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2,3-Butanedione monoxime attenuates the β-adrenergic response of the L-type Ca^{2+} current in rat ventricular cardiomyocytes

[La 2,3-butanodiona monoxima atenúa la respuesta β-adrenérgica de la corriente de Ca^{2+} tipo L en cardiomiocitos ventriculares de rata]

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Abstract

Context: 2,3-Butanedione monoxime (BDM), an uncoupler of cardiac contraction, is commonly used in enzymatic dissociations to prevent hypercontraction of cardiomyocytes and in cardioplegic solutions to decrease oxygen demand during surgery. However, BDM affects multiple cellular systems including the L-type Ca^{2+} current (I_{CaL}). If its phosphatase activity is the mechanism underlying the decrease I_{CaL} in cardiomyocytes is a still unresolved question.

Aims: To study the effects of BDM on I_{CaL} of rat ventricular cardiomyocytes focusing our attention on the response of I_{CaL} to β-adrenergic stimulation.

Methods: The whole-cell patch-clamp method was used to study I_{CaL} in enzymatically dissociated rat ventricular cardiomyocytes.

Results: Extracellular BDM (5 mM) decreased peak I_{CaL} by ≈45%, slowed its fast inactivation but accelerated its slow inactivation. Cardiomyocytes incubated in BDM (≥ 30 min; 5 mM) perfused with normal extracellular solution, showed normal I_{CaL} properties. However, extracellular BDM (in cardiomyocytes incubated in BDM or not) markedly reduced the response of I_{CaL} to isoproterenol (1 μM). BDM also strongly attenuated the increase of I_{CaL} in cardiomyocytes intracellularly perfused with cyclic AMP (50 μM).

Conclusions: The decrease of basal I_{CaL} by BDM is not related to its dephosphorylation action. Its effect on the Ca^{2+} channel occurs most probably in a site in the extracellular side or within the sarcolemmal membrane. Due to its phosphatase action, BDM strongly attenuates the response of I_{CaL} to β-adrenergic stimulation. These actions of BDM must be taken into account both for its use in the dissociation of cardiomyocytes and in cardioplegic solutions and myocardial preservation.

Keywords: 2,3-butanedione monoxime; calcium channels; cardiomyocytes; myocardial preservation; patch-clamp.

Resumen

Contexto: La 2,3-butanodiona monoxima (BDM), un desacoplador de la contracción cardíaca, es comúnmente utilizada en la disociación enzimática para prevenir la hipercontracción de los cardiomiocitos y en soluciones de cardioplejia para reducir la demanda de oxígeno durante la cirugía. No obstante, la BDM reduce la corriente de Ca^{2+} tipo L (I_{CaL}) pero su mecanismo de acción no ha sido dilucidado definitivamente.

Objetivos: Estudiar los efectos de la BDM sobre I_{CaL} de cardiomiocitos ventriculares de rata, centrando la atención en la respuesta de I_{CaL} al isoproterenol (ISO).

Métodos: Se utilizó la técnica de patch-clamp para registrar I_{CaL} en cardiomiocitos ventriculares de rata disociados enzimáticamente.

Resultados: La BDM extracelular (5 mM) redujo I_{CaL} en ≈45% y modificó su inactivación rápida. Los cardiomiocitos incubados en BDM (≥ 30 min; 5 mM) y perfundidos con solución extracelular normal, mostraron I_{CaL} normales. No obstante, la BDM extracelular (en cardiomiocitos incubados en BDM o no incubados), redujo marcadamente la respuesta de I_{CaL} al ISO (1 μM). La BDM atenuó fuertemente el aumento de I_{CaL} en cardiomiocitos perfundidos intracelularmente con AMP cíclico.

Conclusiones: La reducción de I_{CaL} basal por BDM no está relacionada a su actividad desfosforiladora. Su efecto sobre el canal de Ca^{2+} ocurre probablemente en un sitio extracelular. Debido a su acción como fosfatasa, la BDM atenúa fuertemente la respuesta de I_{CaL} al ISO. Estas acciones de la BDM deben ser consideradas tanto para su utilización en la disociación de cardiomiocitos como en las soluciones de cardioplejia y la preservación miocárdica.

Palabras Clave: 2,3-butanodiona monoxima; canales de calcio; cardiomiocitos; patch-clamp; preservación miocárdica.

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INTRODUCTION

Isolated adult cardiomyocytes stand for the experimental model of choice in studies of the biochemical, biophysical, electrical and contractile activities of normal and diseased myocardium and also in pharmacological studies (Bers, 2001). Dissociation methods of adult cardiomyocytes are quite diverse and almost every laboratory uses its own protocol. However, one feature in common to all methods is to try to prevent the well-known "calcium paradox phenomenon" (Daly et al. 1987) that results in hypercontraction and death of cardiomyocytes after calcium re-admission following perfusion of hearts will nominally calcium-free solutions. Because of its ability to uncouple cardiac (and skeletal) muscle contraction 2,3-butanedione monoxime (BDM), was profiled to have "cardioprotective" properties and has been used to prevent hypercontraction of Ca^{2+} -intolerant cardiomyocytes in many dissociation protocols (e. g. Kivistö et al., 1995; Chung et al., 2015; see for review Abi-Gerges et al., 2013). Originally developed as a reactivator of acetylcholinesterase (Wilson and Ginsburg, 1955), BDM has been considered as a "chemical phosphatase". This compound affects serine/threonine protein phosphorylation (Stapleton et al., 1998) and has been shown to affect the activities of proteins like myosin-II light chain kinase (Siegman et al., 1994); BDM is known to increase the equilibrium constant for ATP hydrolysis inhibiting the rate of phosphate release and stabilizing the M.ADP.PI intermediate (Herrmann et al., 1992) whose overall effect is the inhibition of the myosin ATPase rate and a decrease in force production. Yet, BDM is not considered to be a general myosin ATPase inhibitor (Ostap, 2003). BDM has been also used in cardioplegic solutions to suppress contraction in order to preserve the myocardium by decreasing oxygen demand thus preserving energy (ATP) during surgery (Stringham et al., 1994; Vahl et al., 1995; Habazettl et al., 1998; Jayawant et al., 1999; Warnecke et al., 2002; Chambers, 2005; Reichert et al., 2013; Lee et al., 2016).

While these actions of BDM may be useful to obtain high yield of non-contracted myocytes after enzymatic digestion and even to preserve myocardium during cardioplegic arrest, BDM is known to affect other cellular mechanisms such as the activity of connexins (Verrechia and Hervé, 1997), to block the Na-Ca exchanger (Watanabe et al., 2006) and the expression (Borlak and Zwadlo; 2004) and the activity of ionic channels (e.g. Schlichter et al., 1992; Lopatin and Nichols, 1993). It has been shown that BDM promotes inhibition of mitochondrial respiration by acting directly on electron transport chain reducing cell viability (Hall and Hausenloy, 2016). BDM inhibits the L-type Ca^{2+} (I_{CaL}) in cardiac ventricular myocytes (Coulombe et al., 1990) and in guinea-pig taenia caeci (Lang and Paul, 1991); the effects on I_{CaL} could be more marked in ventricular myocytes from spontaneously hypertensive rats (Xiao and McArdle, 1995). The decrease of I_{CaL} was accompanied by an acceleration of its inactivation (Coulombe et al., 1990; Chapman, 1993; Allen and Chapman, 1995). Although Schwinger et al. (1994) suggested that BDM does not affect the β-adrenergic response in human myocardium, Chapman (1993) and Allen and Chapman (1995) showed that, due to its phosphatase activity, BDM interfered with the β-adrenergic response of I_{CaL} in ventricular myocytes. Nonetheless, this has been questioned by Eisfeld et al. (1997) and Allen et al. (1998) who demonstrated that BDM does not interfere with the interaction sites between PKA and cardiac Ca^{2+} channel expressed in HEK 293 cells and *Xenopus* oocytes, respectively. It can be argued, however, that heterologous expression systems do not exactly reproduce native cellular systems leaving open the question of whether BDM affects or not the β-adrenergic response of I_{CaL}.

Regarding the mechanism of the decrease of I_{CaL} by BDM, the prevailing idea has been that due to its phosphatase like activity, this oxime affects the phosphorylated state of the L-type Ca^{2+} channel (Coulombe et al., 1990; Chapman, 1993; Allen and Chapman, 1995). Also, in murine DRG neurons, Huang and McArdle (1992) suggested that the decrease of an L-type Ca^{2+} current by BDM could be related to alterations in PKA regulation of I_{CaL}. On the other hand, Lang and Paul (1991) in guinea-pig taenia caeci cells suggested that blockade of I_{CaL} by BDM could be related to the interactions of this oxime on resting and/or inactivated states of the Ca^{2+} channel. Ferreira et al. (1997) pointed out that the effects of BDM on I_{CaL} and its increase in inactivation time constants could be mechanistically consistent with dephosphorylation but also with a dihydropyridine-like action; nevertheless, they ruled-out an open

channel block as a mechanism of BDM on the L-type Ca^{2+} current. A clear-cut mechanism of I_{CaL} decrease by BDM has not been established.

The aim of the present study was to re-evaluate the effects of BDM on I_{CaL} of rat ventricular cardiomyocytes focusing our attention on the changes in the response of I_{CaL} to β-adrenergic stimulation. Our results show that when cardiomyocytes were incubated in BDM, the β-adrenergic response of I_{CaL} is greatly attenuated.

MATERIAL AND METHODS

Chemicals

2,3-Butanedione monoxime ($C_4H_7NO_2$; PubChem CID: 6409633; CAS Number: 57-71-6; >98%) was purchased from Sigma Aldrich and was prepared in ethanol as stock solution. All other chemicals were also from Sigma Aldrich.

Animals

Experiments were performed using male adult Wistar (7 - 8 weeks) rats according to the procedures approved by the National Center for Laboratory Animal Reproduction (CENPALAB; Santiago de Las Vegas, La Habana, Cuba). Prior to experiment, animals were adapted for seven days to laboratory conditions (controlled temperature 25 ± 2°C, relative humidity 60 ± 10% and 12 h light/dark cycles). Tap water and standard diet for rodents supplied by CENPALAB were freely provided. All procedures were also conducted according to the European Commission guide-lines for the use and care of laboratory animals and approved by the Committee for Animal Care in Research of the Center. The minimum number of animals and duration of observation required to obtain consistent data were employed.

Enzymatic isolation of ventricular cardiomyocytes

Ventricular cardiomyocytes were isolated as previously described in detail (Alvarez-Collazo et al., 2012) and were kept in a K^+-Tyrode solution containing 1 mM Ca^{2+} at room temperature (21 ± 2 °C) and used for experiments for 6 h.

Patch-clamp recordings

Aliquots of cardiomyocytes were transferred to a Petri dish (with the same K^+-Tyrode solution) on the stage of an inverted microscope. Relaxed, non-contracting, cardiomyocytes exhibiting clear striated pattern were selected for patch-clamping. Whole-cell currents were recorded at room temperature (Alvarez-Collazo et al., 2012). Currents were filtered at 3 kHz and digitized at 50-µs intervals, stored on a computer and analyzed off-line with the ACQUIS1 software (version 2.0, CNRS License, France). To study L-type Ca^{2+} currents, K^+ currents were blocked by substituting all potassium by cesium in extracellular and "intracellular" solutions. The extracellular solution contained (in millimolars): 117 NaCl, 20 CsCl, 10 HEPES, 2 $CaCl_2$, 1.8 $MgCl_2$, and 10 glucose, pH 7.4. The standard pipette (intracellular) solution contained (in millimolars): 130 CsCl, 0.4 Na_2GTP, 5 Na_2ATP, Na_2-creatine phosphate, 2.0 $MgCl_2$, 11 EGTA, 4.7 $CaCl_2$ (free Ca^{2+} ≈108 nM), and 10 HEPES, with pH adjusted to 7.2 with CsOH. In the experiments, cells were first left to lie in Petri dishes filled with K^+-Tyrode solution with 1 mM Ca^{2+}. Cells attached to the micropipette could be positioned on the extremity of each of six microcapillaries (i.d. 250 µm) through which the different extracellular Cs^+-containing solutions were perfused by gravity (≈15 µL/min), allowing rapid changes (≈1 s) of the extracellular medium.

Pipette resistance was 1.0 - 1.2 MΩ. Membrane capacitance (Cm) and series resistance (Rs) were calculated on voltage-clamped cardiomyocytes as previously described (Alvarez et al., 2000). Average Cm and uncompensated Rs were 168 ± 8.6 pF and 3.3 ± 0.5 MΩ, respectively (n = 44). Rs could be electronically compensated up to 50% without ringing and was continually monitored during the experiment. Liquid junction potential was compensated before establishing the gigaseal. No leak or capacitance subtractions were performed in the recordings.

For routine monitoring of the L-type Ca^{2+} current (I_{CaL}) a double pulse voltage-clamp protocol was employed: from a holding potential (HP) of -80 mV every 4s the cell membrane was depolarized by a prepulse to -40 mV for 50 ms to inactivate the fast Na^+ current. From this membrane potential, a 200-ms test pulse to 0 mV evoked I_{CaL}. Current-to-voltage relationships (I-V) and availability curves

were obtained using the same prepulse protocol but interpolating 300-ms pulses from -40 to +50 mV between the pre- and test pulses. Pulses for I-V and availability curves were applied at 8 s intervals. The inactivation time course of I_{CaL} was fitted to a double exponential using the fitting procedures of the ACQUIS1 software.

Statistical analysis

Results are expressed as means and standard errors of means. Statistical significance was evaluated by means of paired or unpaired Student's t test according to the experimental situation. Differences were considered statistically significant for $p < 0.05$.

RESULTS

Effects of BDM on basal I_{CaL}

Under control condition, peak I_{CaL} density at 0 mV was 9.2 ± 0.6 pA/pF; its inactivation time course could be fitted to fast (τ_{fast}) and slow (τ_{slow}) exponentials whose values were 5.9 ± 0.3 ms and 53.2 ± 2.2 ms, respectively (n = 12). Extracellularly applied BDM decreased I_{CaL} in a fast (2 - 3 pulses) tonic fashion (Fig. 1) with an IC_{50} near to 5 mM (5.4 ± 0.3 mM; n = 3), close to that reported by Chapman (1993) and Lang and Paul (1991) in cardiomyocytes and smooth muscle cells, respectively. From here on, in all the experiments reported, BDM was used at 5 mM concentration. At this concentration, BDM decreased peak I_{CaL} by 43.1 ± 7.5% and significantly increased τ_{fast} to 7.6 ± 0.9 ms and decreased τ_{slow} to 44.7 ± 2.2 ms (Fig. 2A-B; $p < 0.05$). The action of BDM on I_{CaL} was also rapidly reversed upon washout ("on - off" effect; Fig. 1).

Basal I_{CaL} in cardiomyocytes incubated in 5 mM BDM

In a series of experiments, after enzymatic dissociation, cardiomyocytes were incubated in the same K^+-Tyrode solution (1 mM Ca^{2+}) supplemented with 5 mM BDM at room temperature (21 ± 2°C) for 30 minutes. Aliquots of cardiomyocytes were then transferred to the Petri dish containing K^+-Tyrode solution without BDM. They were quickly patch-clamped (time to achieve whole cell configuration was less than 2 min) and perfused with normal extracellular solution (without BDM). Under these condition, I_{CaL} density was 9.1 ± 1.4 pA/pF (n = 8), not significantly different from that of control cardiomyocytes. τ_{fast} was not different from that of control cardiomyocytes (5.9 ± 1.1 ms) and τ_{slow} was slightly decreased (46.0 ± 3.8 ms), but not statistically significant (Fig. 2A-B). In these BDM-incubated cardiomyocytes, perfusion with an extracellular solution containing BDM (5 mM) had essentially the same "on - off" effect as in control (not incubated) cardiomyocytes; I_{CaL} density at 0 mV was decreased by 43.8 ± 8.0% but τ_{fast} was markedly increased to 9.6 ± 1.0 ms ($p < 0.05$). Contrary to what occurred in control cardiomyocytes, τ_{slow} was increased by BDM to 57.2 ± 7.9 ms; however, this effect was not statistically significant (Fig. 2A-B).

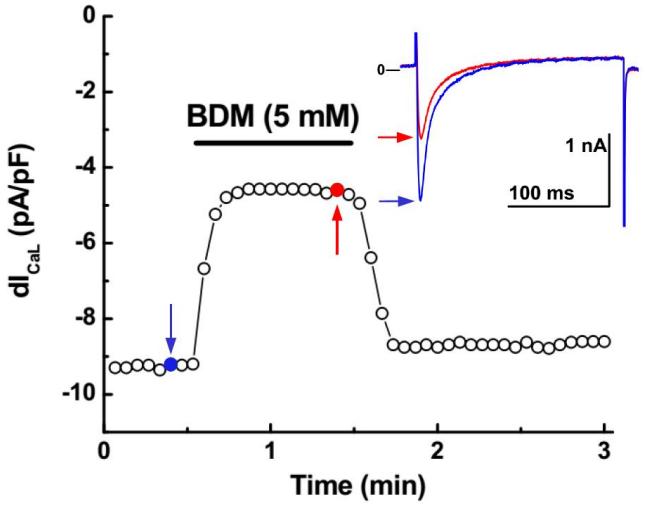

Figure 1. Time course of the effect of extracellularly applied BDM on I_{CaL} in a rat ventricular cardiomyocyte.

BDM (5 mM) decreases I_{CaL} by ≈50% in an "on - off" fashion. The action of BDM was fully reversible upon returning to control solution. The inset shows the current traces corresponding to the blue (Control) and red (stable BDM effect) arrows at different times during the experiment.

BDM does not change I_{CaL} voltage-dependence

Current-to-voltage relationships and availability curves of I_{CaL} were obtained using standardized protocols. Availability curves of I_{CaL} from -80 to 0 mV were fitted to a Boltzmann function ($f_\infty = 1 / 1 + \exp [V_{0.5} / s]$) to obtain the voltage for half inactivation ($V_{0.5}$) and the slope factor (s). In control, not incubated, cardiomyocytes (n = 12) the effects of extracellular BDM (5 mM) were not voltage-dependent; voltage for maximal I_{CaL} (0 mV) or its reversal po-

tential (≈+50 mV) were not shifted (Fig. 3A). Consequently, availability curves were barely modified; $V_{0.5}$ was -29.5 ± 0.3 mV in control and -31.7 ± 0.3 mV in the presence of BDM (Fig. 3B; n = 8). The slope factor, s, was not significantly changed (5.9 ± 0.4 mV vs 5.6 ± 0.3 mV). In BDM-incubated cardiomyocytes (n = 8) perfused with normal extracellular solution (as described above) voltage for maximal I_{CaL} and its reversal potential were similar to those of control cardiomyocytes (Fig. 3C). $V_{0.5}$ and s were not significantly different from control cardiomyocytes (-30.9 ± 0.2 mV and 5.5 ± 0.2 mV, respectively; Fig. 3D). Perfusion of these cardiomyocytes with extracellular BDM (5 mM; Fig. 3D) produced no significant effects on $V_{0.5}$ (-31.0 ± 0.2 mV) and s (5.3 ± 0.4 mV).

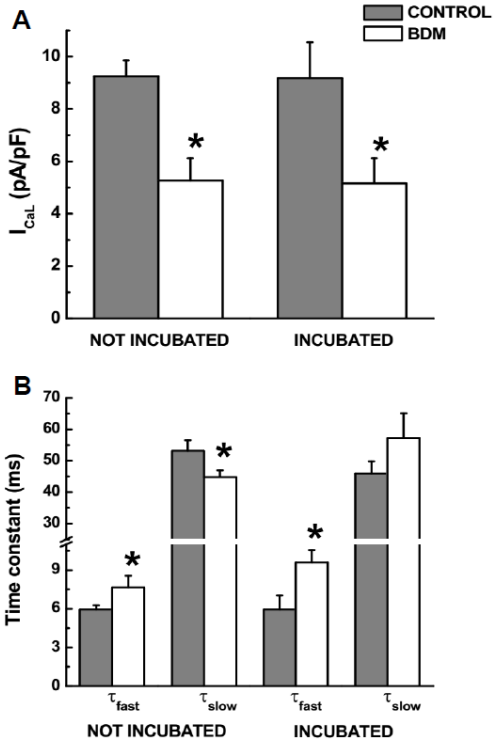

Figure 2. Extracellularly applied BDM decreases I_{CaL} density and changes its inactivation time course.

A. In cardiomyocytes not incubated as well as in cardiomyocytes incubated in 5 mM BDM for at least 30 min, extracellularly applied BDM (5 mM) induced a similar decrease in I_{CaL} density. **B.** Effects of extracellular BDM on fast (τ_{fast}) and slow (τ_{slow}) inactivation time constants. In both not incubated and incubated cardiomyocytes, extracellular BDM significantly increased τ_{fast}. However, in not incubated cardiomyocytes extracellular BDM decreased τ_{slow} but showed a tendency (not statistically significant) to increase τ_{slow} in incubated cardiomyocytes. It is to note that BDM incubation *per se* had no effect on I_{CaL} density or its inactivation time constants when incubated cardiomyocytes are perfused with control extracellular solution.
*p < 0.05 with respect to its control value.

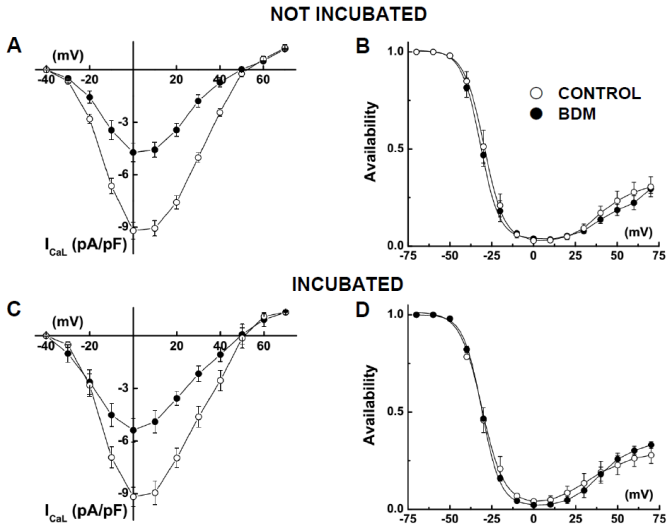

Figure 3. Extracellular BDM did not change the voltage-dependence of I_{CaL}.

A. Current-to voltage relationships, in cardiomyocytes not incubated in BDM, under control condition (○) and in the presence of 5 mM extracellular BDM (●). **B.** Corresponding availability curves. $V_{0.5}$ and s were -29.5 ± 1.7 mV and 31.7 ± 1.6 mV and 5.9 ± 1.1 mV and 5.6 ± 0.3 mV in control and BDM, respectively. **C.** Current-to voltage relationships, in cardiomyocytes incubated in BDM, under control condition (○) and in the presence of 5 mM extracellular BDM (●). **D.** Corresponding availability curves. $V_{0.5}$ and s were -30.9 ± 1.5 mV and 31.0 ± 1.5 mV and 5.5 ± 0.8 mV and 5.3 ± 0.4 mV in control and BDM, respectively.

BDM attenuates the response of I_{CaL} to β-adrenergic stimulation

β-adrenergic stimulation increases I_{CaL} via a well-characterized signaling cascade and is one of the most stable cardiomyocyte response to neuromediators (Bénitah et al., 2010). In order to investigate the possible effects of BDM (5 mM) on the response of I_{CaL} to β-adrenergic stimulation by isoproterenol (ISO, 1 μM) we considered four experimental conditions: A.- Control, not incubated, cardiomyocytes on which ISO was applied (n = 6). B.- Cardiomyocytes incubated in BDM on which ISO was applied (n = 6). C.- Control, not incubated, cardiomyocytes on which BDM was applied and then ISO in the presence of BDM (n = 12). D.- Cardiomyocytes incubated in BDM on which BDM was applied and then ISO in the presence of BDM (n = 8). Cardiomyocytes included in "C" and "D" experimental conditions were the same already presented in the previous sections. Under control conditions (experimental condition "A"), 1 μM isoproterenol (ISO) induced an increase in I_{CaL}, which was stable in 3 - 4 min. Mean increase of I_{CaL} by ISO was 60.8 ± 4.1% (n = 6). τ_{fast} increased from 5.8 ± 0.3 ms to 6.7 ± 0.3 ms ($p < 0.05$) and τ_{slow} decreased from 56.6 ± 3.3 ms to 50.6 ± 2.6 ms ($p < 0.05$; Fig. 4A-B). These effects were similar to those described by our group under similar experimental conditions (Alvarez et al., 2004). ISO did not prevent the decrease in I_{CaL} by BDM. In three cardiomyocytes, BDM (5 mM) applied during the ISO effect still decreased I_{CaL} by 44.3 ± 2.0%. When ISO was applied to BDM-incubated cardiomyocytes (experimental condition "B" as described above), mean increase of I_{CaL} was only 7.4 ± 2.1% (n = 6; $p < 0.05$ with respect to condition "A"). Both τ_{fast} and τ_{slow} showed a tendency to decrease (from 5.9 ± 1.1 ms and 45.9 ± 3.8 ms to 5.5 ± 1.5 ms and 42.5 ± 2.7 ms, respectively) but without statistical significance (Fig. 4A-B). When BDM was applied before ISO in control cardiomyocytes, β-adrenergic response of I_{CaL} was also greatly attenuated. In control cardiomyocytes (experimental condition "C"), ISO under the effect of extracellular BDM, increased I_{CaL} by only 14.2 ± 4.3% (n = 12; $p < 0.05$ with respect to condition "A"); τ_{fast} was increased to 10.4 ± 1.4 ms and τ_{slow} was decreased to 36.6 ± 2.5 ms ($p < 0.05$; Fig. 4A-B). In BDM-incubated cardiomyocytes (experimental condition "D"), ISO was practically without effect under the action of extracellular BDM, I_{CaL} was only increased by 2.8 ± 1.7%. Both τ_{fast} and τ_{slow} showed a tendency to decrease (from 9.6 ± 0.9 ms to 9.3 ± 1.3 ms and from 57.2 ± 7.9 ms to 53.6 ± 8.8 ms, respectively) but without statistical significance (n = 8; Fig. 4A-B).

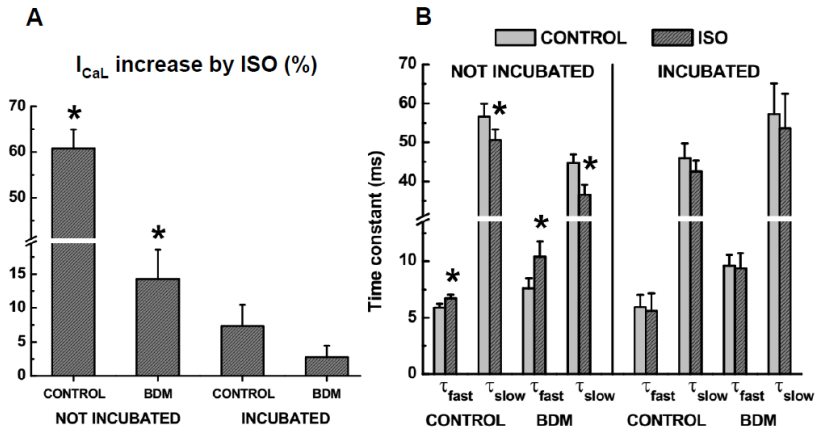

Figure 4. BDM attenuates the response of I_{CaL} to β-adrenergic stimulation.

A. In control cardiomyocytes, not incubated in BDM, isoproterenol (ISO, 1 μM) significantly ($p < 0.05$) increased I_{CaL} density by ≈60%. If ISO was applied after I_{CaL} was decreased by extracellular BDM (5 mM), then I_{CaL} was increased by only ≈15%, but still statistically significant. In cardiomyocytes previously incubated in BDM (5 mM, the β-adrenergic increase in I_{CaL} density was almost abolished. **B.** In control cardiomyocytes, not incubated in BDM, the characteristic response of I_{CaL} inactivation to β-adrenergic stimulation is an increase in τ_{fast} and a decrease in τ_{slow}. This typical response was not changed when ISO was applied after I_{CaL} was decreased by extracellular BDM (5 mM). However, in cardiomyocytes previously incubated in BDM there were no significant changes in τ_{fast} and τ_{slow} after β-adrenergic stimulation.
*$p < 0.05$ with respect to its control value.

From these results, it is clear that BDM attenuates β-adrenergic response of I_{CaL}. We next studied whether BDM was also able to attenuate the response of I_{CaL} to intracellular 3',5'-cyclic adenosine monophosphate (cAMP) a well-known activator of protein kinase A. Not incubated (n = 4) and BDM-incubated (n = 4) cardiomyocytes were patch-clamped with pipettes containing the normal "intracellular" solution but added with 50 µM cAMP. Immediately after patch rupture I_{CaL} was continuously monitored. In not incubated cardiomyocytes, I_{CaL} increased by 167.6 ± 22.0% from its initial value in about 2 min (Fig. 5A; see also, Alvarez-Collazo et al., 2012). In BDM-incubated cardiomyocytes, however, I_{CaL} was barely increased by 10.0 ± 6.0% from its initial value (Fig. 5B). Moreover, in two control, not incubated, cardiomyocytes, application of extracellular BDM after the steady-state cAMP effect, still decreased I_{CaL} by 42 and 48%. The effect was "on - off" but after washout, I_{CaL} never recovered its maximal attained value (Fig. 6).

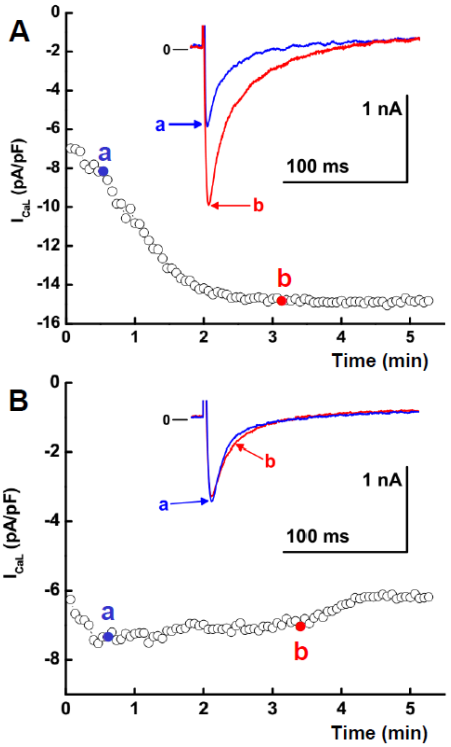

Figure 5. BDM attenuates the response of I_{CaL} to intracellularly applied cyclic adenosine monophosphate (3', 5'-cAMP; 50 µM).

A. Example of a control cardiomyocyte (not incubated in BDM) intracellularly perfused with cAMP in which there is a huge increase in I_{CaL} density. **B.** In a cardiomyocyte previously incubated in 5 mM BDM, there was almost no effect of cAMP on I_{CaL}.

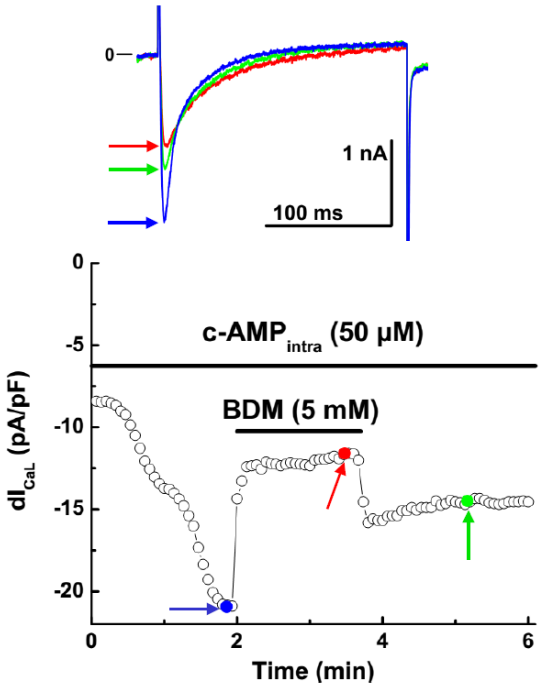

Figure 6. Extracellularly applied BDM is able to decrease I_{CaL} density in cardiomyocytes intracellularly perfused with cAMP.

In a cardiomyocyte not incubated in BDM, after I_{CaL} was maximally increased by intracellular cAMP, extracellular perfusion with 5 mM BDM is still able to decrease I_{CaL} by ≈50%. Upon washout with normal extracellular solution, I_{CaL} never returned to its maximal value previous to BDM. The inset shows the current traces corresponding to the blue (Control), red (stable BDM effect) and green (washout of BDM) arrows at different times during the experiment.

DISCUSSION

The main outcome of the present investigation is that, in isolated rat ventricular cardiomyocytes, BDM attenuates the response of I_{CaL} to β-adrenergic stimulation. Our results also suggest that BDM could inhibit the L-type Ca^{2+} channel by acting on a site in the external side of the sarcolemmal membrane.

Our results show that extracellular BDM decrease I_{CaL} in an "on - off" manner with an IC_{50} around 5 mM, similar to that commonly reported for cardiac myocytes (Coulombe et al., 1990; Chapman, 1993; but see Xiao and McArdle, 1995) and smooth muscle cells (Lang and Paul, 1991) but that is lower than the IC_{50} reported for the inhibition of an L-type Ca^{2+} current in neurons (Huang and McArdle, 1992) and of the human L-type Ca^{2+} channel expressed HEK 293 cells (Eisfeld et al., 1997) and *Xenopus* oocytes (Allen et al., 1998). The decrease of I_{CaL} by BDM in the present

experiments was not voltage-dependent since neither the I-V relationships nor the availability curves were modified by the oxime. This is in agreement with the results of Huang and McArdle (1992) in neurons, Lang and Paul (1991) in smooth muscle cells and Eisfled et al. (1997) in HEK-293 cells expressing the human L-type Ca^{2+} channel. Coulombe et al. (1990) and Ferreira et al. (1997) in rat and guinea-pig ventricular cardiac myocytes, respectively and Allen et al. (1998) in L-type Ca^{2+} channels expressed in *Xenopus* oocytes found a 4 - 6 mV leftward shift in I_{CaL} availability but at much higher concentrations of BDM. In the present experiments, the inactivation time course of I_{CaL} was affected by BDM; τ_{fast} was consistently increased while τ_{slow} was decreased. Similar results were reported by Allen and Chapman (1995) for the exponential and sustained phases of I_{CaL} in guinea-pig ventricular cardiomyocytes using Ca^{2+} or Ba^{2+} as charge carriers. Although the effects of BDM on the fast inactivation of I_{CaL} could be interpreted in terms of dephosphorylation or direct effects on channel gating (e.g. Allen and Chapman, 1995; Ferreira et al., 1997) it should be considered that τ_{fast} is related to the Ca^{2+}-dependent inactivation (CDI; for review see Bénitah et al., 2010), which depends on the Ca^{2+} load of the sarcoplasmic reticulum (SR). It has been reported by Tripathi et al. (1999) that BDM is able to increase the open probability of SR Ca^{2+} channels. Additionally, BDM decreases peak I_{CaL}. It is thus possible that under the action of BDM the SR Ca^{2+} load is decreased and CDI is diminished increasing τ_{fast}. The decrease we observed in τ_{slow} is most probably related to an effect of BDM on channel gating (e.g.; Ferreira et al., 1997). On the other hand, Eisfled et al. (1997) and Allen et al. (1998) reported accelerations in the inactivation time course of I_{CaL} under the action of BDM. It is, however, difficult to explain such a discrepancy since those results were obtained measuring currents through L-type Ca^{2+} channels expressed in heterologous systems using Ba^{2+} as charge carrier.

One important finding of the present results is that incubating cardiomyocytes in a BDM-containing solution did not affect I_{CaL} density or its inactivation time course recorded when cardiomyocytes were perfused with control extracellular solution as described above. This is an expected result since the effect of extracellular BDM on I_{CaL} was quickly established an also rapidly washed out ("on - off"). Moreover, in those cardiomyocytes perfusion with an extracellular solution containing BDM produced essentially the same changes in I_{CaL} properties as in not incubated cardiomyocytes. These results suggest that inhibition of basal I_{CaL} by BDM is probably not related to its (intracellular) phosphatase activity (see Chapman, 1993; Allen and Chapman, 1995) but to a direct action on the L-type Ca^{2+} channel through a site located in the extracellular sarcolemmal interphase. Another possibility is that BDM, due to its lipophilicity, could penetrate the membrane and produce its I_{CaL} blocking action either by directly interacting with the Ca^{2+} channel or by disturbing the lipid domains around the channel. Both possibilities (outside or within the membrane) are consistent with the fast decrease and washout ("on - off") of BDM effect on I_{CaL}. Our results are also consistent with the idea that there is a minor role of PKA in the maintenance of basal I_{CaL} (see for review Weiss et al., 2013). It should be noted here that the results of Eisfled et al. (1997) and Allen et al. (1998), using heterologous expression systems, supported the idea that BDM effects on basal I_{CaL} were not mediated by dephosphorylation. However, as will be discussed below, the intracellular phosphatase activity of BDM, reflected in a decreased β-adrenergic response, is long lasting.

The most important result of the present experiments is that BDM markedly attenuated the response of I_{CaL} to β-adrenergic stimulation. In rat ventricular cardiomyocytes, I_{CaL} usually respond to ISO (1 μM) with a ≥60% increase (see Alvarez et al., 2004; present results). However, our results show that when extracellular BDM was first applied to cardiomyocytes (basal I_{CaL} is decreased) ISO was much less effective in increasing I_{CaL} (≈15%). Furthermore, when cardiomyocytes incubated in BDM (at least 30 min) were patch-clamped and perfused with normal extracellular solution (time to achieve whole cell configuration was less than 2 min), the response of I_{CaL} to ISO was greatly diminished or even abolished (see Fig. 4). These results are agreement with those of Lang and Paul (1991) in smooth muscle cells but are in contrast to those of Chapman (1993) and Allen and Chapman (1995) in cardiomyocytes and Huang and McArdle (1992) in neurons who found that cAMP-dependent phosphorylation could

revert BDM effects on the L-type Ca^{2+} current. In order to clarify this aspect we conducted experiments in cardiomyocytes intracellularly perfused with cAMP to fully activate PKA and the results show that when cardiomyocytes were previously incubated in BDM, the cAMP-mediated increase in I_{CaL} (>160% in control cardiomyocytes) was almost suppressed. Moreover, in cardiomyocytes not incubated in BDM and intracellularly perfused with cAMP, extracellular BDM was still able to decrease the stimulated I_{CaL} by an amount similar to that observed in control conditions.

CONCLUSIONS

Overall the present results indicate that the decrease of basal I_{CaL} by BDM is not related to the dephosphorylation action of this oxime and that this action of BDM on the L-type Ca^{2+} channel occurs most probably in a site in the extracellular side or within the sarcolemmal membrane. However, due to its phosphatase action, BDM strongly attenuates the response of I_{CaL} to β-adrenergic stimulation. The experiments with BDM-incubated cardiomyocytes indicate that intracellular phosphatase-like action of BDM could be long lasting. These actions of BDM must be taken into account both for its use in the dissociation and preservation of isolated myocytes, and for its utilization in cardioplegic solutions and myocardial preservation. We should remark that the concentrations of BDM used in the present experiments were lower than those commonly reported by other authors.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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REFERENCES

Abi-Gerges N, Pointon A, Pullen GF, Morton MJ, Oldman KL, Armstrong D, Valentin J-P, Pollard CE (2013) Preservation of cardiomyocytes from the adult heart. J Mol Cell Cardiol 64: 108-119.

Allen TJ, Chapman RA (1995) The effect of a chemical phosphatase on single calcium channels and the inactivation of whole-cell calcium current from isolated guinea-pig ventricular myocytes Pflügers Arch Eur J Physiol 430: 68-80.

Allen TJ, Mikala G, Wu X, Dolphin AC (1998) Effects of 2,3-butanedione monoxime (BDM) on calcium channels expressed in *Xenopus* oocytes. J Physiol (L) 508: 1-14.

Alvarez-Collazo J, Díaz García CM, López Medina AI, Vassort G, Alvarez JL (2012) Zinc modulation of basal and β-adrenergically stimulated L-type Ca^{2+} current in rat ventricular cardiomyocytes: consequences in cardiac diseases. Pflügers Archiv Eur J Physiol 464: 459-470.

Alvarez JL, Aimond F, Lorente P, Vassort G (2000) Late post-myocardial infarction induces a tetrodotoxin-resistant Na^+ current in rat cardiomyocytes. J Mol Cell Cardiol 32: 1169-1179.

Alvarez JL, Hamplova J, Hohaus A, Morano I, Haase H, Vassort G (2004) Calcium current in rat cardiomyocytes is modulated by the carboxy-terminal ahnak domain. J Biol Chem 279: 12456-12461.

Bénitah JP, Alvarez JL, Gómez AM (2010) L-type Ca^{2+} current in ventricular cardiomyocytes. J Mol Cell Cardiol 48: 26-36.

Bers DM (2001) Excitation-contraction Coupling and Cardiac Contractile Force. Second edition Kluwer Academic Press, The Netherlands: Dordrecht.

Borlak J, Zwadlo C (2004) The myosin ATPase inhibitor 2,3-butanedione monoxime dictates transcriptional activation of ion channels and Ca^{2+}-handling proteins. Mol Pharmacol 66: 708-717.

Chambers DJ (2005) Mechanism of cardiac damage associated with cardiac surgery. Heart Metab 29: 5-9.

Chapmann RA (1993) The effect of oximes on the dihydropyridine-sensitive Ca^{2+} current of isolated guinea-pig ventricular myocytes. Pflügers Arch Eur J Physiol 422: 325-331.

Chung CS, Mechas C, Campbell KS (2015) Myocyte contractility can be maintained by storing cells with the myosin ATPase inhibitor 2,3-butanedione monoxime. Physiol Rep 3: e12445 doi: 1014814/phy212445.

Coulombe A, Lefebvre IA, Deroubaix E, Thuringer D, Coraboeuf E (1990) Effect of 2,3-butanedione monoxime on slow inward and transient outward currents in rat ventricular myocytes. J Mol Cell Cardiol 22: 921-932.

Daly MJ, Elys JS, Nayler WG (1987) Contracture and the calcium paradox in the rat heart. Circ Res 61: 560-569.

Eisfeld J, Mikala G, Varadi G, Schwartz A, Klockner U (1997) Inhibition of cloned human L-type cardiac calcium channels by 2,3-butanedione monoxime does not require PKA-dependent phosphorylation sites. Biochem Biophys Res Comm 230: 489-492.

Ferreira G, Artigas P, Pizarro G, Brum G (1997) Butanedione monoxime promotes voltage-dependent inactivation of L-type calcium channels in heart. Effects on gating currents. J Mol Cell Cardiol 29: 777-787.

Habazettl H, Voigtlander J, Leiderer R, Messmer K (1998) Efficacy of myocardial initial reperfusion with 2,3 butanedione monoxime after cardioplegic arrest is time-dependent. Cardiovasc Res 37: 684-690.

Hall AR, Hausenloy DJ (2016) Mitochondrial respiratory inhibition by 2,3-butanedione monoxime (BDM): Implications for culturing isolated mouse ventricular

cardiomyocytes. Physiol Rep 4: e12606 doi: 1014814/phy212606.

Herrmann C, Wray J, Travers F, Barman, T (1992) Effect of 2,3-butanedione monoxime on myosin and myofibrillar ATPases: An example of an uncompetitive inhibitor. Biochemistry 31: 1222-12232.

Huang GJ, McArdle JJ (1992) Novel suppression of an L-type calcium channel in neurones of murine dorsal root ganglia by 2,3-butanedione monoxime. J Physiol (L) 447: 257-274.

Jayawant AM, Stephenson ES, Damiano RJ (1999) 2,3-Butanedione monoxime cardioplegia: Advantages over hyperkalemia in blood-perfused isolated hearts. Ann Thorac Surg 67: 618-623.

Kivistö T, Makiranta M, Oikarinen E-L, Karhu S, Weckström M, Sellin LC (1995) 2,3-Butanedione monoxime (BDM) increases initial yields and improves long-term survival of isolated cardiac myocytes. Jap J Physiol 45: 203-210.

Lang RJ, Paul RJ (1991) Effects of 2,3-butanedione monoxime on whole-cell Ca^{2+} channel currents in single cells of the guinea-pig taenia caeci. J Physiol (L) 433: 1-24.

Lee BK, Kim MJ, Jeung KW, Choi SS, Park SW, Yun SW, Lee SM, Lee DH, Min YI (2016) 2,3-Butanedione monoxime facilitates successful resuscitation in a dose-dependent fashion in a pig model of cardiac arrest. Am J Emerg Med 34: 1053-1058.

Lopatin AN, Nichols CG (1993) Block of delayed rectifier (DRK1) K^+ channels by internal 2,3-butanedione monoxime in *Xenopus* oocytes. Receptors Channels 1: 279-286.

Ostap EM (2003) 2,3-Butanedione monoxime (BDM) as a myosin inhibitor. J Mus Res Cell Motil 23: 305-308.

Reichert KL, Pereira do Carmo, HR, Lima F, Torina AG, de Souza Vilarinho KA, Martins de Oliveira PP, Silveira Filho LM, Barbosa de Oliveira Severino ES, Petrucci O (2013) Development of cardioplegic solution without potassium: Experimental study in rat. Rev Bras Cir Cardiovasc 28: 524-30.

Schlichter LC, Pahapill PA, Chung I (1992) Dual action of 2,3-butanedione monoxime (BDM) on K^+ current in human T lymphocytes. J Pharmacol Exp Ther 261: 438-46.

Schwinger RH, Bohm M, Koch A, Morano I, Ruegg JC, Erdmann E (1994) Inotropic effect of the cardioprotective agent 2,3-butanedione monoxime in failing and nonfailing human myocardium. J Pharmacol Exp Ther 269: 778-786.

Siegman MJ, Mooers SU, Warren TB, Warshaw DM, Ikebe M, Butler TM (1994) Comparison of the effects of 2,3-butanedione monoxime on force production, myosin light chain phosphorylation and chemical energy usage in intact and permeabilized smooth and skeletal muscles. J Mus Res Cell Motil 15: 457-472.

Stapleton MT, Fuchsbauer CM, Allshire AP (1998) BDM drives protein dephosphorylation and inhibits adenine nucleotide exchange in cardiomyocytes. Am J Physiol 275: H1260-H1266.

Stringham JC, Paulsen KL, Southhard JA, Mentzer M, Belzer FO (1994) Prolonging myocardial preservation with a modified University of Wisconsin solution containing 2,3-butanedione monoxime and Ca^{2+}. J Thorac Cardiovasc Surg 107: 764-775.

Tripathi A, Xu L, Pasek DA, Meissner G (1999) Effects of 2,3-butanedione 2-monoxime on Ca^{2+} release channels (ryanodine receptors) of cardiac and skeletal muscle. J Membr Biol 169: 189-198.

Vahl CF, Bonz A, Hagl C, Timek T, Herold U, Fuchs H, Kochsiek N, Hagl S (1995) Cardioplegia on the contractile apparatus level: evaluation of a new concept of myodardial preservation in perfused pig hearts. Thorac Cardiovasc Surg 43: 185-193.

Verrechia F, Hervé JC (1997) Reversible blockade of gap junctional communication by 2,3-butanedione monoxime in rat cardiac myocytes. Am J Physiol 272: C875-C885.

Warnecke G, Schulze B, Hagi C, Haverich A, Klima U (2002) Improved right heart function after myocardial preservation with 2,3-butanedione 2-monoxime in a porcine model of allogenic heart transplantation. J Thorac Cardiovasc Surg 123: 81-88.

Watanabe Y, Koide Y, Kimura J (2006) Topics on the Na^+/Ca^{2+} exchanger: pharmacological characterization of Na^+/Ca^{2+} exchanger inhibitors. J Pharmacol Sci 102: 7-16.

Weiss S, Oz S, Benmocha A, Dascal N (2013) Regulation of cardiac L-type Ca2+ channel CaV 1.2 via the β-adrenergic-cAMP-protein kinase pathway: old dogmas, advances and new uncertainties. Circ Res 113: 617-631.

Wilson IR, Ginsburg S (1955) A powerful reactivator of alkylphosphate-inhibited acetylcholinesterase. Biochim Biophys Acta 18: 168-170.

Xiao YF, McArdle JJ (1995) Effects of 2,3-butanedione monoxime on blood pressure, myocardial Ca^{2+} currents, and action potentials of rats. Am J Hypertens 8: 1232-1240.

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Concepts or Ideas				X
Design	X	X		X
Definition of intellectual content			X	X
Literature search	X	X	X	X
Experimental studies	X	X	X	X
Data acquisition	X	X		X
Data analysis	X	X	X	X
Statistical analysis	X	X	X	X
Manuscript preparation	X	X	X	X
Manuscript editing			X	X
Manuscript review	X	X	X	X

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