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Eudistomin D and Penaresin derivatives as modulators of ryanodine receptor channels and sarcoplasmic reticulum Ca²⁺ ATPase in striated muscle.

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Nonstandard Abbreviations:

APIII – Antipyrylazo III

BED – 7-bromo-eudistomin D; 9*H*-Pyrido[3,4-*b*]indol-6-ol, 5,7-dibromo-

[Ca²⁺]_{cyt} – Cytosolic Ca²⁺ concentration

CPZ – Cyclopiazonic Acid

EuD, Eudistomin D, Eudistomine D; 9*H*-Pyrido[3,4-*b*]indol-6-ol, 5-bromo-

MBED – N-methyl Bromoeudistomin D; 9-methyl-7-bromoeudistomin D; 9*H*-Pyrido[3,4-*b*]indol-6-ol, 5,7-dibromo-9-methyl-; 5,7-Dibromo-6-hydroxy-9-methylpyrido[3,4-*b*]indole

MPenM; N-Methyl-penaresin, methyl ester; 2-Propenoic acid, 3-(6-bromo-1-methyl-1*H*-indol-3-yl)-, methyl ester, (2*E*)-; (E)-3-(6-bromo-1-*H*-indole-1-Methyl-3-yl)prop-2-enoate

MPenNO; "methyl-penaresin nitroderivative"; 1*H*-Indole, 6-bromo-1-methyl-3-[(1*E*)-2-nitroethenyl]-

Pen; Penaresin; Penaresine; 2-Propenoic acid, 3-(6-bromo-1*H*-indol-3-yl)-, (E)-; (E)-6-Bromoindole-3-acrylic acid;

PenCN; "penaresin dicyano derivative"; Propanedinitrile,2-[(6-bromo-1*H*-indol-3-yl)methylene]-

PenM; Penaresin methylester; methyl (E)-3-(6-bromoindol-3-yl)prop-2-enoate;

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Ryanodine - 1H-Pyrrole-2-carboxylic acid, (3S,4R,4aR,6S,6aS,7S,8R,8aS,8bR,9S,9aS)-
dodecahydro-4,6,7,8A,8B,9A-hexahydroxy-3,6A,9-trimethyl-7-(1-methylethyl)-6,9-
methanobenzo[1,2]pentaleno[1,6-bc]furan-8-ylester

RR – Ruthenium Red

RyR – Ryanodine Receptors

RyR1 – Skeletal RyR

RyR2 – Cardiac RyR

SERCA – Sarco/Endoplasmic Reticulum Ca²⁺ ATPase

SR – Sarcoplasmic Reticulum

V_m – Membrane voltage

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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 n-methylbromoeudistomin D (MBED) 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 sarcoplasmic reticulum 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 P_o " 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 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.

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INTRODUCTION

Ryanodine receptors (RyRs; skeletal muscle RyR1 and cardiac RyR2)/Ca²⁺-release channels are crucial for excitation-contraction (EC) coupling (Fill and Copello, 2002; Fleischer, 2008; Lanner et al., 2010; Meissner, 2004; Sitsapesan and Williams, 1998). After isolation and reconstitution in artificial bilayers, RyRs display complex single-channel kinetics, which includes modal gating (Rosales et al., 2004; Zahradnikova and Zahradnik, 1995). RyRs also exhibit functional heterogeneity in their response to physiological modulators (cytosolic Ca²⁺, Mg²⁺ 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; Copello et al., 2002). RyRs dwelling in the high P_o mode were found to display increased sensitivity to physiological agonists, lower sensitivity to blockers and increased ability to associate with neighboring channels for coupled gating (Copello et al., 2002; Diaz-Sylvester et al., 2011; Gyorke and Fill, 1993; Porta et al., 2012; Zahradnikova et al., 1999). 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 pharmacological 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 (Porta et al., 2011; Rousseau and Meissner, 1989; Sitsapesan and Williams, 1990). However,

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caffeine, as well as other xanthines, are inadequate for binding studies or molecular labeling due to their relatively low affinity for RyRs (Liu and Meissner, 1997). 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 (Francis et al., 2011; Müller and Jacobson, 2011; Riksen et al., 2011; Zahradnik and Palade, 1993).

Among other bioactive agents isolated from marine sponges, the indole alkaloids Bromo-Eudistomin D and Penaresin (Pen) were found to induce Ca^{2+} release from skeletal muscle and heart sarcoplasmic reticulum (Kobayashi et al., 1990; Nakamura et al., 1986). Subsequently, 9-methyl-7-bromoeudistomin D (MBED) was reported to increase the binding of ryanodine and to be an even more powerful Ca^{2+} releaser from cardiac and skeletal SR (Seino-Umeda et al., 1998; Seino et al., 1991). 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 and cardiac RyRs reconstituted into lipid bilayers. Our results suggest that only one of these compounds N-methylbromoeudistomin D (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.

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MATERIALS AND METHODS

Cardiac and skeletal SR microsomes

All procedures involving animals were designed to minimize pain and suffering and conformed to the guidelines of the National Institutes of Health. SIUMED animal research procedures have AAALAC accreditation and PHS assurances numbers 000551 and A3209-01, respectively. The Laboratory Animal Care and Use Committee (LACUC) 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 (Chu et al., 1988; Saito et al., 1984). The R4 fraction of SR is highly enriched in terminal cisternae (TC) of SR, which consists of junctional face membrane (where the RyR1 localize) and the calcium pump membrane, where the Ca²⁺ ATPase protein (SERCA 1a) localize (Fleischer, 2008; Saito et al., 1984). 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 SERCA1a-function in microsome assays (leak and loading respectively). The R2 fraction of SR is enriched in longitudinal tubules of SR which contain high density of SERCA1a 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

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were stored in liquid nitrogen. Every month, SR microsomes were separated into aliquots to be used in experiments. 15 μ l (bilayers) or 100 μ l (loading/release/ATPase assays) aliquots at a concentration of 5-15 mg protein/ml in 5 mM imidazol-Cl 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 Ca²⁺ loading/leak by SR microsomes

Ca²⁺ uptake by cardiac SR microsomes or R4 fractions of skeletal TC microsomes was measured with a spectrophotometer (Cory 50, Varian, Walnut Creek, CA) using the Ca²⁺-sensitive dye antipyrylazo III (APIII), as previously described (Chamberlain et al., 1984a; Chu et al., 1988; Neumann and Copello, 2011; Neumann et al., 2011). Briefly, Ca²⁺ uptake was initiated by adding 40 nanomoles CaCl₂ to 40-100 μ g SR membranes resuspended in 1 ml of buffer [in mM: 100 KH₂PO₄, 5 MgCl₂ (free Mg²⁺ ~0.3 mM), 5 ATP and 0.2 APIII; pH 7.0]. The rate of Ca²⁺ uptake by the skeletal (R4 fractions) and cardiac microsomes was measured in the absence (control) or presence of the RyR blocker ruthenium red (5 μ M) 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 (J_{Ca}), in μ Mole Ca²⁺ • (mg protein)⁻¹ • min⁻¹ was estimated as previously described (Neumann et al., 2011). The rate of Ca²⁺ leak from skeletal R4 fractions preloaded with Ca²⁺ (three pulses of 40 μ M Ca²⁺) was measured after addition of cyclopiazonic acid (CPZ; 20 μ M), which inhibits SERCA, plus 1 μ l dimethyl sulfoxide (DMSO; control) or penaresins/eudistomins in DMSO. Leak experiments were carried out in absence or presence of ruthenium red (5 μ M).

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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 tubule (R2 fractions) were incubated with buffer containing (in mM): 140 KCl, 5 MgCl_2 , 5 HEPES, 2 phosphoenolpyruvate, 0.3 CaCl_2 , and variable amounts of BAPTA for free Ca^{2+} levels of approximately 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

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[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 2½ hours (in 1.5 ml polyethylene vials) with 0.6 ml of solution containing (in mM) 130 KCl, 20 K-phosphate (pH ~ 7.0), 1 EGTA, 1 HEDTA and CaCl₂ levels in order to buffer free [Ca²⁺] 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 (TB). 30 µM cold ryanodine was also added to the solution for measuring non-specific binding (NSB). After incubation, SR microsomes containing bound [³H] ryanodine were pelleted by centrifugation (15,000 rpm) at 4°C for 40 min. 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) with occasional agitation/vortexing. Subsequently, 1.5 ml of liquid scintillation fluid was added to the vials, mixed and left to rest 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 min in a liquid scintillation counter.

RyR channel measurements in planar lipid bilayers.

Planar lipid bilayers made of 50% phosphatidylethanolamine, 40% phosphatidylserine and 10% phosphatidylcholine (Avanti Polar Lipids; total 50 mg/ml), were formed on ~100 µm diameter circular holes in Teflon septa, separating two 1.2 ml

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compartments (Copello et al., 1997). The trans bilayer solution containing HEPES 250 mM and $\text{Ca}(\text{OH})_2$ 50 mM (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 500-1,000 mM CsCl, 1 mM CaCl_2 and SR microsomes (5–15 μg) 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 min. 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 (4-8 min duration 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 (Copello et al., 1997; Rosales et al., 2004; Sitsapesan and Williams, 1994).

Drugs and Chemicals

CaCl_2 standard for calibration was from World Precision Instruments Inc. (Sarasota, FL). Phospholipids (phosphatidylethanolamine, phosphatidylserine and

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phosphatidylcholine) were obtained from Avanti (Alabaster, AL). The agents tested here included Eudistomin D (EuD), a compound originally isolated from *Eudistoma olivaceum* (Kobayashi et al., 1984), and its synthetic derivative n-methyl bromo eudistomin D (MBED), which may be a potent RyR agonist (Seino et al., 1991). We also tested Penaresin (Pen), an agent originally isolated from *Penares sp.* that has also been reported to increase SR Ca²⁺ 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 Dicianoderivative (PenCN), n-methyl penaresin methyl ester (MPenM), and N-methylpenaresin Nitroderivative (MPenNO). All Pen and EuD derivatives were a gift from Dr. M. Große-Bley, Bayer AG Central Research, Leverkusen. The structures of these agents are shown in **Supplemental Figure 1**.

Statistical analysis

Data is presented as means ± 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.

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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 ryanodine receptors isoform 2 (RyR2) 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 Ca²⁺ (in the *trans* chamber) as current carrier.

Initially, all agents were tested in the presence of Mg/ATP and cytosolic [Ca²⁺] ~ 1 μM. Under these conditions, RyR2 have low to moderate activity (Open probability, P_o, ranging from 0.03 to 0.2), but are strongly activated by 10 mM Caffeine (reaching P_o's higher than 0.95). This would allow us to recognize even minor activating effects of high doses of agents that act as caffeine. **Fig. 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. **Fig. 1B and 1C** show 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 the

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other compounds did not produce any effect. Not shown, studies of RyR2 current amplitude versus voltage indicate 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 due to 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 / lumenal surface of the RyR2 channels (results 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 μM Ca²⁺ (in the absence of ATP/Mg). 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 as caffeine does. As shown in **Fig. 2A**, RyR2 channels bathed with 100 nM cytosolic Ca²⁺ 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 effective

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concentration of MBED that produced 50% activation (EC_{50}) was 683 nM, while much higher doses of caffeine were required 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. **Fig. 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 $[Ca^{2+}]_{cyt}$ (EC_{50} ~3 μ M $[Ca^{2+}]_{cyt}$), as previously reported (Copello et al., 1997; Copello et al., 2002; Diaz-Sylvester et al., 2011; Porta et al., 2011). In the presence of 1 μ M MBED or 10 mM caffeine, the $[Ca^{2+}]_{cyt}$ required to activate RyR2 was much lower (EC_{50} were ~81 nM and 93 nM, respectively). Moreover, the maximal P_o reached in the presence 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 $[Ca^{2+}]_{cyt}$ in the absence of other agonists. **Fig. 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** (mid and low panels) in the presence of 5 μ M 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. 2 D** and in its legend, respectively. Time constants 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, which is a practical simplification of a more complex gating behavior (Fill and Copello,

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2002; Sitsapesan and Williams, 1994). Open times of Ca^{2+} -activated channels distributed with $\tau_{o1} = 3.4$ ms and $\tau_{o2} = 42.2$ ms. As evidenced from the channel recordings and dwell open times distribution (Fig. 2C and 2D), significantly longer open events were observed in caffeine-activated ($\tau_{o1} = 18$ ms and $\tau_{o2} = 321$ ms) or MBED-activated channels ($\tau_{o1} = 11$ ms and $\tau_{o2} = 336$ ms). 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, as 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 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 3A** and **3B** show 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 $[\text{Ca}^{2+}]_{\text{cyt}}$ to 2 μM (iv). The sensitivity to Mg^{2+} of

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MBED-activated RyR1 mimics previous observations 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 EC₅₀ for MBED was 479 nM. Notice that RyR1 channels reached on average a $P_o = 0.36$, much lower than the values reported for RyR2 channels ($P_o \sim 0.95$) (**Fig. 1 and Fig. 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 in 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) makes unlikely to detect minor changes in activity. Consequently, we tested the ability of these agents to modulate [³H] ryanodine binding and SR Ca²⁺ leak, which provide consistent indications of RyR1 channel activity. [³H] ryanodine binding studies were carried out with skeletal muscle SR microsomes incubated for 3 hours in buffer containing 1 μ M Ca²⁺ (**Fig. 4C**). MBED and caffeine produced an identically large increase in [³H] ryanodine binding while EuD produced activation to a lesser extent. Pen produced very mild activation and all other derivatives had no significant action on [³H] ryanodine binding (**Fig. 4C**).

Measurements of Ca²⁺ leak from skeletal muscle TC microsomes (R4 fractions) are also appropriate to detect minor effects of agonists on RyR1-mediated Ca²⁺ release. To perform these tests, we first loaded the microsomes with Ca²⁺ (~ 1.5 μ Mole Ca²⁺/mg

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SR protein). Subsequently, we measured the effects of drugs on the release (or leak) after addition of 20 μM of cyclopiazonic acid (CPZ), a SERCA inhibitor. **Figure 5A** shows examples of Ca^{2+} leak under control conditions and in 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 μM) and caffeine (10 mM) had large activating effects. EuD was partially effective, as the maximal concentrations tested (200 μM) did not reach the values observed with caffeine. Maximal doses (200 μM) 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 of 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 (Fill and Copello, 2002; Sitsapesan and Williams, 1998). 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 Sarcoplasmic Reticulum Ca^{2+} ATPase (SERCA) of skeletal and cardiac SR microsomes.

Ca^{2+} uptake by cardiac SR microsomes or skeletal TC microsomes, mainly represent 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 RyRs activity). SR microsomes also have RyR-independent SR Ca^{2+} leak which is more significant in cardiac versus skeletal microsomes (Chamberlain et al.,

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1984b). We have found that MBED and caffeine significantly decreased the rate of SR Ca^{2+} loading by skeletal muscle terminal cisternae, which is expected for RyR1 agonists (**Supplemental Figure 3**). We also observed that MBED decreased the SR loading rate after blocking RyR1 with ruthenium red (**Supplemental Figure 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 Figure 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, [^3H] 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. **Fig. 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, EuD, MBED, Pen and PenM displaying the most significant effects.

The effects of these agents on SERCA were also studied using an ATPase assay. **Figures 6C** and **6D** show examples and summary data of measurements of ATPase activity performed with a Ca^{2+} concentration of 2 μM , 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 SR microsomes. In skeletal muscle SR microsomes (R2 fractions where the ATPase activity is fully inhibited by CPZ), we determined that the IC_{50} for EuD and Pen were 61 and 219 μM respectively (**Supplemental Figure 5**). MBED was a less potent inhibitor of SERCA, estimated IC_{50} were 285 and 720 μM respectively in skeletal and cardiac

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microsomes (**Supplemental Figure 6**). MBED concentrations lower than 1 μM 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 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.

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DISCUSSION

We tested derivatives from alkaloids of marine sponges that are putative RyR agonists. Among them, the eudistomin D (EuD) derivative MBED was a potent agonist of RyRs in skeletal muscle and heart. MBED mimicked caffeine activating effect with a ~1000 times higher potency. Single-channel studies indicated that MBED affects the gating of RyR2 in a caffeine-like 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 are shown in **Table 1**.

Effect of Eudistomins and Penaresins on RyRs

9-methyl 7-bromo eudistomin D (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-Umeda et al., 1998; Seino et al., 1991). Our single-channel studies show that MBED directly activates RyR channels. For single

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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 (Diaz-Sylvester et al., 2011; Porta et al., 2008; Porta et al., 2011). The effects of MBED were indistinguishable from those of caffeine in all our tests, including the changes they induce on 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 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 SR microsomes. These discrepancies are due to the fact that in conditions of high salt (1 M) and 10 μM 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 in order 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 properties but no studies on its effects on SR

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microsomes have been reported (Kobayashi et al., 1984). In our hands, EuD was a mild "caffeine-like" agonist which 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 μM (Kobayashi et al., 1990). We found no effect of Pen (200 μM) on SR Ca^{2+} leak from skeletal 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 them the ability to modulate RyRs. In summary, our data suggest that MBED is the only agent which mimics the effects of caffeine on RyRs both quanti and qualitatively, acting with a 1000-fold higher potency.

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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 (Bruton et al., 2003; Francis et al., 2011; Ishiyama et al., 2008; Liu and Meissner, 1997; 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 the Ca^{2+} ATPase of sarcoplasmic reticulum (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 or no effect) and the previously reported activating effects of Pen on skeletal muscle SR Ca^{2+} release (Kobayashi et al., 1990). Early reports stated that MBED and its analog BED, 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, Ca^{2+}) 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

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skeletal muscle microsomes is ~99% inhibitable by the SERCA blocker cyclopiazonic acid (CPZ). The fact that MBED affects SERCA activity while caffeine does not, might also help explain reported differences between MBED (at 100 μ M levels) and caffeine (10 mM) on Ca^{2+} signaling in smooth muscle (Ohi et al., 2001).

Marine alkaloids as potential tools to study Intracellular Ca^{2+} signaling - Conclusions.

A myriad of marine organisms, including sponges incorporate bromide, which is relatively abundant in sea water, 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 biological activity (Gribble, 2012). This study revised the potential of two groups of alkaloids (indol and beta-carboline derivatives) to modulate two main elements of intracellular Ca^{2+} homeostasis in striated muscle: RyRs and SERCA.

Caffeine produces huge effects on the gating of RyRs, even in the cellular environment (Herrmann-Frank et al., 1999; Porta et al., 2011; Rousseau et al., 1988; Sitsapesan and Williams, 1990). 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 high P_o mode of gating (Fill and Copello, 2002; Zahradnikova et al., 1999; Zahradnikova and Zahradnik, 1995). Moreover, this knowledge could contribute to establishing possible links between the caffeine binding site(s) and RyR mutations

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associated with "leaky channels" and disease (Wei and Dirksen, 2010). As indicated, radiolabelled MBED has been previously utilized in binding experiments (Yoshikawa et al., 1995). However, we still need to develop MBED derivatives suitable for covalent photo-labeling of the RyR molecule and/or probes for fluorescence resonance energy transfer (FRET).

Cellular studies suggest that MBED can enter the cell and induce Ca^{2+} release from intracellular stores (Bruton et al., 2003; Ohi et al., 2001; Seino et al., 1991). Thus, MBED is also an appealing tool for functional studies as low doses of MBED (e.g., 100-200 nM) could be utilized 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, 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 (Lv et al., 2013; Neumann et al., 2011; 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 EC-coupling.

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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,
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UNNUMBERED FOOTNOTES TO THE TITLE

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Paula L. Diaz-Sylvester and Maura Porta have contributed equally to the work.

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FIGURE LEGENDS

FIGURE 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 $V_m = 0$ mV. Luminal Solution contained (in mM): $\text{Ca}(\text{OH})_2$ 50, HEPES 250 (pH =7.4). Cytosolic solution: Tris 100, 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 open probabilities (P_o) estimated from 4-minute recordings under control conditions ($[\text{Ca}^{2+}]_{\text{cyt}} \sim 1 \mu\text{M}$ 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 $P_o \pm$ SEM of RyR2 channels under control conditions (open bars), after subsequent addition of either 10 μM of MBED or 200 μM of the 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 SEM ($n=5$) 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 .

FIGURE 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 (grey 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 ± 48 nM (MBED) and 3.7 ± 0.2 mM

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(caffeine). For both agents, the activation was cooperative; Hill coefficient (n_H) were 1.8 ± 0.3 (MBED) and 2.1 ± 0.2 . **(B)** Mean open probability as a function of the concentration of cytosolic Ca^{2+} ($[Ca^{2+}]_{cyt}$) in the absence of other agonists (open circles) or in the presence of either 10 mM Caffeine (grey triangles; $n=8$) or 5 μ M MBED (black triangles; $n=5$). From the dose-response curves we estimated under control conditions EC_{50} for Ca^{2+} activation was $2.3 \pm 0.1 \mu$ M; $n_H = 2.7 \pm 0.2$. In caffeine, we estimated an $EC_{50} = 81 \pm 12$ nM and $n_H = 1.7 \pm 0.3$, which were not significantly different from those obtained with MBED, $EC_{50} = 93 \pm 6$ nM and $n_H = 2.1 \pm 0.2$. **(C)** Representative single-channel recordings of RyR2 activated by 10 μ M cytosolic Ca^{2+} (top traces), 10 mM Caffeine (middle panel) or 1 μ M 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 $\tau_1^o = 3.4 \pm 0.9$ ms ($48 \pm 5\%$) and $\tau_2^o = 42.2 \pm 7.7$ ms ($52 \pm 5\%$). $\tau_1^c = 0.6 \pm 0.1$ ms ($85 \pm 3\%$) and $\tau_2^c = 2.8 \pm 0.5$ ms ($15 \pm 3\%$). From the analysis of $n=14$ caffeine-activated channels we estimated $\tau_1^o = 18.4 \pm 5.9$ ms* ($23 \pm 3\%*$) and $\tau_2^o = 321 \pm 84$ ms* ($76 \pm 3\%*$), $\tau_1^c = 1.9 \pm 0.5$ ms* ($47 \pm 4\%*$) and $\tau_2^c = 177 \pm 42$ ms* ($53 \pm 4\%*$). With MBED average values ($n=10$ experiments) were $\tau_1^o = 11.3 \pm 3.7$ ms* ($34 \pm 7\%*$) and $\tau_2^o = 336 \pm 84$ ms* ($66 \pm 7\%*$), $\tau_1^c = 3.8 \pm 0.6$ ms* ($33 \pm 5\%*$) and $\tau_2^c = 159 \pm 39$ ms* ($67 \pm 5\%*$). (*the parameters estimated for caffeine-activated RyR2 were significantly different than those of channels activated by Ca^{2+} , with $p < 0.05$ or better).

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FIGURE 3. Effect of addition of cytosolic Mg²⁺ and Ca²⁺ 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 ATP/Mg) and after sequential addition of 5 μM MBED, 1mM Mg²⁺ and 2 μM Ca²⁺ to the cytosolic surface of the channel. The averaged open probabilities of 5 experiments is shown in (B). (C) Dose-response curve of skeletal muscle RyR1 channels exposed to increasing concentrations of MBED (n=7 experiments). The EC₅₀ for MBED was $480 \pm 25 \text{ nM}$; $n_H = 1.3 \pm 0.1$.

FIGURE 4. Lack of effect of Pen on skeletal muscle RyR1 channels. (A) Single-channel recordings of a skeletal muscle RyR1 under control conditions ($[Ca^{2+}]_{cyt} = 0.1 \mu M$; 1 mM total ATP and 1 mM free Mg²⁺) and after addition of 20 μM Pen. The averaged open probabilities of 8 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 exception of MBED (10 μM) and caffeine (20 mM), all other agents were used at 200 μM . Data are means \pm SEM of n=5 determinations.

FIGURE 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 pre-loaded 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 cyclopyazonic acid (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

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indicated drugs are shown in **(B)**. **(C)** Examples of Ca^{2+} 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 (grey 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 Ca^{2+} leak rate (EC_{50}) were 844 ± 88 nM (MBED) and 2.4 ± 0.3 mM (caffeine); n_H were 1.3 ± 01 and $1.6 \pm 0,2$, respectively.

FIGURE 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 (grey) 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 (grey) and cardiac (black) microsomes exposed to the indicated EuD or Pen derivatives. With exception of MBED (10 μM) and caffeine (20 mM), all other agents were used at 200 μM .

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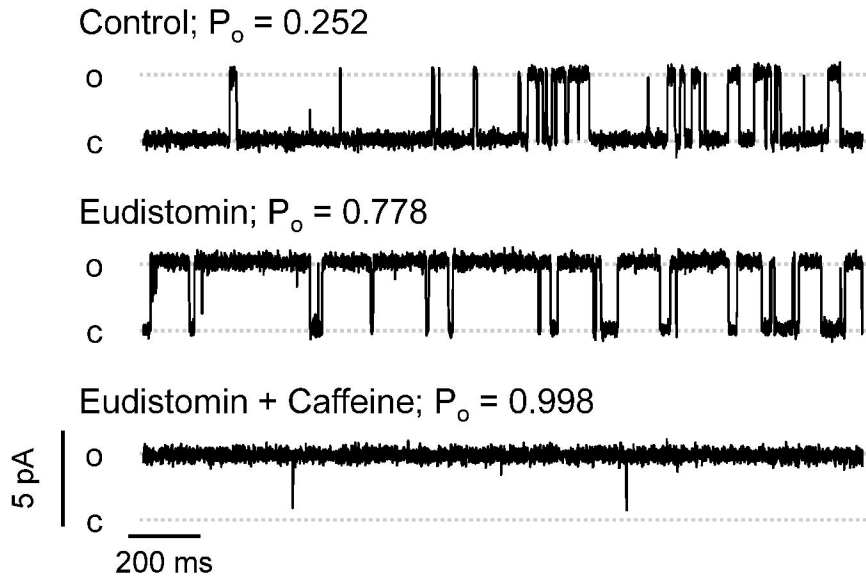
TABLE 1: Effect of EuD and Pen derivatives on RyR function and ATPase activity.

Columns indicate EuD or Pen derivative name, percentage of change in channel activity, SR Ca²⁺ 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 Ca²⁺ release, which was only determined in skeletal microsomes. Dashes represent no effect and N/A not tested.

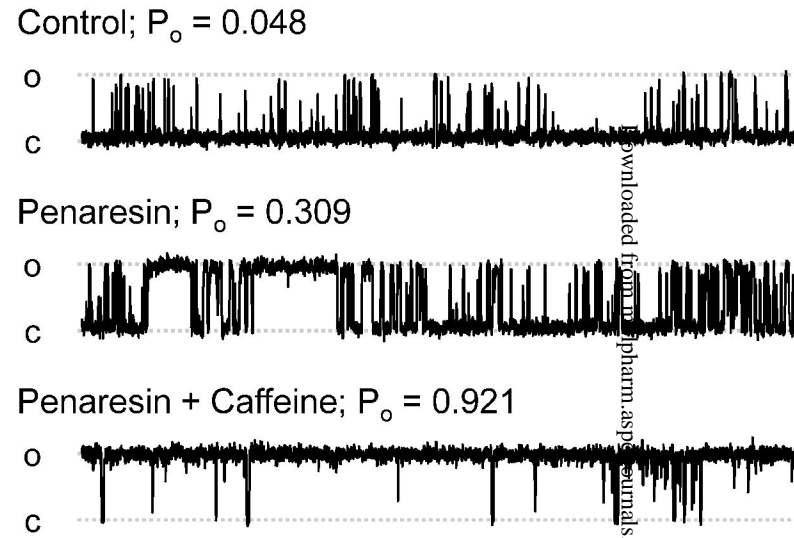
Drug	Skeletal Muscle				Cardiac Muscle		
	RyR1 activity	Release	Ryanodine binding	ATPase activity	RyR2 activity	Ryanodine binding	ATPase activity
Caffeine	↑ (100%)	↑(100%)	↑ (100%)	--	↑ (100%)	↑ (100%)	--
EuD	N/A	↑ (22%)	↑ (39 %)	↓ (-49%)	↑ (86%)	↑ (41%)	↓ (-54%)
MBED	↑ (100%)	↑ (118%)	↑ (118%)	↓ (-24%)	↑ (105%)	↑ (97%)	↓ (-18%)
Pen	--	--	↑ (17 %)	↓ (-61%)	↑ (20%)	--	↓ (-36%)
PenCN	N/A	--	--	↓ (-17%)	--	--	↓ (-24%)
MPenM	N/A	--	--	↓ (-14%)	↑ (53%)	--	↓ (-18%)
MpenNO	N/A	--	--	--	--	--	--
PenM	N/A	--	--	N/A	--	--	N/A

Figure 1

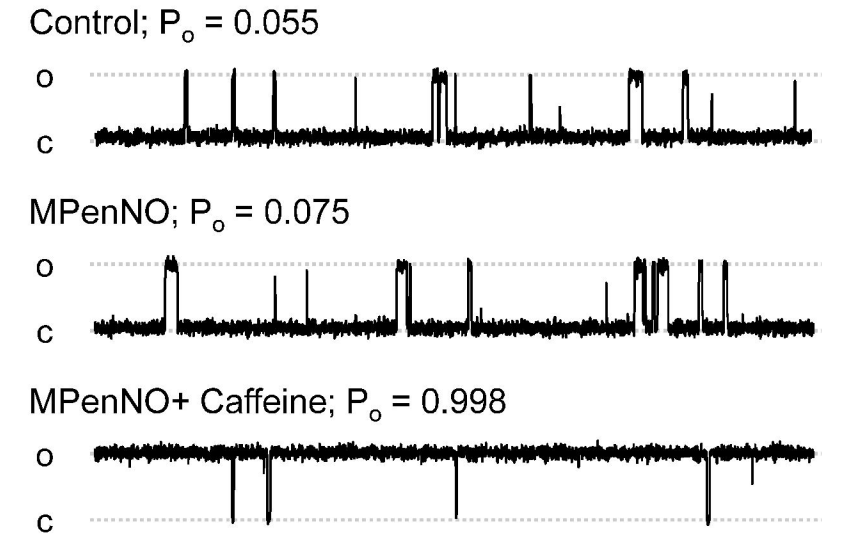
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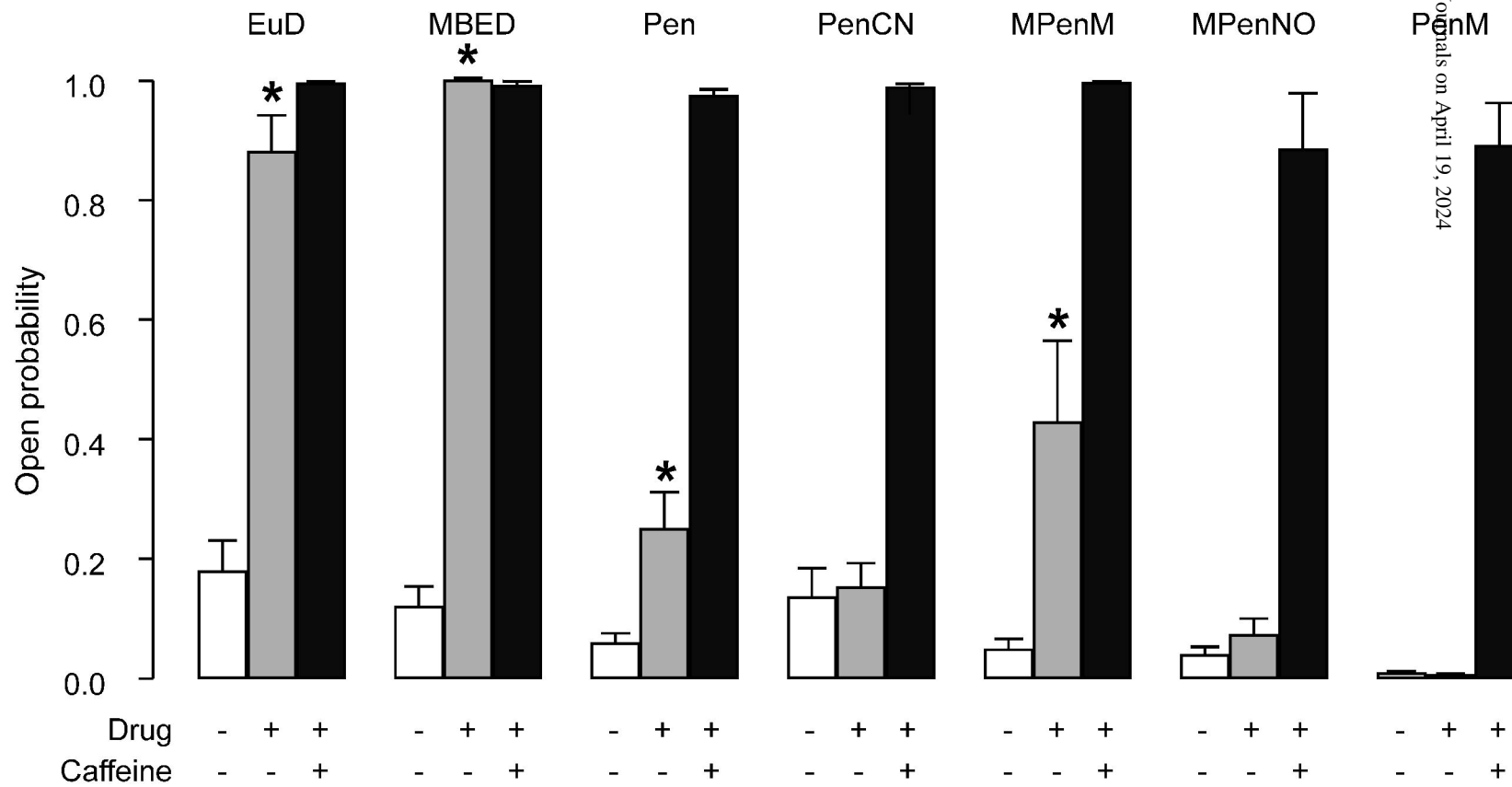
(B)



(C)



(D)



(E)

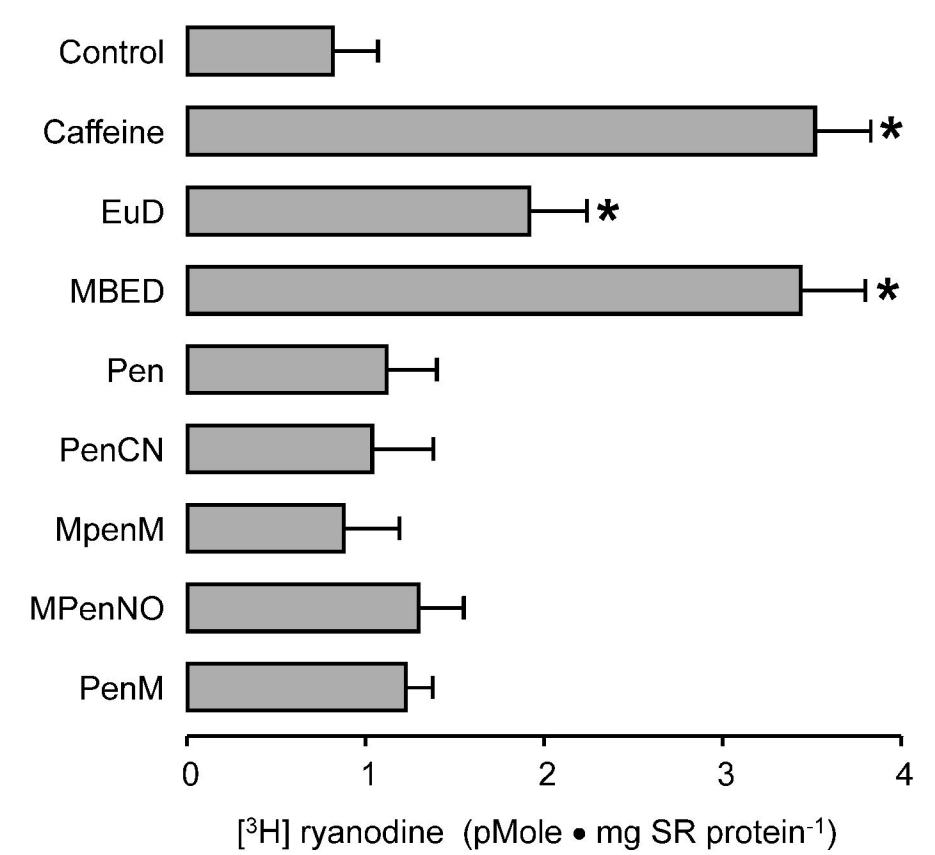
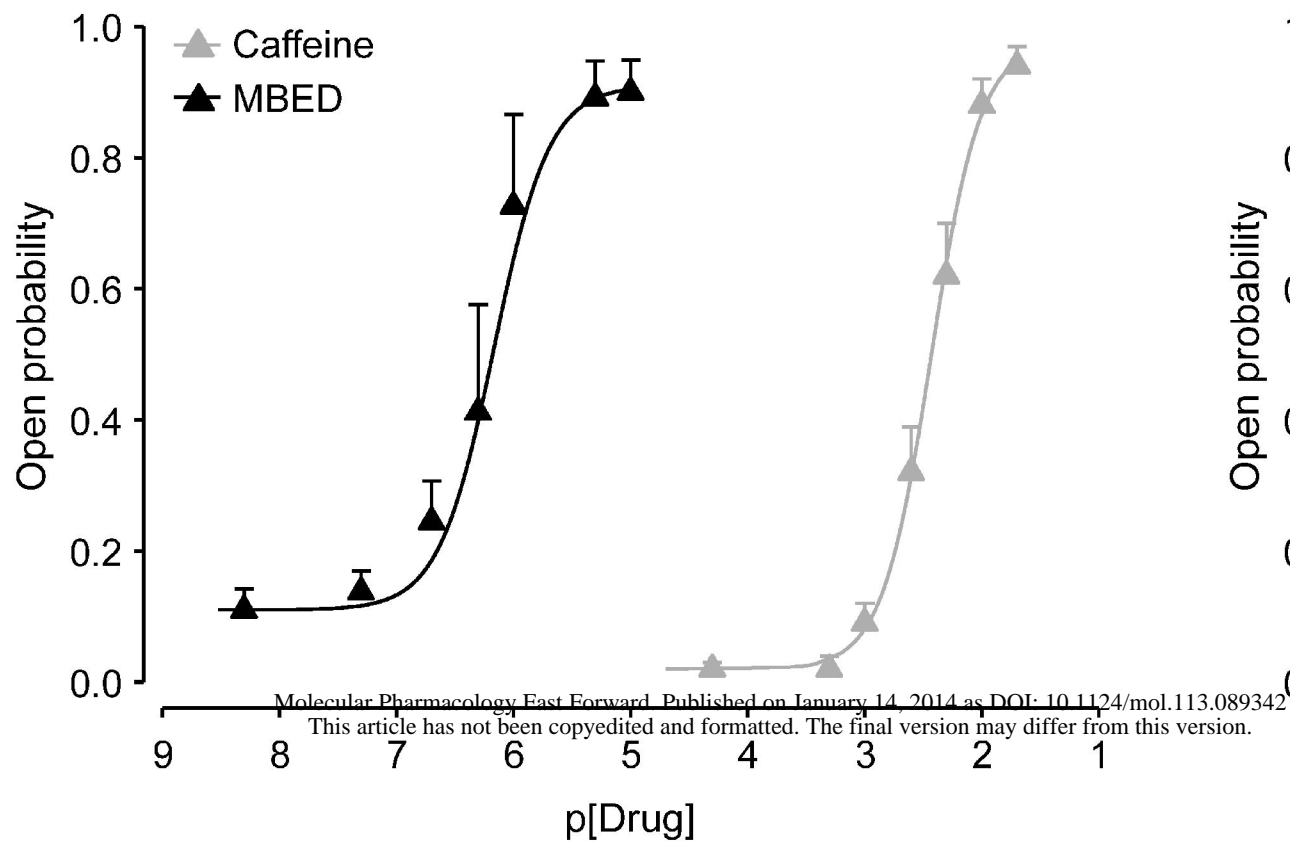
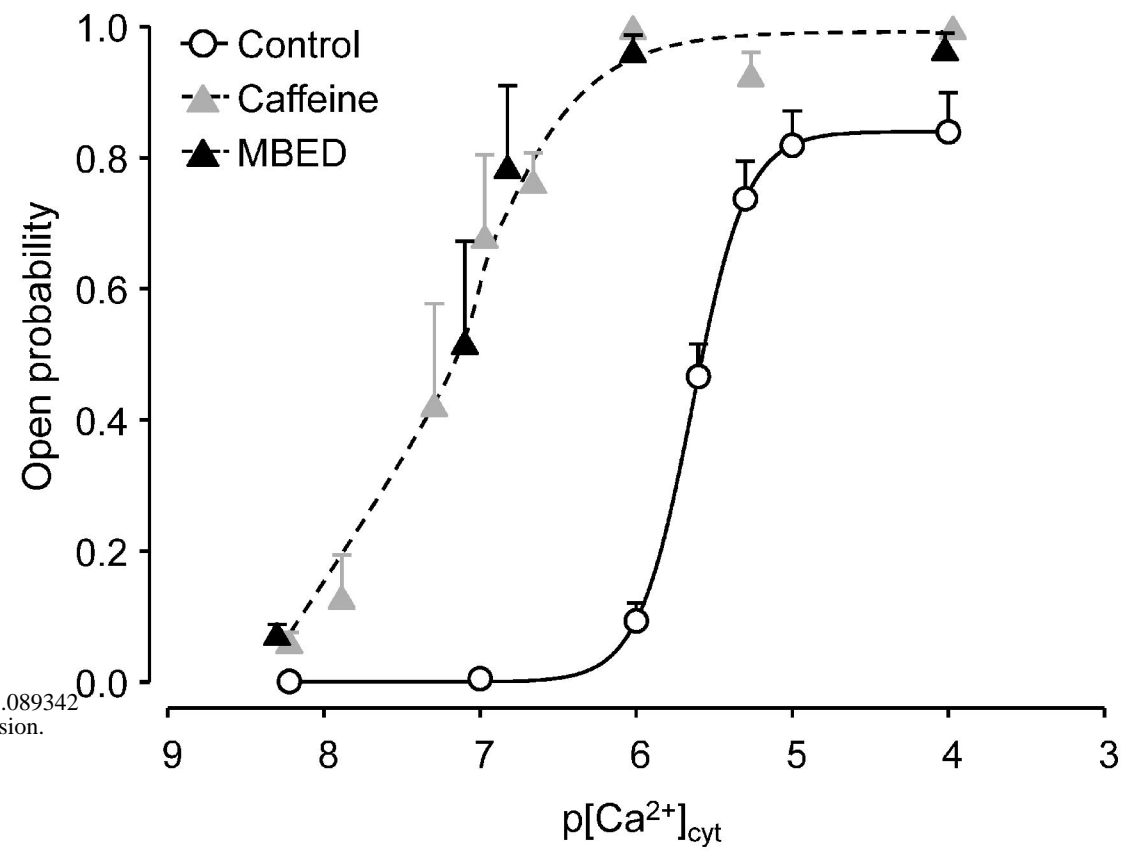


Figure 2

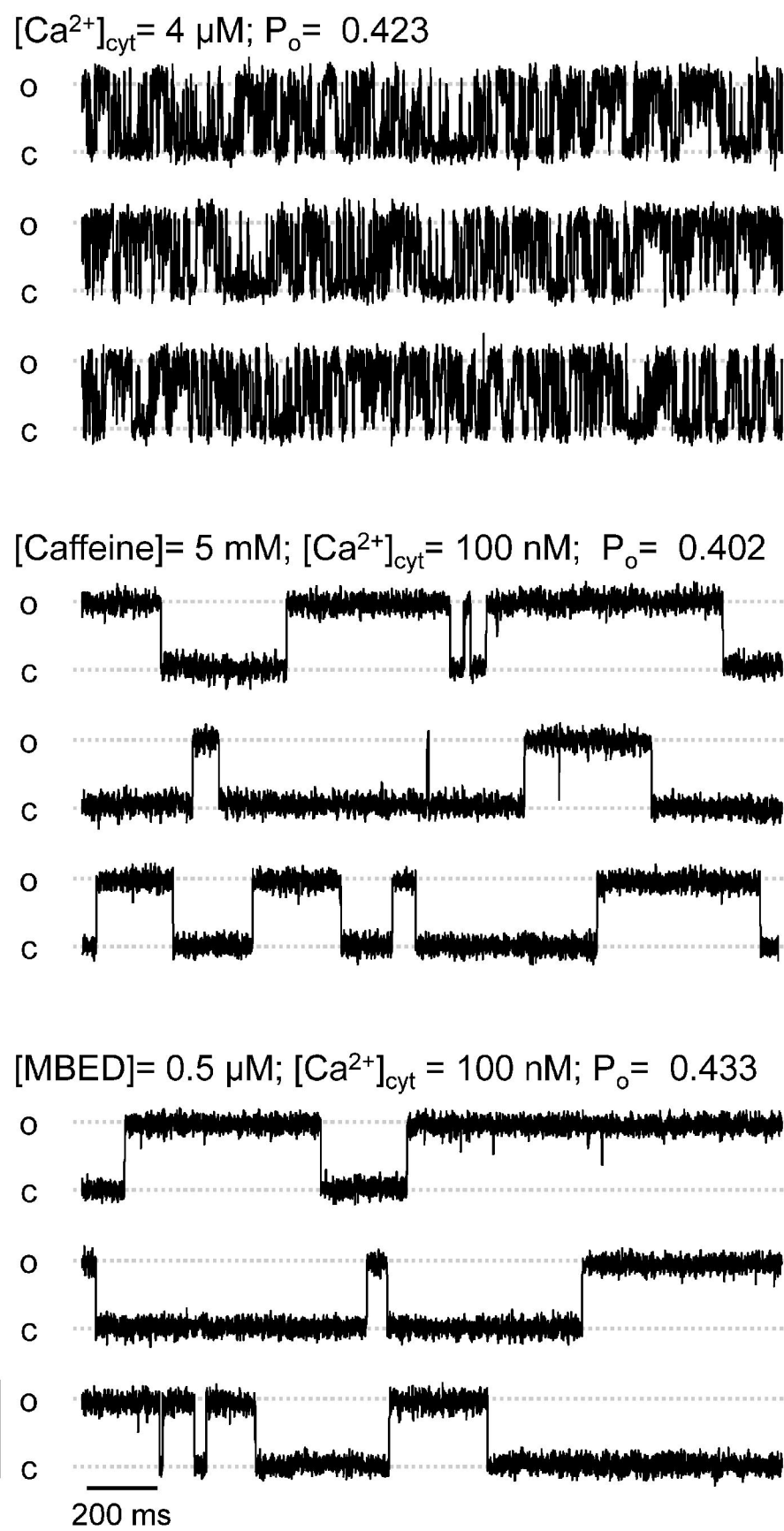
(A)



(B)



(C)



(D)

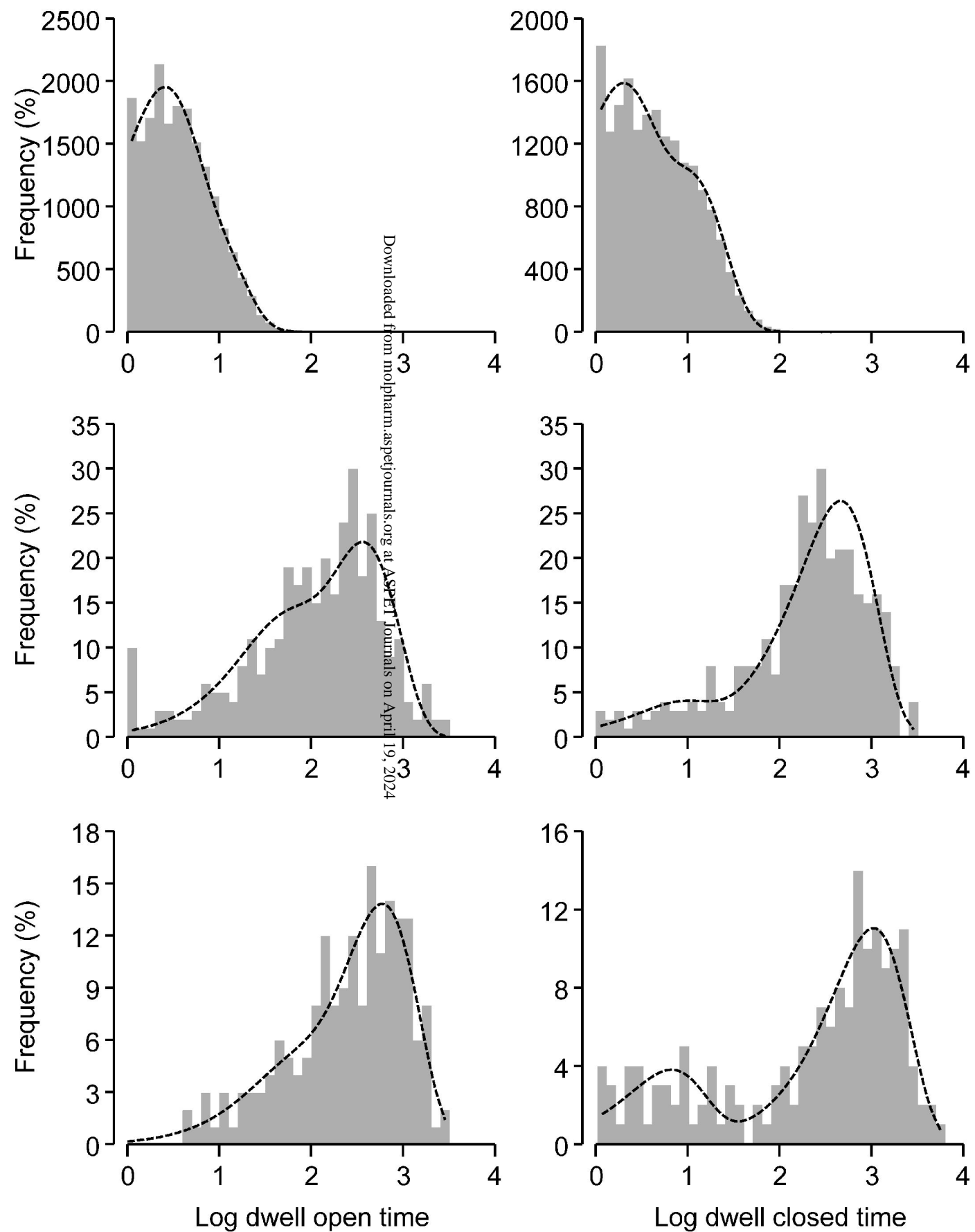
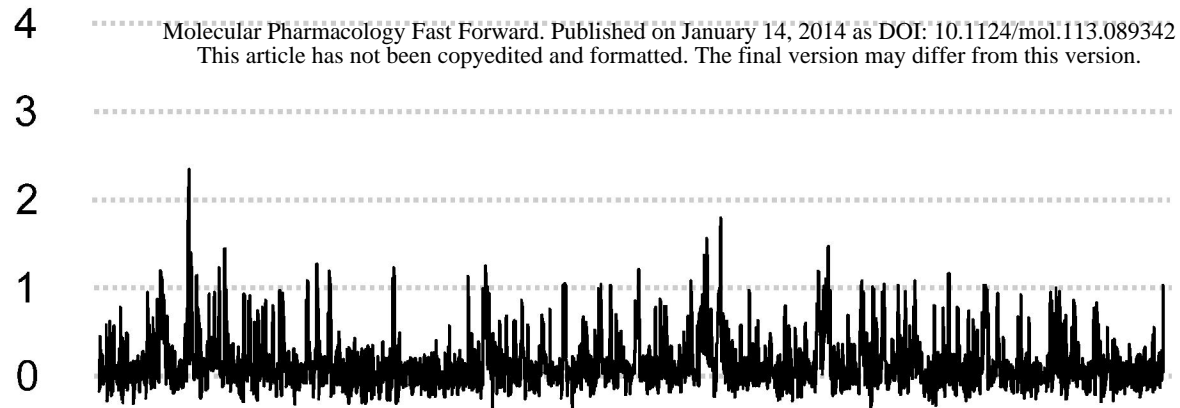


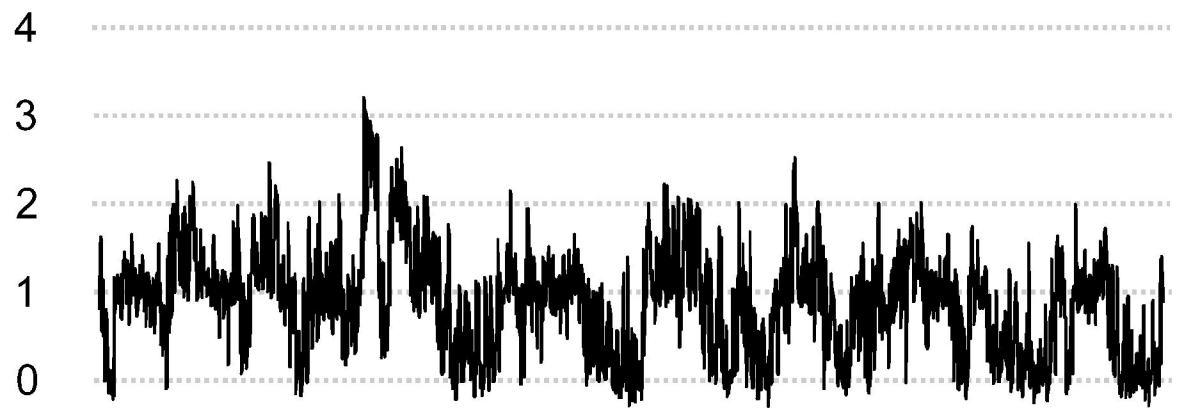
Figure 3

(A)

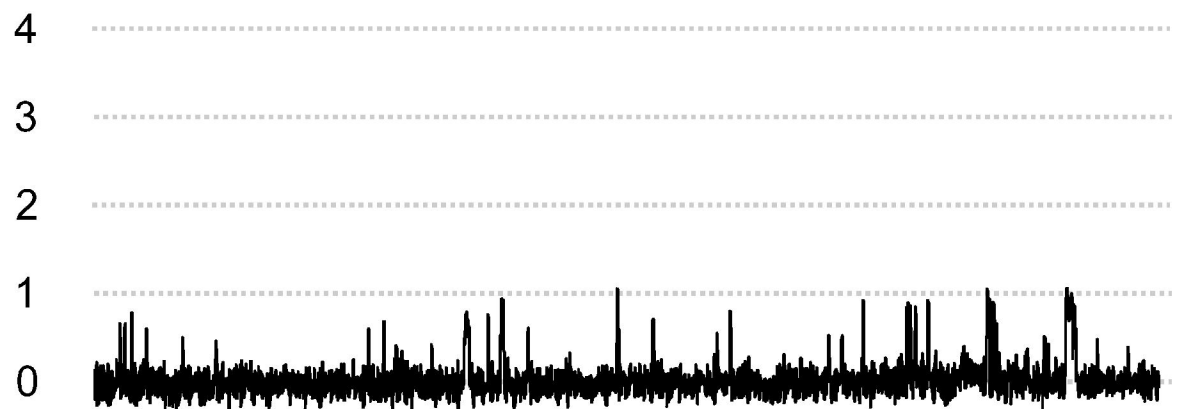
$[Ca^{2+}] = 0.1 \mu M$; $P_o = 0.072$



$[MBED] = 5 \mu M$; $[Ca^{2+}] = 0.1 \mu M$; $P_o = 0.243$



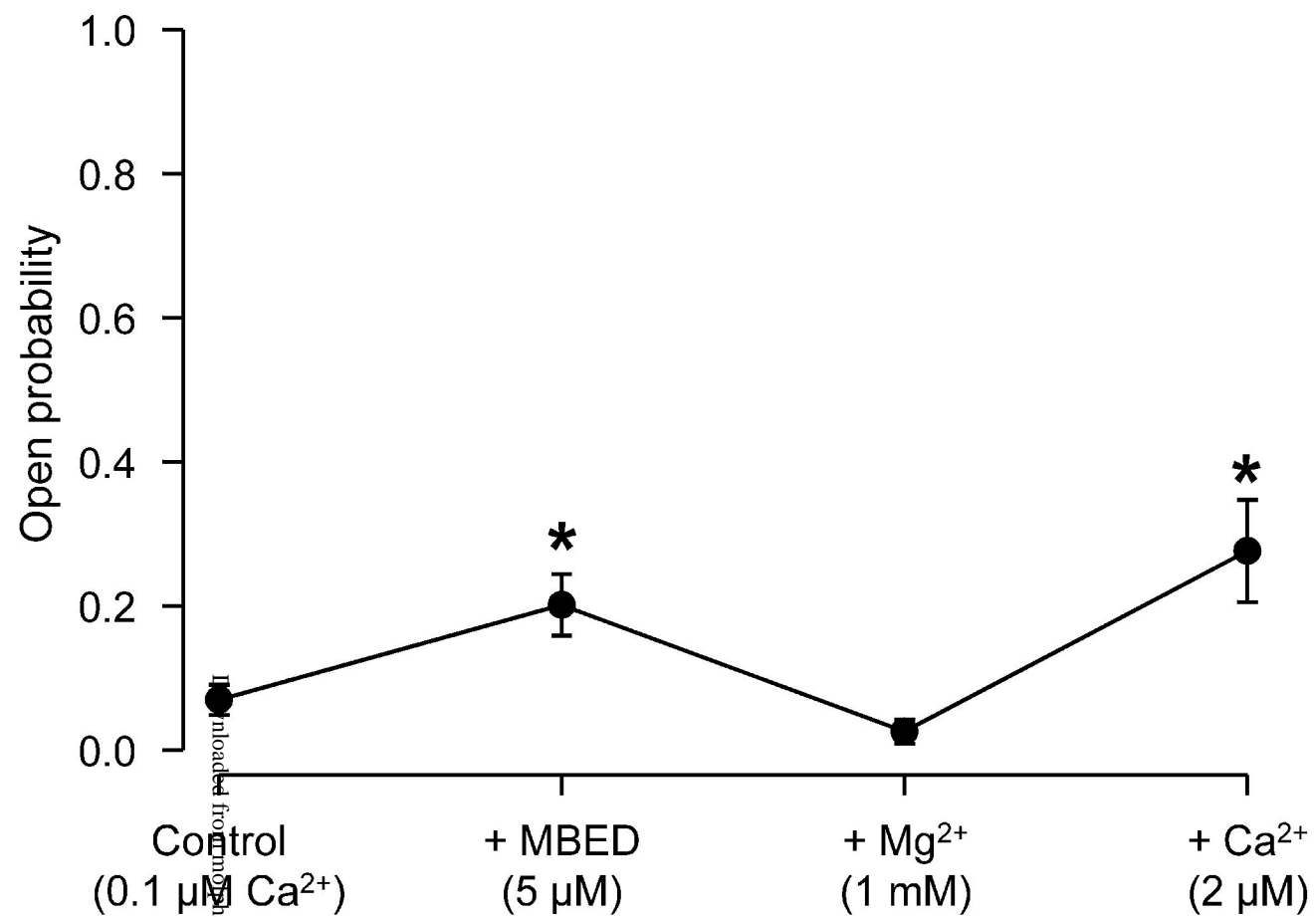
$[MBED] = 5 \mu M$; $[Mg^{2+}] = 1 \text{ mM}$; $[Ca^{2+}] = 0.1 \mu M$; $P_o = 0.005$



$[MBED] = 5 \mu M$; $[Mg^{2+}] = 1 \text{ mM}$; $[Ca^{2+}] = 2 \mu M$; $P_o = 0.437$



(B)



(C)

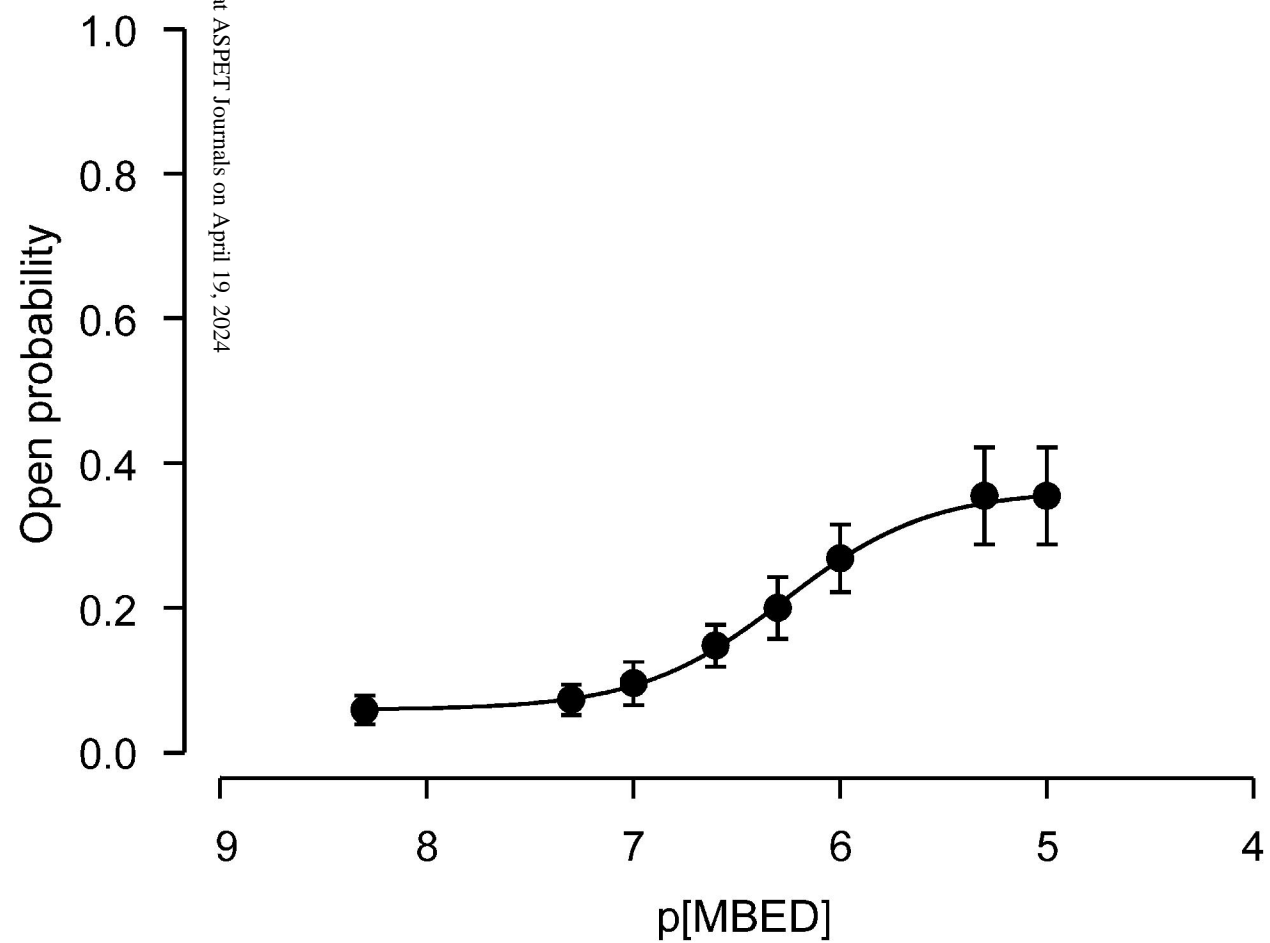
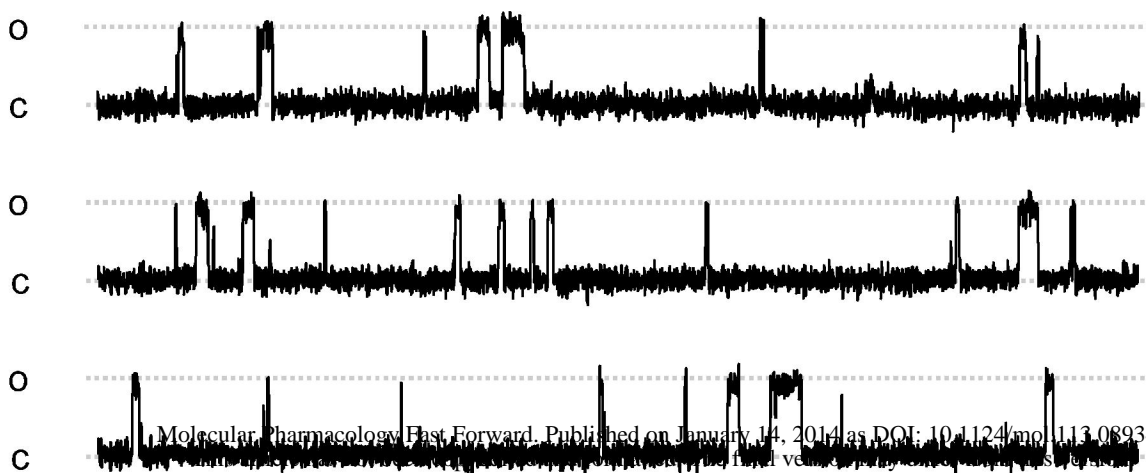


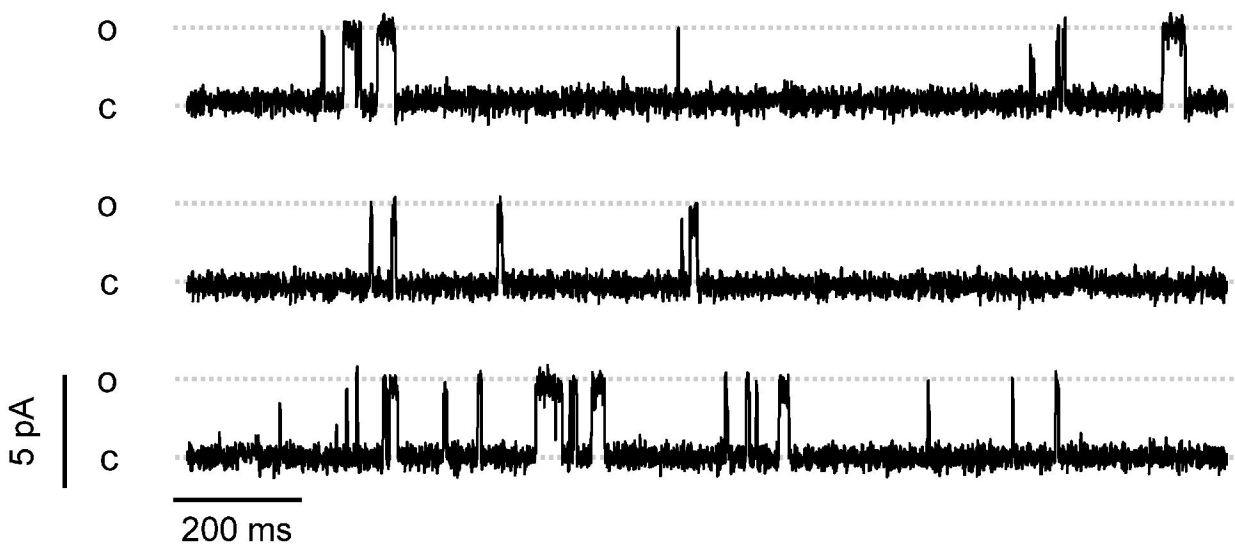
Figure 4

(A)

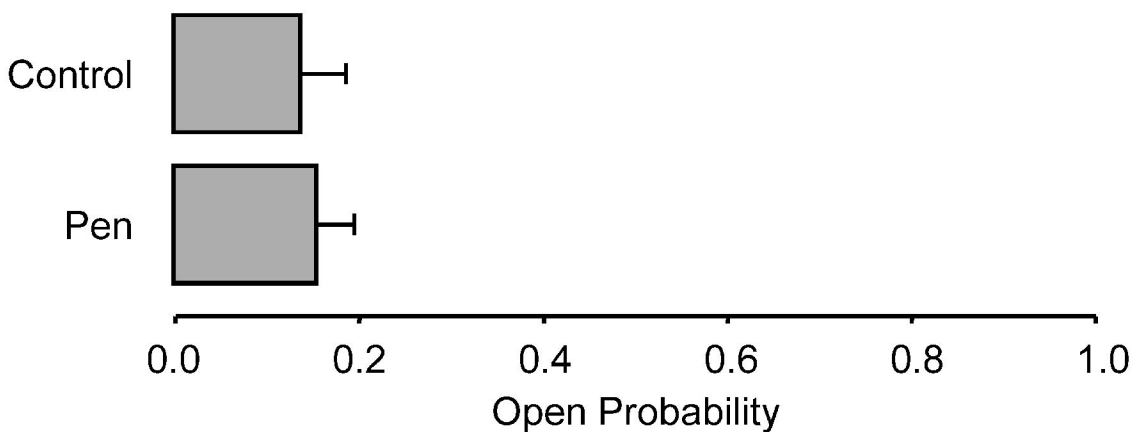
Control; $P_o = 0.116$



Penaresin; $P_o = 0.128$



(B)



(C)

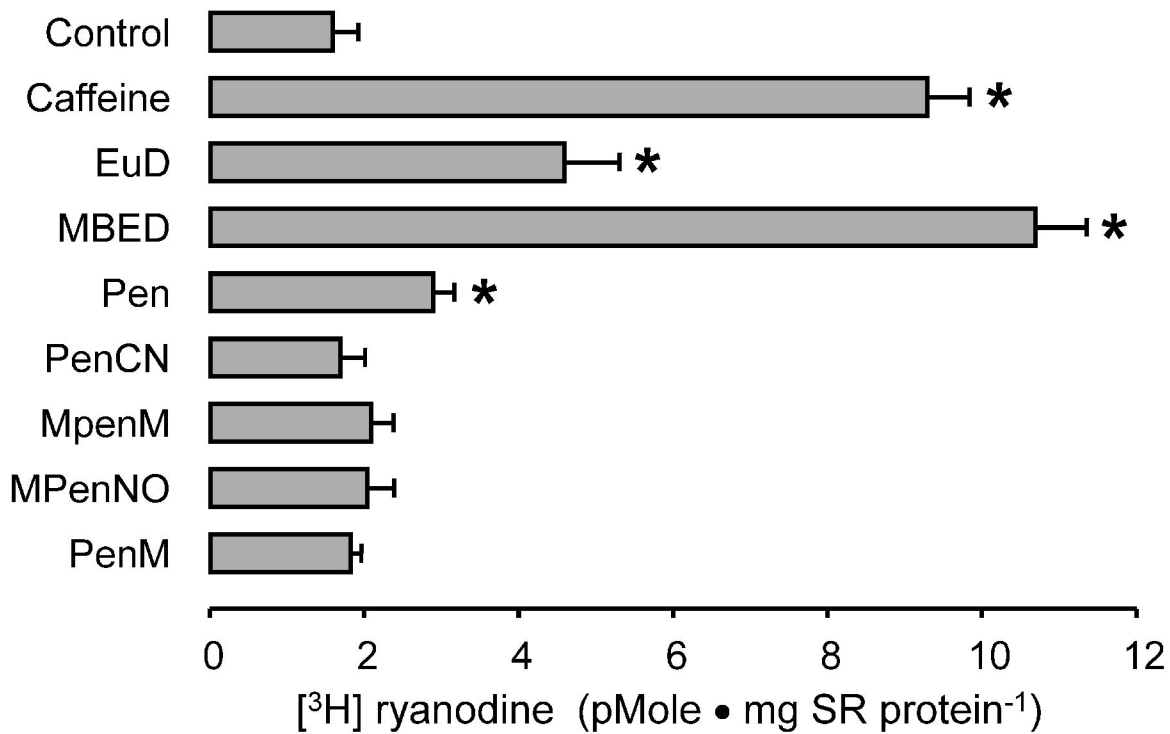
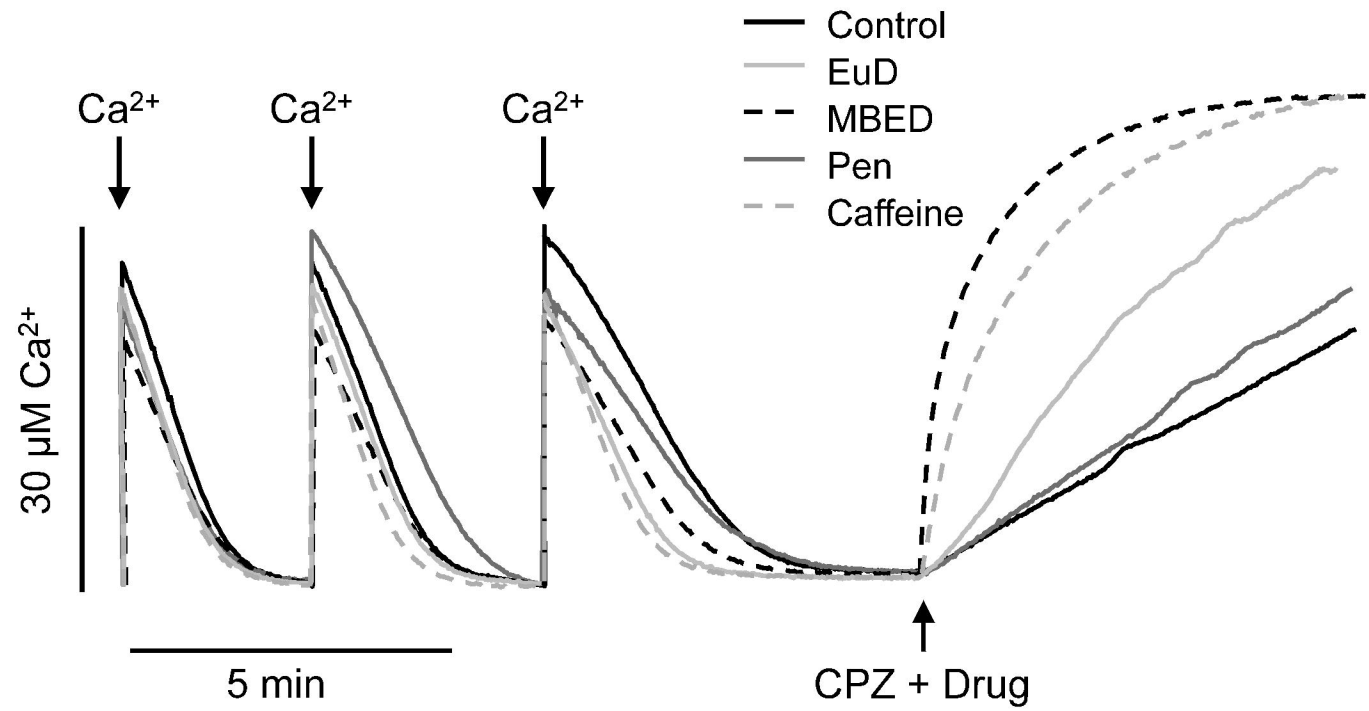
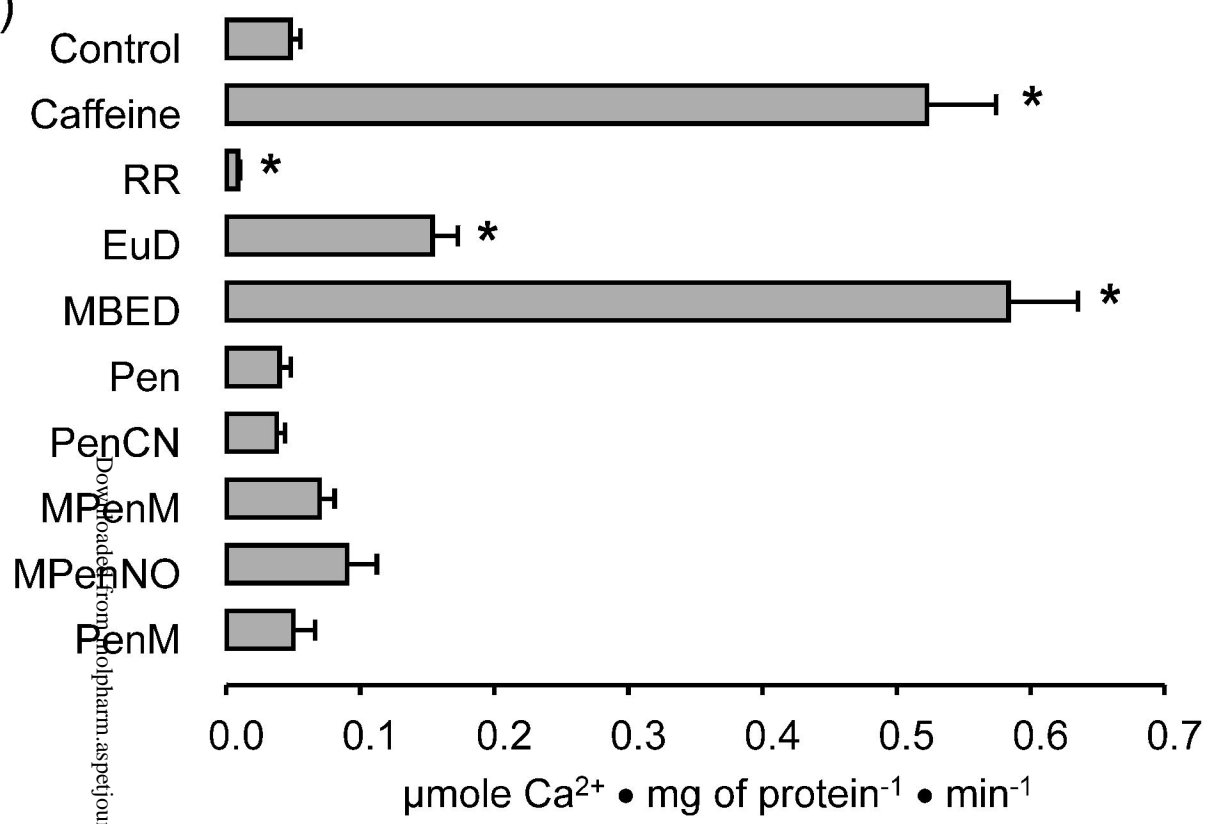


Figure 5

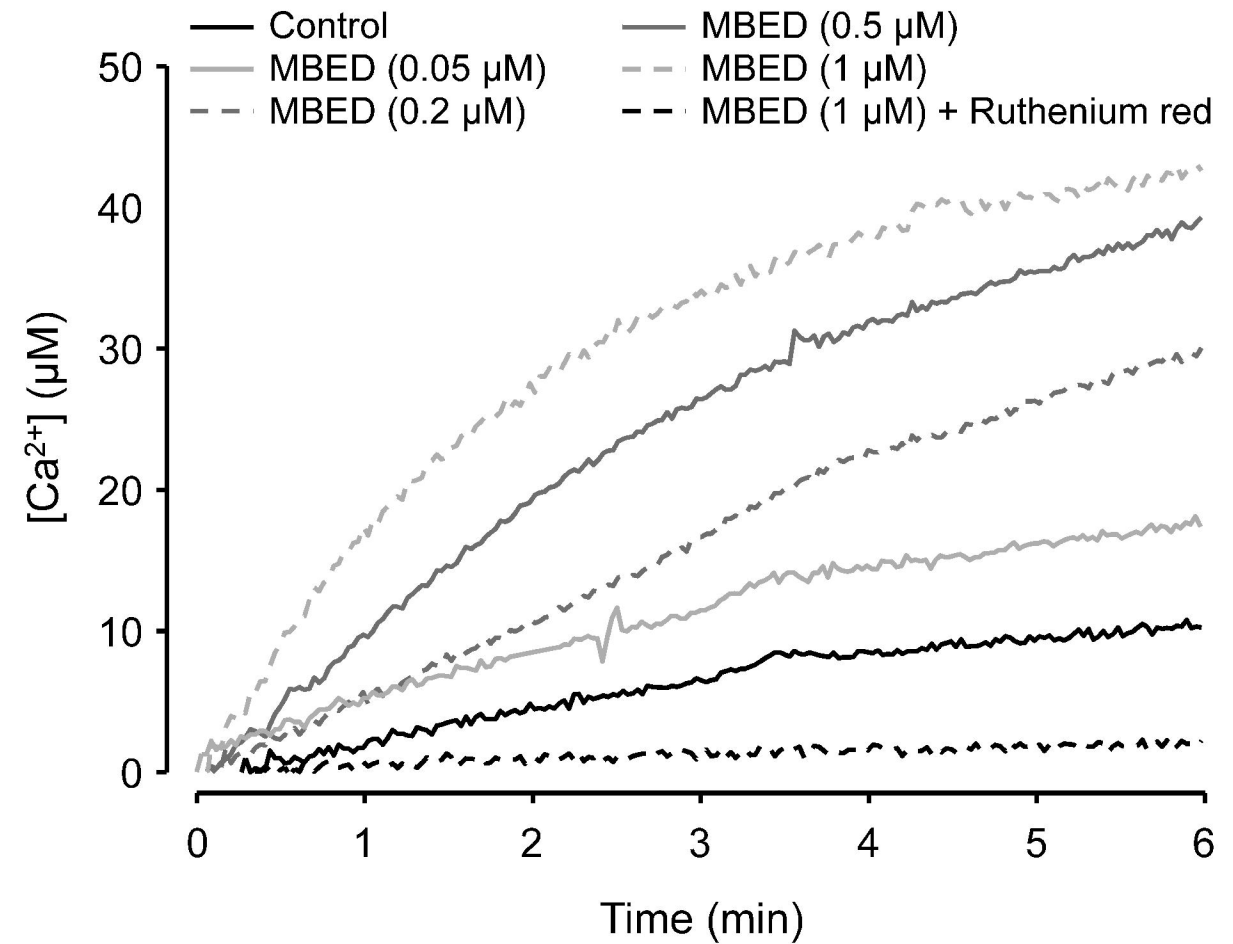
(A)



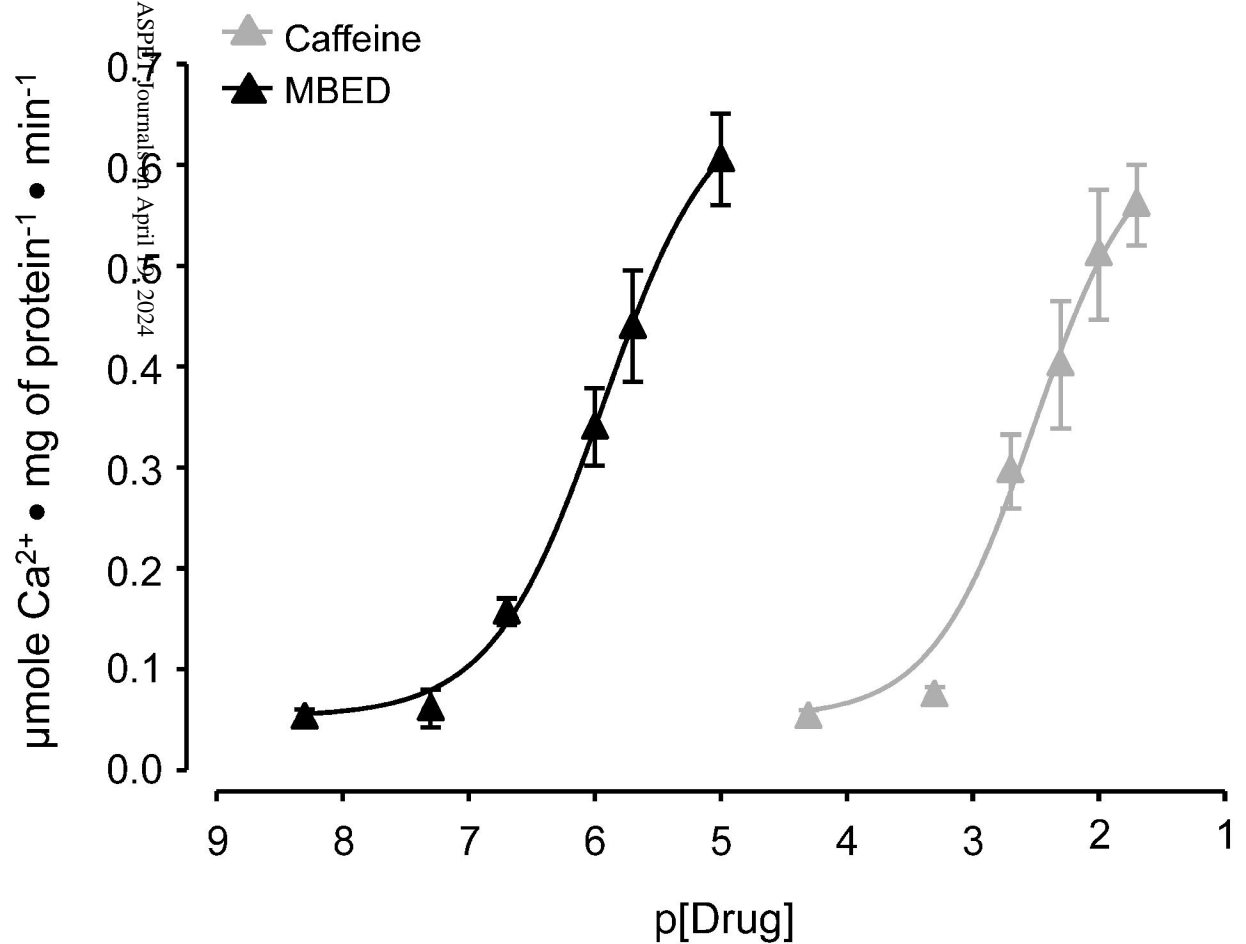
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(C)



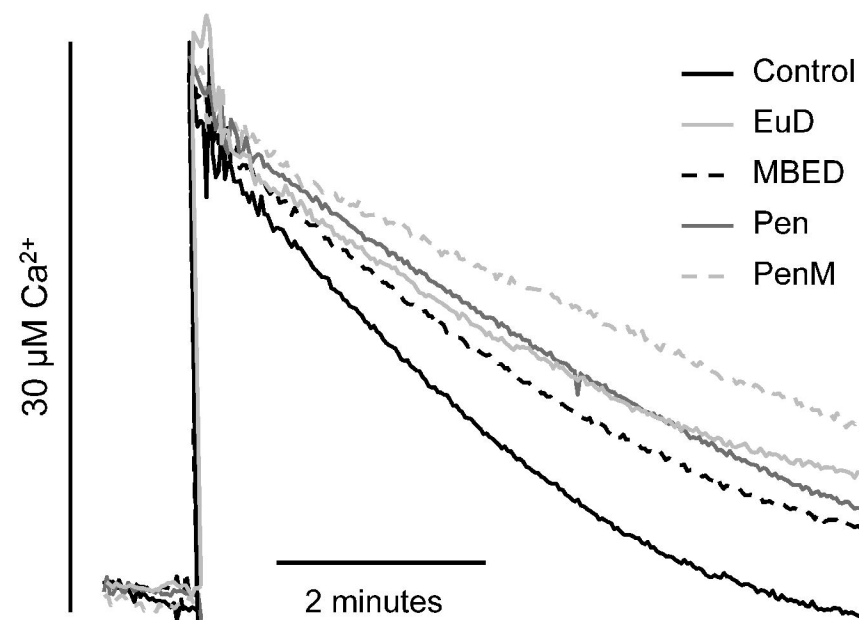
(D)



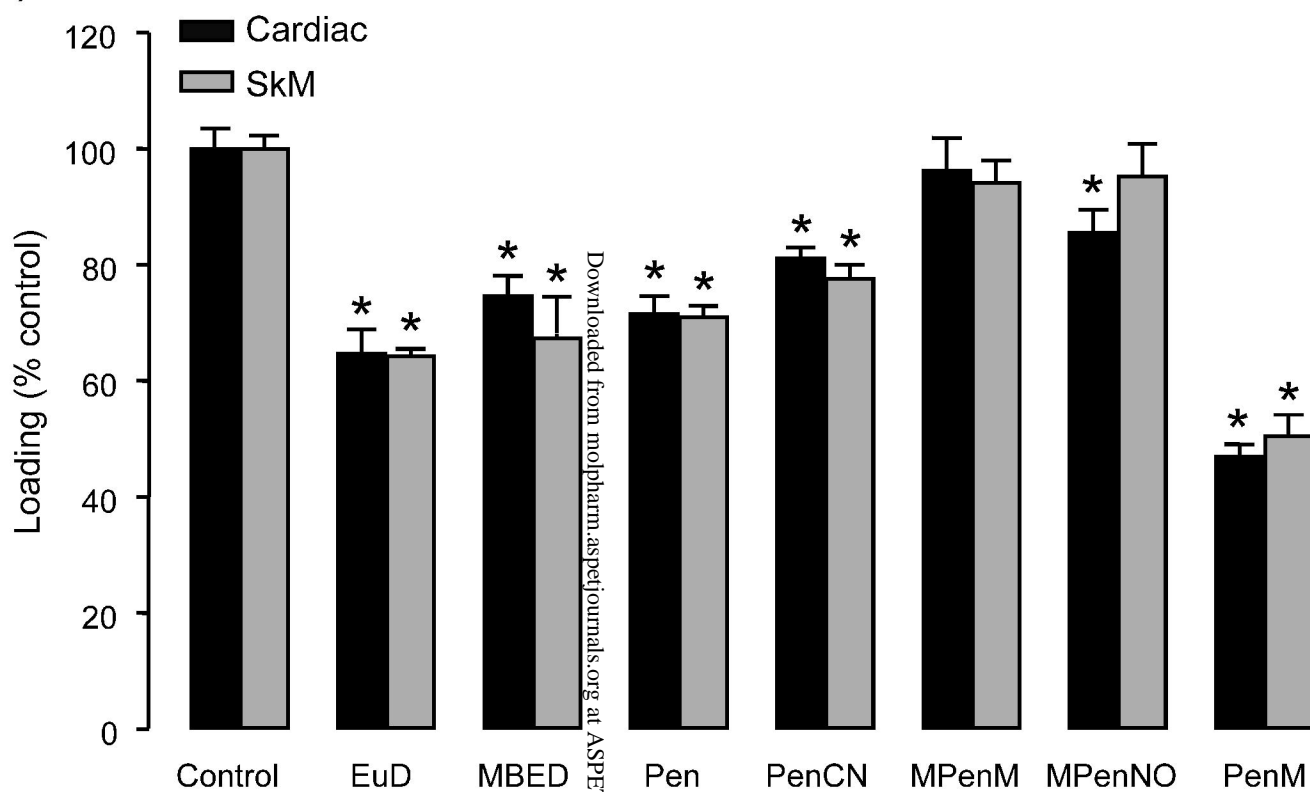
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Figure 6

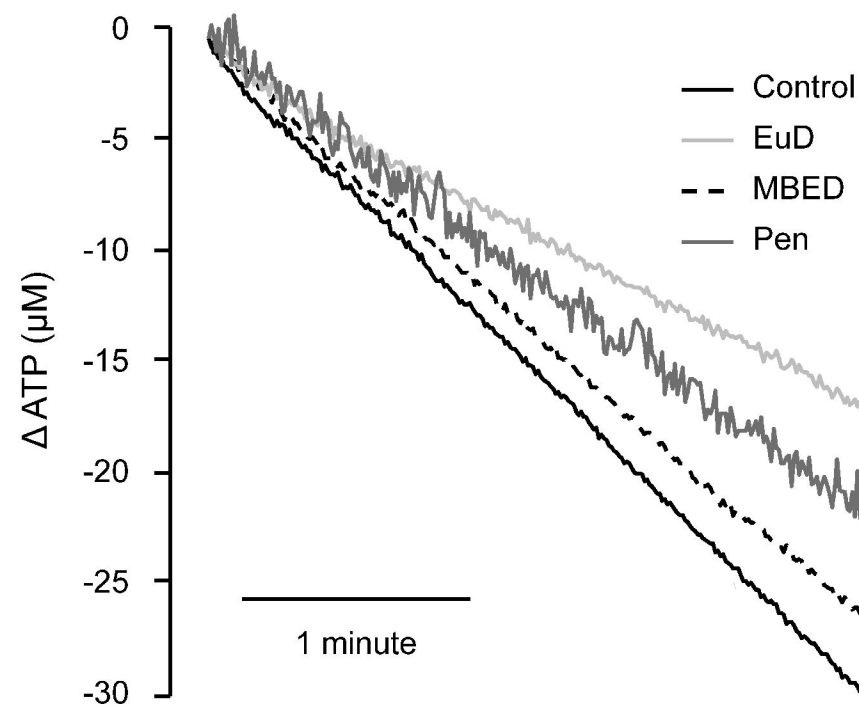
(A)



(B)



(C)



(D)

