Caffeine-Induced Myocardial Injury in Calcium-free Perfused Rat Hearts

RICHARD S. VANDER HEIDE, BS, and
CHARLES E. GANOTE, MD

From the Department of Pathology, Northwestern University Medical School, Chicago, Illinois

Hearts depleted of extracellular calcium become susceptible to injury caused by repletion of extracellular calcium (calcium paradox). It has been suggested that calcium-free perfusion causes weakening of intercalated disks and that the physical stress of contracture may cause sarcolemmal membrane rupture and creatine kinase (CK) release. To further investigate this hypothesis, the effects of caffeine on contracture, cellular morphology, and CK release were studied in control and calcium-free perfused isolated rat hearts. Control hearts perfused with 2.5 mM calcium retained normal ultrastructure for long periods of perfusion. Calcium-free hearts perfused for 12 minutes developed separations of fascia adherens portions of intercalated disks but retained intact nexus junctions. Hearts subjected to 5-minute calcium-free perfusion, followed by calcium repletion, developed a massive CK release and extensive contraction band necrosis (calcium paradox). Ten millimolar caffeine, which causes rapid calcium release from sarcoplasmic reticulum (SR), produced contracture, but not CK release, from control hearts perfused with medium containing 2.5 mM calcium. In calcium-free perfused hearts, caffeine caused sudden CK release accompanied by contracture, development of contraction bands, wide separations of cells at intercalated disks, and sarcolemmal membrane injury. Caffeine-induced injury occurred despite 3 mM amobarbital inhibition of mitochondrial respiration. Hearts perfused with caffeine in the presence of calcium relaxed when made calcium-free and did not release CK. Addition of caffeine following calcium-free perfusion at 22 C, which protects the heart from the calcium paradox, produced a rapid, transient contracture. These results are compatible with the hypothesis that myocardial cell injury in calcium-free hearts is not dependent on repletion of extracellular calcium or mitochondrial function, but can result from contracture following caffeine-induced release of intracellular calcium from the SR. (Am J Pathol 1985, 118: 55–65)

INTRACELLULAR CALCIUM overload may play an important role in myocardial cell injury and death.[1,2] The calcium paradox has been proposed as a model system for the study of calcium overload injury.[3] The calcium paradox occurs when hearts are sequentially perfused at 37 C with calcium-free and calcium-containing solutions.[4] Reperfusion of hearts with a calcium-containing solution following a brief calcium-free perfusion results in irreversible contracture,[5,6] loss of electrical activity,[3,6] a large gain in tissue calcium,[7,8] massive cellular disruption,[3–5,9–11] and myocardial enzyme release.[4,9,12,13]

The events subsequent to the initial entry of calcium into cells are not known. Repletion of calcium initiates a marked increase in nonselective permeability of the sarcolemmal membrane and a release of intracellular enzymes. The release of intracellular enzymes is associated with membrane rupture and contraction band necrosis.[4,14–16] Investigations of the mechanisms which produce nonselective sarcolemmal membrane injury and enzyme release have shown that they occur when contracture is induced in calcium-free hearts or when energy-depleted hearts in anoxic contracture are rendered calcium-free.[14] It has been suggested under the former conditions that hearts perfused with calcium-free media develop weakening of intercalated disk junctions. When such hearts develop contracture, separation of intercalated disk membrane faces leads to sarcolemmal membrane damage and enzyme release. In the latter circumstance, when contracture precedes calcium depletion, membrane damage and enzyme release is thought to develop as intercalated disks separate subsequent to weakening of intercalated disk junctions in

Accepted for publication July 25, 1984.
Address reprint requests to Richard S. Vander Heide, Department of Pathology, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, IL 60611.

the presence of sustained contracture. Because both membrane injury and enzyme release occurred in the absence of calcium repletion and in the presence of severely depleted energy stores, sarcolemmal permeability changes under these conditions could not be attributed to either abnormal calcium fluxes or energy-dependent calcium accumulation. It has been postulated that physical stress associated with cellular contracture is responsible for the membrane injury and enzyme release observed in anoxic calcium-free hearts.[14-16]

If physical stress alone were indeed capable of mediating cell injury in anoxic calcium-free hearts (and not some other component of anoxic injury) then the production of contracture, under conditions not related to anoxia, should also produce sarcolemmal membrane damage and enzyme release. Contracture can be induced in isolated calcium-free rat hearts with caffeine, a drug that stimulates intracellular calcium release from the sarcoplasmic reticulum (SR) and contracture without altering mitochondrial function.[17]

In the present study, caffeine was used for investigation of the mechanism of membrane injury and enzyme release in calcium-free rat hearts. If enzyme release occurs upon addition of caffeine to hearts in the absence of both extracellular calcium repletion and energy depletion, it would provide evidence that physiologic amounts of intracellular calcium, acting as a messenger for contracture, may be responsible for sarcolemmal membrane injury and enzyme release in calcium-free hearts.

Materials and Methods

Perfusion Techniques

Male Sprague–Dawley rats weighing 200–400 g were anesthetized by intraperitoneal injection of sodium pentobarbital and anticoagulated with 1000 IU of heparin sulfate injected intravenously. Hearts were removed and immediately immersed in ice-cold oxygenated Krebs–Henseleit bicarbonate solution. The aorta was cannulated on a triple reservoir nonrecirculating Langendorff apparatus, and the hearts were perfused at 37 C at a pressure of 85 mm Hg. Coronary flow was measured by timed collection of effluent, and aliquots were used for enzymatic analysis. Contractile activity was monitored by either a force-displacement transducer attached to the apex of the left ventricle or with a latex rubber intraventricular balloon coupled to a Gould/Statham P23 ID pressure transducer.

Control perfusion solution was a Krebs-Henseleit bicarbonate buffer[18] containing in millimoles per liter: NaCl, 125; $CaCl_2$, 2.5; $NaHCO_3$, 25; KH_2PO_4, 1.2; KCl, 4.8; $MgSO_4$, 1.2; dextrose, 11. Calcium-free medium was prepared by exclusion of calcium chloride and included 0.1 mM EDTA to ensure removal of contaminants. Solutions were continuously gassed with 95% O_2–5% CO_2.

Table 1 – Caffeine Dose-Response Curve

Conditions	Total CK release (IU/20 min/g wet wt)	Percent damaged myocytes (%)
(1) 5 mM	19.86 ± 3.53, n = 6 $P < 0.001$* $P < 0.001$†	11.5 ± 2, n = 4 $P < 0.01$* $P < 0.001$†
(2) 10 mM	55.39 ± 6.49, n = 6 $P < 0.001$†	33.3 ± 4.5, n = 3 $P < 0.001$†
(3) 20 mM	104.41 ± 4.30, n = 6 $P < 0.001$* $P < 0.001$†	70.5 ± 2.9, n = 4 $P < 0.001$* $P < 0.01$†
(4) 40 mM	141.21 ± 4.09, n = 5 $P < 0.001$* $P < 0.01$†	86.5 ± 3.2, n = 4 $P < .001$* $P < .05$†
(5) 60 mM	143.06 ± 8.19, n = 6 $P < 0.001$* $P < 0.05$†	90.0 ± 2.7, n = 4 $P < .001$* P NS†
(6) Calcium paradox	173.52 ± 4.69, n = 4	98 ± 1, n = 3

* Versus 2.
† Versus 6.

For determination of an effective dose of caffeine for these experiments, a dose-response curve for caffeine was calculated. The results shown in Table 1 indicate that cellular injury was progressive as the dose of caffeine was increased over a 5–40 mM range. A dose of 10 mM was chosen as the lowest dose that produced consistent injury without introducing possible nonspecific effects of higher dose levels of caffeine on the experimental results.

Experimental Protocol

Five groups of experiments were performed as outlined in Table 2. All hearts were initially perfused for 15 minutes with oxygenated control medium. In studies of the calcium paradox the control perfusion period was followed by a 5-minute perfusion with calcium-free medium. Hearts were then reperfused for 15 minutes with control medium containing 2.5 mM calcium. In experiments designed to test the effect of caffeine on calcium-free hearts, hearts were perfused for 5 minutes with calcium-free medium and then for 15 minutes with calcium-free solution containing 10 mM caffeine. To determine whether mitochondrial respiration was required for caffeine-induced enzyme release in calcium-free hearts, we added 3 mM amobarbital to the 10 mM caffeine-containing calcium-free solution. Control experiments were carried out for determination of the

Table 2 – Experimental Protocol

Group 1	2.5 mM Ca^{2+}, O_2 37 C 15 min	Ca^{2+}-free, O_2 37 C 5 min	2.5 mM Ca^{2+}, O_2 37 C 15 min	Fixation for morphology
Group 2	2.5 mM Ca^{2+}, O_2 37 C 15 min	Ca^{2+}-free, O_2 37 C 5 min	Ca^{2+}-free, 10 mM caffeine, O_2 37 C 15 min	Fixation for morphology
Group 3	2.5 mM Ca^{2+}, O_2 37 C 15 min	Ca^{2+}-free, O_2 37 C 5 min	Ca^{2+}-free, 10 mM caffeine, 3 mM amobarbital, O_2 37 C 15 min	Fixation for morphology
Group 4	2.5 mM Ca^{2+}, O_2 37 C 15 min	Ca^{2+}-free, O_2 37 C 5 min	Ca^{2+}-free, 3 mM amobarbital, O_2 37 C 15 min	Fixation for morphology
Group 5	2.5 mM Ca^{2+}, O_2 37 C 15 min	2.5 mM Ca^{2+}, 10 mM caffeine 37 C 5 min	Ca^{2+}-free, 10 mM caffeine, O_2 37 C 15 min	Fixation for morphology

effects of amobarbital on calcium-free hearts. In this group, hearts were perfused with calcium-free solution for 5 minutes and then for 15 minutes with calcium-free solution containing 3 mM amobarbital. In the final series of experiments the effects of adding 10 mM caffeine to the hearts prior to calcium-free perfusion were studied. Hearts were perfused for 5 minutes with control medium containing 10 mM caffeine and subsequently perfused for 15 minutes with calcium-free solution containing caffeine.

Enzymatic Analyses

Creatine kinase (CK) activity was assayed with 0.1-ml aliquots of the coronary effluent with a Gilford 3402 automatic enzyme analyzer and Worthington reagents (Freehold, NJ). The results are expressed as international units of CK activity per minute per gram of initial wet heart weight.

Morphologic Studies

The hearts were fixed at the end of the experiments by perfusion with 50 ml of 1% glutaraldehyde in calcium-free modified Tyrode's solution. Following infusion, the hearts were immersed in the same fixative for an additional 48 hours. Tissues to be examined by light microscopy were transferred into buffered 10% formalin, paraffin-embedded, and stained with hematoxylin and eosin (H&E). From each experimental group shown in Table 1, 3 or 4 hearts were perfusion-fixed for morphologic analysis. From light-microscopic sections of these hearts 500 longitudinally oriented cells present in the midmyocardium were examined with a ×40 dry objective and were classified as to the presence or absence of severe myocardial cell injury. For electron microscopy, tissues were postfixed with 1% osmium tetroxide, stained *en bloc* with aqueous uranyl acetate, dehydrated, and embedded in plastic. Blocks from some hearts were not stained until after sectioning for prevention of uranyl acetate extraction of calcium. Semithin (1-μ) sections of plastic blocks were cut and stained with toluidine blue for light microscopy; thin sections were cut with a diamond knife for electron microscopy.

Statistical Analysis

The results are expressed as the mean ± standard error of the mean (SEM). A Student *t* test was used for determination of statistical significance of differences between experimental groups.

Results

Effect of Extracellular Calcium Repletion on Calcium-Free Hearts (Calcium Paradox)

During the control and a subsequent 20-minute calcium-free perfusion the hearts did not release significant amounts of CK. Readmission of 2.5 mM extracellular calcium following 5 minutes of calcium-free perfusion resulted in a sudden massive CK release which peaked at 1 minute and totaled 172.07 ± 4.77 IU of CK/15 min/g wet wt during the 15-minute reperfusion period (Figure 1).

Effect of Caffeine on Calcium-Free Hearts

To investigate whether membrane injury and CK release could occur in calcium-free hearts without repletion of extracellular calcium, we added caffeine to hearts following a 5-minute calcium-free perfusion at 37 C.

Addition of 10 mM caffeine induced a sudden release

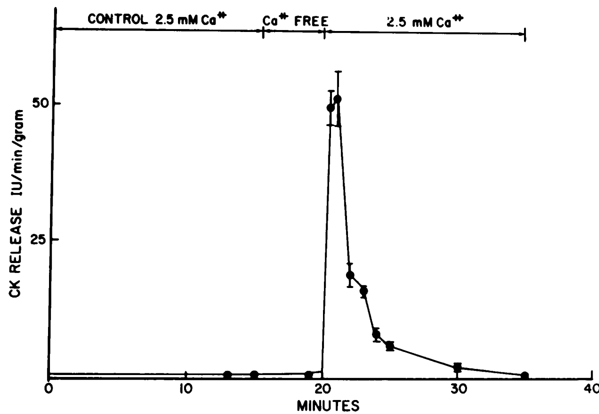


Figure 1 — Creatine kinase release curve from four hearts perfused with calcium-free medium for 5 minutes and then reperfused with 2.5 mM calcium-containing medium (calcium paradox). There was a massive CK release upon repletion of calcium.

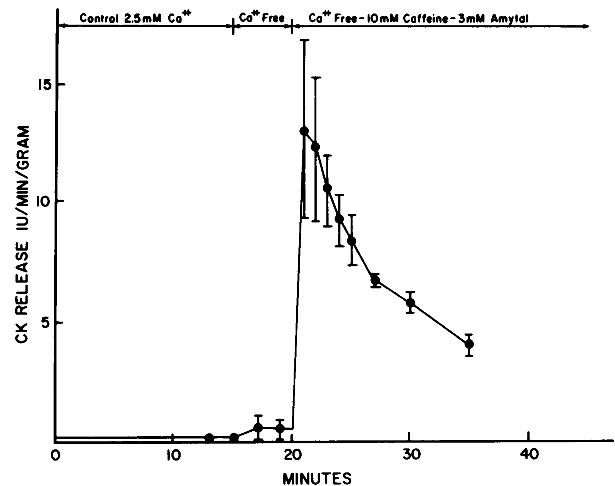


Figure 3 — CK release curve from three hearts exposed to 10 mM caffeine-3 mM amobarbital containing solution following calcium-free perfusion. There was a sudden release of CK from these hearts despite inhibition of mitochondrial respiration with amobarbital.

of CK totaling 75.63 ± 10.34 IU/15 min/g wt during 15 minutes of perfusion (Figure 2). Morphologic studies of caffeine-injured hearts revealed wide separation of myocytes, severe sarcolemmal membrane injury, and contraction band necrosis.

Effect of Amobarbital on Caffeine-Induced Myocardial Injury

To determine whether mitochondrial respiration was required for caffeine-induced injury to occur, we added 3 mM amobarbital to the 10 mM caffeine-containing calcium-free solution. The amount of enzyme release observed under these conditions, 114.52 ± 12.04 IU/15 min/g wet wt, was similar to that observed in hearts exposed to caffeine alone (Figure 3).

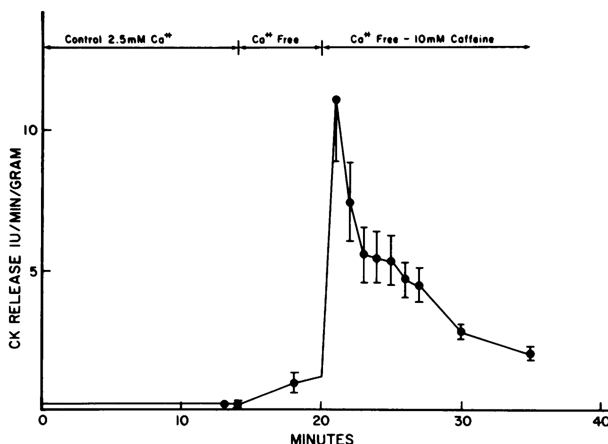


Figure 2 — CK release curve from six hearts exposed to 10 mM caffeine after 5 minutes of calcium-free perfusion. Upon addition of caffeine there was a sudden enzyme release.

Effect of Amobarbital on Calcium-Free Hearts

To determine whether amobarbital alone might cause injury in calcium-free hearts, we exposed hearts perfused with calcium-free medium for 5 minutes to calcium-free solution containing 3 mM amobarbital for an additional 15 minutes. No significant CK release was observed over the 20-minute perfusion (9.46 ± 3.15 IU/20 min/g wet wt).

Effect of Calcium Removal on Hearts in Caffeine-Induced Contracture

Addition of caffeine to control hearts in the presence of 2.5 mM calcium resulted in a caffeine-induced contracture (Figure 4). After removal of calcium from the perfusate while maintaining the presence of caffeine, caffeine-induced contracture is rapidly reversed (Figure 4) and minimal CK release occurs (Figure 5) (11.51 ± 3.73 IU/15 min/g wet wt).

Demonstration of Caffeine Contracture in Calcium-Free Hearts

Because of the severe cardiac injury which occurred after the addition of caffeine to calcium-free hearts, it was not possible to accurately record the development of caffeine-induced contracture. To demonstrate caffeine contracture under calcium-free conditions, the hearts were initially perfused with calcium-free medium at 22 C, and then caffeine was added at 37 C. Hypothermia has been shown to protect hearts from damage associated with the calcium paradox[19-32] and allows

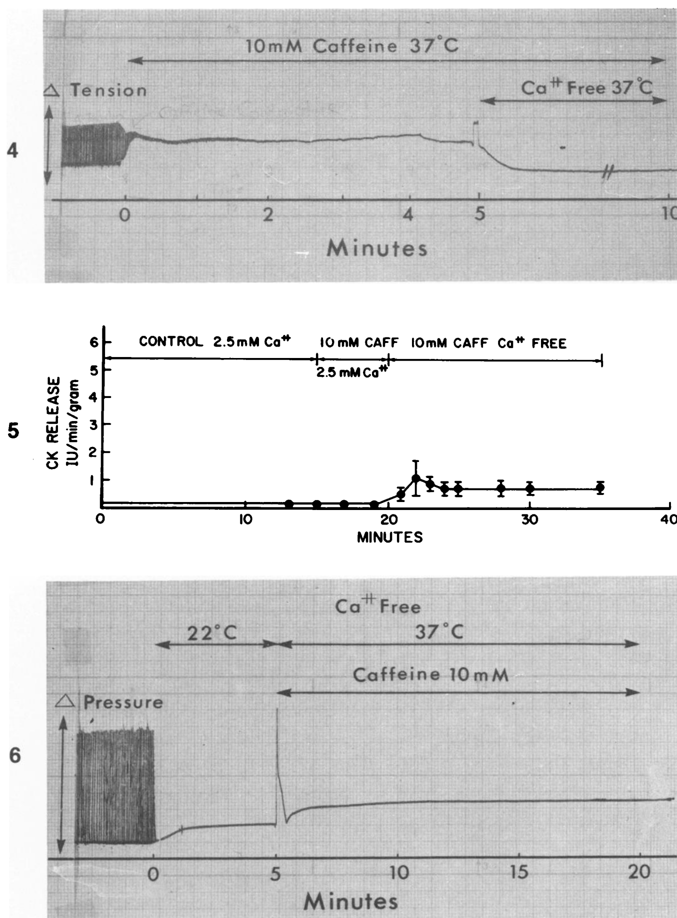


Figure 4 – A force-displacement transducer recording of contractile activity of a heart exposed to control medium with 10 mM caffeine present. Upon removal of extracellular calcium, the caffeine-induced contracture was rapidly reversed. **Figure 5** – Enzyme release curve of four hearts exposed to calcium-free medium after a caffeine-induced contracture. Removal of calcium from the perfusate did not cause a significant release of CK. **Figure 6** – Pressure tracing of a heart containing an intraventricular balloon and perfused with calcium-free medium at 22 C. Addition of 10 mM caffeine to the perfusate at 37 C resulted in the rapid onset of a transient contracture. The initial calcium-free perfusion was at 22 C for prevention of severe injury to the hearts.

recording of pressure changes associated with calcium repletion. The rapid, transient contracture produced by caffeine is shown in Figure 6. No significant CK release was observed over the 20-minute perfusion.

Light Microscopy

Myocardial cells from hearts examined by light microscopy following a 20-minute perfusion with calcium-free medium were relaxed with uniformly spaced z-lines (Figure 7). Intercalated disk junctions were partially separated with narrow gaps between opposing cells (Figure 7).

Hearts reexposed to 2.5 mM calcium following a 5-minute calcium-free perfusion (calcium paradox) contained nearly 100% of myocytes, which were severely injured with single large contraction bands (Table 1). Adjacent myocytes were damaged and widely separated at intercalated disks, and cytoplasmic debris lay in the space between the separated cells (Figure 8).

Hearts exposed to calcium-free medium containing 10 mM caffeine subsequent to 5 minutes of calcium-free perfusion also exhibited severe cellular injury. Individual myocytes were shortened, with single central contraction bands and were widely separated from neighboring cells (Figure 9). When 3 mM amobarbital was added to the calcium-free/caffeine medium, the cellular changes produced were similar but distinct from those seen with caffeine alone. In the presence of both caffeine and amobarbital, individual myocytes were contracted and separated from neighboring cells; however, in contrast to caffeine alone, the myocytes were uniformly contracted and did not exhibit contraction bands (Figure 10).

Hearts made calcium-free following caffeine-induced contracture and subsequent relaxation contained few

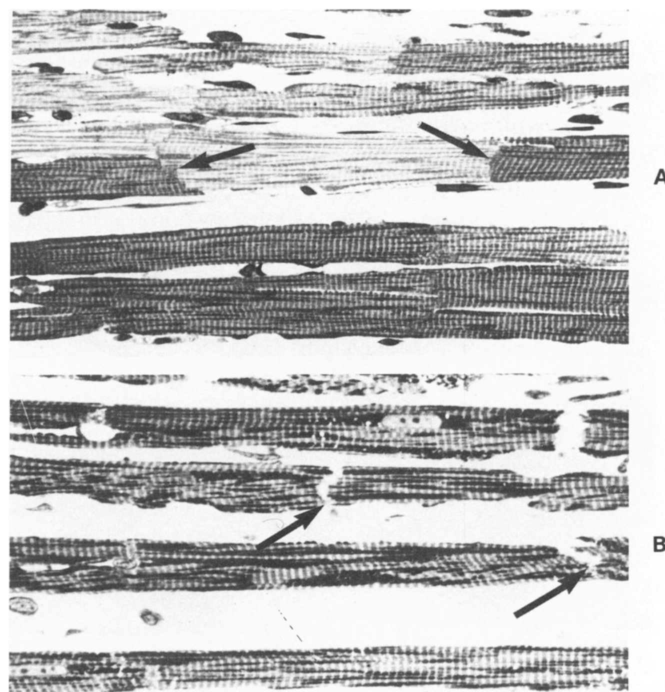


Figure 7A – Light micrograph of a heart perfused for 25 minutes with oxygenated, control Krebs-Henseleit solution. The cells have uniformly spaced sarcomeres, and intercalated disk junctions (*arrows*) appear intact, with no gaps or separations. (Epon-embedded, toluidine blue, ×875) **B** – Light micrograph of a heart perfused for 20 minutes with calcium-free medium at 37 C. The heart is relaxed, with uniformly spaced sarcomeres. The intercalated disks are partially separated, with small gaps and narrow separations of the junctions (*arrows*). (Epon-embedded, toluidine blue, ×875)

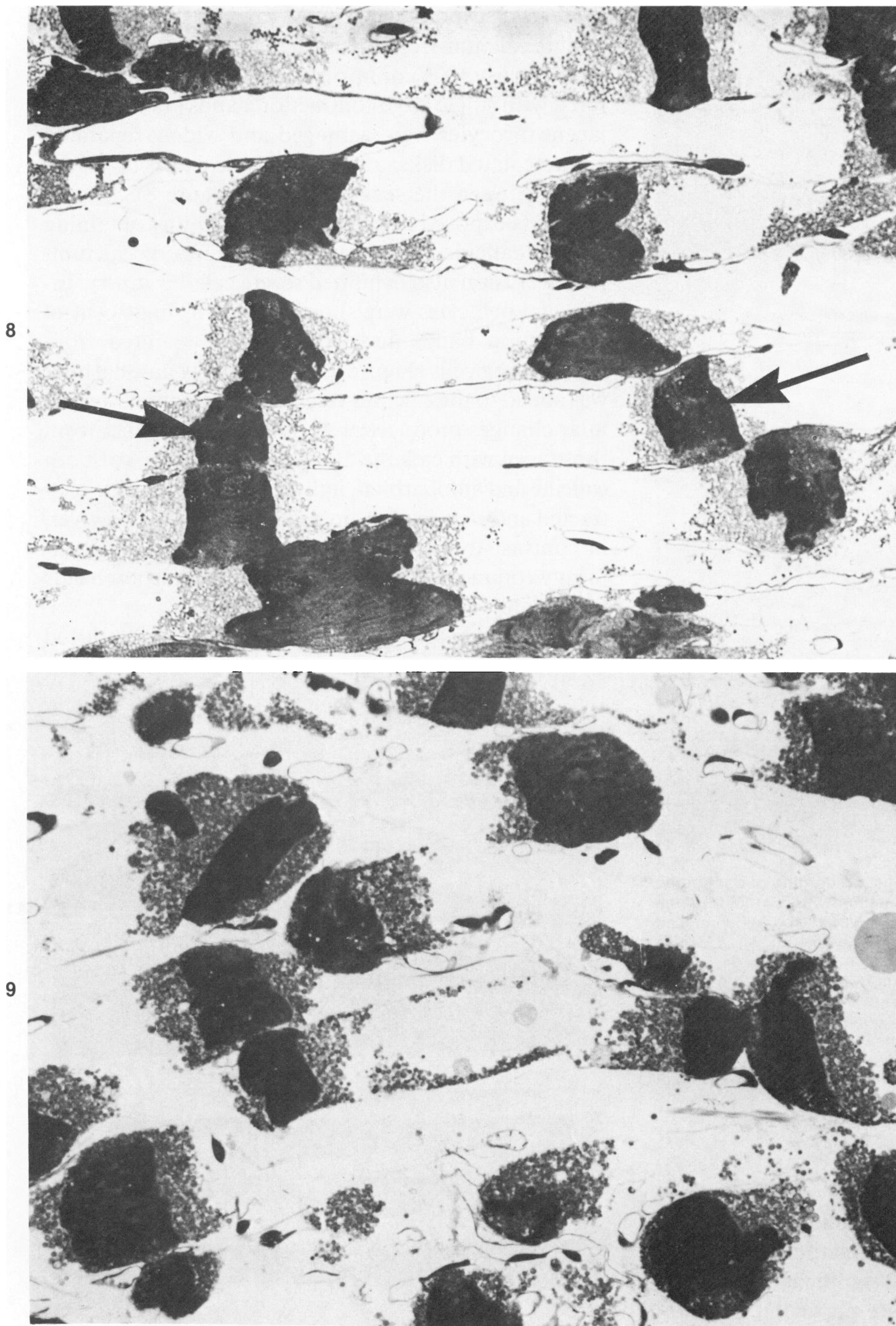


Figure 8 – Light micrograph of a heart sequentially perfused with calcium-free medium followed by calcium-containing medium (calcium paradox). The myocytes are widely separated from neighboring cells and contain large, central contraction bands (*arrows*). Mitochondria and cellular debris can be seen in the spaces between separated cells. (Epon-embedded, toluidine blue, ×1000) **Figure 9** – Light micrograph of a heart perfused for 5 minutes with calcium-free medium and then 15 minutes with 10 mM caffeine added to calcium-free medium. The myocytes exhibit large, central contraction bands and are widely separated from adjacent cells. Mitochondria adhere to the ends of the cells. The appearance of these cells is similar to that of cells after the calcium paradox shown in Figure 8. (Epon-embedded, toluidine blue, ×1000)

obviously damaged cells. The myocytes in these hearts were elongated and appeared relaxed, with widely separated z-bands (Figure 11). Intercalated disks were generally apposed but frequently showed narrow gaps between adjacent cells. Occasionally, widely separated myocytes were observed, which could account for the low level of enzyme release seen in these hearts.

Electron Microscopy

Electron-microscopic studies confirmed and extended the results obtained by light microscopy. Hearts exposed only to calcium-free medium following control perfusion appeared relaxed, with uniformly spaced z-lines and prominent I-bands. The most significant lesion ob-

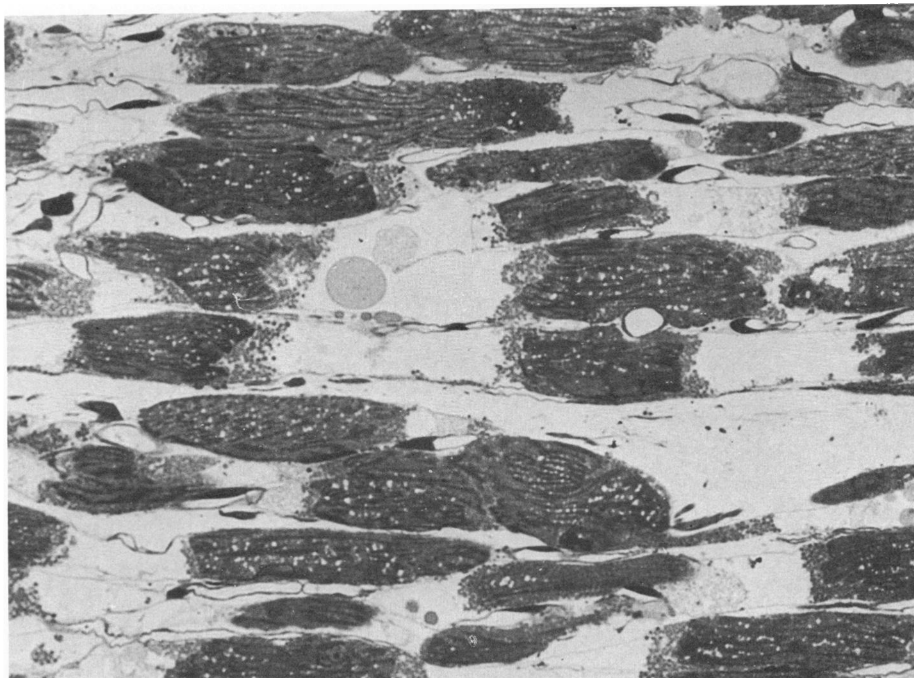


Figure 10—Light micrograph of a heart sequentially perfused for 5 minutes with calcium-free solution and then for 15 minutes with 10 mM caffeine and 3 mM amobarbital added to calcium-free medium. The myocardial cells are in contracture, but do not exhibit contraction bands. The cells are widely separated from adjacent cells at intercalated disk junctions. (Epon-embedded, toluidine blue, ×1000)

served after 10 minutes of calcium depletion involved intercalated disk junctions. The disks were partially separated at the macula and fascia adherens junctions but usually remained intact at nexus junctions (Figure 12).

Hearts perfused for 5 minutes with calcium-free medium followed by 15 minutes of calcium repletion (calcium paradox) exhibited diffuse cellular changes. Individual myocytes contained single, large contraction bands and numerous swollen mitochondria (Figure 13). The mitochondrial matrix was swollen and contained numerous granular dense bodies (calcium accumulation granules) (inset, Figure 13). The identity of the granules was confirmed by observations that they were present only in tissue stained on the sections and were absent in tissue stained *en bloc* with aqueous uranyl acetate, which is acidic and is known to extract calcium. Hearts exposed to 15 minutes of calcium-free medium containing 10 mM caffeine following a 5-minute calcium-free perfusion exhibited severe cellular injury similar to that seen in the calcium paradox (Figure 14). Myocytes contained single, large contraction bands and were widely separated from adjacent cells at intercalated disk junctions. Intercalated disk membrane faces appeared fragmented, with prominent discontinuities (Figure 14).

Mitochondria of caffeine-injured hearts differed from mitochondria of hearts injured by the calcium paradox in that they were contracted with dilated intercristal spaces and moderately dense matrix spaces (inset, Figure 14). Granular matrix densities were absent in mitochondria of caffeine-injured hearts.

Discussion

Caffeine causes calcium release from the SR[17,24-26] but does not alter mitochondrial respiration.[17] Caffeine

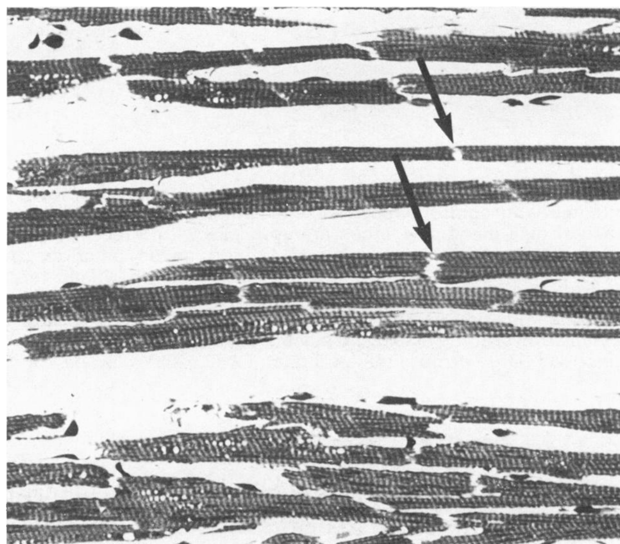


Figure 11—Light micrograph of a heart perfused for 5 minutes with control medium containing 10 mM caffeine and then for 15 minutes with calcium-free medium containing caffeine. Myocardial cells appear relaxed; and except for the presence of small gaps between adjacent cells at intercalated disk junctions (*arrows*), the cells do not appear damaged. There was no enzyme release from this heart. (Epon-embedded, toluidine blue, ×750)

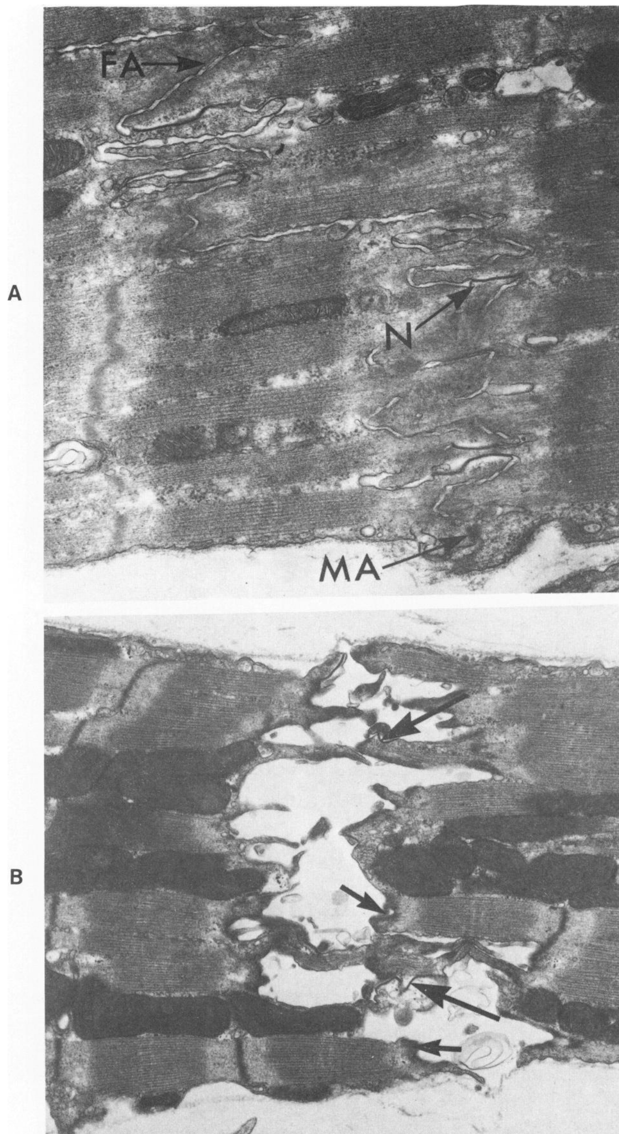


Figure 12A—An electron micrograph from a heart perfused for 25 minutes with control Krebs-Henseleit solution at 37 C. The intercalated disk membrane faces are apposed, with intact fascia adherens (*FA*), macula adherens (*MA*), and nexus junctions (*N*). (×22,100) **B**—Electron micrograph of a heart perfused for 12 minutes with calcium-free solution. The sarcomeres are relaxed, with prominent I-bands. The intercalated disks are completely separated (*small arrows*) at the fascia and macula adherens junction, but nexus junctions (*large arrows*) remain intact. (×13,600)

contracture reflects a raised intracellular calcium concentration considered to be the result of impaired calcium sequestration by the SR[27-30] or changes in the permeability of SR vesicle membranes.[17] Caffeine-induced contracture is rapidly reversible if calcium is removed from the perfusate, indicating that the contracture is not associated with rigor bond formation subsequent to adenosine triphosphate (ATP) depletion.

When caffeine was added to control hearts, a rapid contracture resulted, but there was neither biochemical (CK release) nor morphologic (contraction bands) evidence of cellular injury. Caffeine added to calcium-free hearts perfused at 37 C produced rapid contracture, with contraction bands, associated with severe cellular disruption, membrane injury, and CK release.

These results indicate that neither ATP depletion nor readmission of extracellular calcium is necessary to produce cellular injury in calcium-free hearts and demonstrate that selective release of intracellular calcium from the SR is sufficient to cause cell injury.

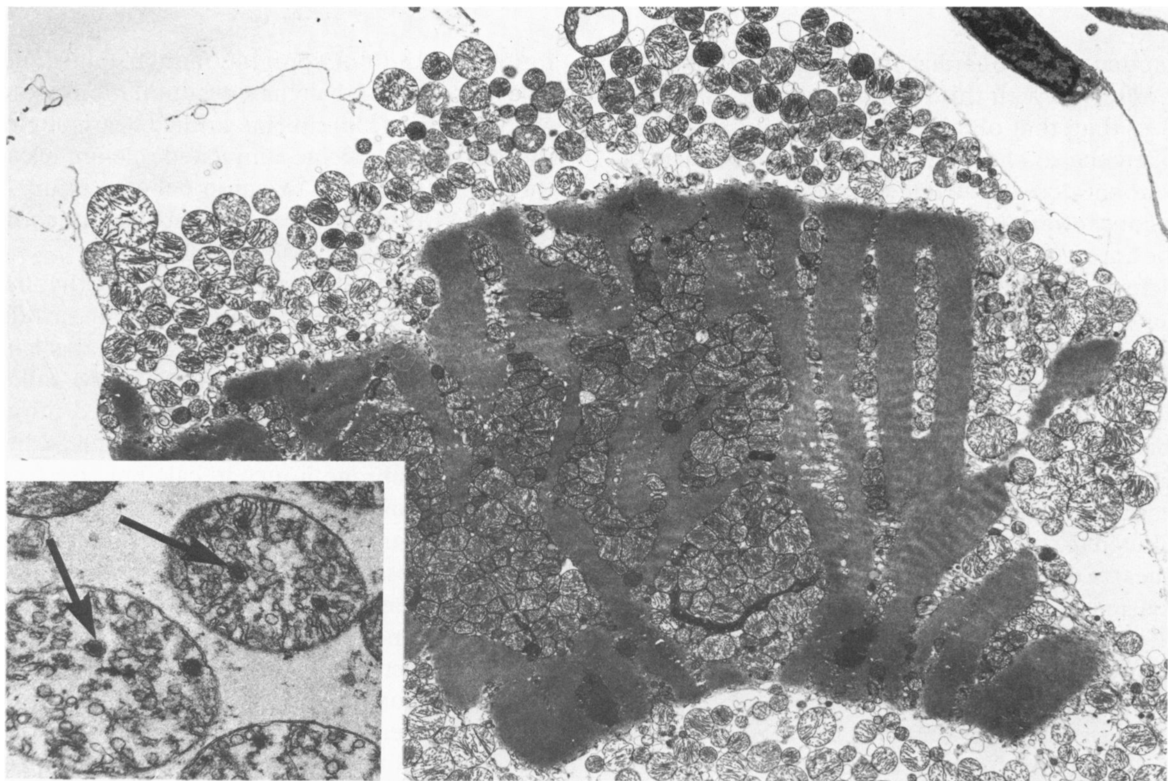
There is some evidence that cell injury resulting from elevated intracellular calcium levels may result from active mitochondrial calcium accumulation.[31,32] The role of mitochondrial respiration as a mediator of injury was investigated by adding amobarbital to the caffeine perfusion of calcium-free hearts. Despite inhibition of mitochondrial respiration by amobarbital, membrane injury and enzyme release similar to caffeine alone was observed. Furthermore, it has recently been shown that addition of the mitochondrial uncoupler 2,4-dinitrophenol (DNP) to calcium-free hearts produces membrane injury and massive CK release.[16,23] Because uncoupled mitochondria can neither phosphorylate adenosine diphosphate nor accumulate calcium ions, the sum of these results suggests that mitochondria probably do not play a central role in the mediation of sarcolemmal membrane injury and enzyme release in calcium-free hearts.

It may be argued that caffeine has a direct toxic effect which could cause membrane injury and CK release in calcium-free hearts. However, the results from experimental group 5 do not support this possibility. In this series, caffeine was present throughout the perfusion period; yet no significant CK release was observed.

Because of the severe injury that developed at 37 C, it was not possible to record accurately the development of contracture during caffeine-induced CK release. Lowering of the perfusate temperature during calcium depletion provides nearly complete protection from the calcium paradox.[19-23] It has been suggested that hypothermic protection results from a temperature-dependent lipid phase transition occurring in sarcolemmal membranes which retards or prevents the deleterious changes produced by calcium depletion.[13,19,21,22] Hypothermia also prevents separation of intercalated disk membranes associated with calcium depletion.[21,23]

To demonstrate caffeine-induced contracture under calcium-free conditions, calcium-free perfusion was carried out at 22 C. When severe injury was prevented by hypothermia, caffeine produced a rapid, transient contracture followed by relaxation (Figure 6).

Although the dose of 10 mM caffeine used in this



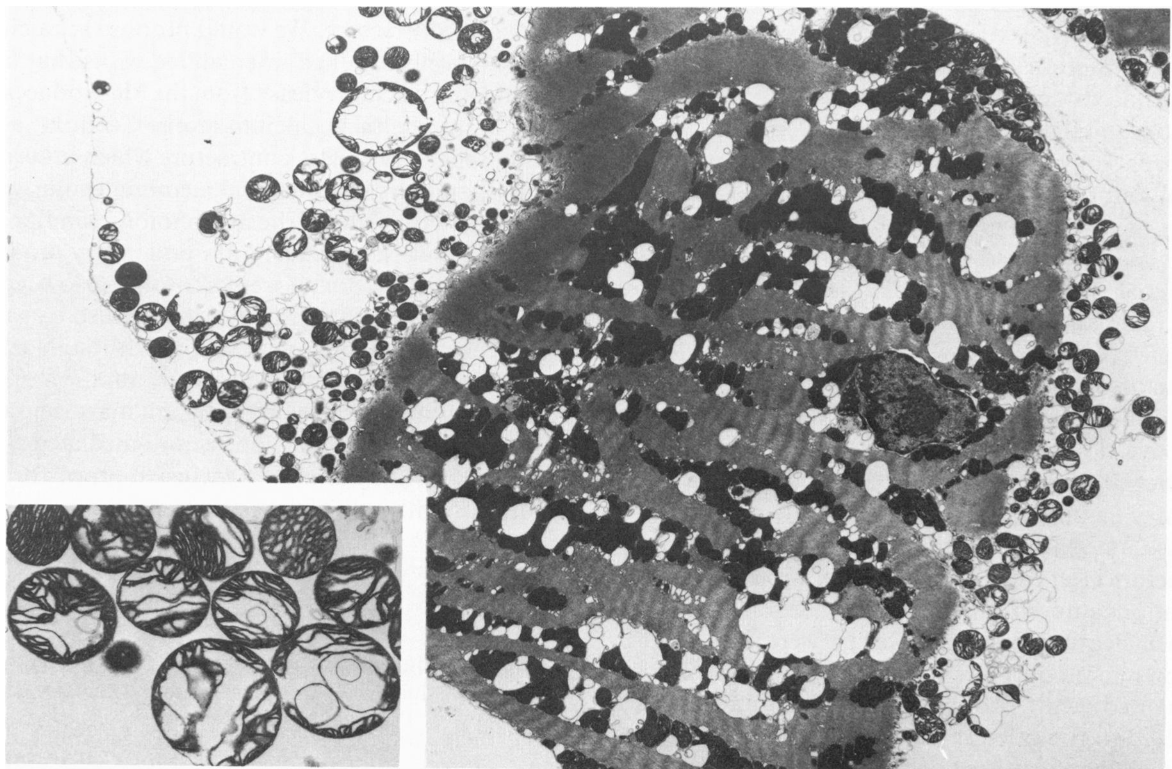


Figure 13 – Electron micrograph of a single myocyte from a heart subjected to the calcium paradox. The cell contains a single, prominent contraction band and has separated from adjacent cells at the intercalated disks. Intercalated disk membrane faces are ruptured, allowing swollen mitochondria to spill into the space between the separated cells. (×3500) **Inset** – High-power view of mitochondria from a heart after the calcium paradox. The matrix spaces are swollen and contain several granular dense bodies, or calcium accumulation granules (*arrows*). (This specimen was stained only on the section with uranyl acetate and lead citrate, ×20,000) **Figure 14** – Electron micrograph of an individual myocyte from a calcium-free perfused heart after caffeine-induced enzyme release. The cell contains a large, central contraction band and is widely separated from neighboring cells. The intercalated disk membrane faces have ruptured, with resultant leakage of mitochondria into the extracellular space. (×4500) **Inset** – Mitochondria from the same heart were often in the condensed configuration with a dense matrix space and dilated intercristal space. Granular dense bodies were not observed under these conditions. (×10,000)

study produced severe cardiac injury, the amount of CK release observed with this dose was significantly ($P < 0.001$) less than that obtained in the calcium paradox. This difference, as shown in Table 1, can be largely attributed to the dose of caffeine selected for these studies. As the concentration of caffeine was increased to 60 mM, the amount of cardiac injury approached that of the calcium paradox. At the highest dose levels of caffeine there was no significant difference in morphologic estimates of injury between caffeine-treated hearts and hearts subjected to the calcium paradox. Despite similar morphologic estimates of damage, there was a small but significant ($P < 0.05$) reduction in the total CK release from caffeine-treated hearts, as compared with the calcium paradox. This difference can possibly be attributed to the mild degree of mitochondrial damage in hearts exposed to caffeine, when cellular damage occurred in the absence of extracellular calcium.

Morphologic Observations

Morphologic changes observed after the addition of caffeine to calcium-free hearts were strikingly similar to those seen in the calcium paradox. In hearts subjected to both types of injury, the myocytes developed single contraction bands and were widely separated from adjacent cells at intercalated disk junctions.

Amobarbital added to caffeine-treated hearts altered the morphology of injured cardiocytes. Cells in the presence of amobarbital developed a uniform contraction of sarcomeres which produced cell shortening and separations at intercalated disks but did not show hypercontracted sarcomeres characteristic of contraction bands. The appearance of such hearts was similar to calcium-free hearts following anoxic or DNP-induced contracture and enzyme release.[14,33] The mechanism of formation of contraction bands is not understood, but the above observations may indicate that continuing mitochondrial respiration and possibly production of ATP may facilitate hypercontracture of sarcomeres. In any case, amobarbital-inhibited hearts developed severe contracture after caffeine treatment with subsequent cell separations and enzyme release.

When hearts from both groups were examined by electron microscopy, sarcolemmal membranes appeared fragmented, with prominent discontinuities. Hearts subjected to the calcium paradox contained damaged cells in which the mitochondria appeared swollen and contained numerous granular matrix densities (calcium accumulation granules). The presence of granular dense bodies indicates that calcium accumulation is the probable cause of mitochondrial damage observed in the calcium paradox. In contrast, mitochondria from caffeine-injured hearts were in the condensed configuration and did not contain granular matrix densities. The absence of calcium accumulation granules in mitochondria from caffeine-injured hearts suggests that the amount of caffeine-stimulated calcium release from the SR is sufficient to cause cellular injury but insufficient to overload and damage the mitochondria or to form granular matrix densities.

The results of the present study show that hearts exposed to calcium-free perfusion become susceptible to injury caused by repletion of extracellular calcium (calcium paradox) or by addition of 10 mM caffeine. Although our results suggest that elevated intracellular levels of calcium resulting from caffeine addition can mediate membrane injury in calcium-free hearts, the exact mechanism of injury production is not known. It may be argued that altered intracellular calcium distribution subsequent to caffeine addition could play a role in the genesis of membrane injury. Furthermore, other calcium-dependent biochemical processes such as calcium-activated neutral proteases and phospholipase activation may contribute to cellular injury after sustained release of SR calcium by caffeine. However, these possibilities seem unlikely because control hearts (Group 5) did not release CK in the presence of caffeine-induced contracture. We would propose in calcium-free hearts with weakened intercalated disks that caffeine-stimulated calcium release from the SR produces a rapid rise in intracellular calcium levels. Calcium, acting as a messenger, produces contracture which in turn causes cell separations, sarcolemmal membrane injury, and CK release. In the light of the morphologic similarities observed between caffeine injury and injury produced by the calcium paradox, it seems reasonable to suggest that calcium-mediated contracture could also cause the injury seen in the calcium paradox. Although cell injury produced in calcium-free hearts by addition of caffeine or repletion of extracellular calcium may occur through the same mechanism of contracture-mediated sarcolemmal membrane damage, conclusive proof for this hypothesis will require further investigation.

References

1. Dhalla NS: Involvement of the membrane systems in heart failure due to calcium overload and deficiency. J Mol Cell Cardiol 1976, 8:661–667
2. Dhalla NS, Das PK, Sharma GP: Subcellular basis of cardiac contractile failure. J Mol Cell Cardiol 1978, 10:363–385
3. Yates JC, Dhalla NS: Structural and functional changes associated with failure and recovery of hearts after perfusion with Ca^{2+}-free medium. J Mol Cell Cardiol 1975, 7:91–103
4. Zimmerman ANE, Hülsmann WG: Paradoxical influence of calcium ions on the permeability of the cell membranes of the isolated rat heart. Nature 1966, 211:646–647

5. Muir AR: A calcium-induced contracture of cardiac muscle cells. J Anat 1968, 102:148-149
6. Ruigrok TJC, Boink ABTJ, Spies F, Blok FJ, Maas AH, Zimmerman ANE: The effect of calcium on myocardial tissue damage and enzyme release, Enzymes in Cardiology: Diagnosis and Research. Edited by DJ Hearse, J DeLeiris. Chichester, John Wiley, 1979, pp 399-416
7. Alto LE, Dhalla NS: Myocardial cation contents during induction of calcium paradox. Am J Physiol 1979, 237:H713-H719
8. Nayler WG, Grinwald PM: The effects of verapamil on calcium accumulation during the calcium paradox. J Mol Cell Cardiol 1981, 13:435-441
9. Muir AR: The effects of divalent cations on the ultrastructure of perfused rat heart. J Anat 1967, 101:239-261
10. Ruigrok TJC, Boink ABTJ, Spies F, Blok FJ, Maas AHJ, Zimmerman ANE: The effect of verapamil on the calcium paradox. Am J Pathol 1980, 98:769-782
11. Frank JS, Langer GA, Nudd LM, Seraydarian K: The myocardial cell surface: Its histochemistry and the effect of sialic acid and calcium removal on its structure and cellular ionic exchange. Circ Res 1977, 41:702-714
12. Hearse DJ, Baker TE, Humphrey SM: Verapamil and the calcium paradox. J Mol Cell Cardiol 1980, 12:733-740
13. Hearse DJ, Humphrey SM, Bullock GR: The oxygen paradox and calcium paradox: two facets of the same problem? J Mol Cell Cardiol 1978, 10:641-688
14. Ganote CE, Safavi S, Kaltenbach JP: Anoxia, calcium and contracture as mediators of myocardial enzyme release. J Mol Cell Cardiol 1981, 13:93-106
15. Ganote CE, Sims MA: Physical stress-mediated myocardial enzyme release and the calcium paradox. J Mol Cell Cardiol 1983, 15:421-429
16. Ganote, Sims MA, Vander Heide RS: Mechanism of enzyme release in the calcium paradox. Eur Heart J 1983 (Suppl H) 4:63-71
17. Blayney L, Thomas H, Muir J, Henderson A: Action of caffeine on calcium transport by isolated fractions of myofibrils, mitochondria, and sarcoplasmic reticulum from rabbit heart. Circ Res 1978, 43:520-526
18. Krebs HA, Henseleit K: Untersuchungen über die Harnstöffbildung im Tierkörper. Hoppe-Seyles Z Physiol Chem 1932, 210:33-66
19. Holland CE, Olsen RE: Prevention by hypothermia of paradoxical necrosis in cardiac muscle. J Mol Cell Cardiol 1975, 7:917-928
20. Boink ABTJ, Ruigrok TJC, de Moes D, Maas AHJ, Zimmerman ANE: The effect of hypothermia on the occurrence of the calcium paradox. Pflugers Arch 1980, 385:105-109
21. Baker JE, Bullock GR, Hearse DJ: The temperature dependence of the calcium paradox: Enzymatic, functional and morphological correlates of cellular injury. J Mol Cell Cardiol 1983, 15:393-411
22. Rich TL, Langer GA: Calcium depletion in rabbit myocardium: Calcium paradox protection by hypothermia and cation substitution. Circ Res 1982, 51:131-141
23. Ganote CE, Sims MA: Parallel temperature dependence of contracture-associated enzyme release due to anoxia, 2,4-dinitrophenol (DNP), or caffeine and the calcium paradox. Am J Pathol 1984, 116:94-106
24. Hunter DR, Haworth RA, Berkoff HA: Cellular calcium turnover in the perfused rat heart: Modulation by caffeine and procaine. Circ Res 1982, 51:363-370
25. Movsesian MA: Calcium physiology in smooth muscle. Prog Cardiovasc Dis 1982, 25:211-224
26. Pretorius PJ, Pohl WG, Smithen CS, Inesi G: Structural and functional characterization of dog heart microsomes. Circ Res 1969, 25:487-491
27. Herz R, Weber A: Caffeine inhibition of Ca uptake by muscle reticulum (Abstr). Fed Proc 1965, 24:208
28. Weber A: Mechanism of the action of caffeine on sarcoplasmic reticulum. J Gen Physiol 1968, 52:760-772
29. Weber A, Herz R: Relationship between caffeine contracture of intact muscle and the effect of caffeine on sarcoplasmic reticulum. J Gen Physiol 1968, 52:750-759
30. Blinks JR, Olson CB, Jewell BR, Braveny P: Influence of caffeine and other methylxanthines on mechanical properties of isolated mammalian heart muscle. Circ Res 1972, 30:367-392
31. Ruigrok TJC, Boink ABTJ, Spies F, Blok FJ, Maas AHJ, Zimmerman ANE: Energy dependence of the calcium paradox. J Mol Cell Cardiol 1978, 10:991-1002
32. Boink ABTJ, Ruigrok TJC, Maas AHJ, Zimmerman ANE: Calcium paradox: Changes in high-energy phosphate compounds of isolated perfused rat hearts, Recent Advances in Studies on Cardiac Structure and Metabolism. Vol II. Heart Function and Metabolism. Edited by T Kobayashi, T Sano, NS Dhalla. Baltimore, University Park Press, 1978, pp 559-564
33. Ganote CE, Grinwald PM, Nayler WG: 2,4-Dinitrophenol (DNP)-Induced injury in calcium-free hearts. J Mol Cell Cardiol, 1984, 16:547-557

Acknowledgments

The authors are grateful to Saideh Safavi for her excellent technical assistance.