J. Membrane Biol. 169, 189–198 (1999)

The Journal of
Membrane
Biology

Effects of 2,3-Butanedione 2-Monoxime on Ca^{2+} Release Channels (Ryanodine Receptors) of Cardiac and Skeletal Muscle

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Received: 31 December 1998/Revised: 9 March 1999

Abstract. Single channel and [^{3}H]ryanodine binding measurements were performed to test for a direct functional interaction between 2,3-butanedione 2-monoxime (BDM) and the skeletal and cardiac muscle sarcoplasmic reticulum Ca^{2+} release channels (ryanodine receptors). Single channel measurements were carried out in symmetric 0.25 M KCl media using the planar lipid bilayer method. BDM (1–10 mM) activated suboptimally Ca^{2+}-activated (0.5–1 μM free Ca^{2+}) single, purified and native cardiac and skeletal release channels in a concentration-dependent manner by increasing the number of channel events without a change of single channel conductances. BDM activated the two channel isoforms when added to either side of the bilayer. At a maximally activating cytosolic Ca^{2+} concentration of 20 μM, BDM was without effect on the cardiac channel, whereas it inhibited skeletal channel activities with $IC_{50} \approx 2.5$ mM. In agreement with single channel measurements, high-affinity [^{3}H]ryanodine binding to the two channel isoforms was increased in a concentration-dependent manner at ≤1 μM Ca^{2+}. BDM was without a noticeable effect at low (≤0.01 μM) Ca^{2+} concentrations. At 20 μM Ca^{2+}, BDM inhibited the skeletal but not cardiac channel. These results suggest that BDM regulates the Ca^{2+} release channels from the sarcoplasmic reticulum of skeletal and cardiac muscle in a concentration, Ca^{2+} and tissue-dependent manner.

Key words: Ca^{2+} release channel — Ryanodine receptor — Sarcoplasmic reticulum — 2,3-butanedione 2-monoxime — Skeletal muscle — Cardiac muscle

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Abbreviations: BDM, 2,3-butanedione 2-monoxime; RyR1, skeletal muscle Ca^{2+} release channel; RyR2, cardiac muscle Ca^{2+} release channel; SR, sarcoplasmic reticulum; P_o, channel open probability.

Introduction

2,3-Butanedione 2-monoxime (BDM) is an effective inhibitor of skeletal muscle and cardiac muscle contraction (for review *see* Sellin and McArdle, 1994). BDM has been suggested to reduce force in cardiac and skeletal muscle by exerting a direct effect on the contractile apparatus and/or by affecting sarcoplasmic reticulum (SR) Ca^{2+} handling (Fryer, Neering & Stephenson, 1988; Maylie & Hui, 1991; Kagawa, Horiuti & Yamada, 1995; Barth et al., 1996; Watanabe, Tomoike & Endoh, 1996; Ebus & Stienen, 1996; Adams, Traffold & Eisner, 1998; Parsons et al., 1997). In fast- and slow-twitch rat skeletal muscle fibers, BDM concentrations of <2 mM appeared to reduce force output by solely reducing the magnitude of the Ca^{2+} transient, whereas higher BDM concentrations also affected the number of cross bridges and decreased the sensitivity of the myofibrils to Ca^{2+} (Fryer et al., 1988). In moderately stretched frog twitch fibers, BDM had a greater effect on twitch tension than the peak amplitude of Ca^{2+} release, which suggested that at low concentrations (a few millimolar) BDM may be a useful inhibitor of the contractile system (Maylie & Hui, 1991). In isolated papillary muscles, BDM at concentrations of <5 mM reduced twitch tension without significantly affecting the size of intracellular Ca^{2+} transients (Blanchard et al., 1990, Watanabe et al., 1996). Other studies, however, have indicated that BDM at a concentration as low as 3 mM either reduced the amounts of Ca^{2+} available for release (Gwathmey, Hajjar & Solaro, 1991) or caused SR Ca^{2+} release from permeabilized (Brotto et al., 1995; Phillips & Altschuld, 1996) and intact cardiac myocytes (Adams et al., 1998). In lobster muscle, 1–3 mM BDM potentiated SR Ca^{2+} release with little effect on force generation (Gyorke, Dettbarn & Palade, 1993). In addition to its effect on the contractile apparatus and the SR, BDM may affect other cellular functions including action potential amplitude and duration, and gap

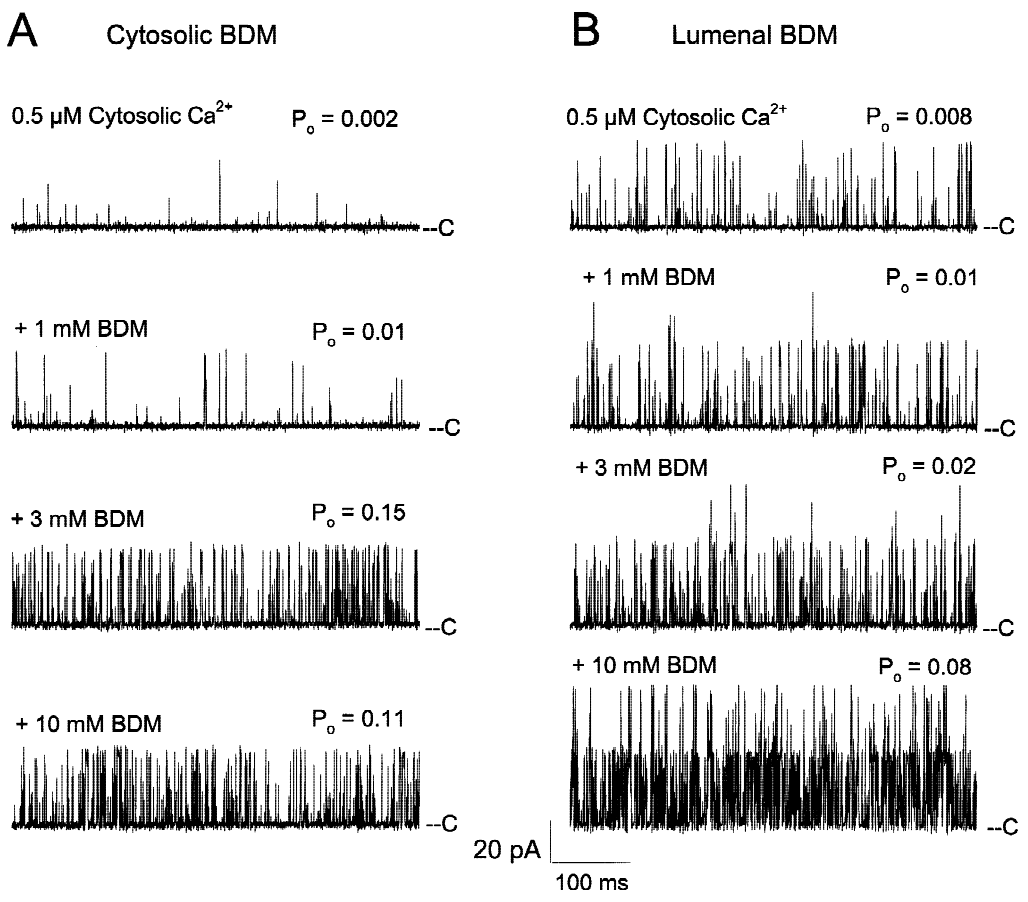

Fig. 1. Activation of skeletal Ca^{2+} release channel by cytosolic (*A*) and lumenal (*B*) BDM at 0.5 μM cytosolic free Ca^{2+}. Channel currents, shown as upward deflections from closed levels (c on right), of one (*A*) and two (*B*) purified RyR1s were recorded at 40 mV in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4 media containing 0.5 μM cytosolic free Ca^{2+} and indicated concentrations of cytosolic (cis) (*A*) or lumenal (trans) (*B*) BDM.

junction and L-type Ca^{2+} currents (Sellin & McArdle, 1994; Verrecchia & Herve, 1997; Eisfeld et al., 1997; Ferreira et al., 1997).

Here, we show that BDM affects SR Ca^{2+} release by modulating the activity of the skeletal muscle and cardiac muscle sarcoplasmic reticulum Ca^{2+} release channels/ryanodine receptors (RyRs). The effects of BDM on single, purified and native skeletal muscle (RyR1) and cardiac muscle (RyR2) RyRs were determined, using the planar lipid bilayer method. The effects of BDM were also investigated in [^{3}H]ryanodine binding measurements. Our results indicate that BDM activates RyR2 at low Ca^{2+} concentrations. A more complex interaction was observed for RyR1 in that BDM could both activate and inactivate the skeletal Ca^{2+} release channel, depending on Ca^{2+} concentration.

Materials and Methods

Preparation of SR Vesicles and Purification and Reconstitution of Ca^{2+} Release Channels

Rabbit skeletal and canine cardiac muscle SR vesicle fractions enriched in [^{3}H]ryanodine binding and Ca^{2+} release activities were prepared in the presence of protease inhibitors (100 nM aprotinin, 1 μM leupeptin, 1 μM pepstatin, 1 mM benzamidine, 0.2 mM phenylmethylsulfonyl fluoride) as described (Meissner, 1984; Meissner & Henderson, 1987). The CHAPS (3-[3-cholamido-propyl)dimethylammonio]-1-propanesulfonate)-solubilized skeletal and cardiac muscle 30 S channel complexes were purified and then reconstituted into proteoliposomes by removal of CHAPS by dialysis (Lee, Xu & Meissner, 1994).

Single Channel Measurements

Single channel measurements were performed by fusing proteoliposomes containing the purified RyRs or SR vesicles with Mueller-Rudin type bilayers containing phosphatidylethanolamine, phosphatidylserine and phosphatidylcholine in the ratio 5:3:2 (25 mg of total phospholipid per ml n-decane) (Tripathy et al., 1998). The side of the bilayer to which the proteoliposomes were added was defined as the cis side. The trans side of the bilayer was defined as ground. Purified channels were recorded in a symmetric KCl buffer solution (0.25 M KCl, 20 mM KHepes, pH 7.4). Channels incorporated into the bilayers using SR vesicles were recorded in symmetric 0.25 M Cs methanesulfonate, 10 mM CsHepes, pH 7.4 solutions. Both recording solutions contained the additions indicated in the text. Electrical signals were filtered at 2–4 kHz, digitized at 10–20 kHz, and analyzed as described (Tripathy et al., 1998). P_o values in multichannel recordings were calculated according to the equation $P_o = \Sigma_{i=1}^{N} iP_i/N$ where N was the total number of channels and P_i their channel open probability. Data acquisition and

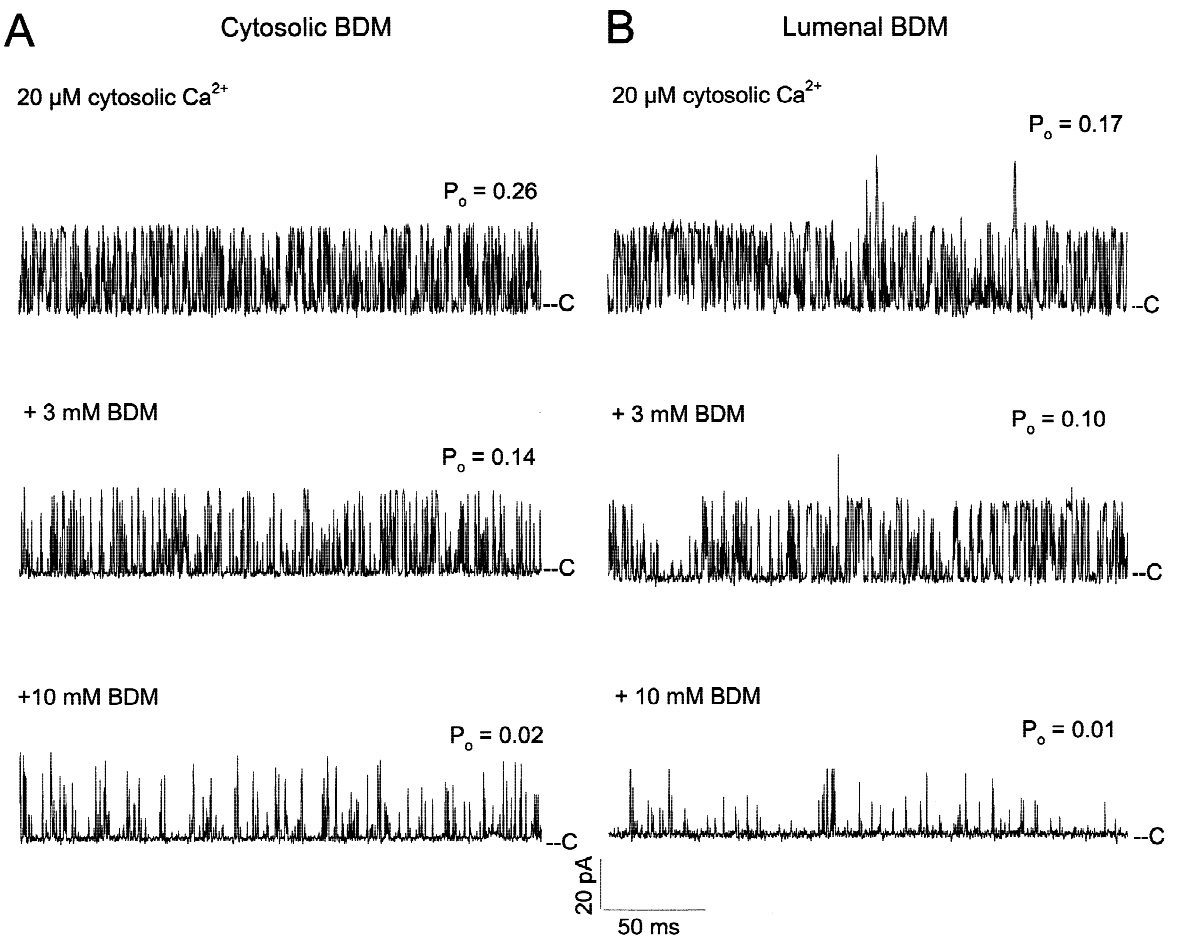

Fig. 2. Inhibition of skeletal muscle Ca^{2+} release channel by cytosolic (*A*) and lumenal (*B*) BDM at 20 μM cytosolic free Ca^{2+}. Channel currents, shown as upward deflections from closed levels (c on right), of one (*A*) and two (*B*) purified RyR1s were recorded at 40 mV as in Fig. 1 in the presence of 20 μM cytosolic-free Ca^{2+}, and indicated concentrations of cytosolic (cis) (*A*) or lumenal (trans) (*B*) BDM.

analysis were performed with a commercially available software package (pClamp 6.0.3, Axon Instruments, Burlingame, CA) using an IBM compatible pentium computer and 12 bit A/D–D/A converter (Digidata 1200, Axon Instruments).

[^{3}H]Ryanodine Binding Measurements

Skeletal and cardiac muscle SR vesicles were incubated for 20 hr at 24°C in media that contained 20 mM imidazole, pH 7, 0.25 M KCl, 0.2 mM Pefabloc SC, 20 μM leupeptin, 1 nM [^{3}H]ryanodine, and the indicated BDM and free Ca^{2+} concentrations. Nonspecific [^{3}H]ryanodine binding was determined using a 1000-fold excess of unlabeled ryanodine. Unbound [^{3}H]ryanodine was separated from protein-bound [^{3}H]ryanodine by filtration of sample aliquots through Whatman GF/B filters presoaked with 2% polyethyleneimine, followed by washing with three 5 ml volumes of ice-cold 0.1 M KCl, 1 mM KPipes, pH 7.0 medium. The radioactivity remaining with the filters was determined by liquid scintillation counting to obtain bound [^{3}H]ryanodine.

Other Assays and Data Analysis

Free Ca^{2+} concentrations were obtained by including in the assay media 100 μM ethylene glycol-bis(β-aminoethyl ether)N,N,N′,N′-tetraacetic acid (or 0.5 mM 1,2-bis(2-aminophenoxy) ethanetetraacetic acid and 1 mM nitriloacetic acid) and the appropriate amounts of $CaCl_2$. Ca^{2+} concentrations of >1 μM were confirmed with a Ca^{2+} selective electrode (World Precision Instruments, Inc., Sarasota, FL). Free Ca^{2+} concentrations of <1 μM were obtained using the stability constants and the mixed solution program published by Schoenmakers et al. (1992). BDM (5 mM) lowered the free Ca^{2+} concentration approximately 2-fold using a Ca^{2+} electrode. However, this change was considered to represent an artifact because it occurred regardless of Ca^{2+} concentration, Ca^{2+} buffer or Ca^{2+} buffer concentration, and was not observed using the Ca^{2+} indicator dye antipyrylazo III.

Results are given as means ± SE. The SE is included within the figure symbols or indicated by error bars if it is larger. Unless otherwise indicated, significance of differences of data was analyzed with Student's *t*-test. Differences were regarded to be statistically significant at $P < 0.05$.

Materials

[^{3}H]Ryanodine was purchased from Dupont NEN (Boston, MA). Unlabeled ryanodine was obtained from Calbiochem (San Diego, CA), and BDM from Sigma (St. Louis, MO). Ryanodine and BDM were prepared as concentrated stock solutions in 0.25 M KCl, 20 mM KHepes, pH 7.4 before their use. All other chemicals were of analytical grade.

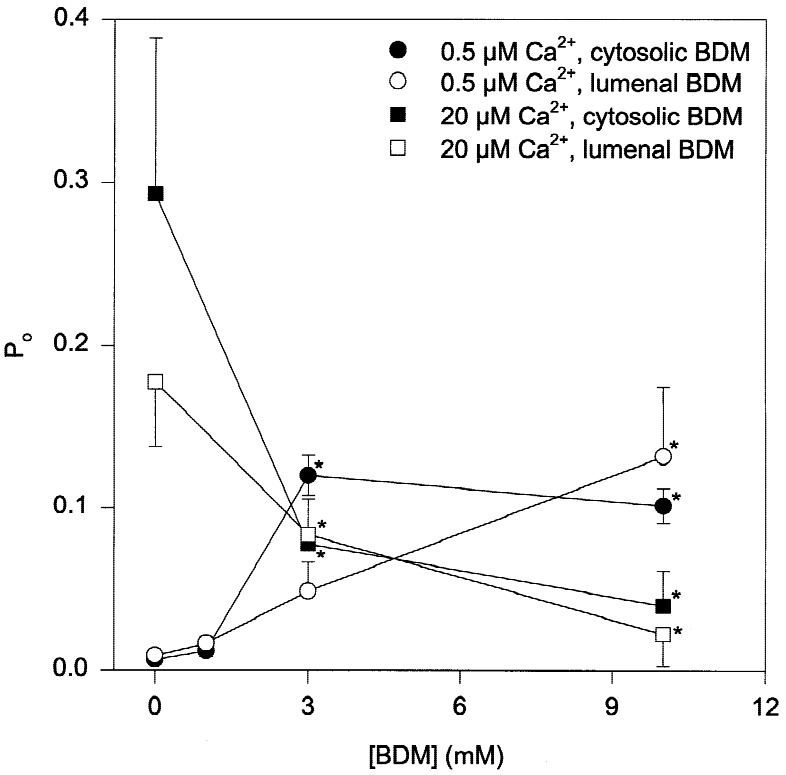

Fig. 3. Concentration dependence of effects of BDM on channel open probability (P_o) of skeletal Ca^{2+} release channel. Channel activities were recorded as in Fig. 1 at a holding potential of 40 mV in the presence of the indicated concentrations of cytosolic Ca^{2+} and cytosolic or lumenal BDM. Data are the mean ± SE of 4–8 experiments. *Significantly different from control (−BDM).

Results

Purified skeletal (RyR1) and cardiac (RyR2) muscle Ca^{2+} release channels reconstituted into proteoliposomes were fused with planar lipid bilayers and recorded with 250 mM K^+ as the current carrier. Single channel conductance was ~770 pS (Tripathy et al., 1998). In agreement with previous single channel measurements, a strong dependence of single channel activities on "cis" Ca^{2+} and ATP suggests that the large cytosolic region (Franzini-Armstrong & Protasi, 1997) of the channels in a majority of our recordings (>98%) faced the side of the bilayer to which the proteoliposomes were added. Channels that could not be activated by ~1–10 μM free cis (cytosolic) Ca^{2+} were discarded. In the single channel recordings described below the effects of BDM on RyR1 and RyR2 were examined at a suboptimally and optimally activating Ca^{2+} concentration of 0.5–1.0 and 20 μM, respectively (Coronado et al., 1994; Meissner, 1994).

Effects of BDM on Single Skeletal Muscle Ca^{2+} Release Channels

In Fig. 1*A* (top trace), a single RyR1 was recorded in the presence of 0.5 μM free cytoslic Ca^{2+} at a holding potential of 40 mV. Infrequent, often not fully resolved channel openings were observed. At 0.5 μM cytosolic Ca^{2+}, the addition of 1 and 3 mM cytosolic BDM increased channel open probability (P_o) from 0.002 to 0.01 and 0.15, respectively (first to third trace of Fig. 1*A*). A further increase in cis BDM to 10 mM was without a significant effect (bottom trace of Fig. 1*A*). Similar results were obtained at negative (*not shown*) and positive holding potentials in Fig. 1*A* and in figures to follow, which suggests that the effects of BDM were not dependent on membrane potential. Fig. 1*B* shows that at 0.5 μM cytosolic Ca^{2+}, the skeletal release channel could be also activated by lumenal BDM. In the recording of Fig. 1*B*, two release channels were present. Single channel open probability increased progressively from 0.008 to 0.08 as lumenal BDM concentration was raised from 0 to 10 mM.

The effects of cytosolic and lumenal BDM on RyR1 were also evaluated at a maximally activating cytosolic Ca^{2+} concentration of 20 μM (Fig. 2). Control P_o was 0.26. Addition of cytosolic BDM decreased P_o of RyR1 by ~45 and 90% at 3 and 10 mM, respectively (Fig. 2*A*). Lumenal BDM was similarly effective in inhibiting RyR1. P_o of two purified skeletal release channels decreased by ~40 and 95% in the presence of 3 and 10 mM BDM, respectively (Fig. 2*B*). The effects observed with the purified RyR1s could also be obtained with RyR1s incorporated into the bilayers using SR vesicles (*not shown*). At 0.5 μM Ca^{2+}, P_o increased approximately 10-fold after the addition of 5 mM cytosolic BDM. At 20

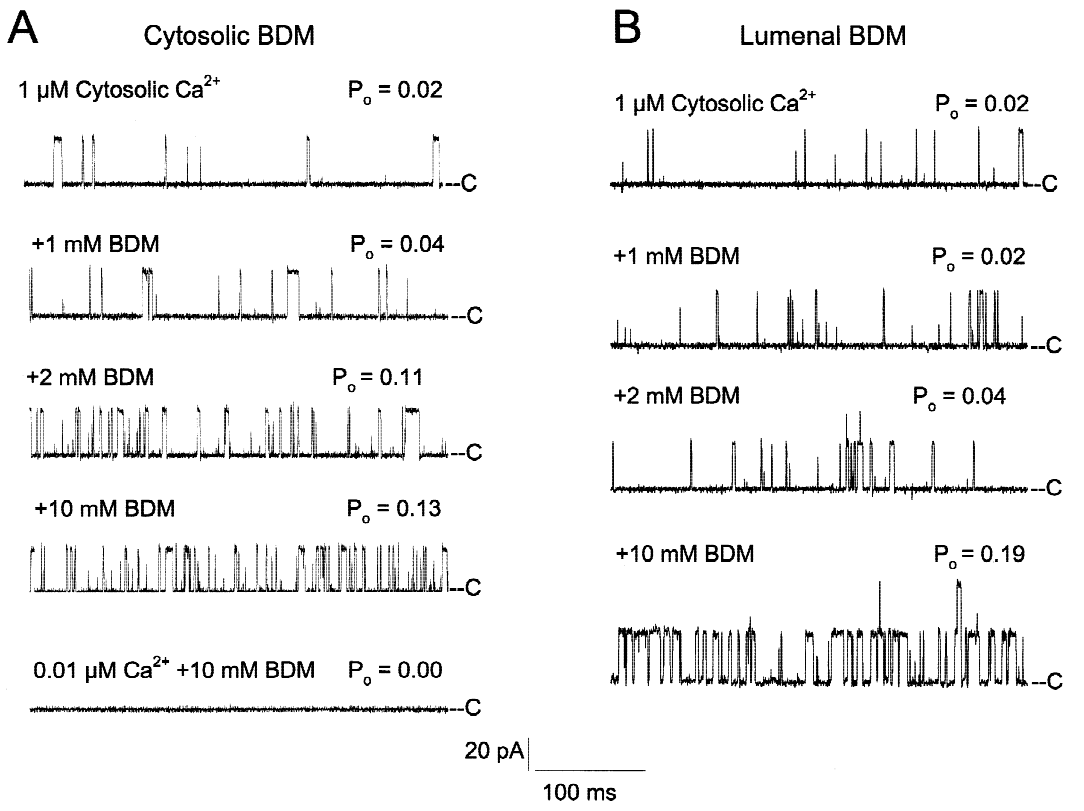

Fig. 4. Activation of cardiac Ca^{2+} release channel by cytosolic (*A*) and lumenal (*B*) BDM at 1 μM cytosolic-free Ca^{2+}. Channel currents, shown as upward deflections from closed levels (c on right), of one (*A*) and two (*B*) purified RyR2s were recorded in symmetrical 0.25 M KCl, 20 mM KHepes, pH 7.4 media containing indicated concentrations of free Ca^{2+} and indicated concentrations of cytosolic (cis) (*A*) or lumenal (trans) (*B*) BDM. The holding potential was 30 mV.

μM Ca^{2+}, addition of 5 mM cytosolic BDM decreased P_o approximately 2.5-fold. BDM did not noticeably activate the purified RyR1 at a free cytosolic Ca^{2+} concentration of ~0.01 μM and was without a significant effect at 1 μM Ca^{2+} (not shown). The latter concentration represented the crossover point of the effects of BDM on [^{3}H]ryanodine binding to RyR1 (*cf.* Fig. 7). Current-amplitude histograms indicated that BDM did not significantly affect single channel conductance (*not shown*).

The concentration dependence of BDM on single channel activities at 0.5 and 20 μM cytosolic Ca^{2+} is compared in Fig. 3. At 0.5 μM Ca^{2+}, cytosolic BDM appeared to be somewhat more effective than lumenal BDM in activating RyR1. A more than tenfold activation of channel activity was observed at a cytosolic BDM concentration of 3 mM. BDM primarily increased P_o by increasing the number of channel events (402 ± 231 and 8875 ± 5332 events/min in the absence and presence of 3 mM cytosolic BDM, respectively, $n = 3$). BDM also prolonged the mean duration of the open events from 0.43 ± 0.04 msec to 0.74 ± 0.09 msec, however, this increase was not significant. Mean duration of closed events was decreased from 107 ± 55 msec to 4.0 ± 1.1 msec. At 20 μM Ca^{2+}, cytosolic and lumenal BDM were similarly effective, both inhibiting RyR1 half maximally at a concentration of ~2.5 mM. Cytosolic BDM (10 mM) primarily decreased P_o by decreasing the number of channel events (23984 ± 8515 to 3617 ± 2253 events/min, $n = 3$). A small (not significant) decrease in the mean open time (from 0.62 ± 0.10 msec to 0.43 ± 0.04 msec) was also observed. Mean closed times were increased by 10 mM cytosolic BDM from 2.0 ± 0.6 to 15.6 ± 6.2 ($n = 3$).

Effects of BDM on Single Cardiac Muscle Ca^{2+} Release Channels

BDM activated the suboptimally Ca^{2+}-activated RyR2. In Fig. 4*A* (top trace), a single purified cardiac muscle Ca^{2+} release channel was recorded in the presence of 1 μM free cytosolic Ca^{2+} at a holding potential of 30 mV. Addition of 1, 2 and 10 mM BDM to the cis side of the bilayer increased channel open probability (P_o) from 0.02 to 0.04, 0.11 and 0.13, respectively (first to fourth trace of Fig. 4*A*). Decrease in cytosolic free Ca^{2+} to ~0.01 μM caused complete channel closing in the presence of 10 mM cytosolic BDM (bottom trace of Fig. 4*A*), which suggests that the activating effects of BDM were

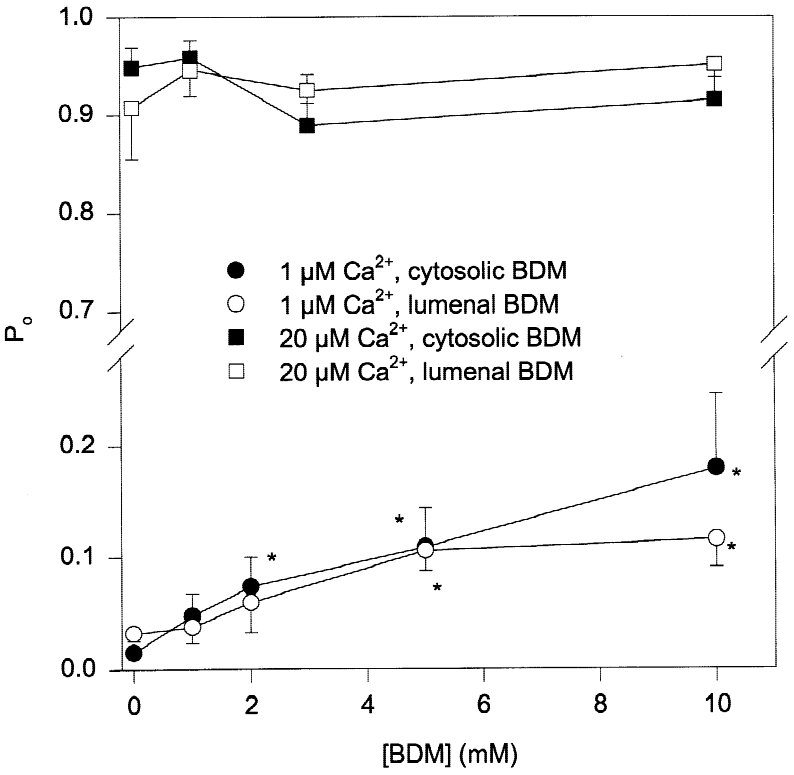

Fig. 5. Concentration dependence of effects of BDM on channel open probability of cardiac Ca^{2+} release channel. Channel activities were recorded as in Fig. 4 at a holding potential of 30 mV in the presence of the indicated concentrations of cytosolic Ca^{2+} and cytosolic or lumenal BDM. Data are the mean ± SE of 4–12 experiments. *Significantly different from control (−BDM).

dependent on the presence of Ca^{2+} in the cis bilayer chamber. As observed for RyR1, BDM did not significantly affect single channel conductance of RyR2 (*not shown*). Time analysis of single channel recordings shows that at 1 μM cytosolic Ca^{2+}, BDM activated RyR1 and RyR2 by a comparable mechanism. Cytosolic BDM activated the cardiac release channel by significantly increasing the number of channel events (757 ± 259 and 3808 ± 944 events/min in the absence and presence of 5 mM BDM, respectively, $n = 4$) without a change of the mean open times (1.4 ± 0.2 and 1.8 ± 0.2 msec, respectively). BDM decreased the mean closed times from 261 ± 110 msec to 38 ± 13 msec.

The cardiac channel could be activated by cytosolic BDM using SR vesicles (*not shown*) or when BDM was added to the SR lumenal side of the purified RyR2 (Fig. 4*B*). In the recording shown in Fig. 4*B*, two purified release channels were present. Single channel open probability increased progressively from 0.02 to 0.19 as lumenal BDM concentration was raised from 0 to 10 mM. On average, BDM was similarly effective in activating RyR2 when added to either the lumenal or cytosolic side of the bilayer (Fig. 5). At 20 μM Ca^{2+}, cytosolic or lumenal BDM were without a significant effect on P_o of RyR2 (Fig. 5).

Reversibility of Effects of BDM

BDM is a nucleophilic compound which dephosphorylates acetylcholinesterase poisoned by organophosphates (Sellin & McArdle, 1994). RyRs are known to be regulated by endogenous and exogenous protein kinases (Witcher et al., 1991; Hain et al., 1995) and it was therefore possible that BDM exerted its effects by altering the phosphorylation states of the RyRs. To test this possibility, channels were recorded before the addition of BDM, in the presence and after removal of the drug. Figure 6*A* shows that addition of 10 mM cytosolic BDM to two RyR1s activated by 20 μM Ca^{2+} decreased P_o from 0.13 to 0.04. Removal of BDM increased channel activities to those before the addition of BDM. Figure 6*B* shows a single RyR2 recorded in the presence of 1 μM cytosolic Ca^{2+}. Addition of 5 mM cytosolic BDM increased P_o from 0.02 to 0.08. Perfusion of the cis (cytosolic) chamber with the control solution returned channel activity close to the control level. The effects of BDM on the purified RyRs were observed in the absence of ATP. Data of Fig. 6 argue, hence, against a dephosphorylation of the channel proteins as the mechanism of the action of the drug.

[^{3}H]Ryanodine Binding

The effects of BDM on RyR1 and RyR2 were also determined in [^{3}H]ryanodine binding measurements using assay media similar to those used in the single channel measurements. A low [^{3}H]ryanodine concentration (1 nM) was used to limit binding to a single high affinity channel binding site. Under these conditions, [^{3}H]rya-

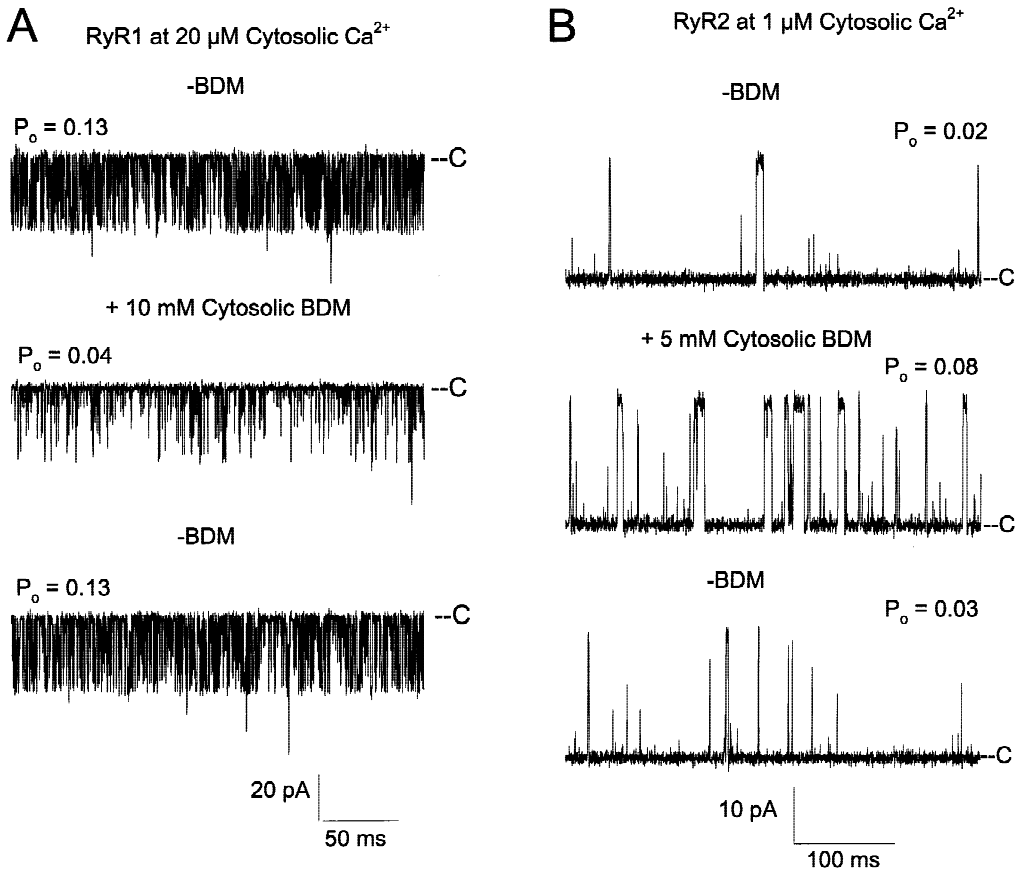

Fig. 6. Reversibility of effects of BDM. Currents from two skeletal (*A*) and a single cardiac (*B*) channels, shown as downward (*A*) or upward (*B*) deflections, were recorded at −40 mV (*A*) and 30 mV (*B*) in the presence of 20 μM (*A*) and 1 μM (*B*) free cytosolic Ca^{2+} before (top traces) and after the addition (middle traces) of the indicated concentrations of cytosolic BDM, and after perfusion with the control medium (bottom traces). A similar reversible activation of RyR1 and RyR2 by BDM was observed in 3 of 3 recordings.

nodine binding has been proven to be a sensitive and convenient method for assessing channel activity (Coronado et al., 1994; Meissner, 1994). Two advantages of [^{3}H]ryanodine binding are that it can evaluate more readily the effects of channel modulators over a wide range of Ca^{2+} concentrations and can sample a much larger population of RyRs than in single channel experiments.

In the absence of BDM, [^{3}H]ryanodine binding to skeletal and cardiac SR vesicles increased as the free Ca^{2+} concentration was raised from submicromolar to micromolar concentrations (Fig. 7). It reached maximum values at ~10–100 μM Ca^{2+}, and declined again with a further increase in Ca^{2+} concentration. The above results are in agreement with previous studies that the cardiac and skeletal muscle release channels have both high-affinity Ca^{2+} activation and low-affinity Ca^{2+} inactivation sites (Coronado et al., 1994; Meissner, 1994). Effects of BDM on [^{3}H]ryanodine binding to RyR1 are shown in Figs. 7*A* and 8. Similar to what was observed in single channel measurements, BDM increased [^{3}H]ryanodine binding to skeletal muscle SR membranes in a concentration-dependent manner at free Ca^{2+} concentrations of ~0.5 μM, whereas at elevated Ca^{2+} concentrations a strong inhibition was observed. As in single channel measurements, the action of BDM appeared to be dependent on Ca^{2+} because at free [Ca^{2+}] <0.1 μM BDM did not substantially increase [^{3}H]ryanodine binding above background levels.

[^{3}H]Ryanodine binding to RyR2 was most profoundly affected by BDM at ~0.5–1 μM free Ca^{2+} (Fig. 7*B*). In this Ca^{2+} concentration range, 1–10 mM BDM increased [^{3}H]ryanodine binding to the cardiac channel in a concentration-dependent manner by approximately 3-fold (Fig. 8). At free Ca^{2+} >1 μM, BDM was without a significant effect. Taken together, the single channel and [^{3}H]ryanodine binding measurements suggest that, at elevated Ca^{2+} concentrations, the skeletal and cardiac Ca^{2+} release channels are differently affected by BDM.

Discussion

The aim of the present study was to determine whether BDM alters SR Ca^{2+} release by a direct functional inter-

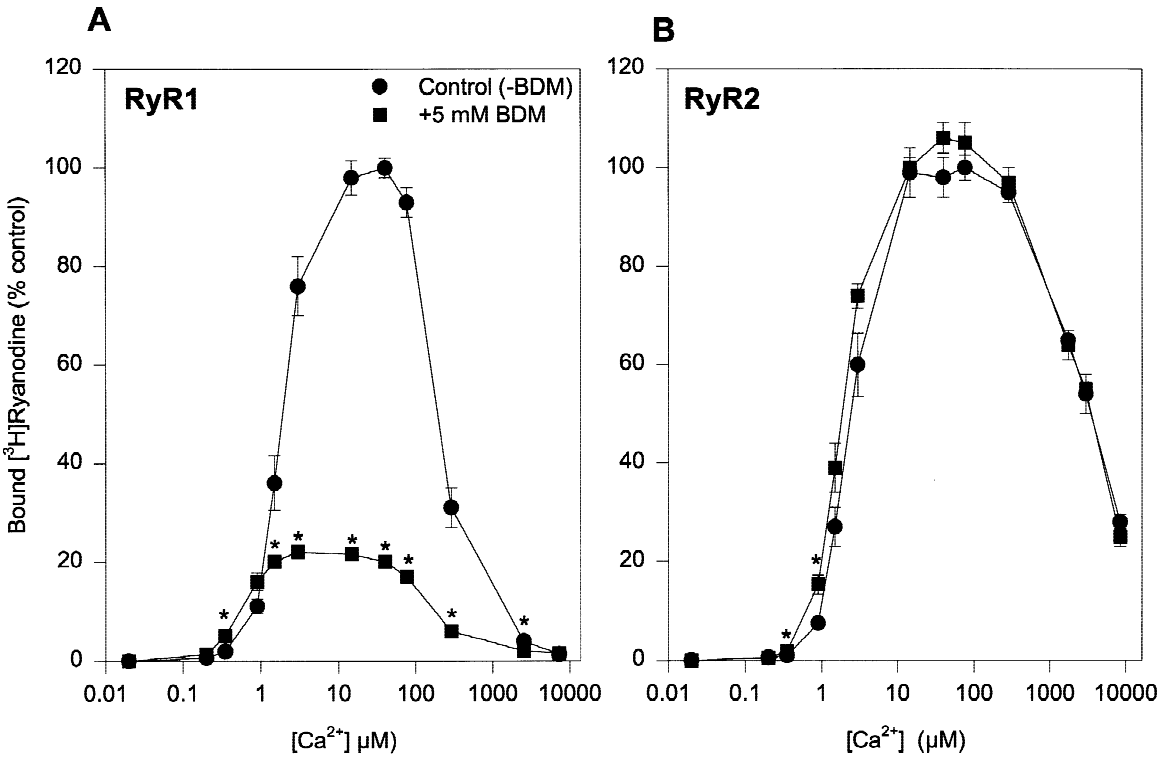

Fig. 7. Effects of 5 mM BDM on Ca^{2+} dependence of [^{3}H]ryanodine binding to skeletal (*A*) and cardiac (*B*) SR vesicles. Specific [^{3}H]ryanodine binding to RyR1 and RyR2 was determined in the absence (●) and presence (■) of 5 mM BDM at the indicated free Ca^{2+} concentrations as described under Materials and Methods. Peak control values (100% = maximum value without BDM) corresponded to 0.8 ± 0.1 and 1.3 ± 0.2 pmol bound [^{3}H]ryanodine/mg protein for RyR1 and RyR2, respectively. Data are the mean ± SE of 3–5 experiments.

action with the skeletal and cardiac muscle SR Ca^{2+} release channels. The two isoforms, known also as ryanodine receptors, play a key role in muscle function by rapidly releasing Ca^{2+} ions in response to an action potential (Sutko & Airey, 1996; Franzini-Armstrong & Protasi, 1997). Two different mechanisms have been implicated in activating the RyRs in cardiac and skeletal muscle. In mammalian skeletal muscle a "mechanical" coupling mechanisms has been formulated, which suggests that a voltage-sensing surface membrane protein complex, the dihydropyridine receptor, activates the SR Ca^{2+} release channel via a direct protein linkage. In contrast, in cardiac muscle the dihydropyridine receptor acts as a voltage-sensitive Ca^{2+} channel (L-type) which permits external Ca^{2+} to enter the cell and serve as a triggering signal for SR Ca^{2+} release (Ca^{2+}-induced Ca^{2+} release). RyR1 and RyR2 have been purified as 30 S channel complexes comprised of four large (Mr ~565,000) and four small (FK506-binding protein, Mr ~12,000) subunits, and have been shown to be regulated by various endogenous and exogenous effectors including Ca^{2+}, Mg^{2+}, ATP, caffeine and ryanodine (Coronado et al., 1994; Meissner, 1994; Sutko & Airey, 1996; Franzini-Armstrong & Protasi, 1997). Of direct relevance to this study is that the cardiac muscle ryanodine receptor is activated by Ca^{2+} to a greater extent than the skeletal muscle receptor (Figs. 2 and 4; Rousseau et al., 1986). Among the exogenous effectors, ryanodine is widely used to assess Ca^{2+} release channel function. Ryanodine is a neutral plant alkaloid that binds with high affinity and specificity to Ca^{2+} release channels in a manner that correlates with their functional states. As a general rule, conditions that open the release channels, such as micromolar to millimolar Ca^{2+}, increase high-affinity [^{3}H]ryanodine binding (Chu et al., 1990; Zimanyi & Pessah, 1991; Xu, Mann & Meisner, 1996). In the present study, [^{3}H]ryanodine binding was used as a rapid and convenient way of assessing RyR activity (Figs. 7 and 8).

This study represents the first demonstration of a direct functional interaction of 2,3-butanedione 2-monoxime with the RyRs of cardiac and skeletal muscle. The compound was originally developed as an antidote of organophosphorous poisoning by being able to remove a phosphate group from the phosphorylated (inactivated) acetylcholine esterase (Sellin & McArdle, 1994). The effects of BDM on the purified RyR1 and RyR2 were reversible and occurred in the absence of ATP, which argues against dephosphorylation of the channel proteins as the mechanism of the action of the drug. Also, BDM does not appear to exert its effects

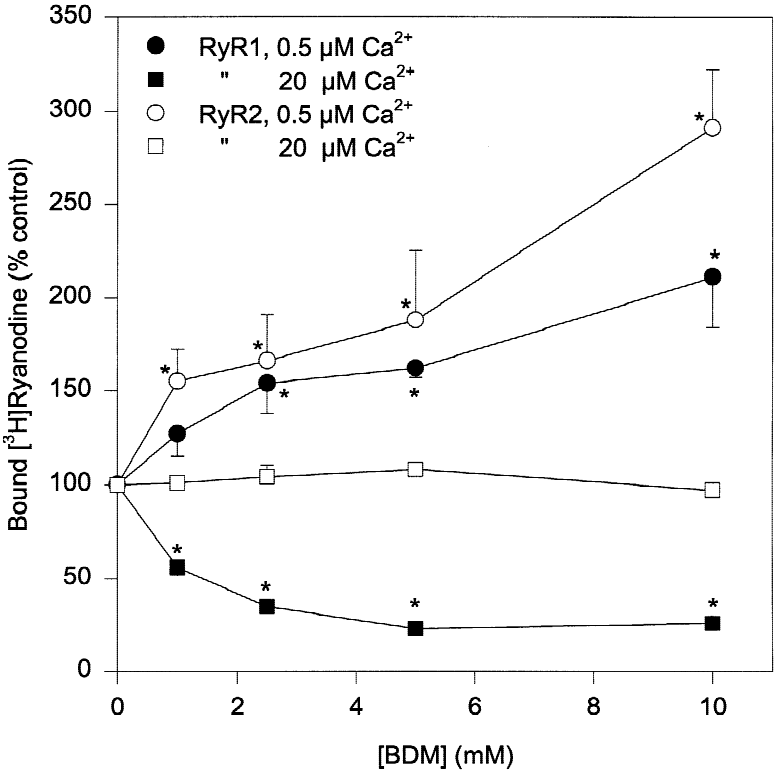

Fig. 8. Concentration dependence of effects of BDM on [^{3}H]ryanodine binding to skeletal and cardiac SR vesicles. Specific [^{3}H]ryanodine binding was determined as described under Materials and Methods in the presence of the indicated BDM and free Ca^{2+} concentrations. The 100% control values correspond to the normalized [^{3}H]ryanodine binding levels in the absence of BDM. Data are the mean ± SE of 4 experiments. *Significantly different from control (−BDM).

through covalent modification of thiols and other groups of the RyRs, as the effects were reversible. BDM exerts its effects when added to intact cells, indicating that it is a membrane permeable compound. We observed a similar activation of RyR1 and RyR2 and inhibition of RyR1 by cytosolic and lumenal BDM, which suggests that the BDM regulatory site(s) may lie in or near the SR membrane.

The results of this study indicate that at suboptimally activating Ca^{2+} concentrations BDM similarly activates the skeletal and cardiac channels by increasing the transition rates of the channels from the closed to the open states. By contrast, major differences in the action of the drug were observed at elevated Ca^{2+} concentrations. In single channel measurements, BDM inhibited the skeletal but not cardiac channel at 20 μM Ca^{2+}. Similarly, [^{3}H]ryanodine binding to RyR1 was decreased by BDM at Ca^{2+} >1 μM, whereas no significant changes in binding to RyR2 were observed.

BDM has been reported to protect the heart from ischemia-reperfusion injury (Fagbemi and Northover, 1995; Habazettl et al., 1996; Tani et al., 1996; Dorman et al., 1996; Zhang et al., 1997) by inhibiting contracture. Our results suggest that a protective effect of BDM due to an inhibition of SR Ca^{2+} release is unlikely because BDM sensitized rather than desensitized the cardiac Ca^{2+} release channel to trigger Ca^{2+}. Our results support observations that BDM decreases SR Ca^{2+} available for release in ferret papillary muscle (Blanchard et al., 1990; Gwathmey et al., 1991) and isolated rat ventricular rat myocytes by stimulating SR Ca^{2+} release (Adams et al., 1998). BDM concentrations ≥3 mM were required to reduce SR Ca^{2+} content in the previous studies as compared to 1–2 mM in our study (Figs. 5 and 8). These differences may be due to the use of intact preparations instead of isolated membranes and proteins. The action of BDM on the cardiac release channel was dependent on Ca^{2+} concentration (Figs. 4 and 7), in agreement with studies using permeabilized cardiac myocytes (Phillips & Altschuld, 1996). The interaction of BDM with the skeletal muscle RyR was more complex in that BDM activated and inhibited the channel depending on Ca^{2+} concentration (Figs. 3 and 7). Consequently, BDM's primary effect in skeletal muscle may be to activate or inhibit the SR Ca^{2+} release channel (Fryer et al., 1988; Maylie & Hui, 1991) or to affect the contractile apparatus (Fryer et al., 1988; Maylie and Hui, 1991; Kagawa et al., 1995; Parsons et al., 1997), depending on the experimental conditions used.

Support by National Institutes of Health grants HL27430 and AR18687 is gratefully acknowledged.

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