BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **229,** 154–157 (1996)
ARTICLE NO. 1772

2,3-Butanedione 2-Monoxime (BDM) Induces Calcium Release from Canine Cardiac Sarcoplasmic Reticulum

Ronald M. Phillips and Ruth A. Altschuld[1]

Department of Medical Biochemistry, The Ohio State University, Columbus, Ohio 43210

Received October 16, 1996

2,3-Butanedione 2-monoxime (BDM) is a well known inhibitor of skeletal and cardiac muscle contraction. Recently, it has been discovered that BDM has an influence on the sarcoplasmic reticulum (SR). We investigated the effects of BDM on the SR in our digitonin lysed myocyte system, which measures accumulated SR Ca^{2+}. While BDM (30 mM) had no effect on SR Ca^{2+} uptake (under conditions that included Ca^{2+} release channel efflux inhibitors), it induced SR Ca^{2+} release (no efflux inhibitors) with a maximal reduction of 72% of SR Ca^{2+} at pCa 6.0. A titration showed that even 5 mM BDM resulted in a 45% reduction at that same pCa. Also, a positive correlation was found between the degree of BDM induced Ca^{2+} release and free Ca^{2+} concentration. Thus, the use of even low concentrations of BDM as an excitation-contraction uncoupler must be approached with caution.

The inhibitory effect of 2,3-butanedione 2-monoxime (BDM) on both skeletal and cardiac muscle contractility has been known for many years. This excitation-contraction uncoupling is both dose dependent and fully reversible upon washout. The mechanism of this inhibitory action, despite numerous recent investigations, is not yet fully understood.

Initially, it was thought that BDM acted primarily at the site of the myofibrils, as evidenced by its inhibition of ATPase activity and reduction of Ca^{2+} sensitivity (1). Later studies revealed that it also had an influence on calcium currents and transients in the cell (2-4). BDM is also known to act as a chemical phosphatase (5), which has led to speculation that dephosphorylation of key Ca^{2+} channel proteins may be involved in its inhibition of contraction. Finally, BDM has been shown to affect the calcium regulatory activities of the sarcoplasmic reticulum (SR).

Several theories have been advanced regarding the action of BDM on the SR. Early observations showed that at low BDM concentrations (1-3 mM) SR Ca^{2+} release was attenuated, suggesting a possible inhibitory effect on the calcium release channel (CRC). Later studies, however, were in agreement that at concentrations greater than 3mM, BDM caused a potentiation of, or even initiated, SR Ca^{2+} release. Steele and Smith (6) showed a BDM induced SR Ca^{2+} release which was ryanodine sensitive in saponin skinned rat trabeculae, and suggested a mechanism involving inhibition of the SR Ca^{2+} ATPase. Györke and colleagues (3) demonstrated similar effects in crayfish cut fibers and lobster microsomes, utilizing tetracaine to block Ca^{2+} release but attributed the results to BDM acting upon the SR CRC. Finally, Brotto *et al* (4) discovered that at a concentration as low as 3 mM, BDM caused Ca^{2+} release from the SR of saponin treated trabeculae of embryonic chick hearts, in a manner independent of free Ca^{2+} concentration.

Since our laboratory has a well established method for directly measuring SR Ca^{2+} accumula-

[1] Corresponding author. Fax: (614) 292-4118.

Abbreviations: BDM, 2,3-butanedione 2-monoxime; SR, sarcoplasmic reticulum; CRC, calcium release channel; BES, *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid; CICR, calcium induced calcium release.

0006-291X/96 $18.00

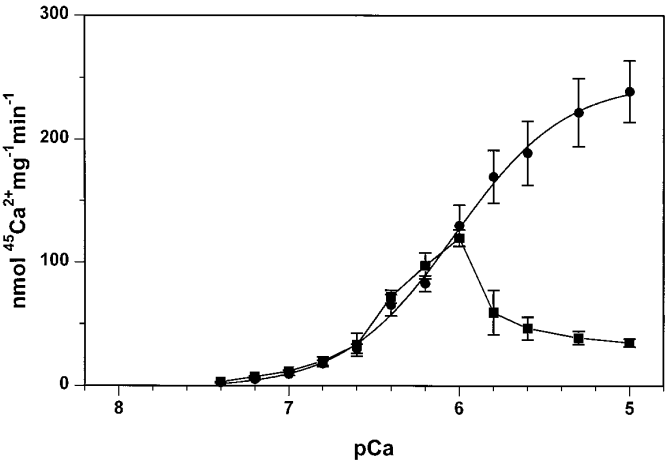

FIG. 1. $^{45}Ca^{2+}$ uptake in digitonin lysed canine ventricular cardiomyocytes. Net calcium accumulation was determined in the absence (■) or presence (●) of the SR calcium release channel efflux inhibitors ruthenium red and procaine as described in the methods section. Data reported as mean ± SEM, n=3.

tion in digitonin lysed cardiomyocytes using isotopically labeled Ca^{2+}, we decided to investigate the effects of BDM on the SR.

MATERIALS AND METHODS

Myocyte isolation. Canine ventricular myocytes were isolated using collagenase perfusion as previously described (7), with the following changes: The medium 199 perfusion was omitted, adenosine was not used at any time during the procedure, and the concentration of glucose in the final perfusion buffer was 4 mM.

Calcium uptake assay. SR calcium uptake was determined as previously described (8). Briefly, myocytes were washed twice and resuspended in the following buffer at room temperature: in (mM) NaCl 50, Na_2HPO_4/NaH_2PO_4 50, glucose 11, *N,N*-bis[2-hydroxyethyl]-2-aminoethanesulfonic acid (BES) 20, rotenone 0.016, EGTA 0.2. The buffer was then supplemented with oligomycin 0.01, phosphocreatine 10, dithiothreitol 1, MgATP 10, and 0.2 units/ml creatine phosphokinase. Where indicated, ruthenium red 0.03, and procaine 10 were added to inhibit SR Ca^{2+} efflux. All cells were lysed with 16 μg digitonin/mg myocyte protein, and pH was adjusted to 7.2. At this point, the lysed myocytes were divided into 1.5 ml aliquots, and the proper quantity of BDM (1 M stock) and/or vehicle (water) was added. Cells were warmed to 37° C during a 5 minute pre-incubation and the appropriate $^{45}Ca^{2+}$ EGTA buffer was added. The cells were incubated for 5 minutes at 37° C, when duplicate 0.5 ml samples were sedimented through a layer of 97% bromododecane/3% dodecane into 0.1 ml of 2N perchloric acid, all layered in a 1.5 ml microcentrifuge tube. SR $^{45}Ca^{2+}$ uptake was determined by counting a 75 μl aliquot of the acid extract using Beckman Ready Caps in a Beckman LS 6800 liquid scintillation counter.

Protein determination. The protein pellets from the previous assay were washed twice with acetone, dried, and dissolved in 1 N NaOH at 100° C. Myocyte protein was then quantified using the method of Lowry (9) with BSA as a standard.

RESULTS AND DISCUSSION

When digitonin lysed cardiomyocytes are challenged with $^{45}Ca^{2+}$-EGTA buffers with varying free Ca^{2+} concentrations, it is possible to generate Ca^{2+} uptake curves like those shown in Fig. 1. The curve with the SR CRC efflux inhibitors ruthenium red and procaine demonstrates a typical saturable dose-response relationship, and can be fitted to a sigmoidal curve function, yielding a V_{max} of 246 $nmol \cdot mg^{-1} \cdot min^{-1}$ and a $K_{0.5}$ of pCa 6.0. Under conditions without efflux inhibitors, similar uptake characteristics are observed until the free Ca^{2+} concentration of pCa 6.0 is reached. At this point, calcium induced calcium release (CICR) begins to occur, and the competition between Ca^{2+} release and uptake results in a net decrease in accumulated SR Ca^{2+}. In light of some recent work involving the effects of BDM on the Ca^{2+} regulating activities of the SR, we decided to examine this drug with respect to our Ca^{2+} uptake system.

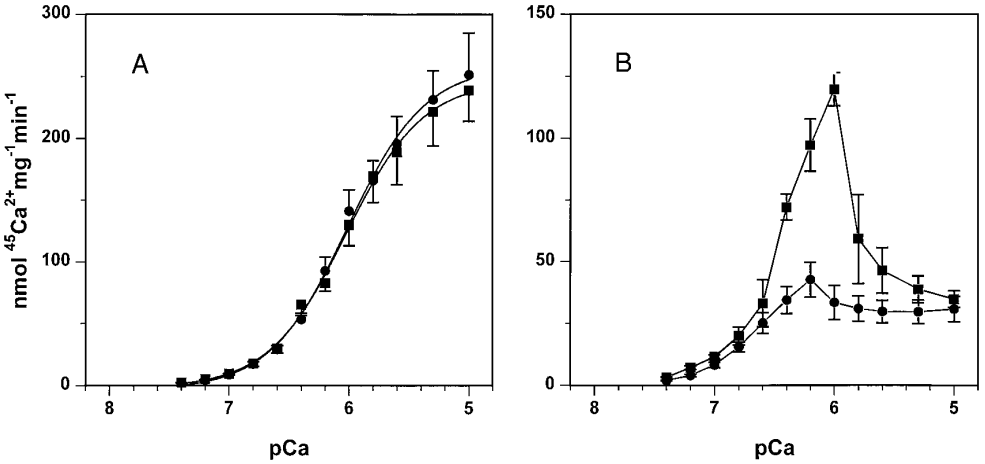

FIG. 2. Effect of BDM on net calcium uptake. In both panels, incubations were performed with (■) or without (●) the addition of 30 mM BDM. (A) Uptake was determined in the presence of efflux inhibitors ruthenium red and procaine. (B) No efflux inhibitors were utilized (note expanded Y axis). For both conditions, n=3.

Fig. 2 summarizes the results of these experiments. Panel A shows that even at a high (30 mM) concentration, BDM has no effect on SR Ca^{2+} uptake when efflux is prevented with ruthenium red and procaine. This indicates that inhibition of the SR Ca^{2+} ATPase is unlikely to be involved in the mechanism of action of this drug. This result concurs with work from another laboratory which reported no BDM inhibition of the SR Ca^{2+} ATPase in crustacean muscle or microsomes (3). When these experiments are repeated in the absence of efflux inhibitors, however, a substantial decrease in accumulated SR Ca^{2+} is observed in the curve with 30 mM BDM (panel B). It is interesting to note that, unlike another study (4), a synergistic relationship between BDM and free Ca^{2+} concentration is observed. The difference between control and BDM curves increases with increasing free Ca^{2+} concentration, reaching a maximum of 72% at pCa 6.0, at which point CICR in the control curve leads to an apparent

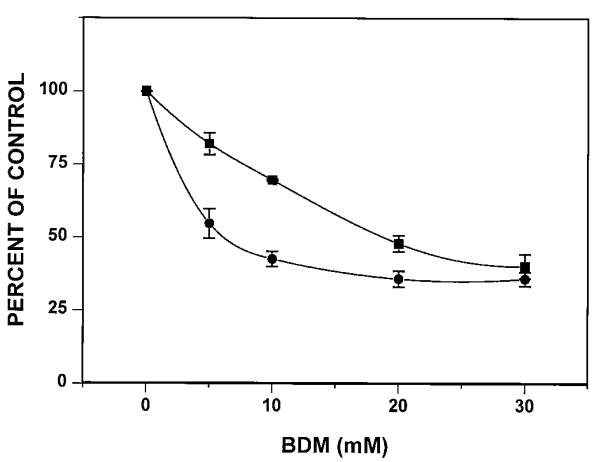

FIG. 3. Concentration dependence and effect of free calcium concentration on BDM induced SR calcium release. Calcium accumulation was determined as previously described, without efflux inhibitors, and at the indicated concentrations of BDM. The titrations were carried out at pCa 6.2 (■), n=4, and pCa 6.0 (●), n=3.

TABLE 1
BDM Titration

	$^{45}Ca^{2+}$ Uptake (nmol · mg^{-1} · min^{-1})	
BDM (mM)	pCa 6.2 (n = 4)	pCa 6.0 (n = 3)
0	80 ± 5	101 ± 7
5	66 ± 7	56 ± 9
10	56 ± 3	43 ± 6
20	38 ± 2	36 ± 4
30	32 ± 2	36 ± 4

convergence of the two. Also, the point of CICR is shifted to a lower Ca^{2+} concentration, from pCa 6.0 (control) to 6.2 (BDM). Together, these results suggest that BDM may induce SR Ca^{2+} release by sensitizing the CRC to trigger Ca^{2+}.

The free Ca^{2+} dependence of BDM induced Ca^{2+} release is also illustrated in Fig. 3. At all BDM concentrations tested (5-30 mM), the decrease in accumulated SR Ca^{2+} is more pronounced at pCa 6.0 vs. 6.2, when normalized to their respective controls (Table 1). It is important to note that even at a BDM concentration as low as 5 mM, the SR Ca^{2+} load is reduced by 17% at pCa 6.2 and 45% at pCa 6.0. Therefore, it is critically important to take free Ca^{2+} concentration, in addition to the BDM induced SR Ca^{2+} release, into account when considering the effect of even low concentrations of BDM on a given system.

ACKNOWLEDGMENTS

The authors would like to thank Lourdes Castillo for her technical expertise in the preparation of myocytes, and Dr. George Billman for supplying the animals used in this study. This work was supported by HL 48835 and HL 36240.

REFERENCES

1. Li, T., Sperelakis, N., Ten Eick, R. E., and Solaro, R. J. (1985) *J. Pharmacol. Exp. Ther.* **232,** 688–695.
2. Gwathmey, J. K., Hajjar, R. J., and Solaro, R. J. (1991) *Circ. Res.* **69,** 1280–1292.
3. Györke, S., Dettbarn, C., and Palade, P. (1993) *Pflügers Arch. Eur. J. Physiol.* **424,** 39–44.
4. Brotto, M. A. P., Fogaça, R. T. H., Creazzo, T. L., Godt, R. E., and Nosek, T. M. (1995) *J. Muscle Res. Cell Motil.* **16,** 1–10.
5. Wilson, I. B., and Ginsburg, S. (1955) *Biochim. Biophys. Acta* **18,** 168–170.
6. Steele, D. S., and Smith, G. L. (1993) *Am. J. Physiol. Heart Circ. Physiol.* **265,** H1493–H1500.
7. Hohl, C. M., and Altschuld, R. A. (1991) *Am. J. Physiol.* **260,** C383–C391.
8. Wimsatt, D. K., Hohl, C. M., Brierley, G. P., and Altschuld, R. A. (1990) *J. Biol. Chem.* **265,** 14849–14857.
9. Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. (1951) *J. Biol. Chem.* **183,** 265–272.