Fig. 2D). However, brief closures were less frequent in the presence of caffeine or MBED

(Fig. 2D; see also Fig. 2 legend). Thus, MBED, like caffeine, affects the kinetics of RyR2 channels by stabilizing conformations that produce long openings and closures, drastically decreasing the frequency of short “flicker” events (both openings and closures). Not shown, in the presence of MBED, there were no significant differences between the P_o values of RyR2 recorded at -20 and $+40$ mV, suggesting that MBED-activated channels are not affected by voltage, as we previously reported for caffeine (Diaz-Sylvester et al., 2011).

Single-Channel Studies, SR Ca^{2+} Release, and $[^3H]$ Ryanodine Binding Indicate That MBED Can Mimic the Activating Action of Caffeine on RyR1 Function. The agonistic effects of MBED and Pen on RyR1 channels from rabbit muscle were studied after reconstitution of the channels from SR membranes into planar lipid bilayers. RyR1 receptors were activated by MBED in 8/13 channel experiments. This heterogeneity in response was also observed for caffeine in previous studies (Copello et al., 2002). Figure 3, A and B, shows examples and summary data of channels bathed with 0.1 μM cytosolic Ca^{2+} displaying low activity (i). Upon addition of MBED (5 μM), the channels significantly activated (ii). Subsequent addition of Mg^{2+} closed the channels (iii), and this was reversed by increasing $[Ca^{2+}]_{cyt}$ to 2 μM (iv). The sensitivity to Mg^{2+} of MBED-activated RyR1 mimics previous observations

Fig. 2. Effect of MBED on the activity, Ca^{2+} sensitivity, and kinetics of cardiac RyR2. (A) Mean open probability as a function of the concentration of caffeine (gray triangles; $n = 8$ experiments) or MBED (black triangles; $n = 5$ experiments). From the dose-response curves we estimated the drug concentrations that produce half-maximal activation (EC_{50}) of RyR2 channels to be $683 \pm 48 \text{ nM}$ (MBED) and $3.7 \pm 0.2 \text{ mM}$ (caffeine). For both agents, the activation was cooperative; n_H was 1.8 ± 0.3 (MBED) and 2.1 ± 0.2 . (B) Mean open probability as a function of the $[\text{Ca}^{2+}]_{\text{cyt}}$ in the absence of other agonists (open circles) or in the presence of either 10 mM caffeine (gray triangles; $n = 8$) or 5 μM MBED (black triangles; $n = 5$). From the dose-response curves we estimated under control conditions that EC_{50} for Ca^{2+} activation was $2.3 \pm 0.1 \mu\text{M}$; $n_H = 2.7 \pm 0.2$. In caffeine, we estimated that $\text{EC}_{50} = 81 \pm 12 \text{ nM}$ and $n_H = 1.7 \pm 0.3$, which were not significantly different from those obtained with MBED, $\text{EC}_{50} = 93 \pm 6 \text{ nM}$ and $n_H = 2.1 \pm 0.2$. (C) Representative single-channel recordings of RyR2 activated by 10 μM cytosolic Ca^{2+} (top traces), 10 mM caffeine (middle panel), or 1 μM MBED (bottom panel). (D) Open and closed time distribution histograms of recordings of RyR2 channels activated by Ca^{2+} (top), caffeine (middle), or MBED (bottom). Average values from similar analysis of $n = 18$ Ca^{2+} -activated channels were $\tau_{o1} = 3.4 \pm 0.9$ milliseconds ($48\% \pm 5\%$) and $\tau_{o2} = 42.2 \pm 7.7$ milliseconds ($52\% \pm 5\%$), and $\tau_{c1} = 0.6 \pm 0.1$ millisecond ($85\% \pm 3\%$) and $\tau_{c2} = 2.8 \pm 0.5$ milliseconds ($15\% \pm 3\%$). From the analysis of $n = 14$ caffeine-activated

with the agonist caffeine (Copello et al., 2002). Similar observations have been obtained with cardiac RyR2 (Supplemental Fig. 2). Figure 3C shows the RyR1 open probability as a function of MBED concentration. From these data, we estimated that the EC_{50} for MBED was 479 nM. Notice that RyR1 channels reached, on average, a P_o of 0.36, much lower than the values reported for RyR2 channels ($P_o \sim 0.95$) (Figs. 1 and 2). These differences in behavior between RyR1 and RyR2 have also been described for caffeine (Copello et al., 2002).

We tested the effect of Pen (200 μ M) in $n = 11$ channel reconstitutions (mostly multichannels). In most cases, as in the example in Fig. 4A, penaresin had no effect. Pen induced partial activation of RyR1 on only one occasion. Overall, the effects of Pen were not significant (Fig. 4B). We think that the heterogeneous behavior of RyR1 and the variability in P_o between recordings (Copello et al., 1997) make it unlikely to detect minor changes in activity. Consequently, we tested the ability of these agents to modulate [3 H]ryanodine binding and SR Ca^{2+} leak, which provide consistent indications of RyR1 channel activity. [3 H]Ryanodine binding studies were carried out with skeletal muscle SR microsomes incubated for 3 hours in buffer containing 1 μ M Ca^{2+} (Fig. 4C). MBED and caffeine produced an identically large increase in [3 H]ryanodine binding, while EuD produced activation to a lesser extent. Pen produced very mild activation, and all other derivatives had no significant action on [3 H]ryanodine binding (Fig. 4C).

Measurements of Ca^{2+} leak from skeletal muscle TC microsomes (R4 fractions) are also appropriate to detect minor effects of agonists on RyR1-mediated Ca^{2+} release. To perform these tests, we first loaded the microsomes with Ca^{2+} (~ 1.5 μ Mole Ca^{2+} /mg SR protein). Subsequently, we measured the effects of drugs on the release (or leak) after addition of 20 μ M CPZ, a SERCA inhibitor. Figure 5A shows examples of Ca^{2+} leak under control conditions and in the presence of caffeine, Pen, EuD, or MBED. Figure 5B summarizes the results comparing the effects of eudistomins and penaresins with those of caffeine. MBED (10 μ M) and caffeine (10 mM) had large activating effects. EuD was partially effective, as the maximal concentrations tested (200 μ M) did not reach the values observed with caffeine. Maximal doses (200 μ M) of Pen or the other penaresin derivatives had no significant effect on the Ca^{2+} leak rate. Figure 5C shows examples of dose response to MBED, and Fig. 5D summarizes data on Ca^{2+} leak rate as a function of caffeine or MBED concentration. From these data, we estimated that the concentrations producing 50% of the maximal SR Ca^{2+} leak rate (EC_{50}) were 844 nM (MBED) and 2.4 mM (caffeine). Notice that the effects of MBED (Fig. 5C) were completely counteracted by ruthenium red, similarly to what is found with caffeine and other agonists (Sitsapesan and Williams, 1998; Fill and Copello, 2002). Thus, our results as a whole indicate that MBED is the only agent that can mimic the action of caffeine on RyR1 and RyR2 channels.

Effects of Eudistomins and Penaresins on SERCA of Skeletal Muscle and Cardiac SR Microsomes. Ca^{2+} uptake by cardiac SR microsomes or skeletal muscle TC microsomes mainly represents the difference between the

active SR Ca^{2+} influx (which depends on SERCA activity) minus the passive Ca^{2+} efflux of Ca^{2+} from the SR microsomes (which depends mainly on RyR activity). SR microsomes also have RyR-independent SR Ca^{2+} leak, which is more significant in cardiac versus skeletal muscle microsomes (Chamberlain et al., 1984b). We have found that MBED and caffeine significantly decreased the rate of SR Ca^{2+} loading by skeletal muscle TC, which is expected for RyR1 agonists (Supplemental Fig. 3). We also observed that MBED decreased the SR loading rate after blocking RyR1 with ruthenium red (Supplemental Fig. 3). In principle, the results may reflect the synergism of a powerful agonist in combination with micromolar Ca^{2+} levels to counteract the effect of a blocker on a RyR (Supplemental Fig. 4). However, a decrease in the rate of loading was also observed with other tested agents that did not display agonistic action when tested on single channels, [3 H]ryanodine binding, or SR Ca^{2+} leak. This suggests that some of the tested drugs might directly inhibit SERCA. Figure 6A illustrates examples of how EuD, MBED, Pen, and PenM slowed down the process of Ca^{2+} uptake by cardiac SR microsomes in the presence of ruthenium red. Figure 6B summarizes the data obtained with all the tested agents and shows that most of them (with exception of MPenM) significantly slow down the Ca^{2+} loading rate, with EuD, MBED, Pen, and PenM displaying the most significant effects.

The effects of these agents on SERCA were also studied using an ATPase assay. Figure 6, C and D, shows examples and summary data of measurements of ATPase activity performed with a Ca^{2+} concentration of 2 μ M, where SERCA reaches near maximal activity (V_{max}). These studies confirmed that EuD and Pen were the agents that produced the highest level of SERCA inhibition, both in cardiac and skeletal muscle SR microsomes. In skeletal muscle SR microsomes (R2 fractions where the ATPase activity is fully inhibited by CPZ), we determined that the IC_{50} values for EuD and Pen were 61 and 219 μ M, respectively (Supplemental Fig. 5). MBED was a less potent inhibitor of SERCA; estimated IC_{50} values were 285 and 720 μ M, respectively, in skeletal muscle and cardiac microsomes (Supplemental Fig. 6). MBED concentrations lower than 1 μ M did not significantly decrease SERCA-mediated ATPase activity. Figure 6B shows that PenM was the most potent inhibitor of SR Ca^{2+} loading. However, we could not use the ATPase assay to test PenM as this compound significantly increased the degradation of NADH, even in the presence of CPZ. This result may reflect that PenM produces an increase in the activity of SERCA-independent ATPases or the activation of microsomal pathways that consume NADH.

Discussion

We tested derivatives from alkaloids of marine sponges that are putative RyR agonists. Among them, the EuD derivative MBED was a potent agonist of RyRs in skeletal muscle and heart. MBED mimicked caffeine's activating effect with an ~ 1000 times higher potency. Single-channel studies indicated that MBED affects the gating of RyR2 in a caffeine-like

channels we estimated that $\tau_{o1} = 18.4 \pm 5.9$ milliseconds* (23% $\pm$ 3%*) and $\tau_{o2} = 321 \pm 84$ milliseconds* (76% $\pm$ 3%*), $\tau_{c1} = 1.9 \pm 0.5$ milliseconds* (47% $\pm$ 4%*), and $\tau_{c2} = 177 \pm 42$ milliseconds* (53% $\pm$ 4%*). With MBED, average values ($n = 10$ experiments) were $\tau_{o1} = 11.3 \pm 3.7$ milliseconds* (34% $\pm$ 7%*) and $\tau_{o2} = 336 \pm 84$ milliseconds* (66% $\pm$ 7%*), $\tau_{c1} = 3.8 \pm 0.6$ milliseconds* (33% $\pm$ 5%*), and $\tau_{c2} = 159 \pm 39$ milliseconds* (67% $\pm$ 5%*). *The parameters estimated for caffeine-activated RyR2 were significantly different than those of channels activated by Ca^{2+} , with $P < 0.05$ or better.

Fig. 3. Effect of addition of cytosolic Mg^{2+} and Ca^{2+} on MBED-activated skeletal muscle RyR1 channels. (A) Multichannel recordings of skeletal muscle RyR1 under control conditions ($[Ca^{2+}]_{cyt} = 0.1 \mu M$; absence of Mg -ATP) and after sequential addition of 5 μM MBED, 1 mM Mg^{2+} , and 2 μM Ca^{2+} to the cytosolic surface of the channel. The averaged open probabilities of five experiments are shown in B. * $P < 0.05$ versus Control. (C) Dose-response curve of skeletal muscle RyR1 channels exposed to increasing concentrations of MBED ($n = 7$ experiments). The EC_{50} for MBED was 480 ± 25 nM; $n_H = 1.3 \pm 0.1$.

manner by stabilizing the high- P_o mode. The parent compound, EuD, only produced partial activation of RyRs at the maximal doses tested. Penaresin and most of its derivatives had, at best, mild agonistic effects on RyRs.

Reportedly, EuD and MBED do not significantly affect adenosine receptors and phosphodiesterases at the doses that can produce activation of RyR channels (Bruton et al., 2003; Ishiyama et al., 2008). Consequently, these potent RyR agonists may represent a good alternative to caffeine for the study of RyR-mediated Ca^{2+} signaling in cells. However, we also found that these compounds, when used at high doses, inhibit the Ca^{2+} ATPase of sarcoplasmic reticulum. A summary of the effects of maximal doses of all the tested agents on RyR function (estimated by measuring SR Ca^{2+} release, $[^3H]$ ryanodine binding, and single-channel activity) and ATPase activity is shown in Table 1.

Effect of Eudistomins and Penaresins on RyRs. MBED has been reported to induce RyR-mediated Ca^{2+} release in skeletal muscle and heart with comparable characteristics to

the caffeine-induced SR Ca^{2+} release (Seino et al., 1991; Seino-Umeda et al., 1998). Our single-channel studies show that MBED directly activates RyR channels. For single RyR2, we have determined that 1–10 μM MBED produces changes in the channel kinetics (open and closed time distributions) that closely mimic those under 5–20 mM caffeine. Considering that channel opening/closure directly reflects binding/unbinding to MBED or caffeine, we can infer that MBED has an on-rate to bind caffeine-binding sites that is ~ 1000 -fold higher than caffeine. However, MBED and caffeine should have similar off-rates to unbind from these sites. In this regard, the action of MBED and caffeine on RyR2 differs from that of divalent cations or ryanoids, where higher on-rates usually produce longer openings, i.e., slower off-rates (Porta et al., 2008, 2011; Diaz-Sylvester et al., 2011). The effects of MBED were indistinguishable from those of caffeine in all our tests, including the changes they induce in RyR2 sensitivity to cytosolic Ca^{2+} and Mg^{2+} . Not shown, when luminal Mg^{2+} (instead of Ca^{2+}) was used as current carrier, activation of RyR2 channels by MBED required

Fig. 4. Lack of effect of Pen on skeletal muscle RyR1 channels. (A) Single-channel recordings of a skeletal muscle RyR1 under control conditions ($[\text{Ca}^{2+}]_{\text{cyt}} = 0.1 \mu\text{M}$; 1 mM total ATP and 1 mM free Mg^{2+}) and after addition of $20 \mu\text{M}$ Pen. The averaged open probabilities of eight experiments are shown in B. (C) Averages of measurements of [^3H]ryanodine binding to skeletal muscle SR microsomes before (control) and after the addition of the indicated drugs. With the exception of MBED ($10 \mu\text{M}$) and caffeine (20 mM), all other agents were used at $200 \mu\text{M}$. Data are means $\pm$ S.E.M. of $n = 5$ determinations; $*P < 0.05$ versus Control.

micromolar levels of cytosolic Ca^{2+} ($n = 3$ experiments), mimicking previous observations with caffeine (Diaz-Sylvester et al., 2011). In contrast to the mild effects originally reported in skeletal muscle (Seino et al., 1991), in our hands, MBED greatly increased [^3H]ryanodine binding to cardiac and skeletal muscle SR microsomes. These discrepancies are due to the fact that, in conditions of high salt (1 M) and $10 \mu\text{M}$ Ca^{2+} (as used in the

previous report), RyRs are highly active and [^3H]ryanodine binding is very high, even in the absence of additional agonists (Ogawa, 1994). Instead, we adjusted the control conditions to keep the channels with a low activity to be able to detect large increases in [^3H]ryanodine binding when exposed to an agonist.

The other agents we tested were much weaker RyR agonists than MBED. EuD has been found to have antiviral and antibacterial properties, but no studies on its effects on SR microsomes have been reported (Kobayashi et al., 1984). In our hands, EuD was a mild caffeine-like agonist that produces partial activation of RyR1 or RyR2 at the maximal concentrations we tested (which were limited by the solubility of the compound). Pen was originally reported as an agent that induces Ca^{2+} release from skinned skeletal muscle fibers with a minimum effective concentration of $40 \mu\text{M}$ (Kobayashi et al., 1990). We found no effect of Pen ($200 \mu\text{M}$) on SR Ca^{2+} leak from skeletal muscle SR microsomes. Single-channel recordings of RyR1 exposed to Pen also failed to detect significant changes in activity. We only detected a minor activating effect of Pen on the [^3H]ryanodine binding assay, suggesting that Pen could have minor agonistic action on RyR1. Likewise, minor activating action of Pen on RyR2 channels was found. The slight discrepancies between results obtained using different testing methodologies ([^3H]ryanodine binding, SR Ca^{2+} leak, loading, single-channel recordings) may reflect the different susceptibility of the RyRs to agonists in the different testing environments or statistical limitations of our samples that preclude detecting small agonistic effects. Previous reports indicated that the potency of caffeine derivatives as RyR agonists depended on their hydrophobicity (Liu and Meissner, 1997). However, esterification of the carboxylic acid, as in PenM, did not increase its potency as a RyR agonist. Among all the tested Pen derivatives, we only detected a significant activating effect of MPenM on RyR2 channel activity. The presence of more electronegative regions in Pen derivatives, such as PenCN and MPenNO, did not confer on them the ability to modulate RyRs. In summary, our data suggest that MBED is the only agent that mimics the effects of caffeine on RyRs both quantitatively and qualitatively, acting with 1000-fold-higher potency.

Eudistomins, Penaresins, and Caffeine Have Additional Targets. Inhibition of phosphodiesterases and adenosine receptors by caffeine and other methylxanthines is known to have much higher affinity than caffeine's agonistic action on RyRs (Liu and Meissner, 1997; Bruton et al., 2003; Ishiyama et al., 2008; Francis et al., 2011; Müller and Jacobson, 2011; Riksen et al., 2011). The opposite appears to be true for MBED, as micromolar levels of MBED (found here to induce maximal activation of RyRs) had no significant effects on phosphodiesterases (Bruton et al., 2003). Likewise, structural studies suggest that significant inhibitory effects of MBED on adenosine receptors would require higher levels than those required to fully activate RyRs (Ishiyama et al., 2008). The ability of Pen and PenM to affect adenosine receptors or phosphodiesterases has not been reported.

Some evidence indicates that MBED may have more than one binding target (Yoshikawa et al., 1995). In this regard, we found that Pen, PenM, EuD, and, to a smaller degree, MBED inhibit SERCA. Indeed, the inhibitory effect of Pen that we found on SERCA may help explain the apparent contradiction between the results of our RyR1 studies (where Pen has little

Fig. 5. Effect of EuD and Pen derivatives on Ca²⁺ leak from skeletal muscle SR microsomes. Skeletal muscle SR microsomes in the absence of drugs (control) or in the presence of EuD or Pen derivatives were preloaded with three shots of Ca²⁺ (indicated by arrows). The loading rate represents the activity of the SR Ca²⁺ ATPase pump (SERCA) minus the activity of RyR1 channels. The rate of Ca²⁺ leak was measured after blocking SERCA by addition of CPZ. Examples of experiments conducted in the presence of caffeine, EuD, MBED, and Pen are shown in A. Averages of the rates of leak from skeletal muscle SR microsomes exposed to the indicated drugs are shown in B. (C) Examples of Ca²⁺ leak from SR microsomes exposed to increasing concentrations of MBED. The data obtained by determining the leak rate in the presence of MBED (black triangles) or caffeine (gray triangles) were used to build the respective dose-response curves shown in D. The estimated concentrations of drug required to reach half-maximal activation of the SR Ca²⁺ leak rate (EC₅₀) were 844 ± 88 nM (MBED) and 2.4 ± 0.3 mM (caffeine); n_H values were 1.3 ± 0.1 and 1.6 ± 0.2 , respectively. RR, ruthenium red. * $P < 0.05$ versus Control.

or no effect) and the previously reported activating effects of Pen on skeletal muscle SR Ca²⁺ release (Kobayashi et al., 1990). Early reports stated that MBED and its analog 7-bromoeudistomin D, even at a concentration of 100 μM , produced no significant inhibitory effects on ATPases, but it is unclear if those studies were carried out under the ideal conditions (ionic strength, Ca²⁺) to highlight drug effects on SERCA (Seino et al., 1991). In our hands, lower MBED doses (10–20 μM) produced significant inhibition of SR load in the presence of ruthenium red and a substantial decrease in ATPase activity, which in our skeletal muscle microsomes is ~99% inhibitable by the SERCA blocker CPZ. The fact that MBED affects SERCA activity while caffeine does not might also help explain reported differences between MBED (at 100 μM levels) and caffeine (10 mM) on Ca²⁺ signaling in smooth muscle (Ohi et al., 2001).

Marine Alkaloids as Potential Tools To Study Intracellular Ca²⁺ Signaling—Conclusions. A myriad of marine organisms, including sponges, incorporate bromide, which is relatively abundant in seawater, into thousands of

organohalogen compounds through metabolic pathways. Nearly 25% of these compounds are halogenated alkaloids, which have been at best partially characterized in their biologic activity (Gribble, 2012). This study revised the potential of two groups of alkaloids (indole and β -carboline derivatives) to modulate two main elements of intracellular Ca²⁺ homeostasis in striated muscle: RyRs and SERCA.

Caffeine produces huge effects on the gating of RyRs, even in the cellular environment (Rousseau et al., 1988; Sitsapesan and Williams, 1990; Herrmann-Frank et al., 1999; Porta et al., 2011). As a high-potency caffeine analog, MBED could be a suitable agent to gain insight into the three-dimensional location of the RyR site(s) to which this compound and caffeine bind, which remain largely unknown. Information on the structural basis of the regulation of RyRs by caffeine will help us understand the stabilization of the high-P_o mode of gating (Zahradnikova and Zahradnik, 1995; Zahradnikova et al., 1999; Fill and Copello, 2002). Moreover, this knowledge could contribute to establishing possible links between the caffeine-binding site(s) and RyR mutations associated with

Fig. 6. Effect of EuD and Pen derivatives on skeletal muscle and cardiac SERCA. (A) Examples of Ca^{2+} loading into cardiac SR microsomes in the absence of drugs (control) or in the presence of EuD or Pen derivatives. (B) Summary of averaged Ca^{2+} loading rates of skeletal muscle (gray) and cardiac (black) microsomes exposed to the indicated EuD or Pen derivatives. (C) The ATPase activity of cardiac SR microsomes is measured as the rate of decrease in absorbance (340 nm) observed as a consequence of the consumption of one molecule of NADH per molecule of hydrolyzed ATP. In the examples, microsomes were in the absence of drugs (control) or in the presence of EuD, MBED, or Pen. (D) Summary of ATPase activities measured in skeletal muscle (gray) and cardiac (black) microsomes exposed to the indicated EuD or Pen derivatives. With the exception of MBED (10 μM) and caffeine (20 mM), all other agents were used at 200 μM . SkM, skeletal muscle (B and D) * $P < 0.05$ versus the respective cardiac or SkM Control.

“leaky channels” and disease (Wei and Dirksen, 2010). As indicated, radiolabeled MBED has been previously used in binding experiments (Yoshikawa et al., 1995). However, we

still need to develop MBED derivatives suitable for covalent photolabeling of the RyR molecule and/or probes for fluorescence resonance energy transfer.

TABLE 1

Effect of EuD and Pen derivatives on RyR function and ATPase activity

Columns indicate EuD or Pen derivative name, percentage of change in channel activity, SR Ca^{2+} release and [^3H]ryanodine binding (relative to caffeine), and ATPase activity (relative to control). The same tests were performed on cardiac and skeletal muscle microsomes except for SR Ca^{2+} release, which was only determined in skeletal muscle microsomes.

Drug	Skeletal Muscle				Cardiac Muscle		
	RyR1 Activity	Release	Ryanodine Binding	ATPase Activity	RyR2 Activity	Ryanodine Binding	ATPase Activity
	%						
Caffeine	↑ (100)	↑ (100)	↑ (100)	—	↑ (100)	↑ (100)	—
EuD	N.D.	↑ (22)	↑ (39)	↓ (−49)	↑ (86)	↑ (41)	↓ (−54)
MBED	↑ (100)	↑ (118)	↑ (118)	↓ (−24)	↑ (105)	↑ (97)	↓ (−18)
Pen	—	—	↑ (17)	↓ (−61)	↑ (20)	—	↓ (−36)
PenCN	N.D.	—	—	↓ (−17)	—	—	↓ (−24)
MPenM	N.D.	—	—	↓ (−14)	↑ (53)	—	↓ (−18)
MpenNO	N.D.	—	—	—	—	—	—
PenM	N.D.	—	—	N.D.	—	—	N.D.

—, no effect; N.D., not determined.

Cellular studies suggest that MBED can enter the cell and induce Ca^{2+} release from intracellular stores (Seino et al., 1991; Ohi et al., 2001; Bruton et al., 2003). Thus, MBED is also an appealing tool for functional studies as low doses of MBED (e.g., 100–200 nM) could be used to mimic the acute cellular effects of a “leaky” RyR, without the interference of adenosine receptor- and phosphodiesterase-mediated pathways characteristically activated by caffeine. Additionally, MBED derivatives may serve as templates for developing pharmacological agents that could stabilize RyR in the closed state, such as the analog 4,6-dibromo-3-hydroxycarbazole (Takahashi et al., 1995). In contrast, Pen derivatives do not appear to have strong potential to generate caffeine-like agonists of RyRs. Still, their effects on SERCA and their very mild agonism of RyRs resemble that of benzothiazepines, agents associated with cardioprotection (Neumann et al., 2011; Lv et al., 2013; Ragone et al., 2013).

In conclusion, we found that MBED, a derivative of the brominated alkaloid eudistomin D, has potent agonistic effects on RyRs and closely mimics the action of caffeine on channel gating, suggesting that MBED has potential for structural and functional studies that may advance our understanding of RyR function during excitation-contraction coupling.

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Authorship Contributions

Participated in research design: Diaz-Sylvester, Porta, Fleischer, Copello.

Conducted experiments: Diaz-Sylvester, Porta, Juettner, Lv, Copello.

Contributed new reagents or analytic tools: Copello, Fleischer.

Performed data analysis: Diaz-Sylvester, Porta, Juettner, Lv, Copello.

Wrote or contributed to the writing of the manuscript: Diaz-Sylvester, Porta, Juettner, Lv, Fleischer, Copello.

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