Parallel Temperature Dependence of Contracture-Associated Enzyme Release Due to Anoxia, 2,4-Dinitrophenol (DNP), or Caffeine and the Calcium Paradox

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Hypothermia during calcium-free perfusion of hearts protects them from injury caused by subsequent calcium repletion at 37 C (calcium paradox). Injury to calcium-free hearts is also associated with contracture caused by anoxia, 2,4-dinitrophenol (DNP), or caffeine. This study was done for the purpose of determining whether hypothermia during calcium-free perfusions protects hearts from contracture-associated injury. Langendorff-perfused rat hearts were studied in four experimental groups: I) Anoxia: Thirty minutes of anoxic perfusion at 37 C was followed by thirty minutes of anoxic calcium-free perfusion at 37–18 C. II) Calcium paradox: Five minutes of calcium-free perfusion at 37–18 C was followed by calcium repletion at 37 C. III, IVa) Caffeine or DNP: Five minutes of calcium-free perfusion at 37–18 C was followed by addition of 10 mM caffeine or 1 mM DNP in calcium-free medium at 37 C or, IVb) 1 mM DNP in calcium-free medium at 22 C. Injury was assessed by measurement of serial releases of creatine kinase (CK) in effluents and by cellular morphology. The results show that progressive hypothermia to 22 C during calcium-free perfusion periods produced a progressive reduction of CK release and morphologic evidence of injury due to anoxia, caffeine, or DNP, which closely paralleled protection of hearts from the calcium paradox. Protection from injury in all experimental groups was associated with preservation of sarcolemmal membrane integrity and prevention of cell separations at intercalated disk junctions. It is proposed that weakening of intercalated disks occurs during calcium-free perfusions and may be a cause of mechanical fragility of the sarcolemma. Hypothermia may protect hearts from contracture-associated injury by preserving the integrity of intercalated disk junctions during periods of extracellular calcium depletion. (Am J Pathol 1984, 116:94–106)

THE STRUCTURAL and functional integrity of the heart is dependent on the presence of extracellular calcium. In the absence of extracellular calcium, the heart becomes susceptible to injury. The readmission of calcium to the heart following a calcium-free interval results in irreversible contracture with a massive release of enzymes.[1,2] The damage caused by calcium repletion is called the calcium paradox.[3] The calcium paradox has been attributed to an intracellular calcium overload due to an uncontrolled entry of calcium into cells following calcium repletion, but the precise mechanisms of calcium-induced myocardial cellular damage have not been established.[4]

Hearts exposed to calcium-free perfusion have also been shown to be susceptible to sarcolemmal membrane damage and enzyme release in the absence of calcium repletion. Injury to calcium-free hearts can be caused by anoxic contracture,[5] physical distension of ventricles by inflating an intraventricular balloon,[6] or by dinitrophenol (DNP),[7] or caffeine-induced contracture.[8] We have proposed that sarcolemmal injury and enzyme release caused by the latter agents is mediated by physical stresses developed during contracture. Dissociation of calcium from sarcolemmal binding sites during calcium-free perfusion is thought to make the sarcolemma fragile by weakening of fascia adherens junctions in intercalated discs. Sarcolemmal rupture occurs as cells dehisce at intercalated disks during induction of contracture.

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Metabolic consequences of intracellular calcium overload are usually considered to be the pathogenesis of membrane injury and enzyme release in the calcium paradox. Since contracture occurs upon calcium repletion to calcium-free hearts, contracture is also a possible mediator of cell injury in the calcium paradox. Although it is clear that myocardial enzyme release caused by anoxia, balloon distension, DNP, or caffeine is not related to an influx of calcium into cells, it has not been possible to distinguish between contracture- or calcium-mediated injury in the calcium paradox. This study attempts to test the hypothesis that contracture may mediate sarcolemmal membrane injury in calcium paradox.

The calcium paradox is highly temperature-dependent,[9] with nearly complete protection from 5 minutes of calcium-free perfusion being afforded by reduction of the temperature during calcium-free perfusion to 22 C.[10,11] If contracture rather than uncontrolled calcium entry into cells were the mediator of sarcolemmal membrane damage and enzyme release in the calcium paradox, then it would be expected that hypothermia would also protect hearts from enzyme release due to anoxia, DNP, or caffeine. A parallel temperature dependence of contracture-induced injury and injury due to the calcium paradox would provide evidence compatible with, but would not prove, the hypothesis that enzyme release in the calcium paradox is mediated by contracture. If, on the other hand, a lack of association between hypothermic protection in contraction-mediated and calcium-induced injury were found, then contracture could be excluded as a cause of enzyme release in the calcium paradox. The present study is of the temperature dependence of contracture-associated injury in calcium-free perfused rat hearts.

Materials and Methods

Male Sprague–Dawley rats weighing 250–350 g were anesthesized by intraperitoneal injection of 55 mg/kg of sodium pentobarbital (Diabutal, Diamond Laboratories, Inc., Des Moines, Iowa). Following intravenous injection of 2000 IU of sodium heparin (Elkins-Sinn, NJ) hearts were quickly excised and immersed in ice-cold Krebs–Henseleit bicarbonate (KHB) solution. After removal of extraneous tissue, the hearts were cannulated by the aorta to the perfusion apparatus. A thin-walled latex balloon was inserted through the mitral valve into the left ventricle and inflated with 0.05–0.1 ml water.

Experimental Design

Four groups of experiments were performed as outlined below. All hearts were perfused for a 15-minute control period with oxygenated KHB buffer at 37 C. Anoxic hearts were then perfused with anoxic medium for 30 minutes at 37 C and then for 30 minutes with anoxic Ca^{2+}-free medium at 37, 34, 25, or 22 C. In the calcium paradox and caffeine experiments, the control period was followed by 5 minutes at Ca^{2+}-free

Group I: Anoxic

2.5 mM Ca^{2+}, O_2 37 C	2.5 mM Ca^{2+}, N_2 37 C	Ca^{2+}-free, N_2 37–22 C	Fixation for morphology
15 min	30 min	30 min	

Group II: Calcium Paradox

2.5 mM Ca^{2+}, O_2 37 C	Ca^{2+}-free, O_2 37–18 C	2.5 mM Ca^{2+}, O_2 37 C	Fixation for morphology
15 min	5 min	20 min	

Group III: Caffeine

2.5 mM Ca^{2+}, O_2 37 C	Ca^{2+}-free, O_2 37–18 C	Ca^{2+}-free, O_2 37 C, 10 mM Caffeine	Fixation for morphology
15 min	5 min	20 min	

Group IV: DNP

a) 2.5 mM Ca^{2+}, O_2 37 C	Ca^{2+}-free, O_2 37–18 C	Ca^{2+}-free, O_2 37 C, 1 mM DNP	Fixation for morphology
15 min	5 min	20 min	
b) 2.5 mM Ca^{2+}, O_2 37 C	Ca^{2+}-free, O_2 22 C	Ca^{2+}-free, O_2 22 C, 1 mM DNP	Fixation for morphology
15 min	5 min	20 min	

perfusion at 37, 34, 25, 22, or 18 C and then 20 minutes of perfusion at 37 C with a medium containing 2.5 mM $CaCl_2$ or 10 mM caffeine. In the final group of experiments, the control period was followed by 5 minutes of Ca^{2+}-free perfusion at 37, 34, 25, 22, or 18 and then 20 minutes of perfusion at 37 or 22 C with medium containing 1 mM DNP. A balloon was placed in the left ventricle of all hearts to measure the level of contracture during the experiment. At the end of the experiments, hearts were fixed for light and electron microscopy. Tissue was embedded in Epon and stained with toluidine blue for light microscopy or thin-sectioned and stained with uranyl acetate for electron microscopy.

Perfusion Fluid

The perfusion fluid was a standard KHB solution[12] containing 2.5 mM $CaCl_2$ and 11 mM dextrose. For anoxic perfusions, dextrose was replaced with 11 mM mannitol. In calcium-free medium, $CaCl_2$ was omitted without osmotic substitution, and 0.1 mM EDTA was added to ensure removal of residual calcium contaminants. Solutions containing 10 mM caffeine or 1 mM DNP were not adjusted for the change in osmolarity. All solutions were appropriately gassed with 95% O_2–5% CO_2 or 95% N_2–5% CO_2 and then brought to a pH range of 7.3–7.4. At the end of the experiments, the hearts were fixed by infusion of 40 ml of 1% glutaraldehyde solution in a modified Tyrode's buffer.[13] In experiments where calcium-free perfusion media were used, $CaCl_2$ was excluded from the fixative, and 0.1 mM EDTA was added.

Perfusion Apparatus

The hearts were perfused on a double-reservoir, gravity-flow, Langendorff apparatus at 80–90 mmHg pressure. The heart was suspended inside of a thermally insulated chamber, and the aortic temperature was monitored with a thermistor probe. It was determined with a second thermistor probe that the right ventricle temperature did not vary by more than 0.5 C from the aortic temperature. Temperature was maintained at 37 C with a Yellow Springs temperature controller (Yellow Springs Instrument Co., Inc., Ohio) connected to the aortic probe. For hypothermic perfusions the temperature was controlled by a separate Gilson water bath (Gilson Instruments, Middleton, Wis). The water bath temperature was adjusted so that the desired aortic temperature was reached within 30 seconds after switching the perfusion fluid. The left ventricle balloon was connected via a polyethylene catheter to a Gould P23 pressure transducer, and pressure was continuously recorded with a Gilson 5/6 recorder.

Enzyme Determination

For enzyme determination, 0.1-ml aliquots of heart effluent were assayed for creatine kinase (CK) activity in a Gilford 3400 automatic enzyme analyzer with Worthington reagents (Freehold, NJ). Enzyme release rates were calculated from the initial wet heart weight and measured coronary flow rates and expressed as international units of CK activity released/min/g (IUCK/min/g). Each experimental point represents the average of five or six hearts except for the anoxic data, where three hearts were used per experimental point. Statistically significant differences in the results were calculated with the use of the Student t test.

Results

Effects of Hypothermia on Anoxic Enzyme Release: Experimental Group I

Hearts develop contracture within 15 minutes of the beginning of anoxic perfusion but do not begin to release enzymes until after 60 to 90 minutes of normothermic anoxia. Hearts made anoxic for 30 minutes and then subjected to anoxic and calcium-free perfusion began a sustained release of enzymes within 2–3 minutes after initiation of calcium-free perfusion. Following an initial period of anoxic perfusion at 37 C, the average total enzyme release of three hearts at 37 C over the subsequent 30-minute period of anoxic and calcium-free perfusion was 114.9 ± 2.3 IUCK ($P < 0.05$ versus 22 C). The effect of hypothermia on enzyme release was assessed by decreasing the temperature of anoxic hearts only during the calcium-free perfusion period. The average total enzyme release at 34 C was 92.2 ± 1.3 IUCK ($P < 0.05$ versus 22 C); at 25 C, 49.3 ± 9.3 IUCK ($P < 0.02$ versus 22 C); and at 22 C, 36.5 ± 10.4 IUCK (Figure 1). The intraventricular pressure of hearts after 30 minutes of anoxia and 4 minutes of calcium-free perfusion was 88 ± 3.8 mmHg at 37 C, 89 ± 5.1 mmHg at 34 C, 80 ± 6.9 mmHg at 25 C, and 81 ± 6.2 mmHg at 22 C. These pressures slowly declined over the period of calcium-free perfusion.

Effects of Hypothermia During Calcium-Free Perfusion on Calcium-, DNP- and Caffeine-Induced Enzyme Release: Experimental Groups II, III, IVa

Hearts were subjected to 5 minutes of calcium-free perfusion in oxygenated medium at 37, 34, 25, 22,

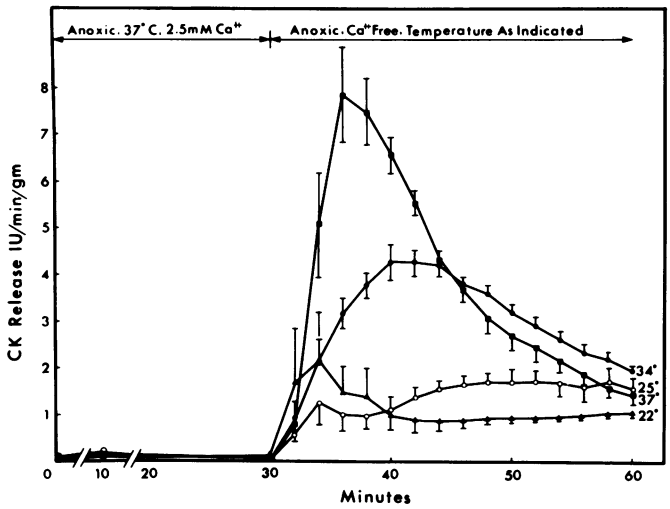


Figure 1—Enzyme release curves from hearts during 30 minutes of anoxic perfusion at 37 C with 2.5 mM calcium in the perfusate and an additional 30 minutes of perfusion with calcium-free perfusate at 37, 34, 25, or 22 C. There was a progressive reduction of enzyme release during calcium-free perfusion with reduction of temperature of the perfusate. Each value is mean ± SEM of a group of three hearts.

and 18 C. Following the calcium-free interval, the hearts were then perfused at 37 with media containing 2.5 mM calcium, 10 mM caffeine, or 1 mM DNP. The media containing caffeine or DNP remained calcium-free. The total amount of CK released over the 20-minute period after switching to the test media was measured from areas beneath enzyme release curves for each of five or six hearts in each group. The results in Figure 2 show that the enzyme release was slightly higher at 34 C than at 37 C for all groups hearts. There was a marked reduction in enzyme release due to calcium ($P < 0.001$) and caffeine ($P < 0.001$) between 34 C and 25 C. Reduction of the temperature of the initial calcium-free interval to 22 C or 18 C resulted in nearly total protection from calcium- or caffeine-induced enzyme release. The enzyme release due to DNP did not show a temperature dependence under these experimental conditions and was not significantly different at 18 C from that at 37 C.

Effect of Sustained Hypothermia on DNP-Induced Enzyme Release: Experimental Group IVb

The experiments described below confirm that 1 mM DNP causes a strong contracture in calcium-free medium at both 22 C and 37 C. This experiment was designed to determine whether enzyme release would occur if hearts were maintained at 22 C after addition of DNP. No significant release of enzymes was observed from DNP-treated hearts if the temperature was maintained at 22 C during both the initial period of calcium-free perfusion and the subsequent 15-minute period of calcium-free perfusion after addition of 1 mM DNP to the perfusate (Figure 3).

Effects of Hypothermia on Contracture

In hearts maintained at 37 C during both the initial period of calcium-free perfusion and a subsequent period of perfusion with calcium, caffeine, or DNP massive injury and large enzyme releases developed. In these severely injured hearts it was not possible to document contracture with an intraventricular bal-

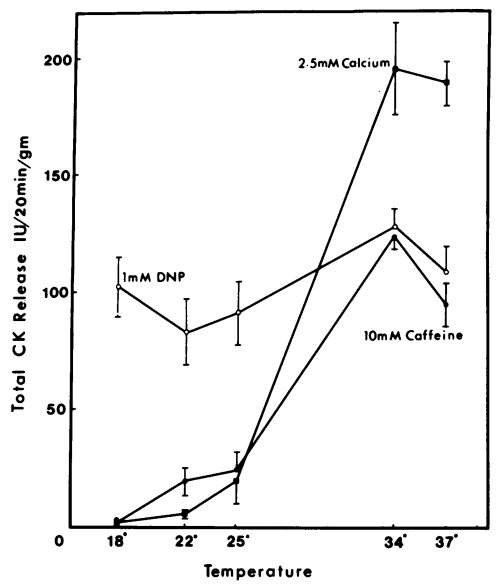


Figure 2—Graph of total enzyme released from hearts perfused for 5 minutes at 37, 34, 25, 22, or 18 C with calcium-free perfusate, followed by addition at 37 C of 1 mM DNP or 10 mM caffeine, in calcium-free solution, or 2.5 mM calcium (calcium paradox). Between 34 and 25 C both caffeine- and calcium-induced enzyme release were markedly reduced. Reperfusion of hearts with calcium-free DNP solution at 37 C caused a similar amount of enzyme release in all groups of hearts. Each point is the mean ± SEM of a group of five or six hearts.

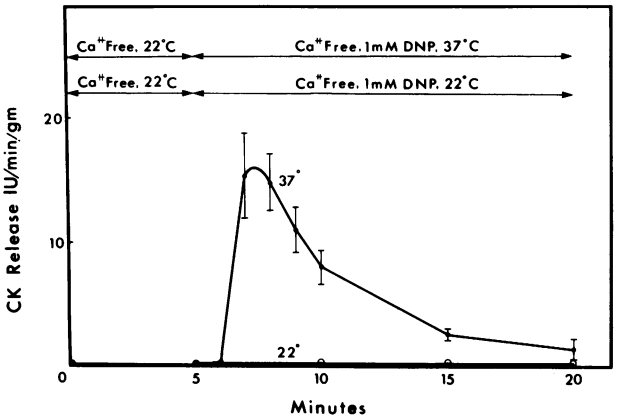


Figure 3—Enzyme release curves from two groups of hearts. Both groups were initially perfused with calcium-free solution for 5 minutes at 22 C. At 5 minutes 1 mM DNP was added either at 37 C or at 22 C. At 37 C DNP induced a large enzyme release, after a brief delay. When the temperature was maintained at 22 C, with DNP there was no enzyme release for 25 minutes of perfusion. At 22 C DNP causes a strong contracture (see Figure 3d). Values are the mean ± SEM for five hearts.

loon. In hearts protected from severe injury measurable contractures did develop. Since 22 C resulted in nearly total protection from injury, this temperature was used to compare contracture curves for each condition (Figure 4 a–d). Readmission of calcium to hearts at 37 C after 5 minutes of calcium-free perfusion at 22 C caused an immediate contracture, which peaked and declined in a few seconds. Resumption of contractions began as the initial contracture was declining, and diastolic pressure returned to baseline within 1 minute (Figure 4a). Addition of 10 mM caffeine at 37 C to calcium-free hearts caused an immediate transient contracture which was completed within 30 seconds. This was followed by a small rise in resting tension; no enzyme release occurred after caffeine (Figure 4b). Addition of 1 mM DNP produced a slowly rising, sustained contracture, which began after a delay of 1–3 minutes. After 2–3 minutes the initial plateau was reached. There was a sustained enzyme release, and the contracture tension slowly declined (Figure 4c). Hearts that were maintained at 22 C during both the initial period of calcium-free perfusion and during addition of 1 mM DNP had a delayed contracture, which began after 6–7 minutes and reached maximal pressure at 8–9 minutes (Figure 4d) but did not release significant amounts of enzymes.

Morphologic Observations

Anoxic Hearts: Experimental Group I

With development of anoxic contracture, hearts showed subendocardial zones of ischemia. Morphologic studies were limited to well-perfused areas of myocardium in the midmyocardial and subepicardial regions of the heart. In calcium-free hearts following anoxic enzyme release at 37 C, nearly every cell was

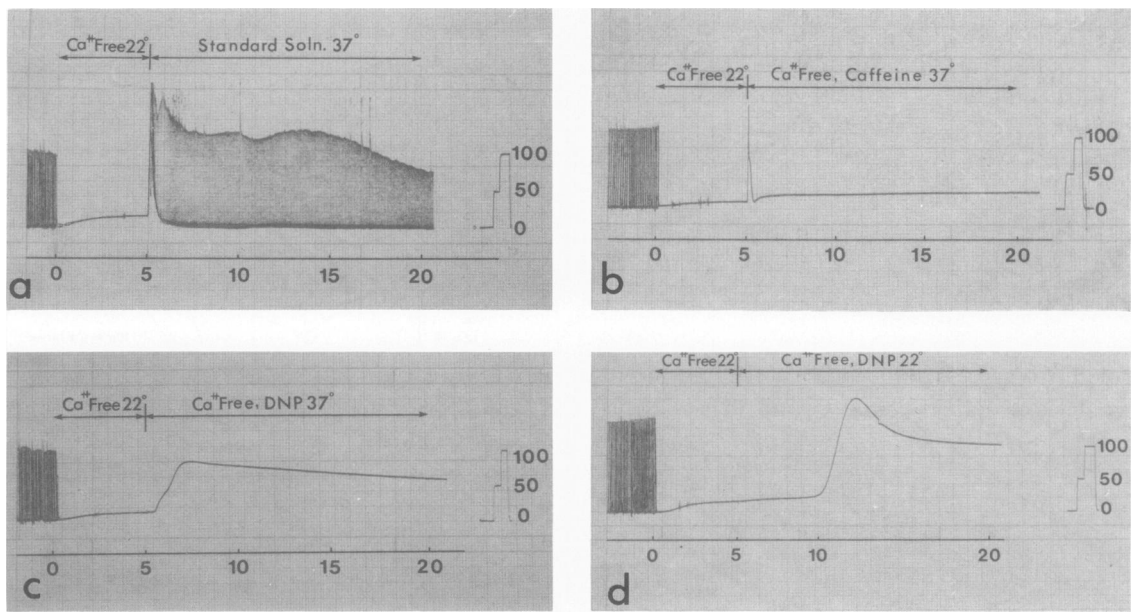


Figure 4—Representative pressure tracings from an intraventricular balloon placed in the left ventricular cavities of hearts. The hearts were perfused initially for 5 minutes with calcium-free solution at 22 C for prevention of induction of a calcium paradox. **a**—Repletion of 2.5 mM calcium at 37 C resulted in an immediate onset of a transient contracture followed by resumption of contractile activity. **b**—Addition of 10 mM caffeine caused an immediate onset of a transient contracture followed by relaxation with only a minimal rise in resting tension. **c**—Addition of 1 mM DNP at 37 C caused, after a brief delay, a slowly rising contracture which was sustained for the duration of the perfusion. **d**—Addition of of 1 mM DNP at 22 C caused a contracture which began after a delay of about 6 minutes and which was sustained for the duration of the perfusion period.

in contracture, with uniformly narrowed Z-band spaces. Both individual cells and small clusters of cells were separated from adjacent cells at intercalated disks. The spaces between separated cells contained cytoplasmic debris (Figure 5a). Electron microscopy confirmed that the cells had separated at intercalated disks and that the intercalated disk membrane faces were damaged (Figure 6). Hearts protected from enzyme release by hypothermia to 22 C were also protected from cell separations (Figure 5b). Intercalated disk membrane faces showed narrow spaces but appeared otherwise intact (Figure 7).

Calcium Paradox: Experimental Group II

Following readmission of calcium to calcium-free hearts, nearly every myocardial cell contained a single central contraction band of condensed sarcomeres (Figure 5e). Intercalated disk membrane faces were damaged and mitochondria spilled into the space between widely separated cells (Figure 8). Hearts protected by hypothermia during the calcium-free interval were also protected from cell separations and contraction bands (Figure 5f). The intercalated disk junctions of protected hearts contained focal membrane separations, but macula and fascia adherens junctions remained intact (Figure 9).

Caffeine-Treated Hearts: Experimental Group III

Hearts subjected to caffeine-induced enzyme release resembled hearts after the calcium paradox. Every myocardial cell contained a single contraction band, and individual cells were separated from neighboring cells (Figure 5g). Hearts protected from caffeine injury contained elongated, relaxed cells with narrow separations of cells at the intercalated disks (Figure 5h).

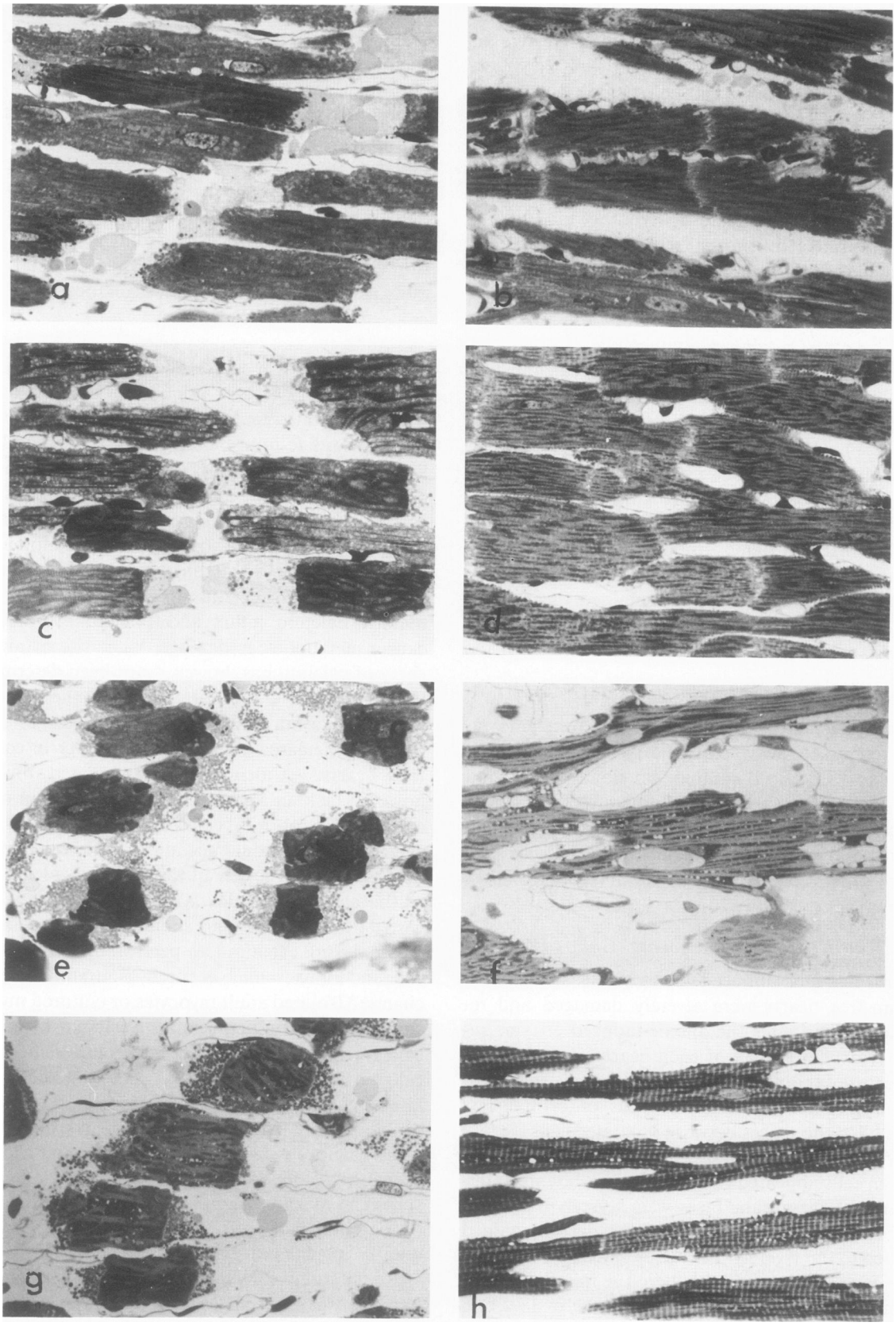
DNP-Treated Hearts: Experimental Goups IVa, IVb

Following DNP-induced enzyme release at 37 C, calcium-free hearts were severely damaged and resembled hearts after the anoxic-induced enzyme release. Nearly every cell of each heart was in marked contracture. Individual cells were widely separated from adjacent cells. Blebs or balls of cytoplasmic debris filled the spaces between separated cells (Figure 5c). By electron microscopy, the cytoplasmic material between cells appeared to be membrane-bound. Intercalated disk membrane faces were severely damaged, but fascia adherens portions of the disks remained attached to the severely contracted sarcomeres (Figure 10). Hearts in experiments where the initial calcium-free interval was maintained at 34, 25, 22, or 18 C and subsequently followed by addition of DNP in calcium-free media at 37 C appeared similar to hearts maintained at 37 C for the entire experimental period. These hearts were not protected from either enzyme release or morphologic evidence of injury. Hearts protected from enzyme release by hypothermia during both the initial period of calcium-free perfusion and after DNP were also protected from morphologic evidence of injury. Individual myocytes were contracted, but cells remained closely apposed to adjacent cells at intercalated disk junctions (Figure 5d). Intercalated disks of cells from protected hearts showed focal separations, but fascia adherens junctions generally remained intact (Figure 11).

Discussion

Exposure of hearts to brief periods of calcium-free perfusion, followed by calcium repletion, results in massive cardiac injury characterized by a sudden release of enzymes and contraction band necrosis (calcium paradox).[3] The accumulation of large amounts of calcium in damaged hearts formed the basis for the theory that calcium-free myocytes develop a selective permeability of sarcolemmal membranes to calcium.[9] Calcium repletion then would result in a massive calcium influx and calcium overload that causes metabolic cell damage.[14] The site or mode of entry of calcium has, however, not been determined.[4] Detachment of the outer lamina of the glycocalyx from the plasma membrane has been suggested as a cause of increased calcium permeability in calcium-free hearts.[15] Calcium-tolerant isolated myocytes, however, lack an external lamina[16] but retain normal calcium control.[17] It seems unlikely that loss of the external lamina would account for the calcium paradox.[4] In a recent review of the mode of calcium entry in the calcium paradox, Grinwald and Nayler suggested that intercellular sodium concentrations increase during calcium-free perfusion, and that initial calcium influxes may occur by sodium/calcium exchange.[4] Isolated adult myocytes or cultured myocytes can develop a sudden influx of calcium by sodium/calcium exchange following sequential incubation in calcium-free and calcium-containing media.[18,19] Isolated myocyte preparations develop contracture but do not suffer massive damage or release enzymes when calcium is resupplied.[20] While sodium/calcium exchange could explain an initial influx of calcium sufficient to cause contracture, sodium/calcium exchange alone does not produce the massive injury seen in the calcium paradox.

An alternative hypothesis to explain the calcium paradox was first suggested by Muir[21] in 1967. In an ultrastructural study describing the morphologic consequences of calcium-free perfusion, he observed that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macula and fascia adherens junctions of intercalated disks separated but that nexus junctions remained intact. Muir proposed that with calcium repletion and contraction, cells become torn apart at intercalated disks, rupturing the sarcolemmal membranes. De Leiris and Feuvray[22] found that calcium-free hearts were fragile and released enzymes with mild trauma or induction of contraction. Further evidence that lesions of the intercalated disks may be related to injury in the calcium paradox was provided by observations that low extracellular sodium concentrations during the calcium-free interval prevented separations of intercalated disks and also protected hearts from the calcium paradox.[2]

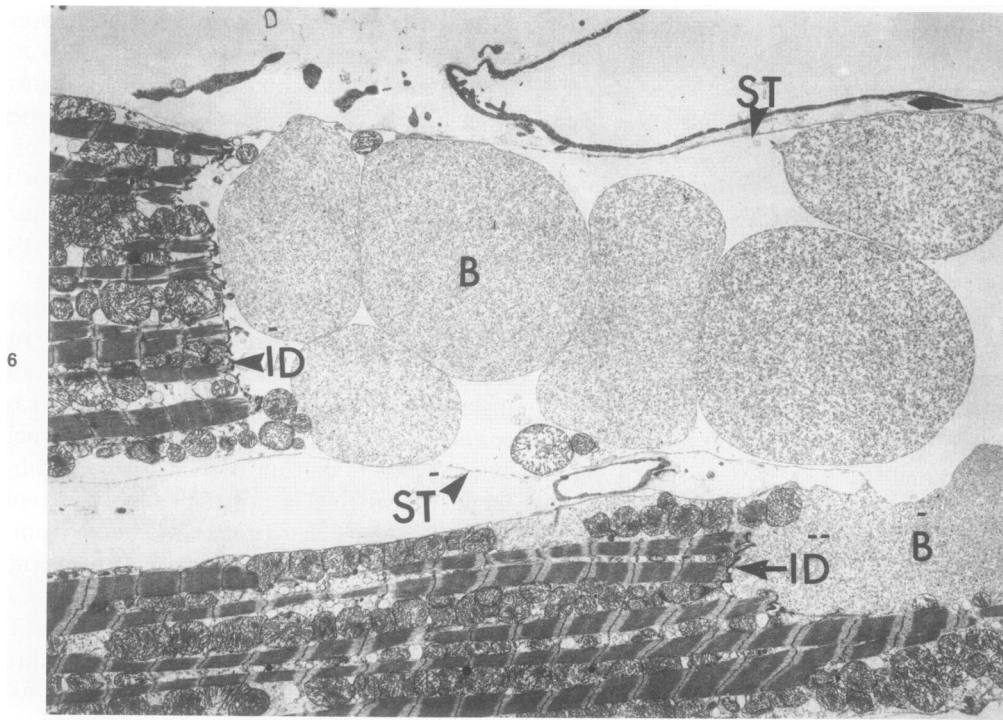
We observed that hearts in anoxic contracture, but still in a stage of reversible injury, developed cell separations, sarcolemmal membrane damage, and a rapid release of enzymes within minutes of exposure to anoxic, calcium-free perfusion medium.[5] It was postulated that physical stresses which developed during contracture caused cell separation and concomitant sarcolemmal damage to intercalated disk junctions weakened by removal of extracellular calcium. Direct evidence that physical stresses could mediate enzyme release from calcium-free hearts was provided by demonstration of a marked exacerbation of enzyme release when hearts were distended by inflation by an intraventricular balloon.[6] More recently, DNP[7,23] and caffeine[8] both were found to induce contracture in calcium-free hearts and cause an enzyme release coincident with contracture. A consistent morphologic feature of hearts after contracture-associated enzyme release has been separation of intercalated disk membrane faces with damage to the plasma membranes.[23]

Although the latter studies indicate that contracture may mediate sarcolemmal membrane injury and enzyme release from calcium-free hearts, they do not distinguish contracture-mediated from calcium overload-induced injury in the calcium paradox. The nearly instantaneous onset of cell damage that occurs in the calcium paradox has made it difficult to determine the sequence of events that occur upon calcium repletion. It is not known whether, upon calcium repletion, there is an initial massive rapid entry of calcium across calcium-permeable plasma membranes that then causes calcium overload injury, or whether the initial calcium entry is only sufficient to cause contracture, which initiates cell separation, causing sarcolemmal membrane rupture, with a secondary release of enzymes and influx of calcium.

Anoxic, DNP, or caffeine-induced contractures do not cause enzyme release from hearts perfused with calcium but do cause cell separations at intercalated disks, membrane rupture, and enzyme release in calcium-free hearts.[23] Since contracture-associated enzyme release in calcium-free hearts caused by anoxia, DNP, or caffeine is not dependent on repletion of calcium, agents that protect the heart from contracture-associated injury must act by a mechanism unrelated to preventing calcium influxes into cells. An approach to determining whether enzyme release in the calcium paradox is mediated by contracture or calcium overload is to study the effects of agents that protect hearts from the calcium paradox.

It can be argued that if contracture were the mediator of enzyme release in the calcium paradox, then agents that protect from the calcium paradox should also protect against contracture-associated enzyme release in calcium-free hearts. The calcium paradox is temperature-dependent,[9,10,11,24] with nearly complete protection occurring from 5 minutes of calcium-free perfusion at 22 C. If hypothermia protects hearts by delaying the development of intercalated disk lesions, then hypothermia should also cause a parallel temperature-dependent protection of calcium-free hearts from anoxic, DNP, or caffeine-induced injury. The present studies were designed to determine whether hypothermia protects calcium-free hearts from contracture-associated injury and to determine the temperature dependence of protection, compared with that occurring in the calcium paradox.

Figure 5 — Light micrographs showing the effects of hypothermia protection on cellular morphology. **a** — Anoxic heart at 37 C for 30 minutes and then for 30 minutes of anoxic and calcium-free perfusion at 37 C. Myocardial cells are contracted and separated at intercalated disks from neighboring cells. **b** — Anoxic hearts at 37 C for 30 minutes, then for 30 minutes of anoxic and calcium-free perfusion at 22 C. The myocardial cells are contracted but remain apposed at intercalated disk junctions. **c** — DNP-treated heart in calcium-free perfusate at 37 C. The cells are in contracture and are separated from adjacent cells at intercalated disks. Blebs of cytoplasm fill the space between separated cells. **d** — DNP-treated heart in calcium-free perfusate at 22 C. The myocardial cells are contracted but remain apposed at intercalated disk junctions. **e** — Calcium paradox at 37 C. Following calcium-free perfusion at 37 C and calcium repletion at 37 C, myocardial cells show extreme contracture with contraction bands. The cells are widely separated at intercalated disk junctions. **f** — Calcium paradox at 22 C. Following calcium-free perfusion at 22 C and calcium repletion at 37 C, the heart resembles normal myocardium. The cells remain apposed at intercalated disk junctions. **g** — Caffeine at 37 C. Calcium-free perfusion at 37 C was followed by caffeine in calcium-free solution at 37 C. The myocardial cells are in severe contracture, with wide separations of cells. **h** — Caffeine at 22 C. Calcium-free perfusion at 22 C was followed by caffeine in calcium-free solution at 37 C. The myocardial cells are relaxed, with elongated cells and wide sarcomere spacings. The cells remain apposed at intercalated disk junctions. (Plastic-embedded, toluidine blue-stained, all ×400)



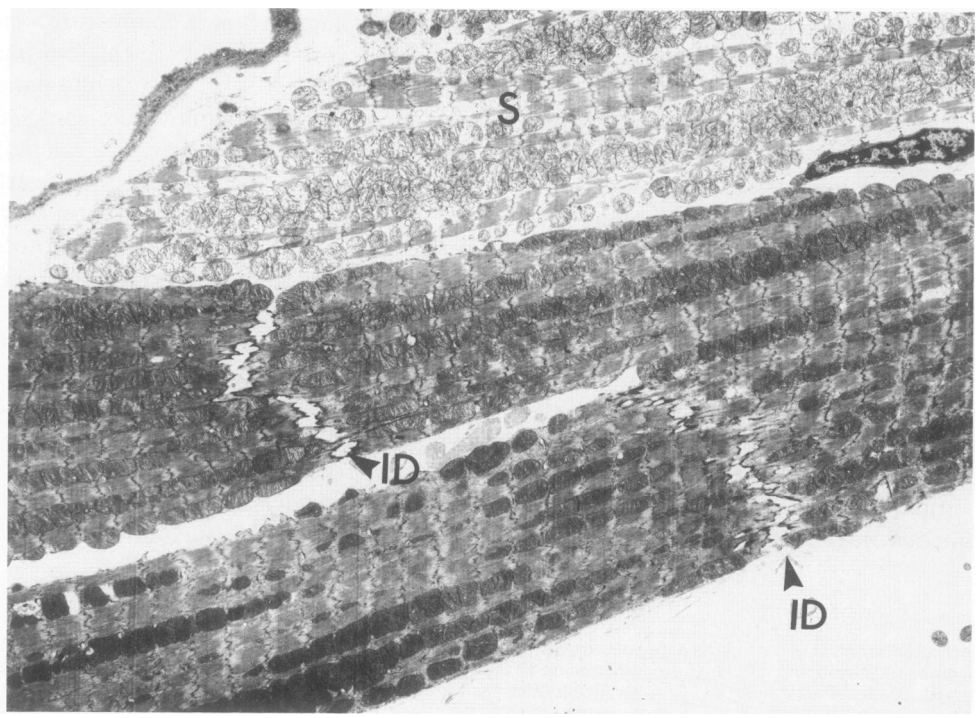


Figure 6 – Electron micrograph of an anoxic and calcium-free heart at 37 C. Following 30 minutes of anoxic perfusion at 37 C, the heart was perfused for an additional 30 minutes with anoxic and calcium-free solution. The cells are separated at intercalated disks (*ID*), and balls and blebs of cytoplasm (*B*) and free mitochondria (*M*) occupy the space between separated cells. Sarcomeres retain their attachments to the internal face of the intercalated disk fascia adherens junctions. The sarcotubular membrane (*ST*) (outer lamina of glycocalyx) remains intact, but the plasma membrane is fragmented. (Uranyl acetate, ×3000) **Figure 7** – Electron micrograph of an anoxic and calcium-free heart at 22 C. Following 30 minutes of anoxic perfusion at 37 C, the heart was perfused for an additional 30 minutes with anoxic and calcium-free solution at 22 C. The cells remain apposed at intercalated disks (*ID*), but there are focal separations of fascia adherens junctions as a consequence of prolonged calcium-free perfusion. The mitochondria show variable degrees of swelling, and some swollen cells (*S*) are present. (Uranyl acetate, ×2800)

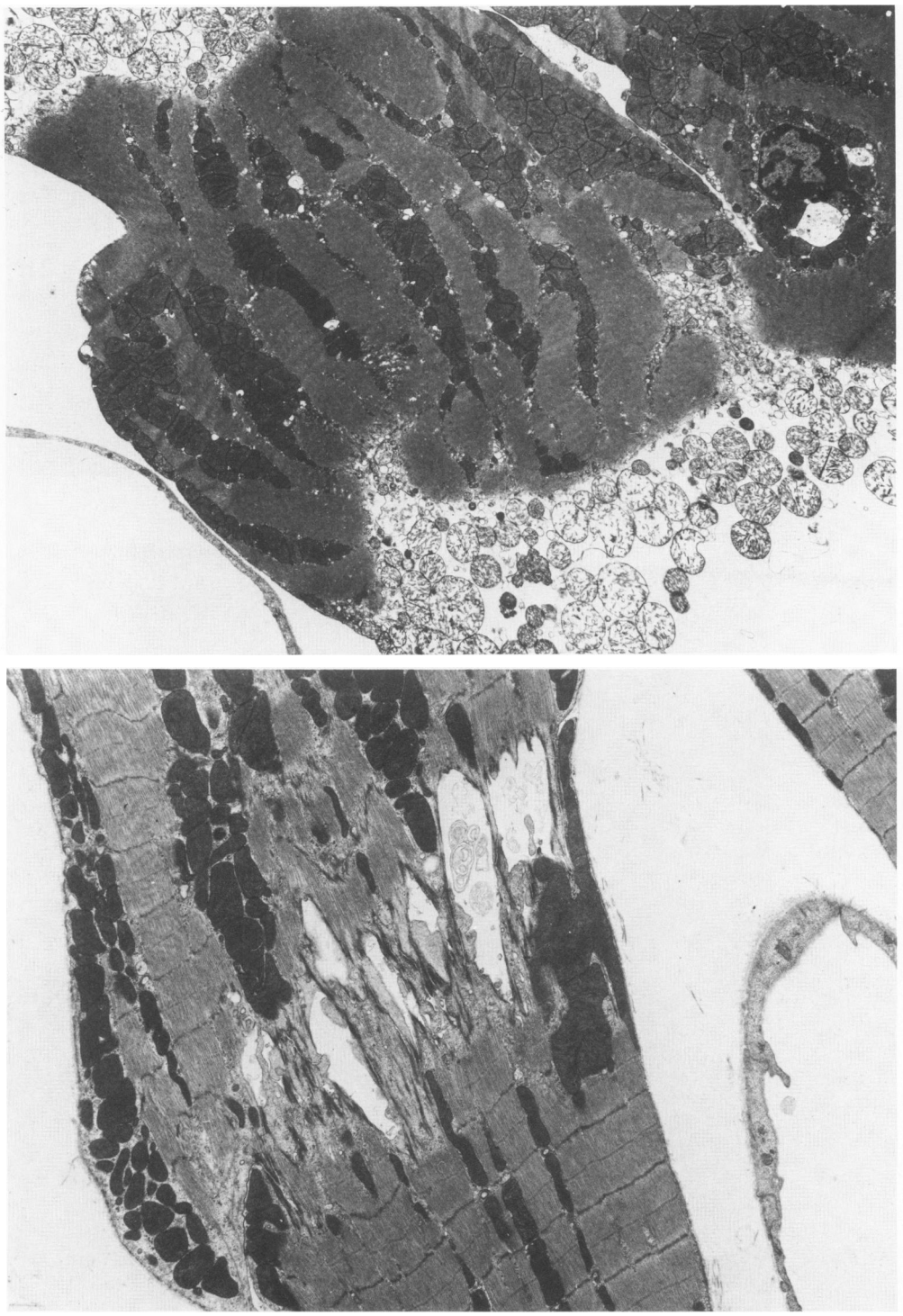


Figure 8—Electron micrograph of a heart subjected to the calcium paradox at 37 C. The cell is hypercontracted with the sarcomeres being condensed into a single contraction band. At either side of the cell the intercalated disk membrane faces have ruptured, and swollen mitochondria have spilled into the space between separated cells. Mitochondria within the contraction band do not appear swollen. (Uranyl acetate, ×4050) **Figure 9**—Electron micrograph of a heart protected from the calcium paradox by calcium-free perfusion at 22 C. The heart was subjected to 5 minutes of calcium-free perfusion at 22 C and 15 minutes of calcium repletion at 37 C. The intercalated disk is distorted and vacuolated, but most fascia adherens junctions are closely apposed. The mitochondria are dense, and sarcomeres show a mild loss of register. (Uranyl acetate, ×7000)

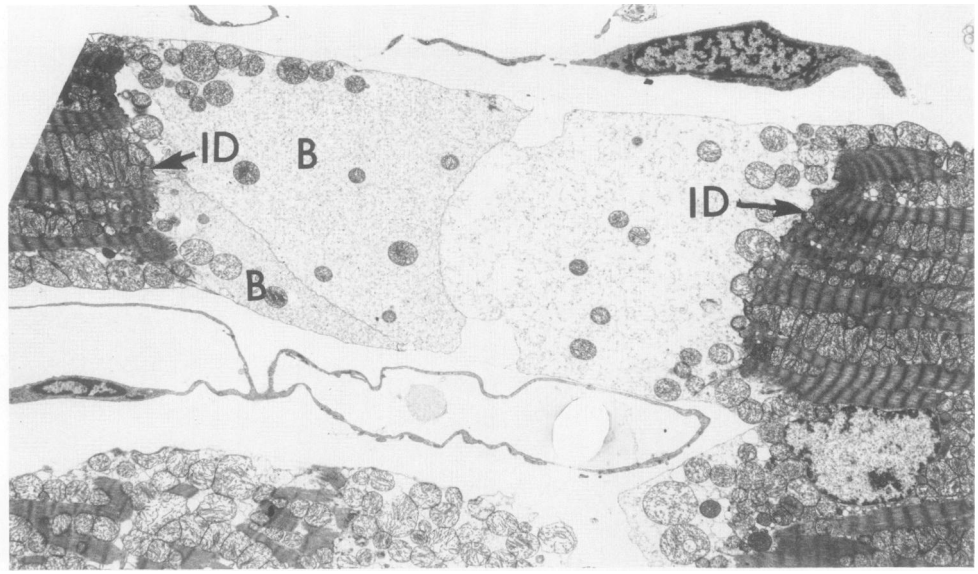


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Figure 10 – Electron micrograph of a DNP-treated heart at 37 C. Following 5 minutes of calcium-free perfusion at 37 C and 15 minutes of DNP perfusion at 37 C, the myocytes are in contracture and are widely separated at intercalated disks. The space between separated cells is filled with large blebs (*B*), derived from intercalated disk membrane faces. The blebs contain flocculent material and swollen mitochondria. (Uranyl acetate, ×2800) **Figure 11** – Electron micrograph of a DNP-treated heart at 22 C. The heart was exposed to 5 minutes of calcium-free perfusion at 22 C and 15 minutes of calcium-free perfusion at 22 C in the presence of DNP. The intercalated disk appears distorted, but the cells remain apposed. Portions of nexus junctions (*N*) are abnormally oriented across the long axis of the cells instead of lying parallel to the lines of stress. Sarcomeres are variably contracted, and mitochondria are swollen. (Uranyl acetate, ×7500)

Anoxic Injury: Group I

The hearts were allowed to develop anoxic contracture in control KHB medium at 37 C and were subsequently made calcium-free at different temperatures. The contracture tension developed by all hearts was comparable at the time of calcium-free perfusion. After 4 minutes of calcium-free perfusion, ventricular pressures ranged from 80 to 90 mmHg for each temperature studied. Despite the similar strength of developed contracture at each temperature studied, progressive hypothermia had a progressive effect in reducing enzyme release during calcium-free perfusion. The slow release of enzymes over the duration of calcium-free perfusion at 25 C or 22 C was probably due to the delayed production of injury, because hypothermia has been shown only to delay the sensitization of the heart to the calcium paradox. After 5 minutes of calcium-free perfusion at 22 C, the heart is totally protected from the calcium paradox,[1,11] but significant injury occurs even at 22 C if the calcium-free perfusion period is extended for 30 minutes or longer.[10,25] Hearts protected from anoxic enzyme release were also protected from separations of cells at intercalated disk junctions and severe sarcolemmal membrane damage as assessed by electron microscopy.

Calcium and Caffeine Injury: Groups II, III

Hypothermia during the period of calcium-free perfusion markedly reduced enzyme release from both the calcium paradox and caffeine-induced injury when caffeine or calcium was added to the perfusate at 37 C. Nearly total protection from these forms of injury was obtained by reducing the temperature of calcium-free perfusion to 22 C. The studies of ventricular function at 22 C demonstrated that hypothermia did not prevent the development of contracture either upon calcium repletion or addition of 10 mM caffeine. The absence of enzyme release, despite development of contracture, can be attributed to hypothermic prevention of intercalated disk injury during the initial calcium-free perfusion period. The absence of enzyme release during the interval after rewarming the hearts, however, was probably due to different mechanisms with calcium repletion, as compared with caffeine treatment. With calcium repletion and rewarming to 37 C, hearts protected from the calcium paradox resumed contractions. Repletion of calcium, therefore, maintained the integrity of intercalated disks during the final perfusion period. With caffeine, after the initial period of hypothermic calcium-free perfusion, the hearts were rewarmed at 37 C during continuing calcium-free perfusion. Calcium-free perfusion at 37 C would, according to our hypothesis, be expected to cause weakening of intercalated disks. The absence of enzyme release with caffeine is explained by the absence of contracture during the final calcium-free interval. In calcium-free media, caffeine produces only a transient contracture followed by relaxation.[8,26] The initial contracture and relaxation caused by caffeine occurred before calcium-free perfusion could have weakened the intercalated disks. This explanation is supported by the morphologic observations of elongated cells with relaxed sarcomeres and apposition of intercalated disk junctions in the caffeine-treated calcium-free hearts.

DNP Injury: Group IV

The initial experiments (Group IVa) did not demonstrate hypothermic protection from DNP-induced enzyme release. The morphology of these hearts revealed comparable degrees of cell separations and membrane damage at intercalated disks at all temperatures studied. The appearance of hypothermic DNP-treated hearts was similar to that of hearts following anoxic enzyme release at 37 C. The occurrence of enzyme release during the period of normothermic calcium-free perfusion with DNP is attributed to the development of a sustained contracture following DNP. DNP-induced contracture is associated with rapid hydrolysis of creatine phosphate and adenosine triphosphate (ATP),[27] so that DNP causes an irreversible rigor contracture that is sustained in the absence of extracellular calcium. Rewarming calcium-free hearts to 37 C in the presence of DNP would cause weakening of intercalated disk junctions. With sustained DNP-induced contracture, cell separations would explain the observed contracture-associated enzyme release during the final period of calcium-free perfusion. This interpretation is supported by the observation that enzyme release did not begin until 3 minutes after switching to calcium-free DNP at 37 C coincident with the development of contracture tension. The additional experiment (Group IVb) maintained hearts at 22 C during both the initial calcium-free perfusion period and subsequent addition of DNP. DNP caused contracture under these conditions, but at 22 C the hearts were protected from both enzyme release and damage to intercalated disks.

Mechanism of Hypothermic Protection

The calcium paradox is exquisitely temperature-sensitive, with nearly complete protection being afforded by reduction of the temperature in a 10-minute period of calcium-free perfusion to 22 C.[11]

Hypothermia only delays injury, and prolongation of calcium-free perfusion periods at the lower temperatures results in development of the calcium paradox.[10,25] An explanation for these observations is that hypothermia may delay the sensitization of cells to the calcium paradox by producing a membrane lipid thermotropic phase transition.[11] Protection could also result from delaying the wash-out of intracellular calcium from cells[2] or from protecting against lesions causing weakening of intercalated disk junctions.

Our studies show that hypothermia causes an effect that protects the heart from the calcium paradox and contracture-associated enzyme release due to anoxia,[5] DNP,[27] and caffeine.[8] Protection from contracture-induced injury seems unlikely to be due to prevention of sarcolemmal permeability changes which specifically affect control of calcium entry into cells, because enzyme releases in these cases occurred without repletion of extracellular calcium. A mechanism of protection of hypothermia in contracture-associated injury consistent with the results of this study would be prevention of intercalated disk separations at macula and fascia adherens junctions with preservation of the mechanical integrity of the contractile elements of the heart. Only 50 μM extracellular calcium is required to prevent the calcium paradox.[10] Hypothermia slows the rate of calcium loss from myocardial cells during calcium-free perfusions.[28] A slowed rate of calcium loss from cells could delay the washout of calcium from the extracellular space and preserve the integrity of intercalated disks.

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