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European Journal of Pharmacology 404 (2000) 247–257

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Maintenance of Ca_i^{2+} transients during prolonged cardiac arrest aids rapid contractile recovery

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Received 13 March 2000; received in revised form 24 July 2000; accepted 28 July 2000

Abstract

We explored the effects of contractile arrest maintained for 24–72 h in the presence of 2,3-butanedione monoxime or a Ca^{2+} channel blocker (nifedipine or verapamil) on contractile activity, Ca_i^{2+} transients, and myofibrillar protein content and ultrastructure in long-term cultures of spontaneously beating adult guinea-pig cardiomyocytes. The contractions were not affected by 5 mM 2,3-butanedione monoxime, but they were strongly or fully suppressed by 10 and 18 mM 2,3-butanedione monoxime, respectively, while the Ca_i^{2+} transients triggered by the maintained spontaneous electrical activity were either not changed at all (5 and 10 mM 2,3-butanedione monoxime) or decreased only slightly (18 mM 2,3-butanedione monoxime). The uncoupling of excitation from contraction by 10–18 mM for 24–72 h did not affect the content of the myofibrillar proteins. Confocal laser microscopy showed that these exposures affected the assembly of myofilaments, giving an overall deranged appearance to the myofibrils. In spite of this effect, the cells' contractile activity was readily regained within 15–60 min upon the washout of 2,3-butanedione monoxime. The 24–72-h exposures to 5 μM nifedipine or 10 μM verapamil, which blocked fully both the Ca_i^{2+} transients and contractility, did not affect the myofibrillar protein content nor their assembly. However, the recovery of contractile activity after exposure to a Ca^{2+}-channel blocker was significantly slower (several days) than after 2,3-butanedione monoxime exposure. Furthermore, cultures exposed to Ca^{2+}-channel blockers also had significantly decreased sensitivity to β-adrenergic stimulation. Altogether, these data indicate the importance of regular Ca^{2+} influx for the maintenance of the functional integrity of adult cardiomyocytes during prolonged periods of contractile arrest.

Keywords: (Guinea pig); 2,3-Butanedione monoxime; Ca_i^{2+} transient; Fura 2-acetoxymethyl (AM) ester; Long-term culture; Contractility; Ultrastructure

1. Introduction

Numerous studies in various model systems have indicated the importance of mechanical load for the functional integrity of cardiac muscle. The effects of a decreased load, specifically that which occurs during cardiac arrest, cannot be studied in complex in vivo models without seriously compromising the animal's heart function. Such investigations therefore virtually require the use of cultured-cell models. Cultured neonatal cardiomyocytes have been used in several studies which have demonstrated that significant decreases in the content of myofibrillar proteins and in the assembly of the atrophied myofibrils occur within 24–48 h of contractile arrest (Byron et al.,1996; Nikcevic et al., 1999; Sharp et al., 1993). The effects that a decrease in external load due to an inhibition of active force have on protein metabolism and the cytoskeletal properties in adult cardiomyocytes have yet to be determined.

In the present study, we have attempted to compare the effects of contractile inactivity per se to the effects of sustained Ca^{2+} influx on the cells' ability to resume normal contractile activity after the arrest. For this purpose we have used two types of excitation–contraction uncoupling agents: 2,3-butanedione monoxime and L-type Ca^{2+} channel blockers (nifedipine and verapamil). 2,3-Butanedione monoxime has been shown to inhibit nearly completely the contractions of cardiac myocytes while the

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0014-2999/00/$ - see front matter
PII: S0014-2999(00)00575-6

Ca_i^{2+} transients triggered by electrical activity were affected only minimally (Backx et al., 1994; Spurgeon et al., 1992; Steele and Smith, 1993), but whether these Ca_i^{2+} transients are maintained during prolonged (> 24 h) exposures of the cardiomyocytes to 2,3-butanedione monoxime remains to be established. In contrast, Ca^{2+} channel blockers inhibit the Ca_i^{2+} transients completely while arresting the contractile activity in a variety of preparations, including cultured adult guinea-pig myocytes (Horackova et al., 2000).

Thus, in this study we aimed to investigate the effect of prolonged (24–72 h) contractile arrest on the content of myofibrillar proteins and their ultrastructural assembly, using a model of spontaneously beating cultured adult guinea-pig ventricular myocytes that we have established in our laboratory (Horackova and Mapplebeck, 1989; Horackova et al., 2000) and that we have shown (Horackova and Byczko, 1997; Horackova et al., 1997) retain their regular myofibrillar striations all through the adaptation to culture conditions without any dedifferentiation or redifferentiation, reestablish intercellular contacts within 10–14 days via adherence junctions, and resume synchronized contractile activity.

Our present data demonstrate that a prolonged (24–72 h) contractile arrest due to exposure to 2,3-butanedione monoxime or Ca^{2+} channel blockers does not affect significantly the total myofibrillar protein content. Perhaps more significant was our unexpected finding that Ca^{2+} channel blockers greatly impaired the return of the myocytes' electrical and contractile activities upon the removal of these agents. By contrast, the Ca_i^{2+} transients were maintained during the entire period of exposure to 2,3-butanedione monoxime (up to 72 h), indicating that regular spontaneous electrical activity occurred in these long-term cultured adult cardiomyocytes in spite of the inhibition of their contractions. The contractile activity returned almost immediately after the removal of 2,3-butanedione monoxime, indicating that maintaining Ca_i transients is critically important for the preservation of the functional integrity of adult cardiomyocytes. The effects of 2,3-butanedione monoxime are discussed in view of its importance as a possible cardioplegic agent as indicated by numerous studies (Fagbemi and Northover, 1995; Habazett et al., 1996; Stowe et al., 1994, 1996; Stringham et al., 1993).

2. Methods

2.1. Solutions and chemicals

The Ca^{2+}-free standard HEPES-buffered Tyrode's solution contained 120.5 mM NaCl, 3.8 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose, and 10 mM HEPES; pH was adjusted to 7.4 with NaOH. This solution is referred to hereafter as Ca^{2+}-free Tyrode's solution.

2.2. Cardiac myocyte preparations

Details of the isolation of adult guinea-pig cardiomyocytes have been presented elsewhere (Horackova et al., 1993, 1997). Briefly, the aorta of anesthetized guinea pigs (300–350 g) was cannulated and perfused retrogradely for 5 min at 35°C with Ca^{2+}-free Tyrode's solution. To this solution was then added 0.16–0.18% collagenase (Worthington type II, Freehold, NJ), 0.004% trypsin (Sigma, St. Louis, MO), 0.4% type F (fatty acid free) albumin, and 25 μM $CaCl_2$. The concentration of collagenase was chosen to yield an optimal dissociation process. This perfusate was recirculated for 3–5 min, then the ventricles were removed and sliced into small strips which were incubated in a shaker bath for several 15-min periods. After gentle centrifugation, cells were pooled and this cell suspension was used for plating after the cells were counted in a special 1-ml counting chamber (Sedgewick rafter; Graticules; Turnbridge Wells, England) At least 98% of the cells in a preparation are myocytes ($\sim 8–12 \times 10^6$ cells/animal), of which > 80% are rod shaped and > 90% are viable (as evidenced by exclusion of Trypan Blue).

2.3. Culture techniques

Cells were cultured in Eagle's minimum essential medium with Earle's salts, supplemented as described previously (Horackova et al., 1993). Cytosine 1-β-D arabinofuranoside (10 μM) was added to the culture medium to minimize the growth of fibroblasts and laminin (5 μg/0.1 ml) was used to coat each 1.2-cm-diameter glass coverslip. We used a plating density (10^5 myocytes/cm^2) which we established in a previous study (Horackova et al., 1997) to be optimal for the culturing of guinea-pig myocytes. Plated cells were maintained in an incubator at 37°C under a 95% air–5% CO_2 atmosphere, and culture media were replaced twice a week. Cultures used in these experiments were 3–4 weeks old, as at this stage they are fully interconnected and very stable in their synchronized beating rates (Horackova et al., 1997). We used the same cultures (grown from myocytes isolated and plated on the same day) to investigate as many structural and functional parameters as possible, in order to minimize the variation between the individual cultures.

2.4. Recording of contractile activity in myocytes

The details of this technique have been described previously (Horackova et al., 1993). Briefly, 1.2-cm-diameter glass coverslips with cultured myocytes were placed in a 0.5-ml bath and perfused at 3 ml/min at 37°C. The contractile activity (shortening) of the myocytes was recorded by a video edge-detecting system. The system recorded the movement of an intercellular boundary (edge) between two or more cells, which represented synchronised contractile activity of the whole confluent layer of each individual culture.

2.5. Measurements of $[Ca^{2+}]_i$

Estimations of $[Ca^{2+}]_i$ were performed using Fura 2-acetoxymethyl (AM) ester fluorescence. Cultured myocytes were loaded with 4 μM Fura 2-PE3-AM (Teflabs, Austin, TX) for 90 min at room temperature under an O_2 atmosphere. The myocytes were then superfused with control Tyrode's solution for 30 min at 37°C on the microscope stage. The measurements of Ca_i^{2+} transients were performed using a PTI-system (Photon Technology International, South Brunswick, NJ) that measures fluorescence at the wavelengths 340 and 380 nm and at the emission wavelength of 510 nm.

After an equilibration period of 15–30 min, cultured cells were exposed to the various pharmacological agents. The Ca_i^{2+} transients were presented as a ratio of emission at 340/380 nm.

2.6. Immunohistochemical examination of myocyte cultures

Antibodies against the myofibrillar protein myomesin and a marker for phalloidin (which specifically labels F-actin) were employed to investigate the morphological and immunohistochemical properties of the cultured adult myocytes, as described previously (Horackova et al., 1997). Cultured cells grown on glass coverslips were fixed for 10–15 min at room temperature in 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). After three washes in phosphate buffered saline (PBS), they were incubated in 0.5% Triton X-100 for 10 min, followed by exposure to acetone for 3–5 min at −20°C, then air dried. They were stained with rhodamine-conjugated phalloidin (Molecular Probes, Eugene, OR) (1:20 dilution) followed by a second labeling with monoclonal antibody against myomesin (a generous gift from Dr. M. Eppenberger-Eberhardt, Swiss Fed. Inst. Tech., Zürich, Switzerland). Anti-myomesin was used undiluted (Horackova and Byczko, 1997) overnight at 4°C. After that, the cells were exposed for 2 h to the secondary antibody, fluorescein (FITC)-conjugated sheep anti-mouse IgG (Jackson Immuno Research Lab., West Grove, PA) diluted 1:20.

2.7. Confocal microscopy

Cultured cells were examined with a Zeiss LSM 410 inverted confocal laser scanning microscope equipped with a krypton/argon (488/568/647 nm) laser and a 40× Zeiss Axiovert 100/1.3 oil Zeiss Plan-Neofluar objective. Simultaneous dual-excitation by double band beam splitter (DBSP) 488/568 nm and dual-channel emission detection by DBS2 FT 560 was used with FITC (bandpass filter 515–540 nm) and rhodamine (bandpass filter 575–640 nm). Optical sectioning was done at a scanning speed of 4 s/frame, and the cultures were sectioned at 0.5–1 μm (20–40 sections). These sections were reconstructed by computer software into a composite image.

2.8. Myofibrillar protein extraction and electrophoresis

Myofibrillar proteins were extracted from cultured cardiomyocytes as previously described (Pelouch et al., 1995; Horackova et al., 2000), with slight modifications. Briefly, cells in 3–6 wells (3×10^5 cells/well) were washed twice in PBS and subsequently lysed in 900 μl of extraction buffer 1 (50 mM sodium potassium phosphate buffer [pH 7.4], 10 mM ethylenediaminetetraacetic acid (EDTA), and 1% Triton X-100). Following centrifugation at $13{,}000 \times g$ for 30 min in a microfuge, the pellet containing the myofibrillar proteins was resuspended in 100 μl of extraction buffer 2 (100 mM sodium potassium phosphate buffer [pH 7.4], 1.1 M KCl) and vortexed gently for 4 h. Samples were centrifuged at $13{,}000 \times g$ for 30 min, and the supernatant containing the total myofibrillar proteins was retained for quantitation using Bio-RadD_C Protein Assay (Bio-Rad Lab., Mississauga, Ontario, Canada).

Polyacrylamide gel electrophoresis (SDS-PAGE) of the extracted protein was performed as previously described (Horackova et al., 2000). Briefly, 5–10 μg of total myofibrillar protein was mixed with an equal volume of sample loading dye (62.5 mM Tris [pH 6.8], 10% glycerol, 2% sodium dodecyl sulfate, 0.05% bromophenol blue, and 5% 2-mercaptoethanol) and boiled for 5 min. The samples were electrophoresed on 4–20% Tris–glycine gradient gels at 150 V for 1.5 h. Gels were stained in a solution of 0.25% Coomassie brilliant blue R-250 for 2 h, then destained, dried and scanned using an Abaton Scan 300/GS scanner. Densitometric analysis of myosin, actin, and tropomyosin was performed using NIH Image software on a Macintosh Performa 5200CD microcomputer.

2.9. Statistical analysis

Data collected from matched pairs of cultures (control vs. treated) were analyzed by paired two-tailed Student's *t*-tests. Values of $P \leq 0.05$ were regarded as statistically significant. Data are expressed as percentage of control mean ± standard error of the mean (S.E.M.).

3. Results

3.1. Effects of 2,3-butanedione monoxime and the Ca^{2+} channel antagonists nifedipine or verapamil on contractions and Ca_i^{2+} transients in cultured adult guinea-pig cardiomyocytes

The effects of 2,3-butanedione monoxime on the contractile activity and the Ca_i^{2+} transients were dose-dependent. In preliminary experiments, we established that the effects reached a steady state within 5 min and these acute effects were fully reversed within 10 min of washout. These periods were used in the present experiments of acute exposures to three concentrations of 2,3-butanedione

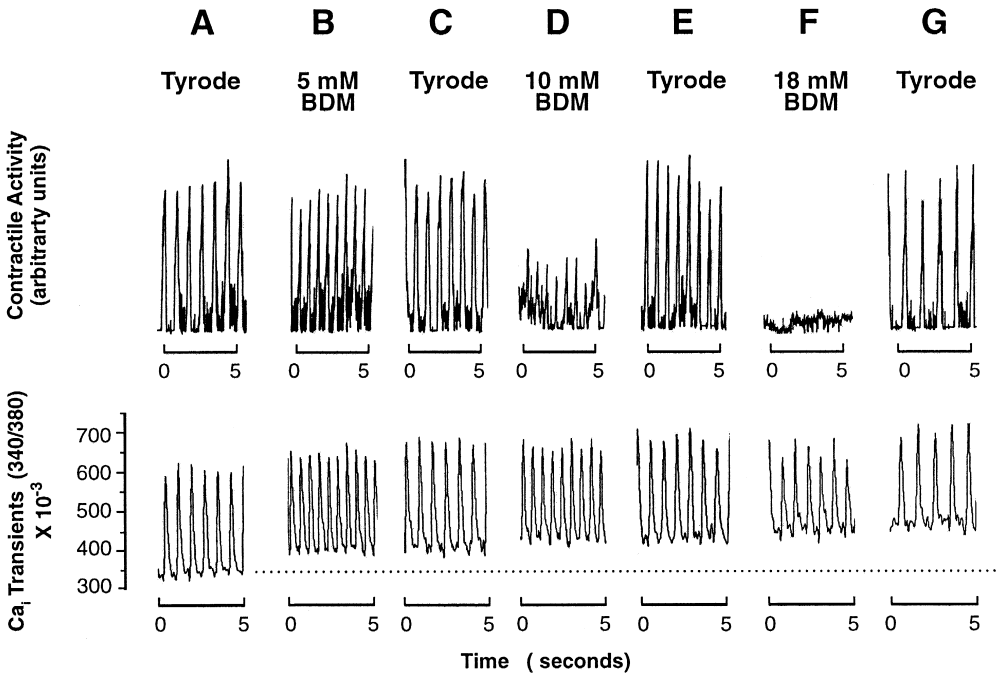

Fig. 1. The acute effects (5 min) of 5 mM (B), 10 mM (D) and 18 mM (F) 2,3-butanedione monoxime (BDM) and their reversals after 10 min in Tyrode's solution (C and G) in a spontaneously beating 25-day-old culture of adult guinea-pig cardiomyocytes: contractile activity (upper panels) and Ca_i^{2+} transients (expressed as the fluorescent ratio of Fura-2AM at 340/380 nm). The dotted line represents the original baseline of Ca_i^{2+}. Similar results were obtained in another nine cultures.

monoxime. Fig. 1 represents the observed effects. Exposure to 5 mM 2,3-butanedione monoxime did not significantly affect the amplitude of the contractions or the Ca_i^{2+} transients in the spontaneously beating cultures of adult

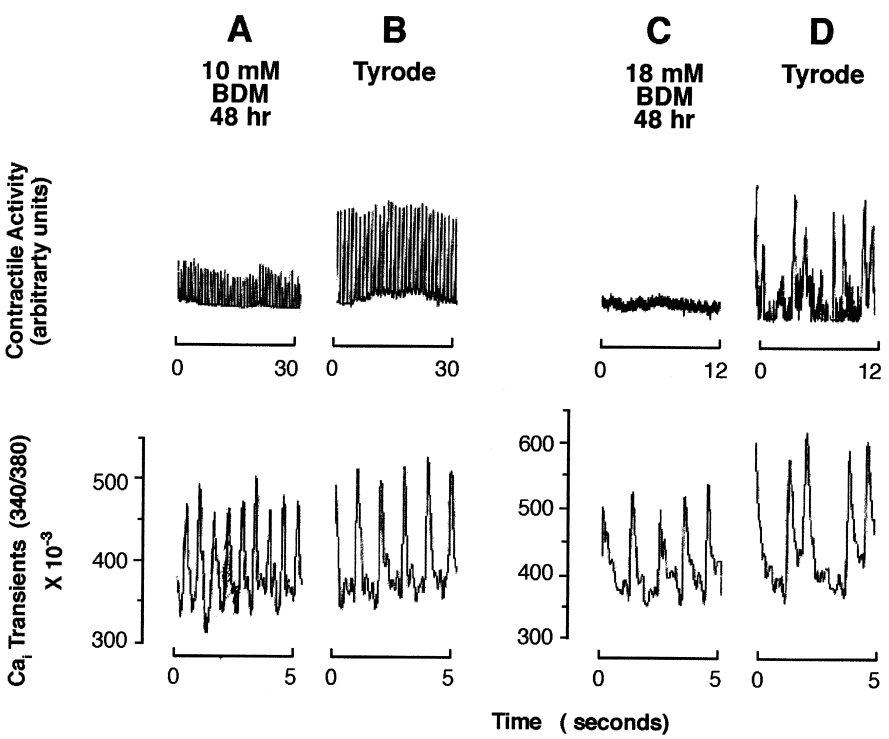

Fig. 2. The effects of prolonged (48 h) exposures to 10 (A) and 18 mM (C) 2,3-butanedione monoxime (BDM) and their reversal after 20-min washout with Tyrode's solution (B and D) in a spontaneously beating 28-day-old culture of adult guinea-pig myocytes: contractile activity (upper panels) and Ca_i^{2+} transients (lower panels). Similar results were obtained in another three cultures.

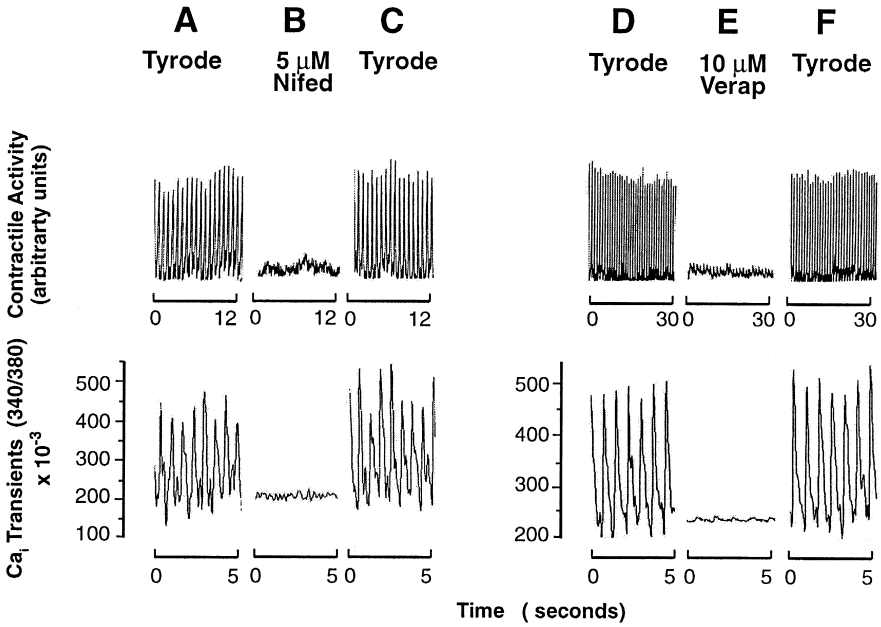

Fig. 3. Spontaneous contractile activity (upper traces) and Ca_i^{2+} transients (lower traces) of a 26-day-old culture of adult guinea-pig cardiomyocytes before the drug application (A and D). The acute effects (5 min) of 5 μM nifedipine (B) or 10 μM verapamil (E) were followed by a 10-min washout period (C and F). Similar results were obtained in another four cultures.

guinea-pig cardiomyocytes (Fig. 1B). A total of 10 mM 2,3-butanedione monoxime decreased the contractions to $32 \pm 2.8\%$ of controls ($n = 10$, $P < 0.05$), while the Ca_i^{2+} transients were not affected significantly (Fig. 1D). A total of 18 mM 2,3-butanedione monoxime fully suppressed the contractions (Fig. 1F), while the Ca_i^{2+} transients triggered

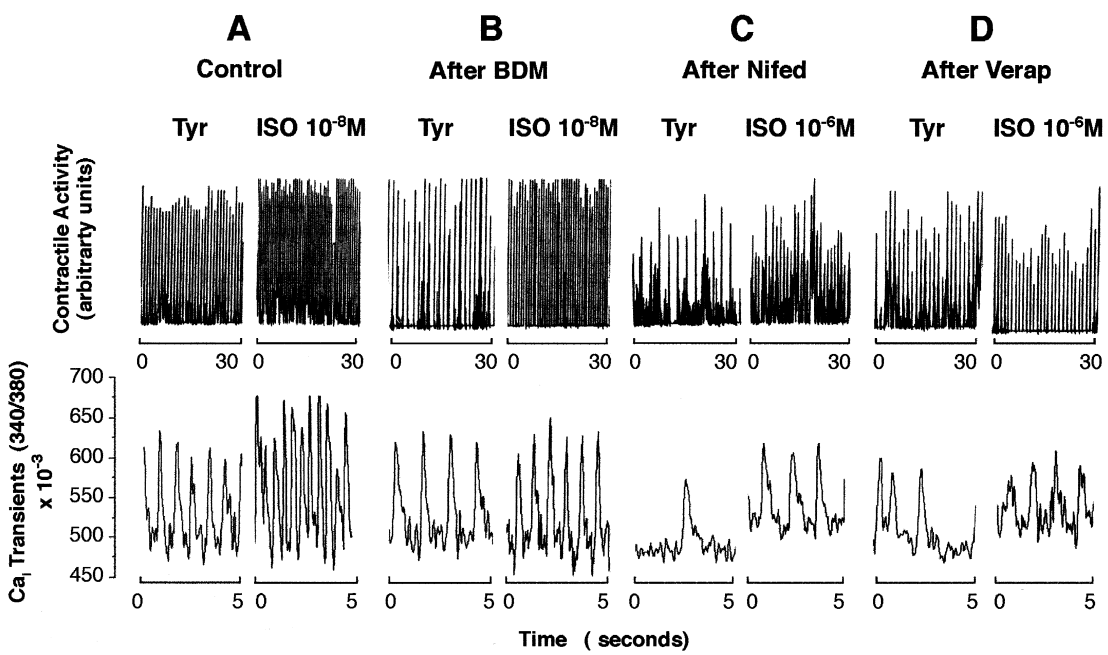

Fig. 4. Spontaneous contractile activity (upper traces) and Ca_i^{2+} transients (lower traces) of a 30-day-old culture of adult guinea-pig cardiomyocytes in Tyrode's solution (left panels) and in the presence of isoproterenol (right panels). The control spontaneously beating culture (A) and a culture after a 15-min washout of 10 mM 2,3-butanedione monoxime (BDM) exposure for 72 h (B). Note there is a similar response (positive chronotropic effect) to 10^{-8} M isoproterenol in both of these cultures. In contrast, the contractile activity and the Ca_i transients were still decreased and irregular after a 24-h wash of 48-h exposure to 5 μM nifedipine (C) and a 48-h wash from 72-h exposure to 10 μM verapamil (D). These cultures (C and D) exerted a slight chronotropic effect only at a much higher concentration (10^{-6} M) of isoproterenol. Similar results were obtained in three other cultures, while other cultures exposed to Ca^{2+}-channel blockers regained their activity after an even longer delay (see text).

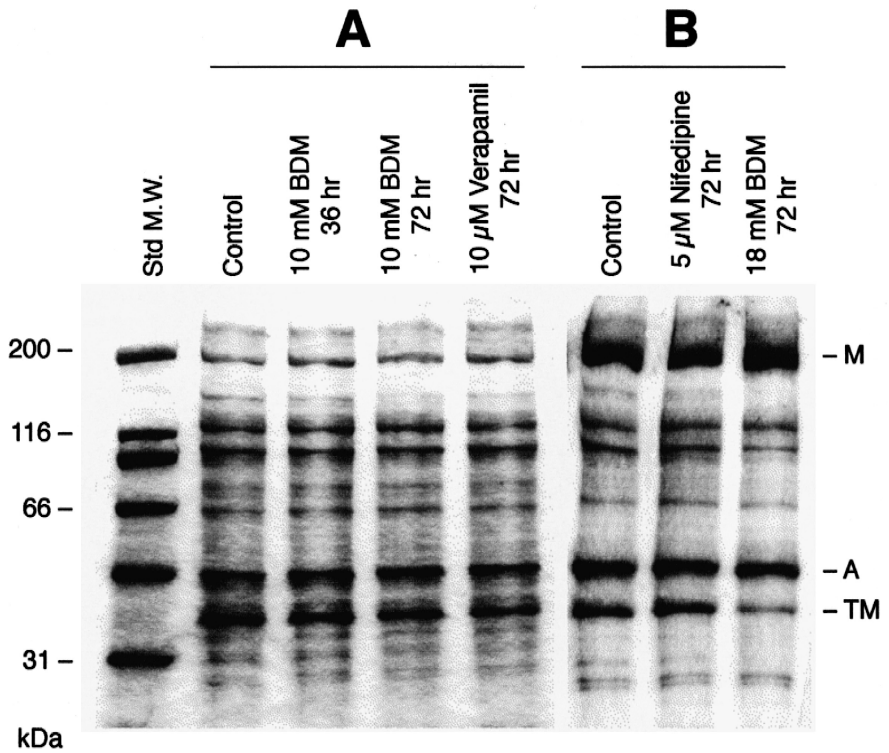

Fig. 5. Proteins of the myofibrillar fraction from 21-day-old (A) and 28-day-old (B) cultures of adult guinea-pig cardiomyocytes. Equal volumes of myofibrillar fraction (10 μg) were separated on SDS gel and stained with Coomassie blue. The samples representing individual drug treatments were compared to the respective untreated controls. M = myosin; A = actin; TM = tropomyosin.

by the spontaneous electrical activity were decreased by only about 20%, i.e., to 81.5 ± 9.2% of controls ($n = 10$, $P < 0.05$). Similar effects were observed during prolonged exposures. The Ca_i^{2+} ransients were maintained during exposure to 10 and 18 mM 2,3-butanedione monoxime, and the return of contractile activity occured within 15–60

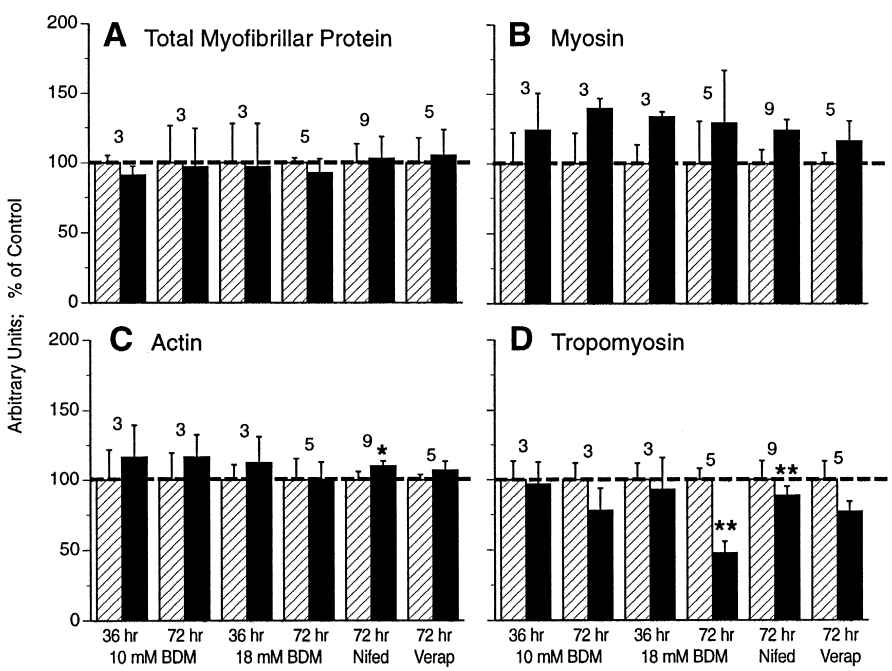

Fig. 6. The content of the total myofibrillar protein (A), myosin (B), actin (C), and tropomyosin (D) in 21–28-d-old cultures of adult guinea-pig cardiomyocytes exposed to various drugs for 36 or 72 h as indicated in the lower panels. Nifedipine was used at a concentration of 5 μM and verapamil at 10 μM. The number of the respective groups is given above the bars. The experimental values are expressed as percentage of controls ± S.E.M. $^{*}P \leq 0.05$, $^{**}P \leq 0.01$. Controls, □; treated, ■.

min upon the removal of 2,3-butanedione monoxime (Fig. 2), although the rate was slightly irregular after the exposure to 18 mM 2,3-butanedione monoxime (Fig. 2D).

Acute exposures (5–30 min) to Ca^{2+} channel antagonists — 5 μM nifedipine or 10 μM verapamil — stopped the contractile activity and inhibited the Ca_i^{2+} transients within 5 min, and these effects were fully reversible upon washout (Fig. 3). However, the return of the Ca_i^{2+} transients and the contractile activity after sustained (24–72 h) contractile arrest were dramatically different in the presence and the absence of Ca_i^{2+} transients, i.e. upon exposure to 2,3-butanedione monoxime and Ca^{2+} channel

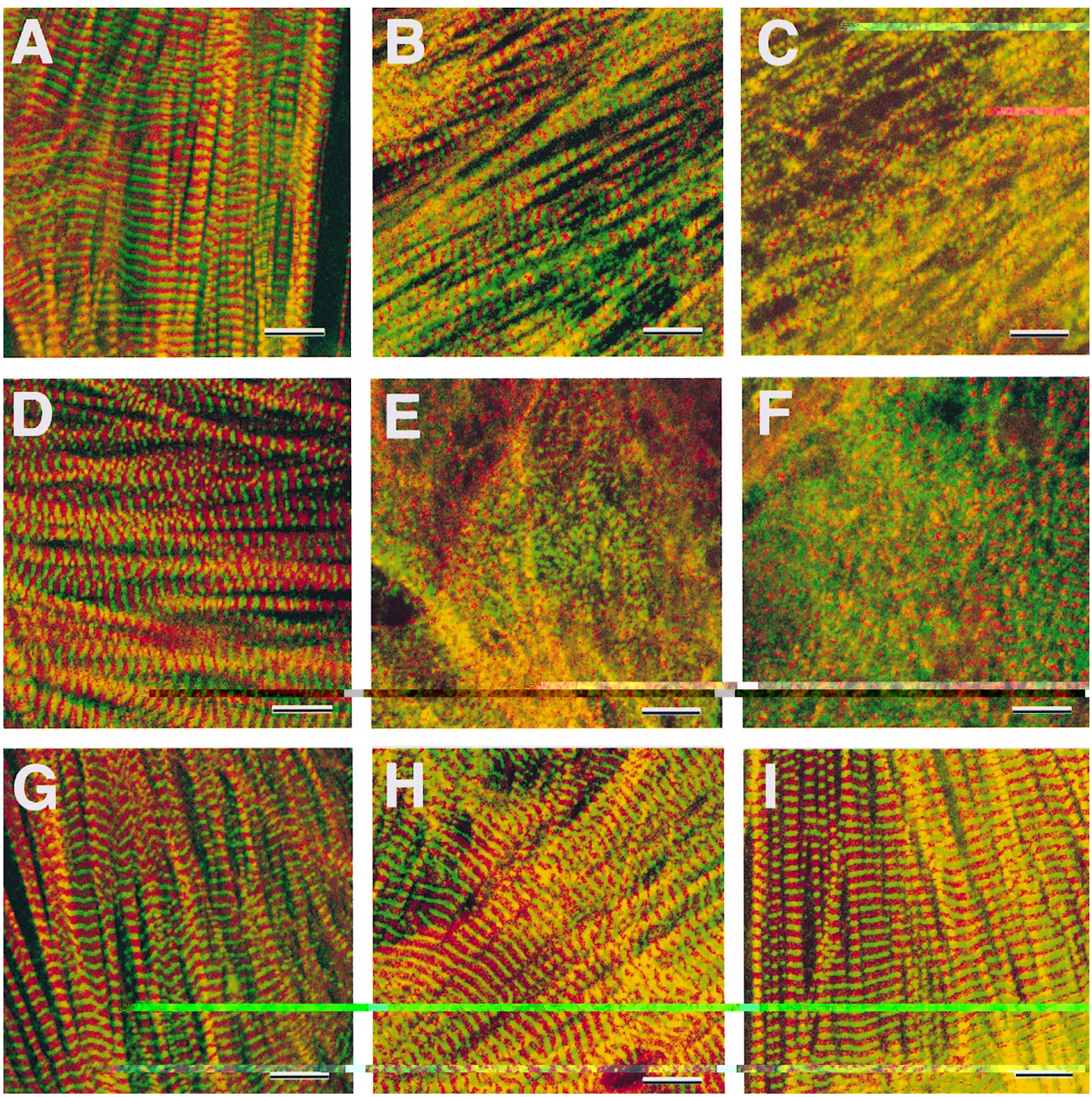

Fig. 7. The combined composite confocal images (15 sections, 1 μm/section) of double-labeled myofilaments in 24-day-old cultures of adult guinea-pig cardiomyocytes (all cultures are from the same preparation/animal): red (phalloidin–rhodamine) staining F-actin and green (FITC conjugated with anti-myomesin antibody) labeling myosin filaments; the yellow represents the overlap of these myofilaments. Control (A); 36-h exposure to 10 and 18 mM 2,3-butanedione monoxime (B and C, respectively); 72-h exposure to 5 μM nifedipine (D); 72-h exposure to 10 and 18 mM 2,3-butanedione monoxime (E and F, respectively); 72-h exposure to 10 μM verapamil (G); 6-day reversal of 72-h exposure to 10 and 18 mM 2,3-butanedione monoxime (H and I, respectively); Scale: 10 μm. Note that this overlap in neighboring myofibrils varies somewhat, presumably due to variability in the stretch imposed on the individual myofibrils in each cell by the surrounding cells. This effect is even more pronounced in the presence of 2,3-butanedione monoxime (B, C, E, F), indicating that some of these myofibrils are semicontracted or stretched more than others (B, C) under exposure to 2,3-butanedione monoxime (BDM). This effect is even more pronounced at longer exposures (E, F), where the composite images of the sarcomeres appeared in a punctate pattern.

blockers, respectively. Thus, while after 24–48 h — and even after 72 h — of sustained exposure to 10 mM 2,3-butanedione monoxime, a regular contractile activity returned within 15–60 min with an average of 20 ± 10 min ($n = 12$) of washout (Fig. 2B and Fig. 4B). This rate of recovery was not significantly different from the untreated controls. In contrast the return of the Ca_i^{2+} transients and the contractions after 24–72 h exposures to either of the Ca^{2+}-channel blockers was greatly delayed, taking at least 24–48 h (Fig. 4C and D) and often even longer (72–96 h). The average time needed for return of regular functional activity after exposure to Ca^{2+} blockers for to 72 h was 50 ± 8 h ($n = 15$), i.e. significantly ($P < 0.05$) longer than after 2,3-butanedione monoxime.

Furthermore, sustained exposures to verapamil or nifedipine rendered the myocytes much less sensitive to β-adrenergic stimulation by isoproterenol. While cultures previously exposed to 10 mM 2,3-butanedione monoxime exhibited the maximal chronotropic effect (similar to that of controls) at 10^{-8} M isoproterenol (Fig. 4A and B), the sensitivity of the cultures previously exposed to Ca^{2+} channel blockers was greatly decreased and the chronotropic effect was much less evident, even at 10^{-6} M isoproterenol, the lowest concentration they responded to (Fig. 4C and D). In addition, the Ca_i^{2+} transients and the contractile activity of these cultures were usually rather irregular, even 24–48 h after removal of the Ca^{2+} channel blockers (Fig. 4D).

3.2. Effects of contractile arrest on the myofibrillar proteins in cultured adult cardiomyocytes

The total myofibrillar protein content levels were not affected significantly by any of the treatments (Fig. 6A). The contents of the three main contractile proteins of interest — actin, myosin, and tropomyosin — were determined by SDS gel analysis (Fig. 5). The only major effect observed the significant decrease by 52% in tropomyosin after 72-h exposure to 18 mM 2,3-butanedione monoxime (Fig. 6D). Nifedipine after 72-h exposure decreased tropmyoin by only 10% while it increased slightly ($< 10\%$) the level of actin (Fig. 6C).

3.3. Effects of long-term arrested contractile activity on myofibrillar structure determined by confocal laser microscopy

In this part of the study, we have used 36- and 72-h exposures to 10 mM 2,3-butanedione monoxime, 18 mM 2,3-butanedione monoxime, 5 μM nifedipine, or 10 μM

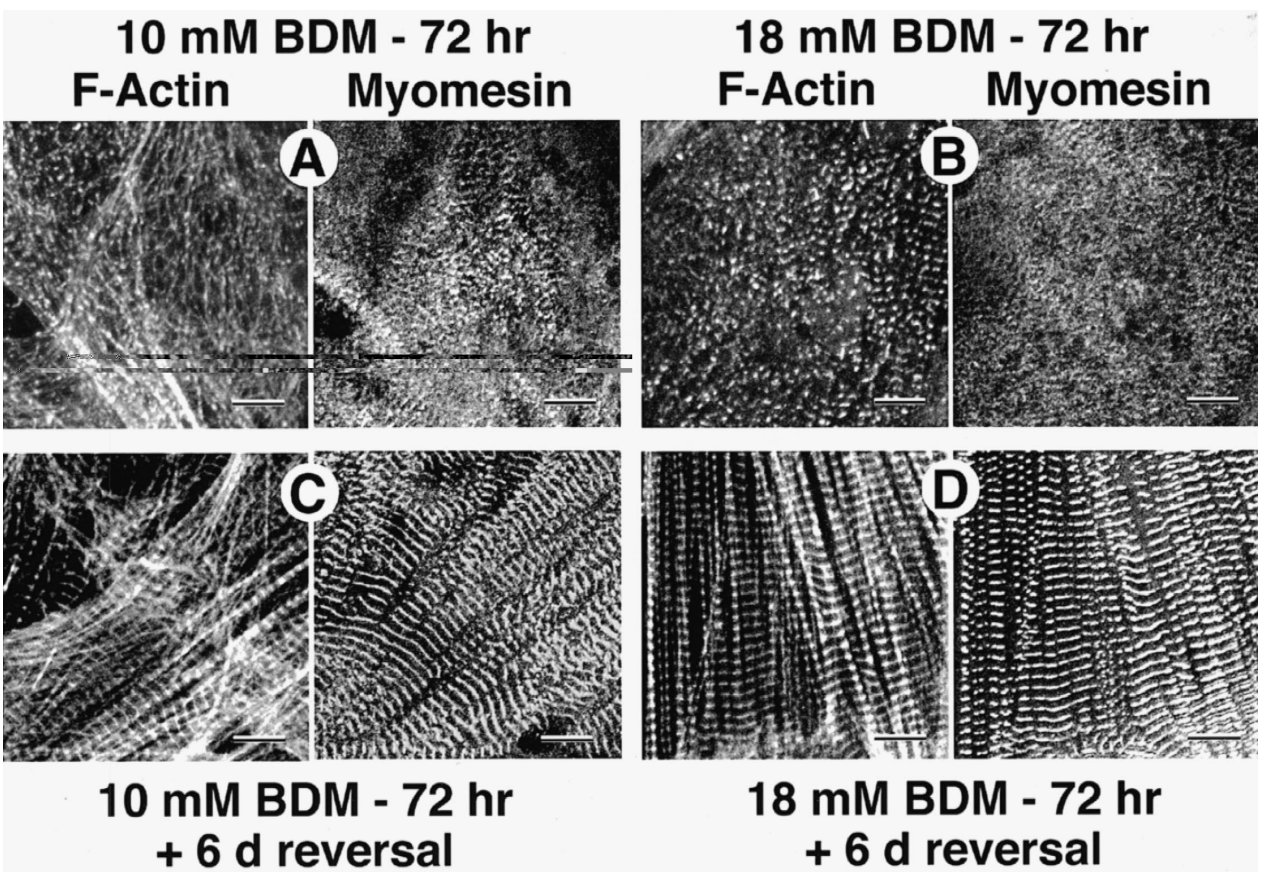

Fig. 8. (A and B) The individual images of F-actin and myomesin shown in Fig. 7E and F, illustrating the effects of 72-h exposure to 10 and 18 mM 2,3-butanedione monoxime (BDM), respectively. (C and D) Six-day reversal of these effects; the same images as shown in Fig. 7H and I. Scale: 10 μm.

verapamil. Fig. 7 demonstrates the regular striated appearance of myofibrils and the actin and myosin myofilaments as visualized in 21–28-day-old cultured cardiomyocytes, using specific immunolabelling and confocal microscopy (Fig. 7A). A 36-h exposure to 10 mM 2,3-butanedione monoxime produced a slight alteration of the sarcomeres' assembly (Fig. 7B), which was even more pronounced after 36-h exposure to 18 mM 2,3-butanedione monoxime. It appeared as if some sarcomeres within the confluent layer shortened while others were stretched, giving a deranged appearance to the myofibrils (Fig. 7C). This process was greatly enhanced after 72-h exposure to both 2,3-butanedione monoxime concentrations (Fig. 7E and F). It should be noted, however, that any disalignment of the individual sarcomeres within the 10–15-μm-thick composite image of the individual 10–15 sections could be visually misinterpreted somewhat due to the three-dimensional complexity of the preparation. Nevertheless, upon the removal of 2,3-butanedione monoxime, the myofilaments' regular arrangement and the striated pattern of the myofibrils was fully recovered within a few days (Fig. 7H and I). We have not investigated the time course of this reversal in detail, as it varied among individual cultures. Indeed, at 6 days, it appeared that the myofibrils were larger and wider ('hypertrophic-like') than before 2,3-butanedione monoxime exposure (compare the control in Fig. 6A vs. 2,3-butanedione monoxime in Fig. 7H and I). Surprisingly, in contrast to the effects of 2,3-butanedione monoxime, there were no visible effects on the myofibrillar arrangement of the same cultures after 72-h exposure to 5 μM nifedipine (Fig. 7D) or to 10 μM verapamil (Fig. 7G). Fig. 8 shows the changes in the structural arrangement of actin (F-actin, represented by staining for phalloidin–rhodamine) and of myosin (represented by anti-myomesin immunoreactivity) after 72-h exposures to 10 mM or 18 mM 2,3-butanedione monoxime (Fig. 8A and B) and the reversal of these effects after 6 days (Fig. 8C and D).

4. Discussion

The present study provides evidence of the important role that maintaining regular Ca^{2+} influx during prolonged contractile arrest (24–72 h) has in the maintenance of the functional properties of cultured adult cardiomyocytes. We have attempted to determine the effects of acute and prolonged exposures to 2,3-butanedione monoxime and to compare these with the effects of Ca^{2+} channel blockers on the structural and functional properties of these myocytes. We have demonstrated first, that 2,3-butanedione monoxime affected the myocytes' contractile activity in a dose-dependent fashion (with 10 mM 2,3-butanedione monoxime reducing it almost completely) but affected Ca_i^{2+} transients very little, even after prolonged (72 h) exposures, whereas 5 μM nifedipine or 10 μM verapamil abolished both contractility and Ca_i^{2+} transients. Second, neither 2,3-butanedione monoxime nor the Ca^{2+} channel blockers affected the content of myofibrillar proteins. Third, the ultrastructural assembly of the myofilaments was deranged by 2,3-butanedione monoxime, but it was not affected by the Ca^{2+} channel blockers. Fourth, in spite of the specific effect of 2,3-butanedione monoxime on the alignment of the myofilaments, the mechanical activity returned almost immediately (in 15–60 min) upon the drug's removal, even after 72-h exposure to 10 mM 2,3-butanedione monoxime, whereas the return of contractile activity after exposure to nifedipine or verapamil was greatly delayed, taking at least 24–48 h and often 3–4 days before a slow and mostly irregular activity reappeared. Finally, the sensitivity to β-adrenergic stimulation was greatly decreased after exposure to either of the Ca^{2+} channel blockers. Thus, altogether our data indicate that it is the maintenance of Ca^{2+} homeostasis rather than contractile activity per se which is responsible for the long-term functional integrity of adult cardiomyocytes.

With regard to protein metabolism and myofibrillar ultrastructure, our present data are in contrast with those obtained in studies of cultured neonatal cardiomyocytes for 24–48 h (Byron et al., 1996; Sharp et al., 1993), which indicate a significant reduction in myofibrillar proteins, leading to a disappearance of myofibrils and an atrophy of these myocytes in the presence of either verapamil or 2,3-butanedione monoxime. The differences between our data and those from the neonatal cultures may reflect not only the different rates in protein turnover in neonatal and adult myocytes but also the greater influence of mechanical activity (independent of alterations in $[Ca^{2+}]_i$) on the myocytes' protein synthesis during the early development of the neonatal heart (Byron et al., 1996). In addition, the neonatal cultures were used during the first 24–96 h after their plating, i.e. during an active proliferative stage. On the other hand, a recent study (Nikcevic et al., 1999) demonstrated that, although mechanical activity, rather than calcium regulates the initiation of α-myosin heavy chain (MHC) translation in neonatal myocytes, it is calcium, rather than mechanical signals that appears to be important for the message localization. In this respect, such a calcium-dependent mechanism may also have been at least partially responsible for the differences in the recovery rate from 24- to 72-h exposures to 2,3-butanedione monoxime or Ca^{2+} channel blockers observed in our experiments, being the much faster ($\leq$ 60 min) after contractile inhibition with 10 mM 2,3-butanedione monoxime (Ca_i^{2+} transients unaffected), and much slower (several days) when Ca_i^{2+} transients are fully inhibited by verapamil or nifedipine. It should be noted, however, that after acute exposures to all these compounds, the return of the functional activity occurred immediately upon their washout (Figs. 1 and 3).

The effect of 2,3-butanedione monoxime as an agent uncoupling excitation from contraction has been demonstrated in various experimental models of skeletal and

cardiac muscles (Fryer et al., 1988; Gwathmey et al., 1991; Perreault et al., 1992). Although there is still some controversy about the precise mechanism of its action, there is general agreement that it decreases the efficiency of cross-bridge attachment by slowing the kinetics of this process (Backx et al., 1994). Although some interaction between actin and myosin exists in the presence of 2,3-butanedione monoxime, this represents a relatively weak, but maintained force, since the rate of dissociation of the cross-bridges is also decreased (Lammerich et al., 1994). Thus, contractile activity is inhibited by 2,3-butanedione monoxime, while Ca_i^{2+} transients are not significantly affected (Backx et al., 1994; Spurgeon et al., 1992; Steele and Smith, 1993; and the present study). Other studies indicate that 2,3-butanedione monoxime may affect Ca^{2+} release and reuptake by sarcoplasmic reticulum (Fryer et al., 1988; Steele and Smith, 1993). In addition, the sensitivity of the myofilaments to Ca^{2+} was reported to be decreased in several experimental models (Gwathmey et al., 1991), as well as in the human heart (Perreault et al., 1992). All of the above effects are likely be to some degree species dependent. Using an in vitro motility assay, (Sata et al., 1995) demonstrated the Ca^{2+} regulation of the sliding of cardiac actin filaments (integrated with tropomyosin–troponin complex) on myosin filaments; this Ca^{2+} regulation was completely abolished in the absence of tropomyosin–troponin complex. It is therefore tempting to hypothesize that the decrease in tropomyosin during exposure to 2,3-butanedione monoxime observed in our experiments may be one of the mechanisms contributing to the altered interaction of actin and myosin reported by many investigators.

In terms of the energy demand in the contracting myocardium, 2,3-butanedione monoxime exerts a beneficial effect, in that it decreases the energy consumption by uncoupling contraction from excitation (Stringham et al., 1993). It has also been demonstrated that 2,3-butanedione monoxime could be useful in the prevention of oxygen paradox (Boban et al., 1993). In addition to these acute effects, several studies (Fagbemi and Northover, 1995; Habazett et al., 1996; Stowe et al., 1994, 1996; Stringham et al., 1993) indicated a significant improvement of cardiac function during and after 16–24-h low-temperature perfusion (storage) of rat or guinea-pig hearts in the presence of 2,3-butanedione monoxime over the conventionally used cardioplegic solutions containing high [KCl] and/or low $[Ca^{2+}]$. Our findings indicate that, in spite of the greater energy consumption in the presence of 2,3-butanedione monoxime (due to maintained Ca^{2+} influx and SR Ca^{2+} release) than in the presence of Ca^{2+}-channel blockers, the maintainance of Ca^{2+} cycling seems to be the most important factor facilitating the recovery of cardiac function after contractile arrest. The main advantages of 2,3-butanedione monoxime seem to be (1) the maintenance of normal ionic gradients and ion-pump function, (2) prevention of Ca^{2+} overload (high [KCl]) or underload (low $[Ca^{2+}]$) as well as membrane depolarization (high [KCl]) leading to dysrhythmias, and (3) most importantly, faster recovery of normal contractility and maintained sensitivity to β-adrenergic stimulation.

In conclusion, our data — particularly the rapid return of contractility upon removal of 2,3-butanedione monoxime but not the Ca^{2+} channel blockers — strongly indicate that the maintenance of Ca_i^{2+} transients during prolonged contractile arrest is essential for the preservation of normal cardiac function. Furthermore, they indicate that the presence of normal Ca^{2+} influx during 24–72-h exposure to 10 mM 2,3-butanedione monoxime in these electrically active preparations should provide better protection during mechanical arrest than Ca^{2+} channel blockers (or any agents causing changes in Ca_i^{2+} homeostasis) by assuring an immediate return of normal contractile function upon 2,3-butanedione monoxime removal. Tentatively, we hypothesize that the greatly delayed return of mechanical activity and the Ca_i transients after exposures to Ca^{2+} channel blockers may be due to an alteration of Ca^{2+} channels availability in the absence of regular Ca^{2+} cycling and/or to depletion of Ca^{2+} content in SR stores. This hypothesis must be examined in future studies. Although our data are very suggestive, it will also be important to further examine the beneficial effects of 2,3-butanedione monoxime for cardiac preservation, using in vivo models to validate the potential successes of such treatment during the reperfusion of ischemic tissue, cardiac surgery, or heart preservation for transplantation.

Acknowledgements

We would like to thank Mr. P. King for his editorial assistance in preparing the manuscript. This work was supported by grants from the New Brunswick Heart and Stroke Foundation and the Medical Research Council of Canada (MT 4128) to Dr. M. Horackova.

References

Backx, P.H., Gao, W.D., Azan-Backx, M.D., Marban, E., 1994. Mechanism of force inhibition by 2,3-2,3-butanedione monoxime in rat cardiac muscle: roles of $[Ca^{2+}]_i$ and cross-bridge kinetics. J. Physiol. 476, 487–500.

Boban, M., Stowe, D.F., Kampine, J.P., Goldberg, A.H., Bosnjak, Z.J., 1993. Effects of 2,3-2,3-butanedione monoxime in isolated hearts: protection during reperfusion after global ischemia. J. Thorac. Cardiovasc. Surg. 105, 532–540.

Byron, K.L., Puglisi, J.L., Holda, J.R., Eble, D., Samarel, A.M., 1996. Myosin heavy chain turnover in cultured neonatal rat heart cells: effects of $[Ca^{2+}]_i$ and contractile activity. Am. J. Physiol. 271, C1447–C1456, (Cell Physiol. 40).

Fagbemi, O.S., Northover, B.J., 1995. Long-term preservation of the rat isolated heart with staurosporine and 2,3-2,3-butanedione monoxime. Transplantation 59, 947–951.

Fryer, M.W., Gage, P.W., Neering, I.R., Dulhunty, A.F., Lamb, G.D.,

1988. Paralysis of skeletal muscle by 2,3-butanedione monoxime, a chemical phosphatase. Pfluegers Arch. 411, 76–79.

Gwathmey, J.K., Hajjar, R.J., Solaro, R.J., 1991. Contractile deactivation and uncoupling of crossbridges: effects of 2,3-butanedione moxoxime on mammalian myocardium. Circ. Res. 69, 1280–1292.

Habazett, H., Palmisano, B.W., Bosnjak, Z.J., Stowe, D.F., 1996. Initial reperfusion with 2,3 2,3-butanedione monoxime is better than hyperkalemic reperfusion after cardioplegic arrest in isolated guinea pig hearts. Eur. J. Cardiothorac. Surg. 10, 897–904.

Horackova, M., Byczko, Z., 1997. Differences in the structural characteristics of adult guinea pig and rat cardiomyocytes during their adaptation and maintenance in long-term cultures: confocal microscopy study. Exp. Cell Res. 237, 158–175.

Horackova, M., Mapplebeck, C., 1989. Electrical, contractile, and ultrastructural properties of adult rat and guinea-pig ventricular myocytes in long-term primary cultures. Can. J. Physiol. Pharmacol. 67, 740–750.

Horackova, M., Huang, M.H., Armour, J.A., Hopkins, D.A., Mapplebeck, C., 1993. Cocultures of adult ventricular myocytes with stellate ganglia or intrinsic cardiac neurons from guinea pigs: spontaneous activity and pharmacological properties. Cardiovasc. Res. 27, 1101–1108.

Horackova, M., Byczko, Z., Maillet-Frotten, L., 1997. Immunohistochemical analysis of the adaptation of adult guinea-pig cardiomyocytes in long-term cultures and in cocultures with cardiac neurons: a novel model for studies of myocardial function. Mol. Cell. Biochem. 172, 227–238.

Horackova, M., Morash, B., Byczko, Z., 2000. Altered transarcolemmal Ca^{2+} transport modifies the myofibrillar ultrastructure and protein metabolism in cultured adult ventricular cardiomyocytes. Mol. Cell. Biochem. 204, 21–33.

Lammerich, A., Holzhütter, H.G., Janiak, R., Storch, E., Günther, J., 1994. Interpretation of the inotropic effect of 2,3-2,3-butanedione monoxime on the isometric twitch of guinea-pig papillary muscle. Gen. Physiol. Biophys. 13, 377–392.

Nikcevic, G., Heidkamp, M.C., Perhonen, M., Russell, B., 1999. Mechanical activity in heart regulates translation of α-myosin heavy chain mRNA but not its localization. Am. J. Physiol 276, H2013–H2019, (Heart Circ. Physiol. 45).

Pelouch, V., Milerová, M., Ošťádal, B., Hučín, B., Šamánek, M., 1995. Differences between atrial and ventricular protein profiling in children with congenital heart disease. Mol. Cell. Biochem. 147, 43–49.

Perreault, C.L., Mulieri, L.A., Alpert, N.R., Ransil, B.J., Allen, P.D., Morgan, J.P., 1992. Cellular basis of negative inotropic effect of 2,3-butanedione monoxime in human myocardium. Am. J. Physiol. 263, H503–H510, (Heart Circ. Physiol. 32).

Sata, M., Sugiura, S., Yamashita, H., Fujita, H., Momomura, S., Serizawa, T., 1995. MCI-154 increases Ca^{2+} sensitivity of reconstituted thin filament: study using a novel in vitro motility assay technique. Circ. Res. 76, 626–633.

Sharp, W., Terracio, W.L., Borg, T.K., Samarel, A.M., 1993. Contractile activity modulates actin synthesis and turnover in cultured neonatal rat heart cells. Circ. Res. 73, 172–183.

Spurgeon, H.A., Dubell, W.H., Stern, M.D., Sollott, S.J., Ziman, B.D., Silverman, S.H., Capogrossi, M.C., Talo, A., Lakatta, E.G., 1992. Cytosolic calcium and myofilaments in single rat cardiac myocytes achieve a dynamic equilibrium during twitch relaxation. J. Physiol. 44, 83–102.

Steele, D.S., Smith, G.L., 1993. Effects of 2,3-butanedione monoxime on sarcoplasmic reticulum of saponin-treated rat cardiac muscle. Am. J. Physiol. 265, H1493–H1500, (Heart Circ. Physiol. 34).

Stowe, D.F., Boban, M., Graf, B.M., Kampine, J.P., Bosnjak, Z.J., 1994. Contraction uncoupling with butanedione moxoxime vs. low calcium or high potassium solutions on flow and contractile function of isolated hearts after prolonged hypothermic perfusion. Circulation 89, 2412–2420.

Stowe, D.F., Graf, B.M., Fujita, S., Gross, G.J., 1996. One-day cold perfusion of bimakalim and 2,3-butanedione monoxime restores ex situ cardiac function. Am. J. Physiol. 271, H1884–H1892, (Heart Circ. Physiol. 40).

Stringham, J.C., Paulsen, K.L., Southard, J.H., Fields, B.L., Belzer, F.O., 1993. Improved myocardial preservation by modification of the University of Wisconsin solution with 2,3-butanedione monoxime and calcium. Transplant. Proc. 25, 1625–1626.