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The Myosin ATPase Inhibitor 2,3-Butanedione monoxime Dictates Transcriptional Activation of Ion Channels and Ca^{2+}-Handling Proteins

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ABSTRACT

2,3-Butanedione monoxime (BDM) is a reversible myosin ATPase inhibitor with phosphatase-like activity. It is being evaluated for its therapeutic benefit in organ preservation in addition to its use to prompt cardioplegic arrest. Its effects on transcriptional regulation of ion channels and Ca^{2+}-handling proteins, surprisingly, are basically unknown. We therefore studied expression of genes coding for ion channels and Ca^{2+}-handling proteins in explanted hearts and cultures of Ca^{2+}-tolerant cardiomyocytes. In addition, we studied the effect of daily treatment with 250 mg of BDM/kg of body weight for up to 72 h. Furthermore, Ca^{2+}-tolerant cardiomyocytes were isolated and cultured in the presence of 15 mM BDM and harvested 24 or 72 h after dosing. It is noteworthy that a modest to highly significant increase in transcript level of ion channels, ion exchangers, Ca^{2+}-binding proteins, and cytoskeletal proteins was observed after treatment of rats with BDM. Likewise, with cultures of cardiomyocytes, statistically significantly increased transcript levels of potassium and sodium ion channels as well as some ion exchangers (PMCA2 and 4) were noted, even though expression of genes coding for Ca^{2+}-binding and cytoskeletal proteins was repressed. This preponderance of transcriptional up-regulation of cardiac-specific genes suggests a mechanism of action whereby unilateral dephosphorylation of coded proteins resulted in a feedback loop of regulation (e.g., transcriptional activation of coding genes).

Butanedione monoxime is a reversible myosin ATPase inhibitor with phosphatase-like properties. It was originally designed to rescue cholinesterase activity in case of poisoning with organophosphates, but its dephosphorylating action proved to be relatively nonspecific and was not limited to acetylcholinesterase (Xiao and McArdle, 1995a). Further studies with BDM demonstrated significant lowering of the blood pressure in normo- and hypertensive rats (Xiao and McArdle, 1995b). The authors attributed the reversible effect to direct and indirect actions of BDM on the cardiac and vascular smooth muscle and particularly inhibition of inward Ca^{2+}-currents.

Because of its ability to uncouple skeletal and cardiac muscle contraction, BDM was profiled for cardioplegic arrest. Several studies point to beneficial effects on the preservation and protection of cardiomyocytes after hypothermic and hyperkalemic cardioplegic arrest (Dorman et al., 1996), including amelioration of myocardial edema and atrioventricular conduction delay after reperfusion (Jayawant et al., 1999) as well as normalization of myocyte contractile function (Dorman et al., 1996). The cardioprotective properties of BDM are linked to an inhibition of cross-bridge force development, the reduction of myofilament Ca^{2+}-sensitivity, and the attenuation of intracellular Ca^{2+}-transients (Jayawant et al., 1999) and was shown to be time-dependent (Habazettl et al., 1998; Voigtlander et al., 1999). Moreover, BDM was more efficient in atria compared with the chambers of the heart (Schwinger et al., 1994). BDM has been shown to improve right atrial function in a porcine model of allogenic heart transplantation (Vahl et al., 1995; Thum and Borlak, 2001; Warnecke et al., 2002).

BDM is still evaluated for cardioplegic arrest and in organ preservation to improve storage of transplants, but a systematic study on the transcriptional regulation of ion channels and other Ca^{2+}-handling proteins is still lacking. Nonetheless, this information is needed because BDM interferes with ion channel function. Indeed, BDM inhibits the L-type Ca^{2+}-channel (Eisfeld et al., 1997) and reduces the opening probability, thereby increasing the closed time of this particular channel (Allen and Chapman, 1995). BDM was also reported to activate skeletal as well as cardiac ryanodine receptors (RYR) in single-channel measurements (Tripathy et al.,

ABBREVIATIONS: BDM, 2,3-butanedione monoxime; RYR, ryanodine receptors; SD, Sprague-Dawley; DEPC, diethyl pyrocarbonate; PCR, polymerase chain reaction; MHC, myosin heavy chain; PKA, protein kinase A.

1999) and blocked the transient outward K^+ current (I_{to}), whereas the inward rectifier K^+ current (I_k) was shown to be relatively recalcitrant toward the effect of BDM (Xiao and McArdle, 1995a).

We previously reported BDM's ability to modulate expression of some major heart-specific genes in transplanted hearts and cultures of cardiomyocytes (Thum and Borlak, 2001), but we did not study the regulation of genes coding for ion channel and Ca^{2+}-sequestering proteins. We now report the effect of BDM on ion channel gene expression in vivo after single and repeated treatment of Sprague-Dawley (SD) rats and compare our findings with results obtained from cultures of Ca^{2+}-tolerant cardiomyocytes. Overall, we aimed to further our understanding of the mode of action of BDM on the transcriptional regulation of ion channel and other Ca^{2+}-handling coding genes.

Materials and Methods

Animals

All animal procedures described in this report were approved by the local authorities, and the investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (National Institutes of Health publication no. 85-23, revised 1996).

Chemicals

BDM, a 2,3-butanedione monoxime, was obtained from Fluka (Buchs, Germany) and was of >99% purity. Ketamine and rompun were purchased from Medistar (Hannover, Germany) and heparin was purchased from Roche (Mannheim, Germany). All chemicals used were of highest purity.

Experimental Design

In Vivo Studies. Male SD rats weighing 230 ± 9 g were obtained from Charles River Laboratories (Sulzfeld, Germany). Food and water were given ad libitum. BDM was dissolved in freshly autoclaved water. Rats ($n = 3$ per time point) were treated daily with a single i.p. dose of 250 mg/kg of body weight. The dose selection is based on literature findings in which dose-response relationships with regard to animal behavior and survival were investigated (Brightman et al., 1995; Xiao and McArdle, 1995a) The first group of animals was killed 24 h after the first injection (single dose), whereas the second group received BDM for 2 days further and thus were killed 72 h after the first injection.

Heart Explantation and Isolation of Cardiomyocytes. Rats ($n = 3$) were anesthetized with 0.1 ml of ketamine per 100 g og body weight and 50 μl of rompun per 100 g of body weight. In addition, 2.000 international units of heparin were given i.p. before surgery. The heart was anatomically prepared and immediately frozen to await further analysis.

Isolation and Cultivation of Cardiomyocyte Cultures. Isolation of cardiomyocytes was done essentially as described by Thum and Borlak (2001). Cells were then examined and photographed under phase contrast microscopy to assess their quality and quantity and showed a purity of >95%. Cardiomyocytes were cultured in the presence of 15 mM BDM. They were kept at 37°C at 5% CO_2 and harvested shortly after isolation as control, as well as 24 and 72 h after treatment, respectively.

RNA and cDNA. Total RNA was isolated from the right and left ventricles of the explanted hearts and from cultured cardiomyocytes using the NucleoSpin RNA kit from Macherey-Nagel GmbH (Düren, Germany), according to the manufacturer's recommendation. The integrity of isolated RNA was verified using a 1.0% agarose gel.

Two micrograms of total RNA was used for reverse transcription. RNA and random primer (Promega) were preheated for 10 min at 70°C and then chilled on ice for 2 min. 5× reverse transcriptase-Avian myoblastosis virus buffer (Promega), dNTPs (10 mM; Promega), RNAsin (Promega), Avian myoblastosis virus buffer (Promega), and diethyl pyrocarbonate (DEPC)-H_2O were added to a final volume of 20 μl. Then, reverse transcription was carried out for 60 min at 42°C and was stopped by heating to 95°C for 5min. The resulting cDNA was diluted with 80 μl of DEPC and then frozen at −20°C to await further analysis.

Thermocycler RT-PCR. PCR reactions for ion channel- and transcription factor-genes were done with a 20-μl reaction mixture containing HotStarTaq Master Mix (QIAGEN, Valencia, CA), DEPC, 1 μl of cDNA, and a 1.0 μM concentration of the 3′- and 5′-specific oligomers (synthesized by Invitrogen GmbH, Karlsruhe, Germany). PCR reactions were carried out in a thermal cycler (T3; Biometra, Göttingen, Germany) using the following conditions: 95°C for 15 min and 94°C for 30 s, followed by the individual conditions for each gene as outlined in Table 1.

PCR reactions were done within the linear range of amplification and were separated using a 1,5% agarose gel. They were visualized by ethidiumbromide under UV transillumination. Quantification of PCR products was done with the software of the Kodak Image Station version 3.5.

Statistical Analysis

We used Hottelings T^2 test with the SPSS software package (version 10.0; SPSS Inc., Chicago, IL). Whenever significant results were computed, further pair-wise t tests were done. With this approach, experiment-wide error rate of multiple testing could be controlled. Results were considered significant at $p < 0.05$.

The stars without brackets (Figs. 2–5) simply describe significant differences between control versus treatment groups, whereas the brackets describe comparisons in vitro or in vivo or a comparison between both systems. Note that we used the mean values of both ventricles for comparison between in vitro and in vivo.

Results

Effects of BDM in Cultures of Ca^{2+}-Tolerant Cardiomyocytes. Approximately $11.9 \pm 0.3 \times 10^6$ cells/heart were isolated. The purity of cardiomyocyte cultures was >98%, as judged by phase contrast light microscopy. Cardiomyocytes were rod-shaped with well-defined cross-striations. It is important to note that cardiomyocytes were not beating. In the absence of BDM, cardiomyocytes developed cytoskeletal defects with loss of rod-shape morphology (Thum and Borlak, 2001). Controls were defined as freshly isolated cells and were frozen immediately to await further analysis.

Expression of Ion Channels. Initially and in the absence of BDM, expression of ATP-dependent K^+ channel (Kir6.1) was reduced to 40% of control but returned to normal upon treatment with BDM (Figs. 1 and 2).

Likewise, BDM restored expression of G-protein regulated K^+ channel (Kir3.4), which was initially reduced to 50% and below the limit of detection after 72 h. The beneficial effects of BDM were not apparent after 72 h of culture, because expression levels were 5% of control. BDM rescued expression of the cardiac Na^+ channel (Nav1.5). In untreated cultures, expression was 50% (24 h) and thereafter not detectable (72 h). BDM elicited an increase in cultures (24 h) of approximately 2.5-fold, but the beneficial effects of BDM were not apparent after 72 h (e.g., expression of Nav1.5 was 5% of control). A 50% decline in transcript level of RYR-2 was observed after 24 h in cultures and further decreased to 5% at 72 h. With BDM, RYR-2 expression remained unchanged

after 24 h but declined to approximately 50% of control, 72 h in culture.

As depicted in Fig. 1, RYR-3 was below the limit of detection after 24 h in cultures and returned thereafter to approximately 50% of control values. Treatment of cultures with BDM evoked a 1.5-fold increase (24 h). Nonetheless, and after 72 h in culture, expression of RYR-3 declined to approximately 50% of control values.

Expression of Ion Exchangers. Expression of NCX-1 was reduced to approximately 50% (24 h) and decreased further to below the limit of detection after 72 h in culture (Figs. 1 and 3). BDM had no apparent effect. Likewise, SERCA2b expression was 50% of control after 24 and 72 h in culture. With BDM, expression remained either unchanged (24 h) or was basically unchanged (72 h). PMCA1 expression was initially below the limit of detection (24 h) but returned to expression levels 2.5-fold above control. BDM rescued expression levels to nearly 50% of control (24 h) and elicited a 300% increase above control after 72 h of culture. Likewise, PMCA2 was reduced to 50% of control (24 h) and below the limit of detection after 72 h of culture. BDM elicited initially an increase of nearly 2-fold in PMCA2 expression (24 h), but expression declined to 50% of control after 72 h. It is noteworthy that PMCA4 expression was increased by 700% and 600% of control. After 72 h in culture, levels dropped to control values or were slightly above upon BDM treatment (see Fig. 3).

At first, expression of the Na-K-ATPase was 50% of control. No further treatment-related effect was observed after 72 h in culture. With phospholamban, expression was nearly 1.5-fold of control after 24 h in culture but declined to 50% of control after 72 h in culture. With BDM, no clear effect was obvious even though expression of phospholamban was 70% of control after 24 h in culture.

Expression of Cytoskeletal Proteins In the absence of BDM, expression of α-cardiac actin was 80 and 40% of control at 24 h and 72 h, respectively. With BDM, expression was either unchanged (24 h) or 20% of control (72 h) (Figs. 1 and 4). As shown in Fig. 4, expression of α-skeletal actin was markedly decreased. In untreated cultures, levels were 50% (24 h) and 10% (72 h) of control. With BDM, expression was nearly 10% at both time points. α-MHC expression was reduced to 50 and 20% after 24 and 72 h of cultures. Overall, BDM had little effect on expression of α-MHC transcripts. Expression of β-MHC was 2-fold (24 h) and 50% of control after 72 h in culture. BDM repressed β-MHC expression to approximately 50% of control after 24 h and 72 h in culture.

Expression of Ca^{2+}-Binding Proteins. In the absence of BDM, expression of calsequestrin was either unchanged (24 h) or hardly detectable (72 h). With BDM, expression of calsequestrin was reduced by half (24 h) and was near the limit of detection thereafter (72 h) (Figs. 1 and 5). Expression of calmodulin was moderately increased (1.5-fold, 24 h) and later reduced to 50% of control (72 h). With BDM, expression was initially 50% of control but basically no change was observed after 72 h in culture. Compared with freshly isolated cardiomyocytes, expression of calreticulin was increased by 1.5-fold (24 and 72 h). With BDM, a similar 1.5-fold increase was observed after 72 h in culture.

Effects of BDM in Vivo. Treatment with BDM was well tolerated. After explantation, the heart was examined macroscopically and weighed. No pathological change was observed, and heart weight was 0.62 ± 0.02% and 0.61 ± 0.05% of total body weight 24 and 72 h after treatment, respectively.

Expression of Ion Channels. Compared with control, expression of the ATP-dependent K^+ channel (Kir6.1) increased by approximately 10- and 14-fold in the right and left ventricles at 24 and 72 h after treatment, respectively (Figs. 2 and 6). BDM also elicited an approximately increases of approximately 2.5- and 2-fold in the gene expression of G-protein regulated K^+ channel (kir3.4) in the right and left

TABLE 1
Oligonucleotide primers used in the reverse transcription-PCR

Gene (Accession Number)	Forward Primer	Reverse Primer	Product Length	Cycler Program
			bp	
Calsequestrin (u33287)	tca aag acc cac cct acg tc	cca gtc ttc cag ctc ctc ag	352	57°C, 60 s/30 cycles
Calreticulin (NM 022399.1)	atgaccccacagattccaag	gcataggcctcatcattggt	339	55°C, 60 s/33 cycles
Calmodulin (AF 178845.1)	gaagcaggccagtcaaagac	cgaatttggaagccaacact	348	55°C, 60 s/33 cycles
SERCA 2a (van den Bosch et al., 1994)	tcc atc tgc ctg tcc at	gcggttactccagtattg	196	55°C, 60 s/35 cycles
SERCA 2b (van den Bosch et al., 1994)	tcc atc tgc ctg tcc at	aga cca gaa cat atc act	324	55°C, 60 s/35 cycles
PMCA 1 (j03753)	tgc ctt gtt ggg att tct ct	cac tct ggt tct ggc tct cc	351	55°C, 60 s/35 cycles
PMCA 2 (j03754)	att gat gga gct ggg atc ag	act tca ccg tgg aca cct tc	350	57°C, 60 s/35 cycles
PMCA 4 (u15408)	agc agt tgc gtc agt cag aa	gct ttg tag agg gct gtt gg	351	57°C, 60 s/38 cycles
Na-K-ATPase (NM012504)	tgt gat tct ggc tga gaa cg	agg aca gga aag cag caa ga	299	57°C, 60 s/35 cycles
NCX-1 (x68191)	tgt ctg cga ttg ctt gtc tc	tca ctc atc tcc acc aga cg	364	55°C, 60 s/35 cycles
Kir 3.4 (L35771)	gga tgg cag aca gga aag ag	acc acc tgt tgg agc tgt tc	351	56°C, 60 s/35 cycles
Kir 6.1 (D42145)	tga gtc tag gac gcg ttg tg	cac cag cca cca cat gat ag	351	55°C, 60 s/35 cycles
Nav 1.5 (M27902)	cag ctg aca tga cca aca cc	ttg agc agc atc tcc aac ac	349	55°C, 60 s/35 cycles
RYR 2 (af130880)	cca aca tgc cag acc cta ct	ttt ctc cat cct ctc cct ca	348	57°C, 60 s/35 cycles
RYR 3 (af130881)	cgt ctc tgg tgt cat ggc ta	cac act tca tat ccg gct ca	345	57°C, 60 s/38 cycles
Phospholamban (x71068)	gct gag ctc cca gac ttc ac	gcg aca gct tgt cac aga ag	339	57°C, 60 s/30 cycles
Troponin T (NM012676.1)	gggtacatccagaaggctca	gtgcctggcaagacctagag	374	55°C, 60 s/33 cycles
Troponin I (NM017144.1)	atctccgcctccagaaaact	cagtagtgcctgcatcatgg	352	55°C, 60 s/33 cycles
α-Cardiac Actin (X80130)	actcctatgtaggtgacgaggc	gacgttatgagtcacaccgtcg	337	57°C, 60 s/33 cycles
α-Skeletal Actin (V01218)	atctcacgttcagctgtggtca	accaccggcatcgtgttggat	182	55°C, 60 s/33 cycles
α-MHC (X15938)	ggaagagcgagcggcgcatcaagg	ctgctggacaggttattcctca	304	65°C, 60 s/35 cycles
β-MHC (X15939)	gccaacaccaacctgtccaagttc	ttcaaaggctccaggtctcagggc	202	55°C, 60 s/35 cycles
GAPDH (J02624)	ggccaaggtcatccatga	tcagtgagcccaggatg	353	55°C, 60 s/32 cycles

ventricles 24 h after treatment. Seventy-two hours after dosing, expression in the right ventricle was further increased by 3-fold, whereas mRNA expression in the left ventricle remained nearly unchanged. BDM produced an increase of nearly 2.5-fold in the cardiac Na^+ channel (Nav1.5) expression in right and left ventricles after 24 h and 72 h of treatment. Expression of the gene coding for RYR-2 increased approximately 3.5-fold in right and left ventricles 24 h after dosing. Thereafter, levels of RYR-2 transcripts were 4-fold in the right ventricle and nearly 3-fold in the left ventricle after 72 h of dosing. Compared with control, BDM produced an increase of nearly 2-fold in RYR-3 gene expression in the right ventricle 24 h after dosing that increased further to nearly 4.5-fold after 72 h. The initial 3.5-fold increase in gene expression in the left ventricle was followed by a decline to 150% of control values. BDM produced an approximate 6-fold (24 h right ventricle and 72 h left ventricle) and 8-fold (72 h right ventricle and 72 h left ventricle) increase in L-type Ca^{2+} channel gene expression compared with control values.

Expression of Ion Exchangers. In both ventricles, expression of NCX-1 was increased by 3-fold after 24 and 72 h of treatment (Figs. 3 and 6). BDM had little effect on SERCA 2a mRNA expression, producing an increase of approximately 2-fold in both ventricles 24 h and 72 h after treatment. As shown in Fig. 3, expression of SERCA2b was increased 2.5- and 1.5-fold in the right and left ventricles, respectively, after 24 h of treatment. Although expression remained unchanged in the right ventricle, it increased further to 2-fold in the left ventricle after 72 h of treatment. Compared with control, BDM elicited an increase in transcript of PMCA1 of approximately 3- and 4-fold in the right and left ventricles, respectively, 24 h after treatment. Expression declined thereafter to approximately 2-fold after 72 h of dosing. In the right ventricle, expression of PMCA2 was 2.5- and 3.5-fold at 24 and 72 h after dosing. With left ventricles, expression was 4-fold (24 h) and 3-fold (72 h). In the case of PMCA4, increases of 2-fold (right ventricle) and 3-fold (left ventricle) were observed 24 h after dosing. In the right ventricle, expression was similar to control but remained unchanged in the left ventricle after 72 h of treatment. Furthermore, BDM caused an increased expression of Na-K-ATPase (ATP1A1) by 5- and 6-fold in the right and left ventricles, respectively, 24 h after treatment. After 72 h of treatment, ATP1A1 gene expression did not change in the right ventricle but was increased by 4-fold in the left ventricle. In addition, phospholamban was increased by 3.5-fold in the right and left ventricles 24 h after treatment. Transcript levels remained approximately 300% above control after 72 h of treatment.

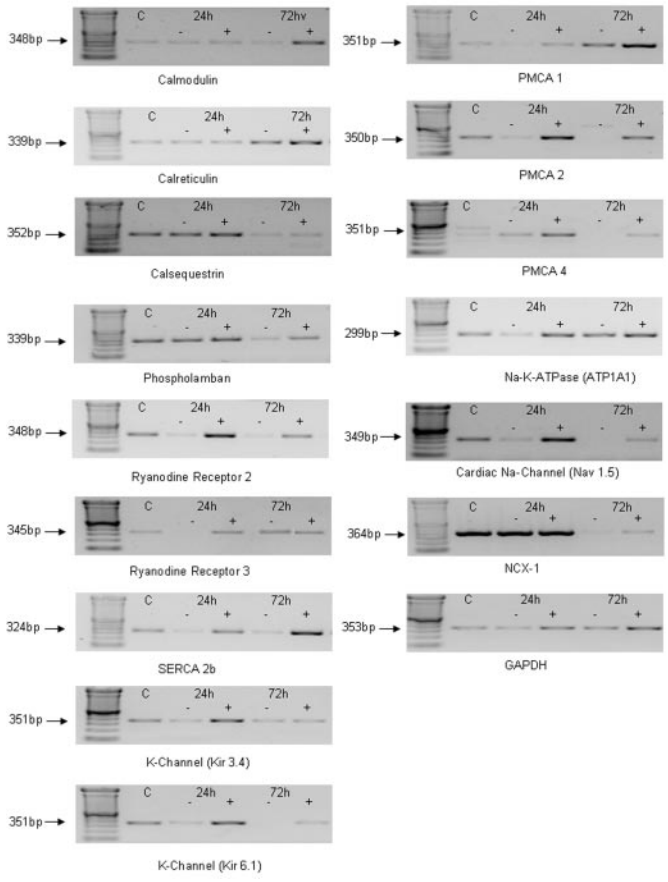

Fig. 1. Examples of ethidium bromide-stained PCR amplification products in cultures of Ca^{2+}-tolerant cardiomyocytes after treatment with BDM.

Expression of Genes Coding for Cytoskeleton. Gene expression of α-skeletal actin was increased by 2.5-fold in both ventricles 24 h after dosing and remained increased 2-fold after 72 h of treatment (Figs. 4 and 6). In contrast, expression of α-cardiac actin was 80 and 60% in right and left ventricles after 24 and 72 h of treatment, respectively. As shown in Fig. 4, BDM had little effect on α-MHC gene expression in either ventricle after 24 and 72 h of treatment. It is noteworthy that gene expression of β-MHC was 200% (24 h) and 500% in right and 400% in left ventricles (72 h) upon treatment with BDM. Gene expression of Troponin T was increased 5-fold (24 h) and 3-fold (72 h) upon treatment with BDM in right and left ventricle, respectively. BDM produced only a 3-fold increase of the gene coding for Troponin I in right ventricle (24 and 72 h) and a 4-fold increase in left ventricle (24 and 72 h).

Expression of Genes Coding for Ca^{2+}-Binding Proteins. Calsequestrin expression was increased nearly 4-fold in both ventricles after 24 and 72 h of treatment (Figs. 5 and 6). Likewise, expression of calmodulin was increased by 3- and 4-fold in the right and left ventricles (24 h) and approximately 4-fold in both ventricles after 72 h of treatment. BDM produced an increase of approximately 6-fold in the gene expression of calreticulin 24 h after treatment and a further 7-fold increase after 72 h.

Discussion

BDM was studied extensively for its effects on rhythm, conduction, and blood pressure. It is currently being evaluated as a cardioprotective agent in transplantation medicine. Its ability to regulate transcription of ion channel and calcium handling proteins of the heart is basically unknown. We therefore studied the effects of BDM in cultures of cardiomyocytes and in vivo in male SD rats. We demonstrate an array of effects in cultures of calcium-tolerant cardiomyocytes and in explanted hearts of male SD rats. Overall, we observed increased transcript levels of ATP-dependent and G-protein-regulated potassium channels, as well as induction in the gene expression of the sodium ion channel. In particular, repressed transcript levels of ion exchangers, calcium binding proteins, and cytoskeletal proteins returned to normal or

were increased in cultures of cardiomyocytes (except for PMCA4 and phospholamban) after treatment with BDM. Thus, BDM rescued expression levels of major ion channels but had little effect on gene expression of cytoskeletal and calcium binding proteins. Although BDM produced similar changes in vivo in explanted rat hearts after 24 and 72 h of treatment, some differences were observed when results from cultured cardiomyocytes were compared with results from in vivo studies. In particular, we observed opposite effects when gene expression of calsequestrin, calreticulin, and calmodulin was compared with results from in vivo studies. Likewise, in the case of β-MHC, results from cultures of cardiomyocytes differ compared with findings from in vivo studies after treatment with BDM. The heart should be considered part of a complex cardiovascular system. The function of the heart in vivo is, on the one hand, influenced by the peripheral resistance (afterload) and, on the other hand, by the end diastolic pressure (preload). This volume load and the resulting stretch forces cannot be adequately simulated in vitro. Even though transcript changes for some genes were similar in vivo and in vitro, the underlying changes may be traced back to different molecular events. Likewise, the differences in response to BDM treatment in vivo and in vitro, as seen for some of the ion channels, ion exchangers, and Ca^{2+}-handling proteins, may aid dissection of direct and indirect effects. Furthermore, there was little difference when left and right ventricles were studied in parallel, even though ventricular gene expression differed slightly for PMCA 1, 2, 4 and SERCA2a and SERCA2b.

An unexpected finding was the up-regulation of ventricular β-MHC after 72 h of treatment. This may or may not be a dose-related effect, but other investigators have used identical doses to profile BDM (Brightman et al., 1995; Xiao and McArdle, 1995a). Our findings fit well with BDM's mode of

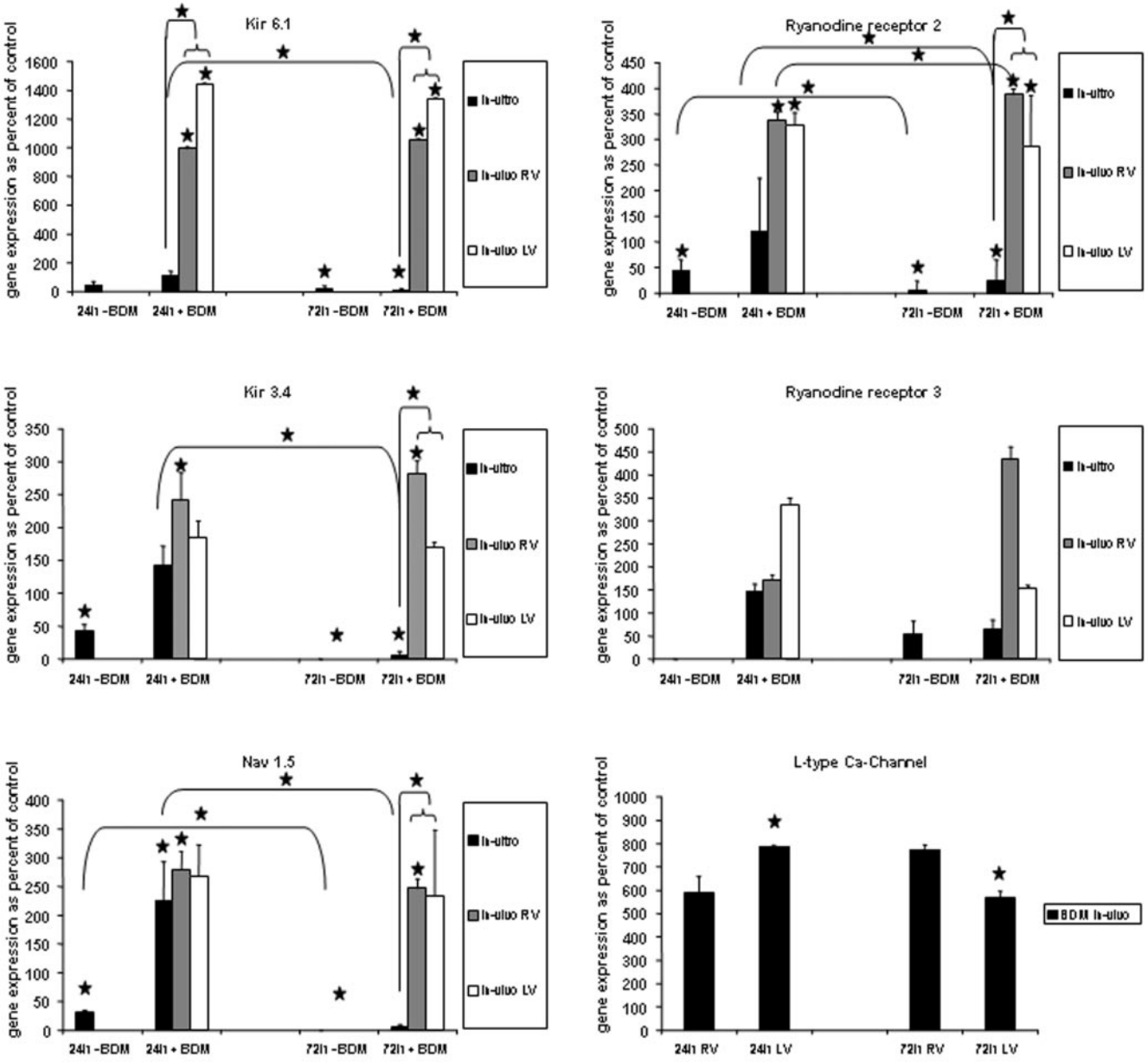

Fig. 2. mRNA expression of ion channels in cultures of Ca^{2+}-tolerant cardiomyocytes and in Sprague-Dawley rats after treatment with BDM.

action, which essentially can be attributed to chemical phosphatase activity and particularly serine-threonine protein dephosphorylation as reported by Stapleton et al. (1998). Indeed, BDM has a dual function (i.e., profiles as electrogenic and "chemical phosphatase"). Several heart-specific proteins (Allen and Chapman, 1995; Allen et al., 1998; Duthe et al., 2000; Watanabe et al., 2001) are influenced by BDM's ability to dephosphorylate amino acid residues, and we propose

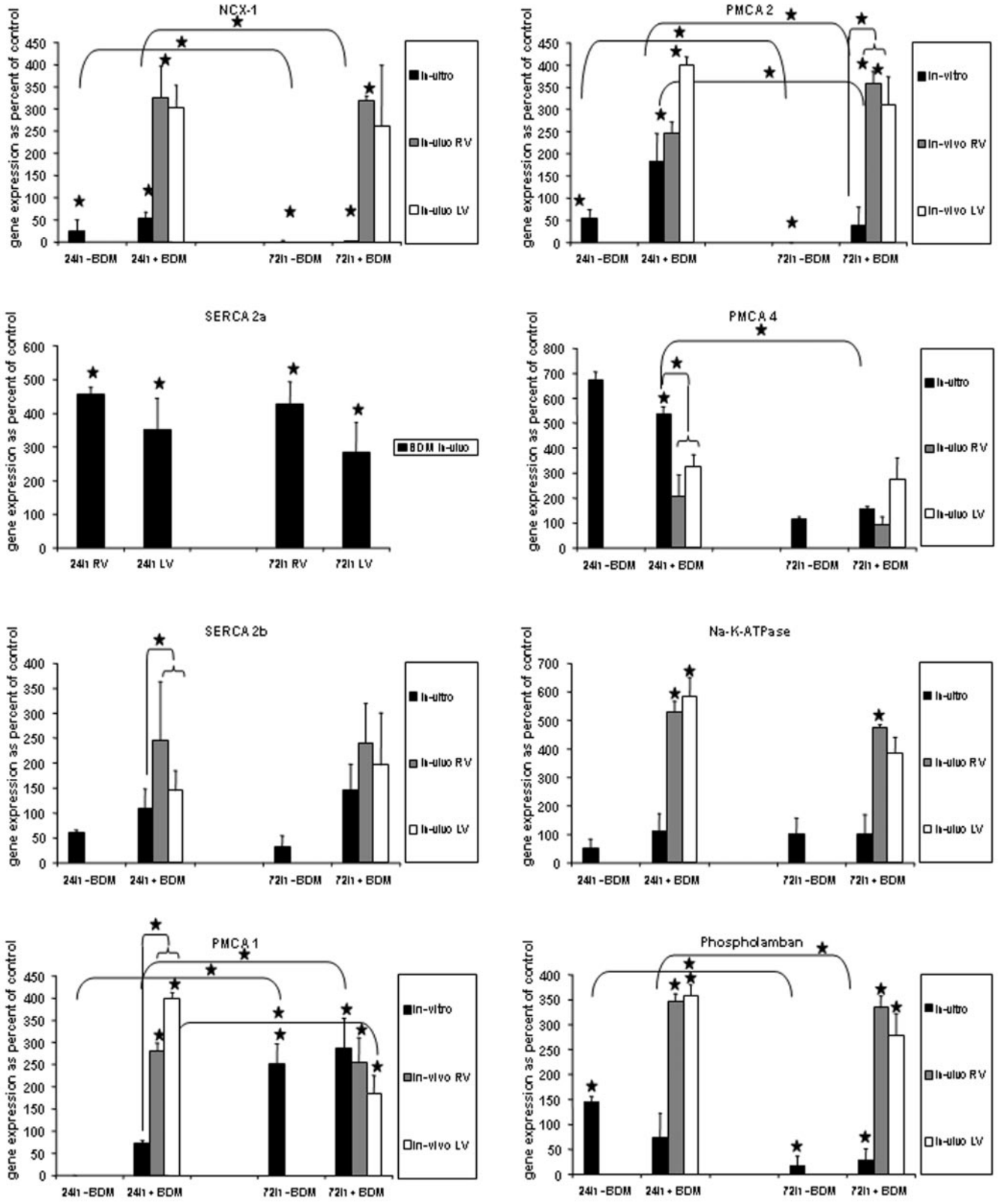

Fig. 3. mRNA expression of ion exchanger in cultures of Ca^{2+}-tolerant cardiomyocytes and in Sprague-Dawley rats after treatment with BDM.

changes in protein phosphorylation to impact transcriptional activation of coded genes, as shown in this study and as discussed below.

BDM drives nonspecific protein dephosphorylation of many cellular proteins, including ion channels. For instance, Eisfeld et al. (1997) demonstrated inhibition of cloned human

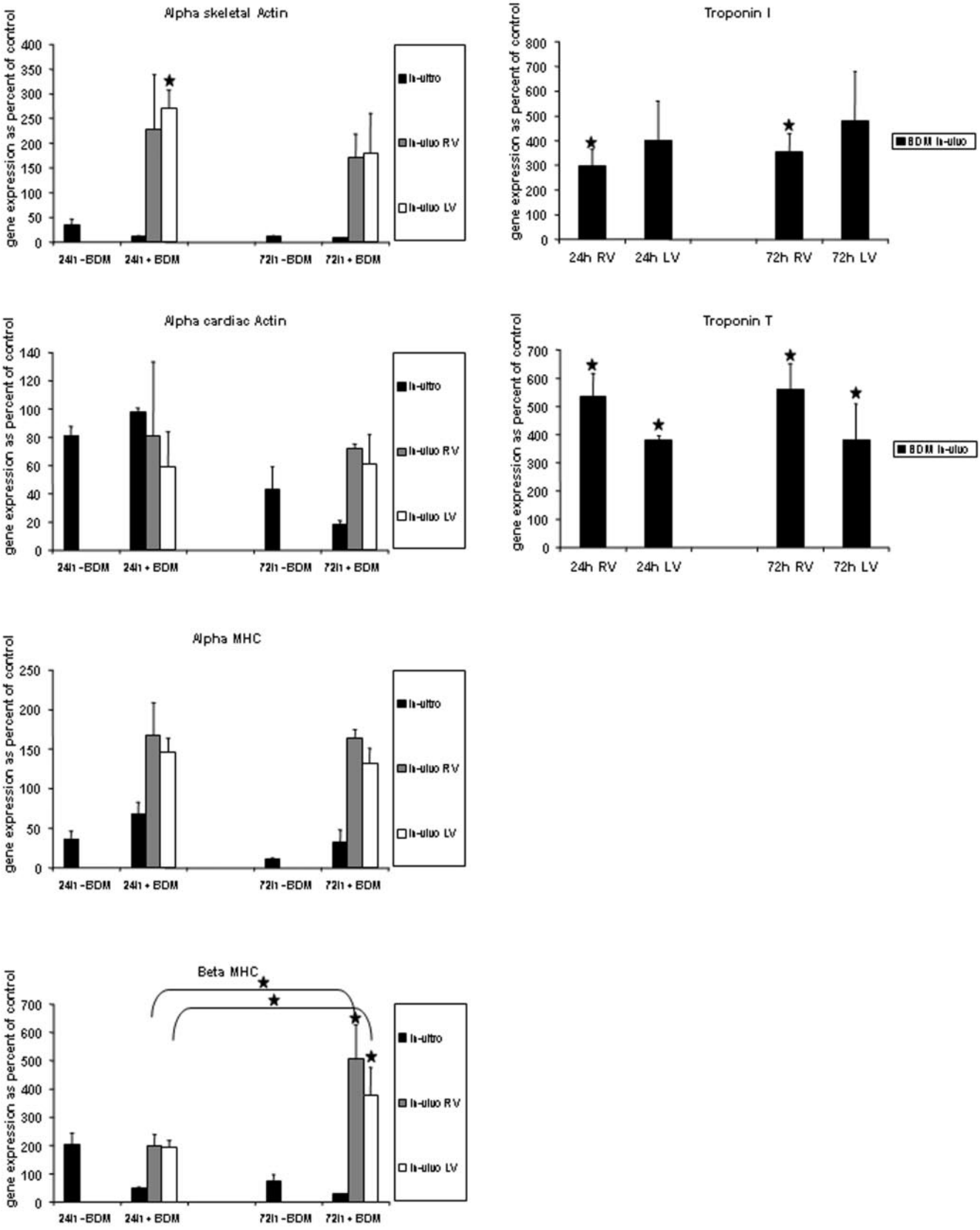

Fig. 4. mRNA expression of cytoskeletal proteins in cultures of Ca^{2+}-tolerant cardiomyocytes and in Sprague-Dawley rats after treatment with BDM.

L-type calcium channels by BDM, although BDM's phosphatase site of action was different from the phosphorylation site of protein kinase A. Likewise, activation of protein kinase A partially reversed the effects of BDM on the transient outward potassium current of rat ventricular cardiomyocytes (Xiao and McArdle, 1995a) and modulation of Kv1.5 current did depend on protein kinase A, tyrosine kinase, and tyrosine phosphatase activity (Mason et al., 2002). These authors concluded that currents encoded by Kv1.5 are regulated by protein kinase A and protein tyrosine phosphatase. In the study of Schlichter et al. (1992), BDM produced a rapid reversible block of potassium current with a half block of about 5 mM in human T-lymphocytes, and BDM inhibited I_{to} in a concentration-dependent manner as suggested by Xiao and McArdle (1995a). I_{to} recovered almost completely at 2 min after washout of BDM, and this suggests a mode of action of oximes that might be explained by protein dephosphorylation. Furthermore, BDM did not suppress the inward-rectifier potassium current of rat ventricular myocytes.

Patel et al. (2000) studied slow inactivation gating of sodium channel α-subunits. In particular, these authors coexpressed sodium channel subunit and protein kinase C in *Xenopus laevis* oocytes and demonstrated phosphorylation of the channel inactivation gate as the mechanism of action, although phosphorylation of other sites in the channel may account for isoform-specific differences. Likewise, activation of protein kinase A (PKA) increased sodium current derived from the human cardiac sodium channel and phosphorylation in addition to putative sarcoplasmic reticulum retention signals required for PKA-mediated potentiation of cardiac sodium current (Zhou et al., 2002). There are additional reports on the role of ion channel phosphorylation by protein kinases, and there is overwhelming evidence for phosphorylation to cause activation of ATP-sensitive potassium channels (Light et al., 2000; Han et al., 2002), but long-term activation of protein kinase C strongly attenuates cardiac delayed-rectifying potassium current when the corresponding changes in capacitance are taken into account (Lo and Numann, 1998). The opening of the ryanodine receptor also depends on phosphorylation by PKA and PKA hyperphosphorylation may result in defective channel function becuase of increased sensitivity to calcium-induced activation, as observed in cardiac disease (Marx and Marks, 2002).

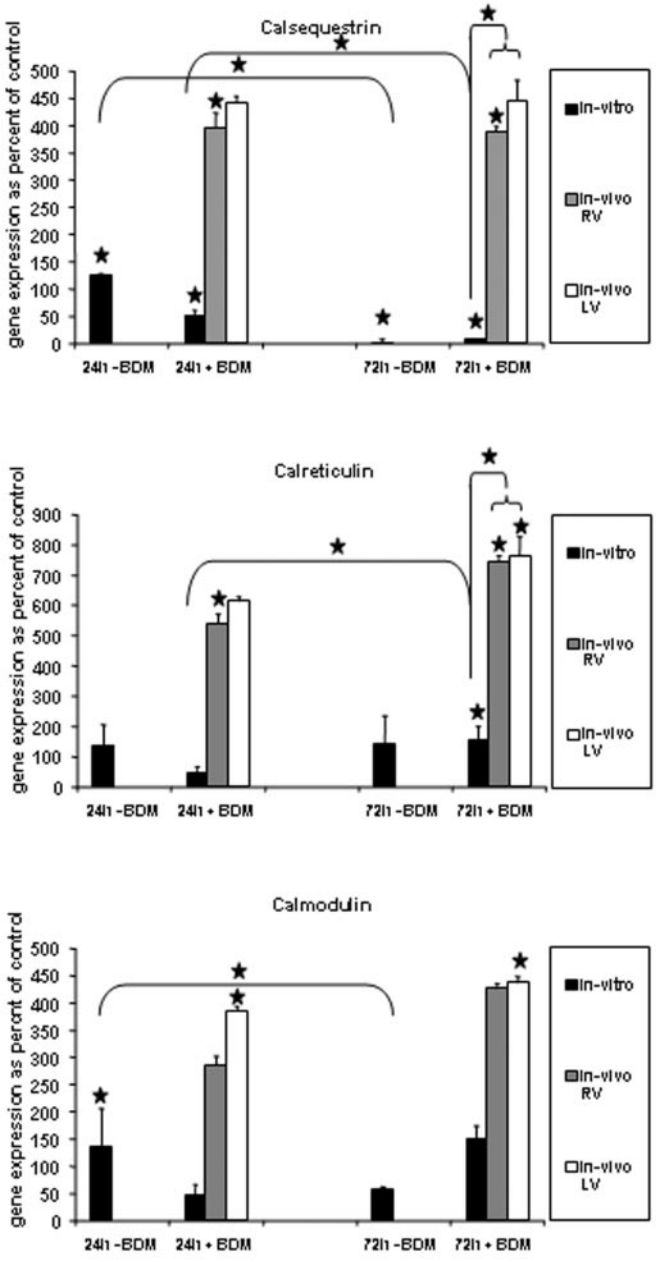

Fig. 5. mRNA expression of Ca^{2+}-sequestrating proteins in cultures of Ca^{2+}-tolerant cardiomyocytes and in Sprague-Dawley rats after treatment with BDM.

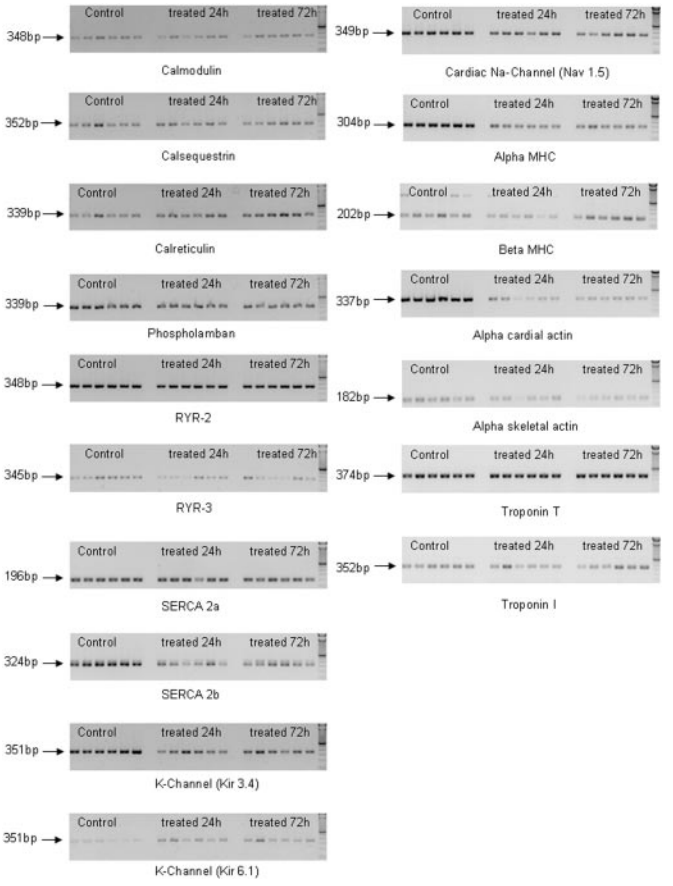

Fig. 6. Examples of ethidium bromide-stained PCR amplification products of genes coding for Ca^{2+}-sequestrating proteins, ion channels, and cytoskeletal proteins in Sprague-Dawley rats after treatment with BDM.

We observed increased expression of genes coding for ion channels and ion exchangers and propose this effect to be an adaptive response for high levels of the corresponding dephosphorylated proteins (Kobayashi et al., 1997; Nikcevic et al., 1999; Sugden, 2001).

This may or may not be a feedback mechanism for the control of gene expression, but there is experimental evidence for some dephosphorylated proteins to dictate specifically transcriptional activation of their coding genes. Indeed, dephosphorylation of the transcription factor NFAT1 resulted in transcriptional activation of the Ca^{2+}-dependent phosphatase calcineurin (Okamura et al., 2000). Similar, dephosphorylation of the inositol 1,4,5-triphosphate receptor by calcineurin resulted in transcriptional activation of the coded gene (Genazzani et al., 1999). Nonetheless, BDM produces an array of effects that might alter gene expression in different ways. For instance, blockage of translation at initiation leads to an increase in α-MHC mRNA from the nonpolysomal fraction (Nikcevic et al., 1999) and dephosphorylation of RNA polymerase II at positions SER2 and SER5 also impacts transcription cycle (Hausmann and Shuman, 2002). Other post-transcriptional mechanisms may include ATP depletion (Stapleton et al., 1998) and decreased phosphorylation of the inhibitory subunit of troponin I as well as phospholamban (Zimmermann et al., 1996).

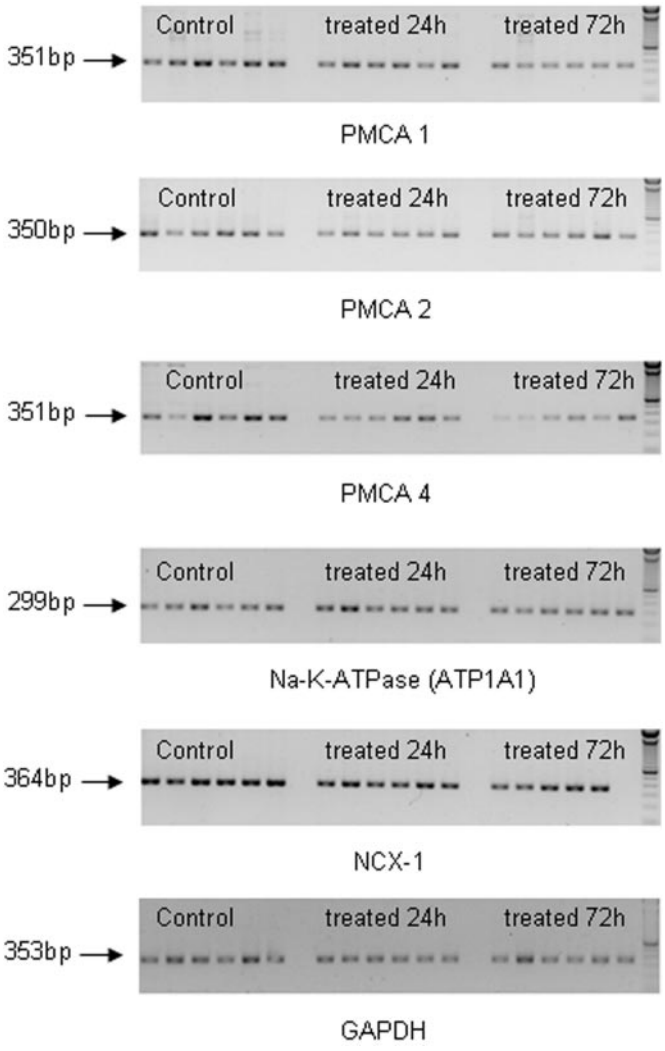

Fig. 7. Examples of ethidium bromide-stained PCR amplification products of genes coding for exchangers in Sprague-Dawley rats after treatment with BDM.

BDM's ability to modulate calcium homeostasis remains controversial. The observed calcium antagonistic effect can be explained, at least in part, by L-type calcium channel blockade and calcium influx into the sarcoplasmatic reticulum (Huang and McArdle, 1992; Otun et al., 1993; Zhu and Ikeda, 1993; Lyster and Stephenson, 1995). It is suggested that the lengthening effect of BDM on action potential duration results mainly from the simultaneous reduction of both the slow inward calcium current and the transient outward current (Coulombe et al., 1990).

We observed induction in the expression of genes coding for the L-type Ca^{2+}-channel, calsequestrin, calreticulin, and calmodulin in explanted rat hearts after treatment with BDM. It would seem that cardiomyocytes aim to restore normal intracellular Ca^{2+} after inhibition of L-type Ca^{2+}-channels. As a consequence of L-type Ca^{2+} channel inhibition, intracellular calcium levels fall (Maesako et al., 2000). This Ca^{2+}-antagonistic effect may result in reduced blood pressure, myocardial calcium currents, and action potential in rats (Xiao and McArdle, 1995a). In addition, BDM promoted voltage-dependent inactivation of L-type calcium channels in parallel with charge interconversion between intramembranous charges (Ferreira et al., 1997) and reduced open probability of single L-type cardiac channels in cell-attached patches from guinea pig ventricular myocytes to cause a fall in channel availability (Allen and Chapman, 1995). Therefore, we observed highly significant induction of the gene coding for L-type Ca^{2+} channel. Furthermore, transcriptional up-regulation of Ca^{2+}-handling proteins increases cytosolic and sarcoplasmic Ca^{2+}-buffer capacity but should not reduce intracellular Ca^{2+} as reported for calsequestrin (Terentyev et al., 2003).

In summary, we show BDM to increase transcript level of genes coding for ion channels, ion exchangers, and calcium binding proteins, but there were differences in response when BDM was studied in vitro and in vivo. Our findings are highly suggestive of an adaptive response of heart muscle cells toward unscheduled and indiscriminate dephosphorylation. Further studies are now needed to understand this proposed feedback mechanism between enhanced protein dephosphorylation and transcriptional activation of the corresponding genes, as shown in this study.

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