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Effects of Diltiazem on Isoproterenol- or Ca-Induced Ventricular Myocardial Cell Injuries in Isolated Perfused Rabbit Heart: An Electron Microscopic Study

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ABSTRACT The ultrastructural changes of isoproterenol- and those of Ca-induced ventricular cell injuries were compared in rabbits and the effect of diltiazem on these injuries was studied by electron microscopy.

In comparison with the controls, the isoproterenol-treated (Group A), the Ca-treated (Group B), and the diltiazem-posttreated (Groups E and F) showed severe myocardial cell damage, such as sarcolemmal disruption, mitochondrial swelling, intramitochondrial electron-dense granules, membranous structures along mitochondrial cristae, thickening or close packing of the Z-lines, separation of cell junctions, frayed myofibrils, clumping of chromatin, and intracellular fluid accumulation. These ultrastructural changes were more pronounced in the Ca-treated (Groups B and F) than in the isoproterenol-treated (Groups A and E) animals. In contrast, the diltiazem-pretreated groups (Groups C and D) showed relatively intact myocardial ultrastructure. However, intramitochondrial electron-dense granules could be frequently found, and particularly the diltiazem-pretreated and Ca-treated group (Group D) showed intracellular fluid accumulation.

The results of this study could suggest the following: 1) isoproterenol-induced myocardial cell damage is similar to Ca overload, 2) pretreatment with diltiazem could reduce the deleterious effects of isoproterenol-induced myocardial cell damage, but it could not prevent the effects of Ca overload completely, and 3) posttreatment with diltiazem could not provide any beneficial effect either on the isoproterenol-induced or on the Ca-overloaded mycoardial cell damage, and 4) the beneficial effects of diltiazem are probably derived from the enhanced buffering function of mitochondria to cytosolic Ca or from selective inhibition of transsarcolemmal Ca influx.

Catecholamines have been known to cause ischemic myocardial lesions (Rona, 1985); ischemia causes alteration in ultrastructure (Jennings et al., 1975; Schaper et al., 1979) or function (Shen and Jennings, 1972; Katz, 1973; Kübler and Katz, 1977; Daly et al., 1984) of myocardial cells. Shen and Jennings (1972) suggested a possible association between the genesis of tissue damage in ischemic and reperfused mycoardium and a massive increase in Ca content. Recently, there has been ample evidence that Ca is involved in the progression of these events which are precipitated by ischemia and become exacerbated upon reperfusion and reoxygenation (Katz and Reuter, 1979; Nayler et al., 1980; Nayler, 1981; Flameng et al., 1984).

Since the introduction of a new family of drugs named 'Ca-channel blockers' or 'Ca-antagonists' by Fleckenstein in 1969, these compounds (such as verapamil, nifedipine, and diltiazem) proved to be effective in protecting myocardial cells from experimental ischemia (Nayler et al., 1980; Jolly et al., 1981; Ashraf et al., 1982; Flaim and Zelis, 1982; Zamanis et al, 1982; Hamm and Opie, 1983; Kanaya et al., 1983; Watts et al., 1985; Sashida and Abiko, 1986). When given before or at the start of a period of ischemia, these compounds could reduce cytosolic Ca accumulation (Watts et al., 1980; Bourdillon and Poole-Wilson, 1982; Vaghy et al., 1982; Fleckenstein, 1983).

This study was undertaken: 1) to compare the effects of isoproterenol and Ca on the ultrastructure of rabbit myocardial cells, 2) to establish the effect of diltiazem, a Ca-channel blocker, on isoproterenol- or Ca-induced myocardial injury, and 3) to determine possible mechanisms of action of diltiazem.

MATERIALS AND METHODS

Heart Perfusion

Adult healthy New Zealand white rabbits of either sex (1.7 to 2.3 kg of body weight), seven for controls and

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five for each experimental group, were kept in a constant condition and given food and water ad libitum. They were stunned by a blow to the neck and their hearts were rapidly excised. After removing extraneous fat and connective tissue in oxygen saturated buffered saline, the heart was perfused for 10 min with a modified Tyrode solution (containing in mM: NaCl 140.0, KCl 4.4, $CaCl_2$ 1.0, $MgCl_2$ 1.0, HEPES 3.0, and glucose 10.0), maintained at 37°C, pH 7.4, bubbled with oxygen by non-recirculating Langendorff technique as described by Döhring and Dehnert (1985). The perfusate was delivered via aortic cannula at a mean flow rate of 18 ml/min and at a mean pressure of 80 cm water.

Treatment

After perfusion with Tyrode solution, the hearts (except for controls) were treated as follows: 1. Isoproterenol-treated (Group A): perfused with 2.0 μM isoproterenol for 10 min. 2. Ca-treated (Group B): perfused with saline solution containing 4.0 mM $CaCl_2$ for 10 min. 3. Diltiazem-pretreated and isoproterenol-treated (Group C): perfused with 7.5 μM diltiazem, followed by perfusion with 2.0 μM isoproterenol, for 10 min, respectively. 4. Diltiazem-pretreated and Ca-treated (Group D): perfused with 7.5 μM diltiazem, followed by perfusion with saline solution containing 4.0 mM $CaCl_2$, for 10 min, respectively. 5. Isoproterenol-treated and diltiazem-posttreated (Group E): perfused with 2.0 μM isoproterenol, followed by perfusion with 7.5 μM diltiazem, for 10 min, respectively. 6. Ca-treated and diltiazem-posttreated (Group F): perfused with saline solution containing 4.0 mM $CaCl_2$, followed by perfusion with 7.5 μM diltiazem, for 10 min, respectively.

All of the perfusates at a final pH of 7.2 to 7.4, were bubbled with oxygen, maintained at 37°C, and delivered by the same method as in the control group. Isoproterenol and diltiazem (Sigma Chemical Co., St. Louis, MO) were freshly prepared from dilution of stock solutions with Ca-free saline solution, and kept away from light.

The dose of isoproterenol was based on previous studies (Hisatome et al., 1985; Opie et al., 1985). Moderate to severe degree of myocardial cell damage with slight variations as described by Schaper et al. (1979) were produced at this dose. The concentration of $CaCl_2$ was determined by our preliminary study in which various concentrations (2, 4, 8, 16, and 32 mM) of $CaCl_2$ were tried. Severe ultrastructural changes of myocardial cells with slight variation were produced above 4mM $CaCl_2$. Concentrations of $CaCl_2$ higher than 16 mM caused poorly perfused ultrastructure with considerable variation. The dose of diltiazem was based on previous studies (Polenda et al., 1982; Watts et al., 1986).

Electron Microscopy

Following the treatment described above, the heart was subjected to perfusion with 2.5% glutaraldehyde in 0.1 M phosphate buffer, pH 7.4, at 4°C for 20 min, and was then immersed in the same fixative for 2 h.

The lateral free wall of the left ventricle adjacent to the apex was selected and sliced in ice-cold phosphate buffer. Wedge sections were excluded for electron microscopy. One or two slices were selected, carefully minced, postfixed in 1% phosphate-buffered osmium tetroxide, dehydrated in graded ethanols, infiltrated with propylene oxide, and was embedded in Epon 812. Thick sections were cut at 2 μm and stained with toluidine blue for light microscopy. Silver to gold sections were cut and stained with aqueous uranyl acetate and lead citrate. Double-stained sections were examined with Jeol 200CX transmission electron microscope. Ca-containing intramitochondrial granules were revealed by floating unstained sections on copper grids in 0.2 M EDTA, pH 8.0, at 60°C for an hour.

RESULTS

Control Group

All seven hearts of the control group showed relatively well preserved ultrastructure with slight variation. Sarcomeres were relaxed and longitudinally placed myofibrils were separated by numerous mitochondria (Fig. 1). A-, H-, I-, and M-bands and Z-lines were clearly resolved. Mitochondrial structure was intact and associated with a few lipid droplets. Sarcoplasmic reticulum was sparse but glycogen granules were abundant. Sarcolemma was infrequently scalloped and surrounded by a rather indefinite layer of moderately electron-dense material. Oval or spindle-shaped nucleus was centrally located and chromatin granules were evenly dispersed in the nuclear matrix. Nuclear membrane was frequently indented and nucleolus was relatively apparent. Intercalated discs (cell junctions) ran across the myofibrils in a typical stepwise fashion (Fig. 2).

Group A

All five hearts of the isoproterenol-treated group showed similar ultrastructural changes with slight variation in degree of change. Myofibrils were contracted and Z-lines were thickened (Fig. 3). Frayed myofibrils were seen frequently. Mitrochondria were swollen, matrices cleared, and cristae were distorted. Membranous dense structures could be frequently found along the mitochondrial cristae. Electron-dense granules appeared in the matrices. Clumping of chromatin (Fig. 4) could be found, but the nuclear membrane was intact. Glycogen granules were reduced and lysosomes were found among swollen mitochondria. In places, lipid droplets were engulfed by lysosomes (Fig. 5) and the numbers of lipid droplets slightly increased. Sarcolemma was distorted and slightly vesicular (Fig. 6). Cell junctions were separated (Fig. 7).

Group B

All five hearts of the Ca-treated group showed similar ultrastructural changes. Myofibrils were severely contracted and Z-lines were closely packed together (Fig. 8). The thickness of the Z-lines varied from very faint to a very thickened, densely packed appearance. Mitochondria were swollen, cristae were distorted, and contained electron-dense granules and membranous dense structures. Glycogen granules were depleted and frayed myofibrils were frequently found. Intrasarcoplasmic fluid accumulation was apparent. The sarcolemma was severely distorted and separated from underlying myofibrils (Fig. 9), but nuclear changes were not apparent.

Group C

All hearts except one of the diltiazem-pretreated and isoproterenol-treated group showed relatively well preserved ultrastructure. Myofibrils were relaxed as in the

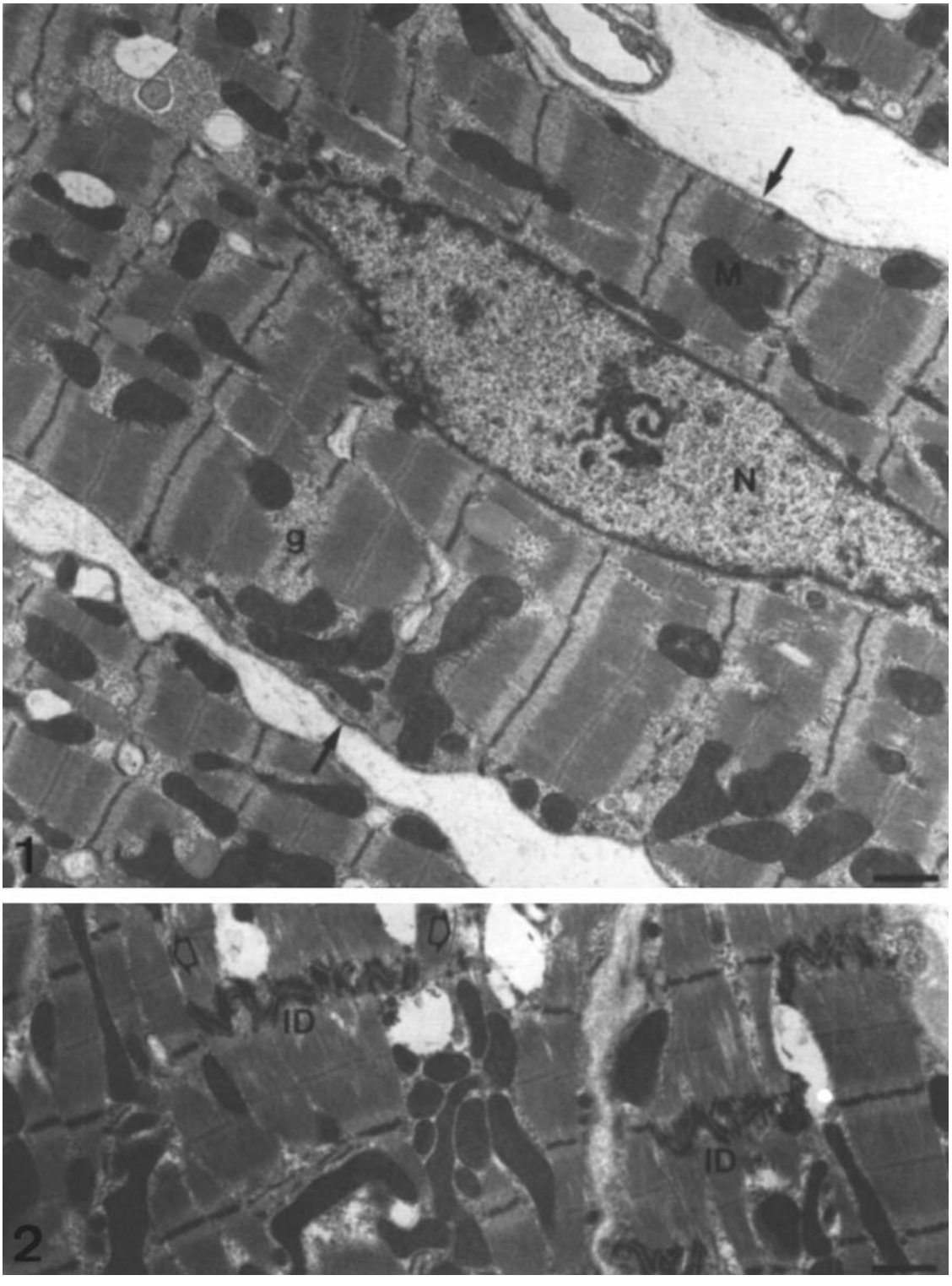


Fig. 1. Control group. Sarcomeres are relaxed and myofibrils are separated by numerous mitochondria (M). Glycogen granules (g) are abundant and sarcolemma (arrow) is intact. N = nucleus; bar = 1.06 μm.

Fig. 2. Control group. Cell junction (ID) shows a typical stepwise fashion and nexuses (open arrows). Bar = 1 μm.

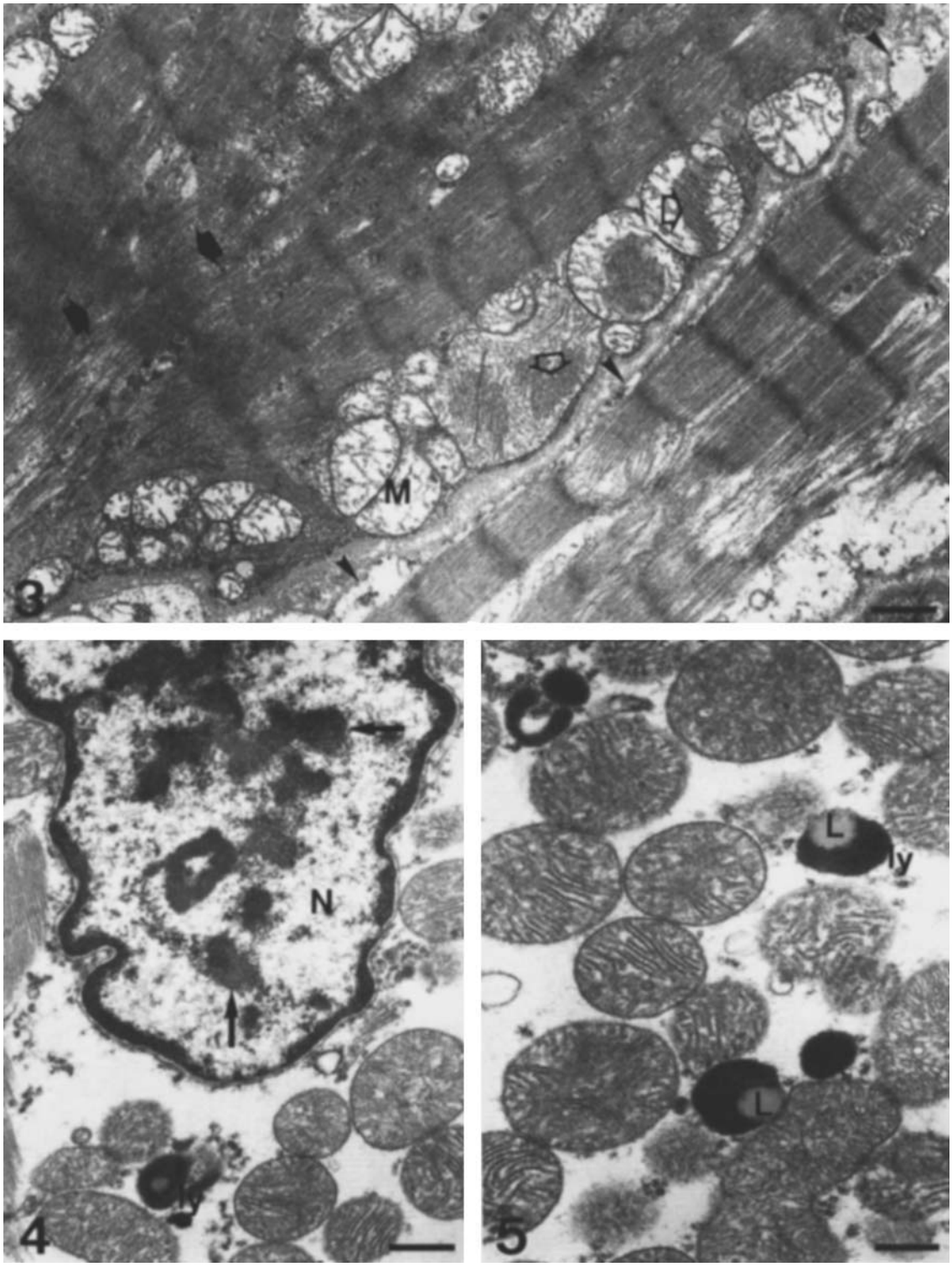


Fig. 3. Isoproterenol-treated group. Mitochondria (M) are swollen and contain numerous dense structures (open arrows). Sarcomeres are severely contracted and Z-lines are thickened (arrows). Sarcolemma (arrowheads) is also distorted. Bar = 1 μm.

Fig. 4. Isoproterenol-treated group. Clumping of chromatin (arrows) is apparent, but nuclear membrane is intact. N = nucleus; ly = lysosome; bar = 0.56 μm.

Fig. 5. Isoproterenol-treated group. Lipid droplets (L) are engulfed by lysosomes (ly). Bar = 0.42 μm.

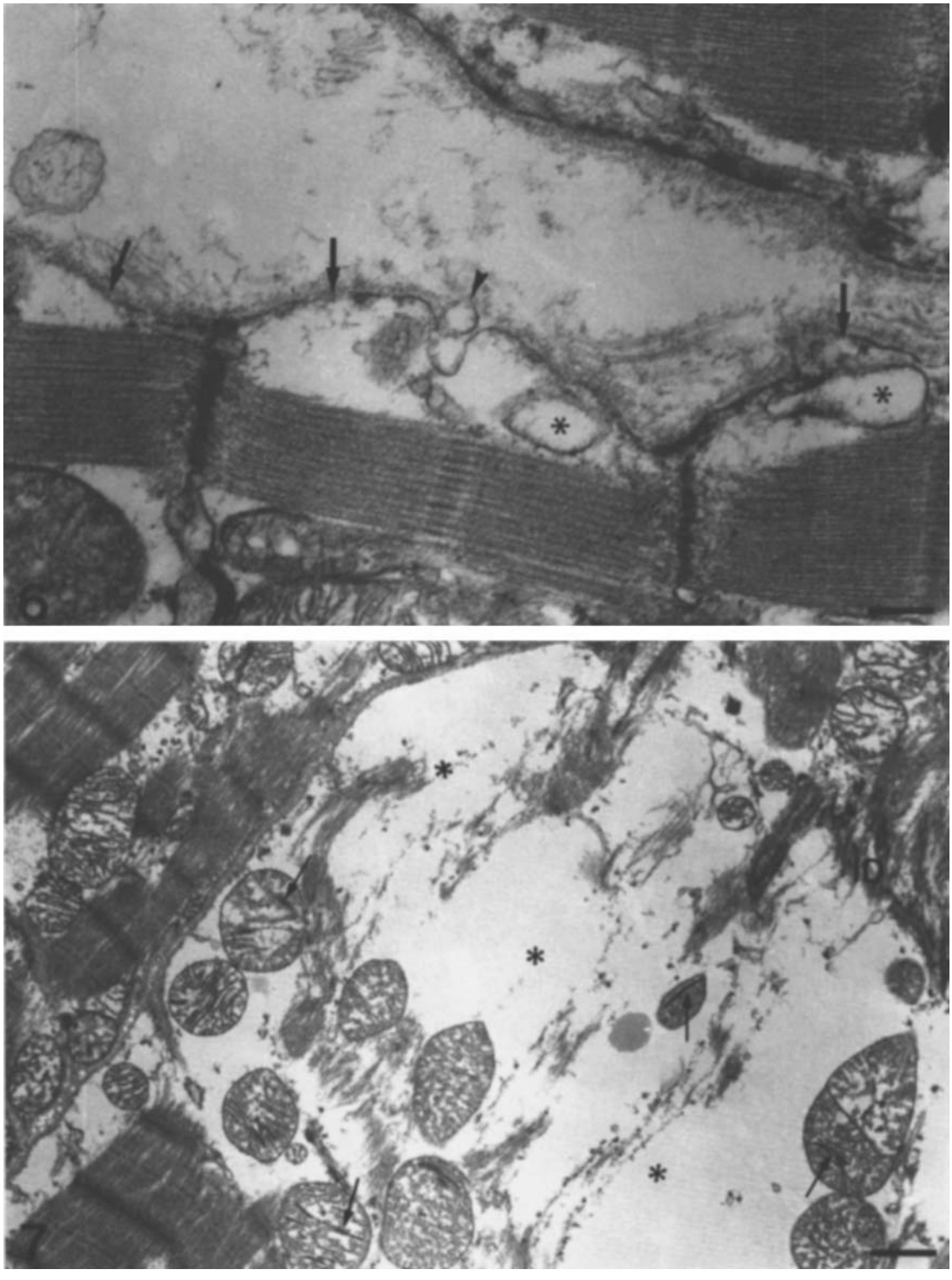


Fig. 6. Isoproterenol-treated group. Sarcolemmal breaks (arrows), exocytotic vesicle (arrowhead) and sarcolemmal vesicles (asterisks) are seen. Bar = 0.25 μm.

Fig. 7. Isoproterenol-treated group. Mitochondria contain dense structures (arrows). Cell junction (ID) is severely disintegrated and myofibrolytic area (asterisks) is seen. Bar = 1 μm.

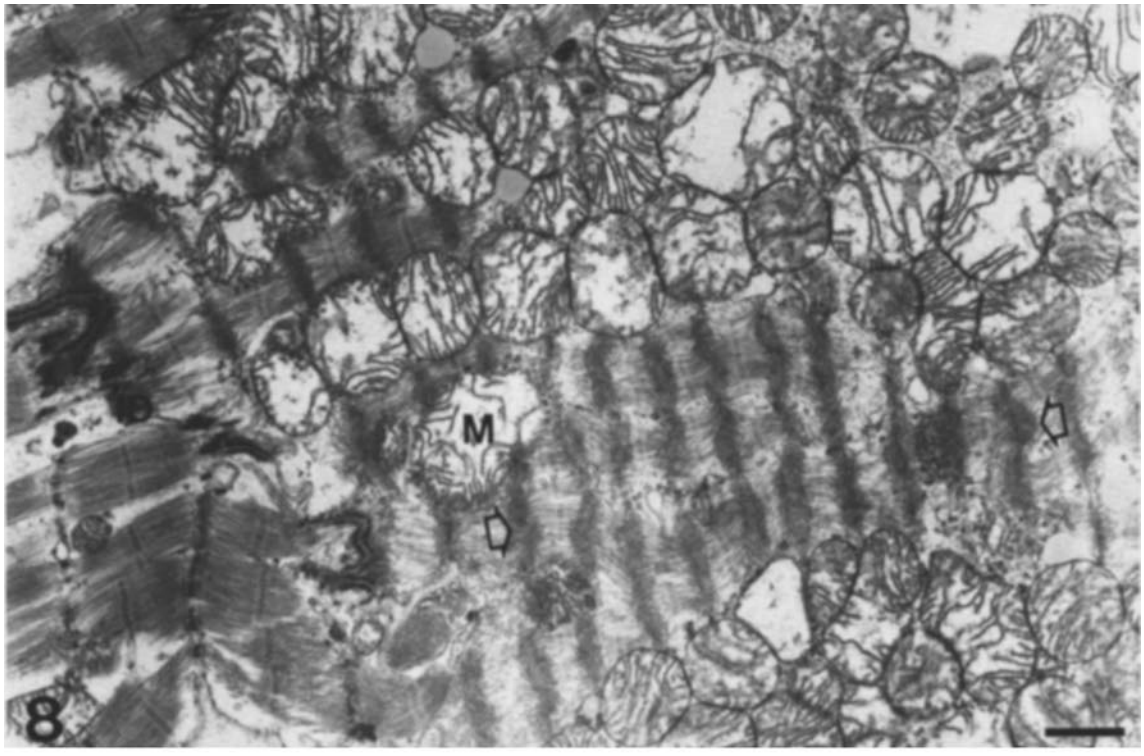


Fig. 8. Ca-treated group. Severely contracted sarcomeres are seen in contraction band lesion (open arrows). Cell junction (ID) is also separated. M = mitochondria; bar = 1 μm.

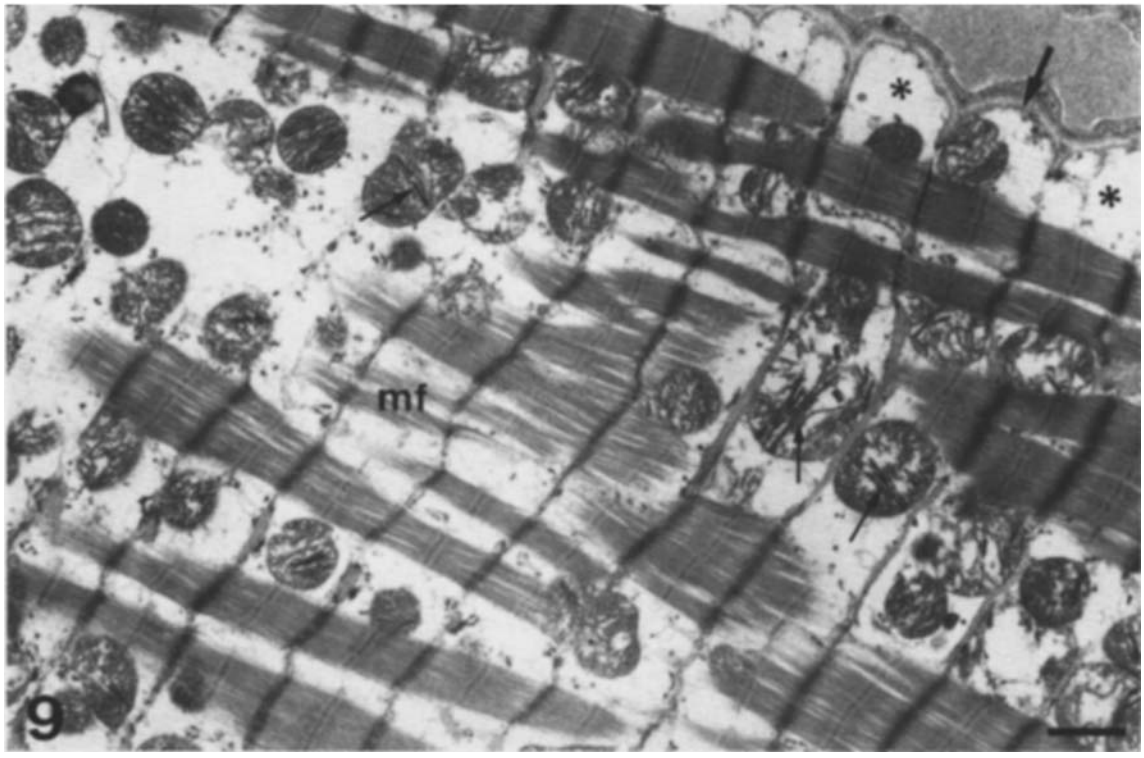
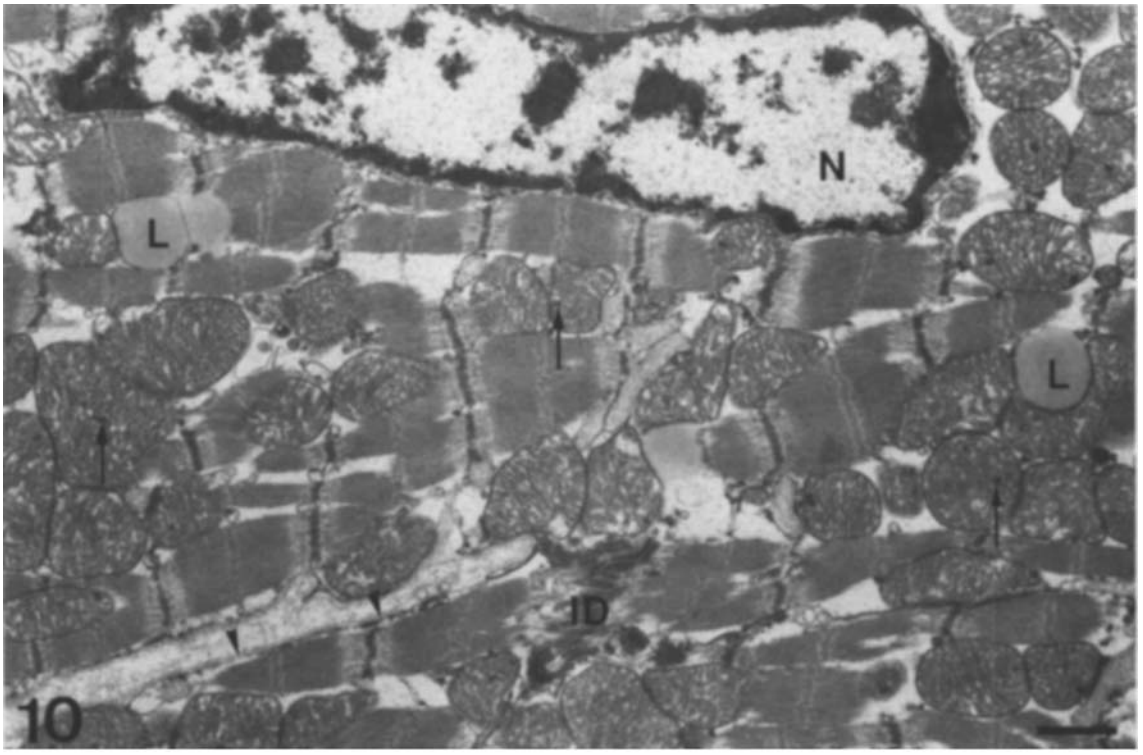


Fig. 9. Ca-treated group. Mitochondria contain dense structures (thin arrows). Myofibrils (mf) are frayed and sarcolemma (thick arrow) is separated from underlying myofibrils by blebs (asterisks). Bar = 1.25 μm.



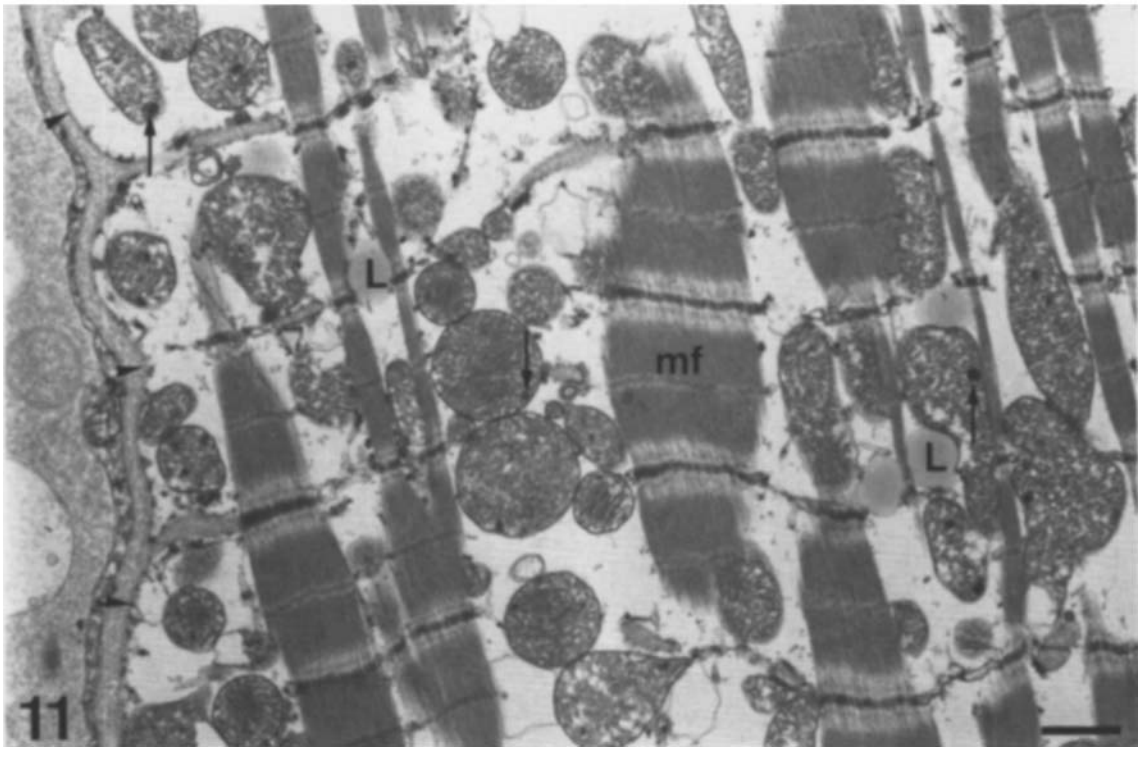


Fig. 10. Diltiazem-pretreated and isoproterenol-treated group. Sarcomeres are relaxed. Mitochondria are slightly swollen and contain electron-dense granules (arrows), but their structures are intact. Sarcolemma (arrowheads) is relatively intact. L = lipid droplets; N = nucleus; ID = cell junction; bar = 1 μm.

Fig. 11. Diltiazem-pretreated and Ca-treated group. Myofibrils (mf) are frayed and mitochondria contain electron-dense granules (arrows). Sarcolemma (arrowheads) is relatively intact, but intracellular fluid accumulation is apparent. L = lipid droplet; bar = 1 μm.

TABLE 1. Major ultrastructural changes

	Control	A	B	C	D	E	F
Sarcomere	Intact, relaxed	Destructed, contracted	Destructed, contracted	Infrequently destructed, relaxed	Infrequently destructed, relaxed	Destructed, contracted	Destructed, contracted
Z-line	Intact	Thickened	Closely packed	Intact	Intact	Thickened	Thickened
Mitochondria	Intact	Destructed	Destructed	Intact	Intact	Destructed	Destructed
Intramitochondrial granules	Rare	Less frequent	Less frequent	Frequent	Frequent	Less frequent	Less frequent
Membranous dense structure	None	Frequent	Less frequent	None	None	Infrequent	Infrequent
Sarcolemma	Intact	Distorted, vesicular	Severely distorted, vesicular	Intact but slightly vesicular	Intact	Distorted, vesicular	Distorted, vesicular
Clumping of chromatin	None	Frequent	Infrequent	Infrequent	Infrequent	Infrequent	Infrequent
Glycogen	Abundant	Depleted	Depleted	Abundant	Abundant	Depleted	Depleted
Cell junction	Intact	Separated	Separated	Intact	Intact	Separated	Separated
Intracellular fluid accumulation	None	Inapparent	Apparent	Inapparent	Slight	Apparent	Apparent

control group. Intramitochondrial electron-dense granules were seen more frequently (Fig. 10) than in the control or in the isoproterenol-treated group; however, the mitochondrial ultrastructure was intact. Clumping of chromatin was infrequently found. One heart showed focal frayed myofibrils, intrasarcoplasmic fluid accumulation, and swollen mitochondria, but internal structure of the mitochondria was relatively intact.

Group D

Three hearts of the diltiazem-pretreated and Ca-treated group showed relatively well preserved ultrastructure except intramitochondrial electron-dense granules, intrasarcoplasmic fluid accumulation and frayed myofibrils (Fig. 11). The sarcolemma was slightly more scalloped than in the control group.

Two hearts showed similar changes with considerable variation. Frayed myofibrils, mitochondrial swelling, and focal sarcolemmal distortions could be found, but mitochondrial cristae were relatively intact. Intramitochondrial electron-dense granules were frequently found.

Group E

All hearts except one of the isoproterenol-treated and diltiazem-posttreated group showed severe ultrastructural changes. Myofibrils were contracted and frayed myofibrils were frequently found. Mitochondrial swelling, matrix clearing, cristal distortion and membranous dense structures could be found frequently (Fig. 12). Lipid droplet accumulation increased but glycogen granules were slightly depleted. Intrasarcoplasmic fluid accumulation was apparent. The sarcolemma was distorted and separated from underlying myofibrils (Fig. 13). Cell junctions were also separated. One heart showed relatively well preserved ultrastructure as in the control group.

Group F

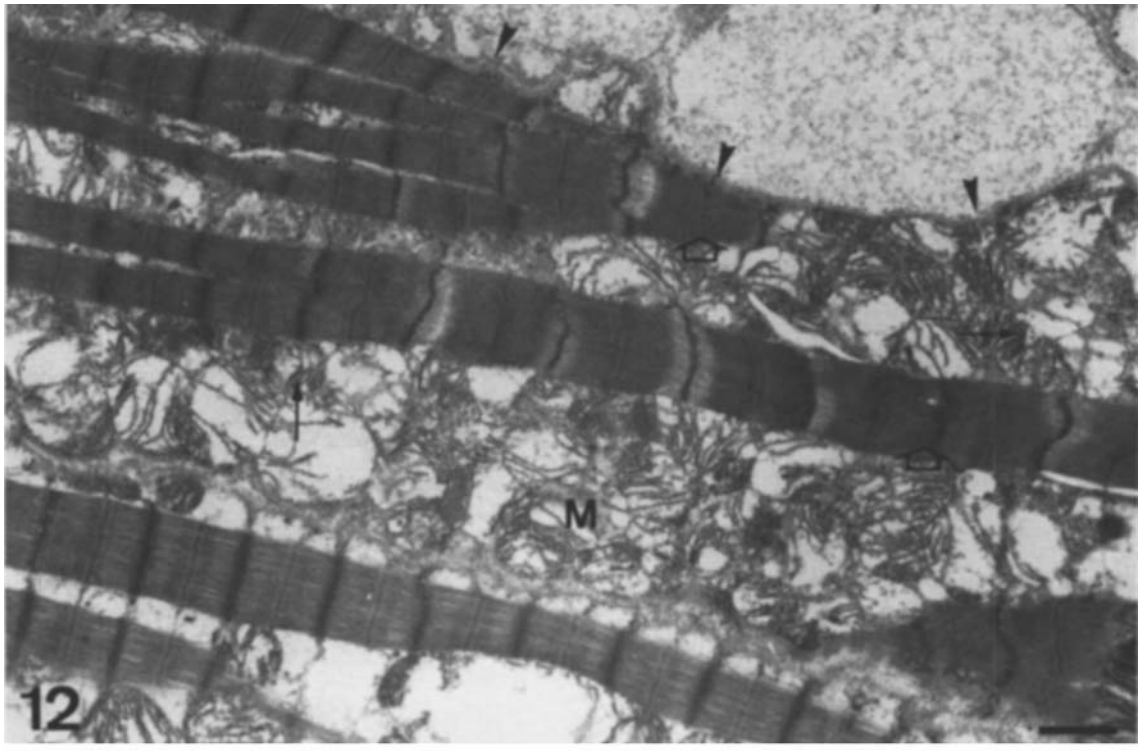
All hearts of the Ca-treated and diltiazem-posttreated group showed severe ultrastructural changes with slight variations. Myofibrils were severely contracted (Fig. 14) as in the Ca-treated group. Mitochondria were swollen and cristae were distorted (Fig. 15), but intramitochondrial electron-dense granules appeared less frequently than in the Ca-treated group. Glycogen depletion, frayed myofibrils, intrasarcoplasmic fluid accumulation and membranous dense structures could be found,. The sarcolemma was distorted and separated from underlying myofibrils (Fig. 16). Cell junctions were also separated.

The major ultrastructural changes observed in this study are summarized in Table 1.

DISCUSSION

In this study, the effects of isoproterenol (2.0 μM) and Ca (4 mM) on myocardial cells of the left ventricular free wall of rabbit heart and the effect of diltiazem (7.5 μM) on the isoproterenol- or Ca-induced myocardial cell injuries have been described. As summarized in Table 1, severe ultrastructural changes in myocardial cells (Schaper et al., 1979), similar, if not identical, to those in human conditions, were produced in the isoproterenol-treated (Groups A and E) and in the Ca-treated (Groups B and F) groups. Particularly, contraction bands, distortions both of mitochondria and sarcolemma were more pronounced in the Ca-treated groups (Groups B and F).

Contraction bands in this study derived from hypercontraction of sarcomeres from individual myocardial cells associated with thickening of the Z-lines, were characterized by complete disruption of the normal striation pattern throughout the entire myocardial cell. The thickness of the Z-lines varied from a very faint to



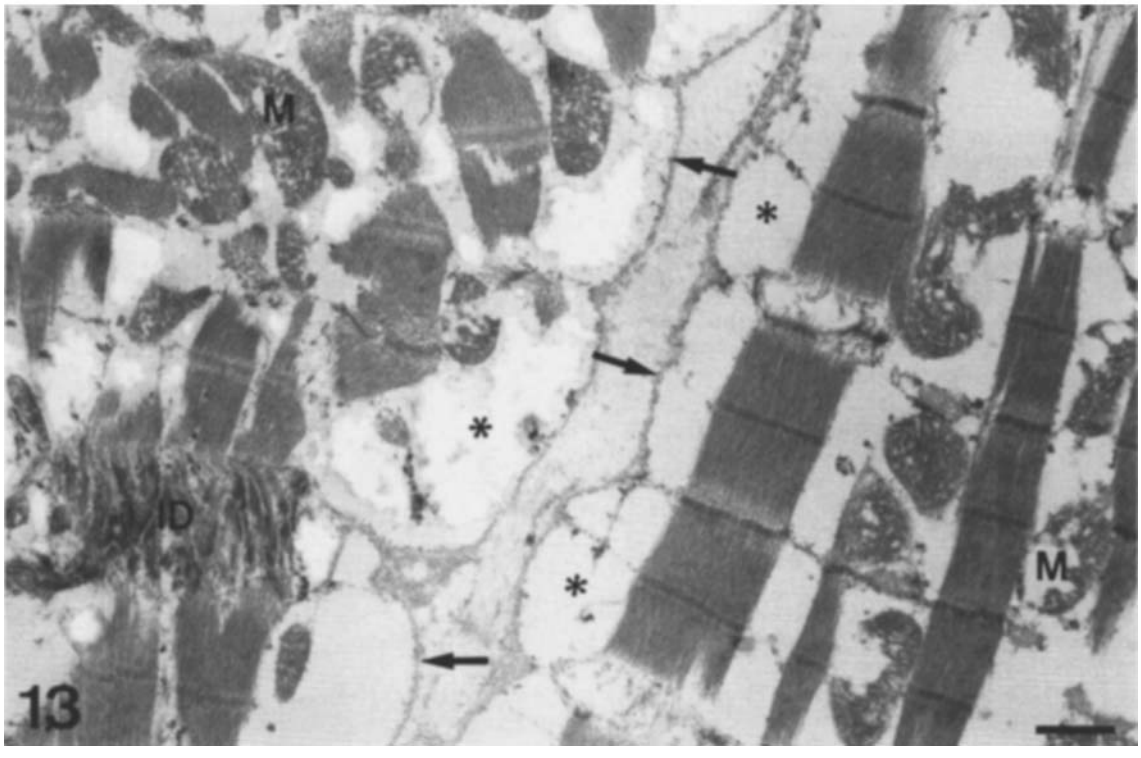


Fig. 12. Isoproterenol-treated and diltiazem-posttreated group. Sarcomeres are contracted (open arrows). Mitochondria (M) contain dense structures (arrows) and sarcolemma (arrowheads) shows only indefinite layer of moderately electron-dense material. Bar = 1.25 μm.

Fig. 13. Isoproterenol-treated and diltiazem-posttreated group. Sarcolemmas (arrows) are separated from underlying myofibrils by large blebs (asterisks) and cell junction (ID) is also disintergrated. M = mitochondria; bar = 0.79 μm.

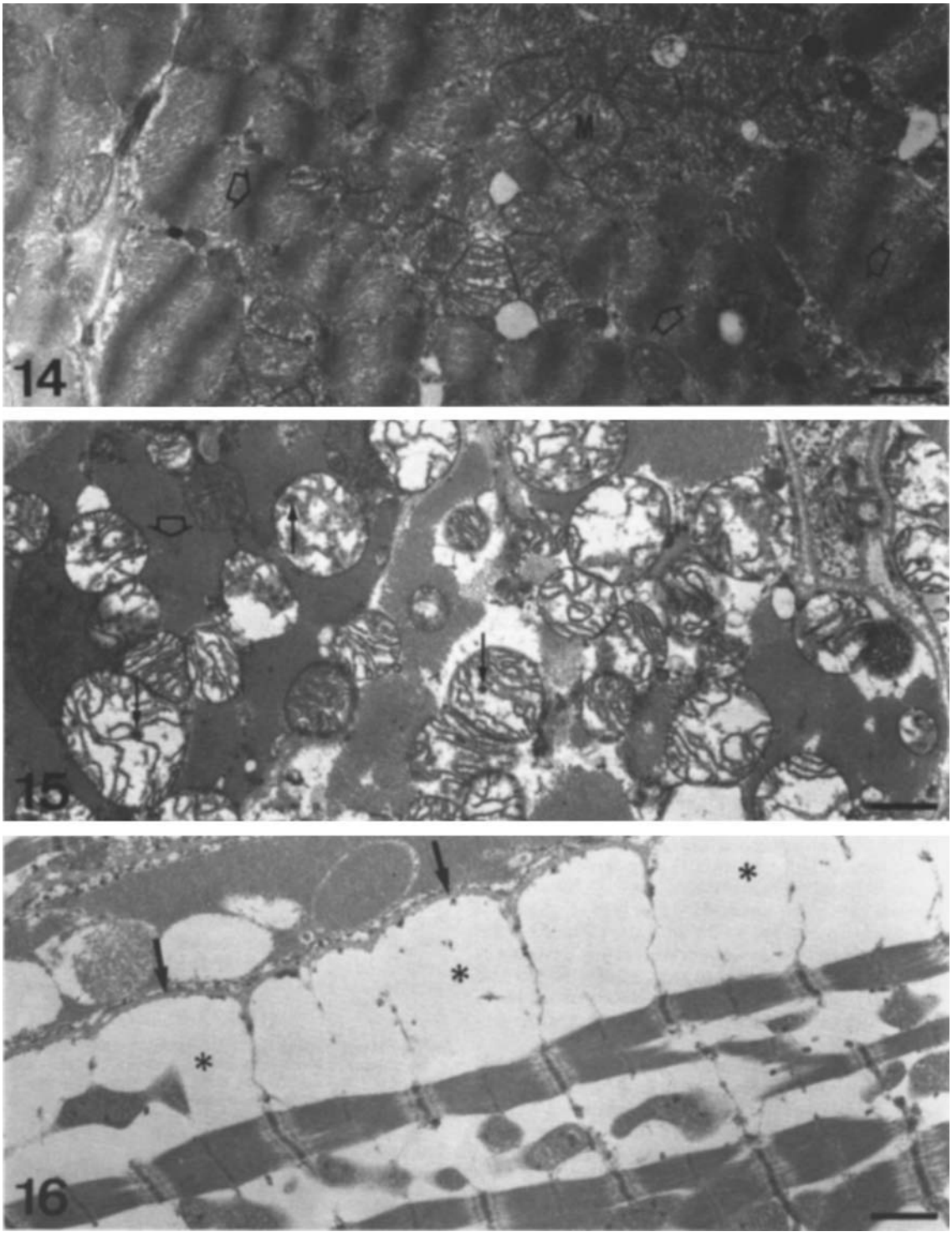


Fig. 14. Ca-treated and diltiazem-posttreated group. Sarcomeres are severely contracted and Z-lines are thickened (open arrows). M = mitochondria; bar = 1 μm.

Fig. 15. Ca-treated and diltiazem-posttreated group. Swollen mitochondria contain electron-dense granules (arrows). Open arrow indicates contracted myofibrils. Bar = 1 μm.

Fig. 16. Ca-treated and diltiazem-posttreated group. Sarcolemma (arrows) is severely distorted and widely separated from underlying myofibrils by large blebs (asterisks). Bar = 1 μm.

a very pronounced appearance. These lesions were termed 'holocytic' (Todd et al., 1985b) to emphasize the total involvement of the myocardial cell. These lesions were frequently associated with sarcolemmal disruption and separation from underlying myofibrils. In some cases intracellular fluid accumulated. This might be closely related to sarcolemmal disruption in which sarcolemmal function as a water barrier could be attenuated. Increased cytosolic Ca could activate Ca-dependent proteases and induce proteolysis of the sarcolemma. In myocardial cells, Ca-dependent proteases have been identified (Toyo-Oka and Masaki, 1979; Goll et al., 1983). But a preliminary study indicated that intracellular edematous change does not contribute significantly to the cardiac hypertrophy as previously reported (Dhalla et al., 1983; Okumura et al., 1983; Panagia et al., 1985). The 'paradiscal' lesions, another pattern of contraction bands, characterized by a single dense transverse band located adjacent to the intercalated disk, has been identified previously (Todd et al., 1985a), but such a lesion was not apparent in this study. Although it has been reported that the 'holocytic' lesions required a proportionally higher concentration and/or longer exposure of catecholamine (Todd et al., 1985b) and appeared later in time (Todd et al., 1985a), the underlying pathophysiology of these two contraction bands and their relation are unclear. Two primary cellular mechanisms have been proposed for the development of these band lesions (Grossman and Barry, 1980). These include 1) severe ATP depletion with impaired dissociation of actin-myosin crossbridges and 2) increased cytosolic Ca during energy depletion. The authors consider that the presence of these lesions could be indicative of severe injury to the myocardial cells, and disagree with Humphrey and Vanderwee (1986) who concluded that the contraction bands cannot be related to the severity of myocardial cell damage.

Intramitochondrial electron-dense granules and membranous dense structure along cristae in the mitochondria were other peculiar features of isoproterenol- and Ca-induced myocardial cell damage. The former change is thought to be caused by the loss of ability of the sarcolemma to bind Ca (Borgers and Piper, 1986) and the latter change may be derived from lipid accumulation during ischemia (Jennings, 1969). Neely and Feuvray (1981) described that the breakup of mitochondrial cristae might form aggregates of lipid membrane particles.

As indicated, the isoproterenol-induced myocardial cell damages are similar to Ca overload. Although the sarcolemmal Ca accumulation may be transient during ischemia (Borgers and Piper, 1986), the localized accumulation of Ca could activate the various membrane located phospholipidases and proteases (Allan and Welman, 1980), resulting in the partial destruction of the sarcolemma and loss of its selective permeability. This would permit Ca to flood into the cytosol and impair mitochondrial function resulting in swelling, vacuolization, and cristal distortion. Thus, it appears that Ca plays a crucial role in isoproterenol-induced myocardial cell damage (Shen and Jennings, 1972; Horak and Opie, 1982). This is further supported by the data obtained from the diltiazem-pretreated groups in this study. They showed little change in the myocardial ultrastructure. Intramitochondrial electron-dense granules which have been shown to be related with Ca accumulation (Shen and Jennings, 1972; Buja et al., 1976), were a particularly noticeable feature in these groups. As previously described, these granules could appear as a result of myocardial cell damage. However, in the diltiazem-pretreated conditions, the mitochondrial ultrastructure were intact, so it could be supposed that these granules may be derived either from active uptake of Ca by mitochondria or from selective inhibition of Ca release from mitochondria (Vaghy et al., 1982), enhanced probably by diltiazem, to lower the cytosolic Ca concentration. It is unclear that these two effects are generated concomitantly or individually. However, in this way it could be possible that mitochondria remove bound Ca from troponin, thereby producing myocardial relaxation (Affolter et al., 1976). The diltiazem-pretreated and Ca-treated group (Group D) showed intracellular fluid accumulation. In contrast to the control and the diltiazem-pretreated groups (Groups C and D), the diltiazem-posttreated groups (Groups E and F) showed severe ultrastructural changes in the myocardial cells. These results suggest that pretreatment with diltiazem could not prevent the deleterious effect of Ca overload completely, but it could reduce these effects to a certain extent. It has been reported that Ca-channel blockers do not prevent excessive Ca influx (Nayler et al., 1979) and ischemia-induced loss of mitochondrial function (Weishaar et al., 1979). Although this study is indirect and only morphological, it seems likely that the beneficial effects of diltiazem are probably derived either from enhanced buffering function of mitochondria to cytosolic Ca or from selective inhibition of transsarcolemmal Ca influx.

In conclusion, isoproterenol-induced myocardial cell damages are similar to Ca overload, and it would be expected that the pretreatment with diltiazem could provide beneficial effects against isoproterenol-induced myocardial cell damage to a certain extent.

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