Energy Dependence of Contraction Band Formation in Perfused Hearts and Isolated Adult Myocytes

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Aggregation of sarcomeres into contraction bands is a prominent feature of the oxygen paradox, the calcium paradox, and caffeine injury to calcium-free perfused hearts. For investigation of the mechanism of contraction banding, it was necessary to devise a method of evaluating the degree of sarcomere contraction and to define objectively a contraction band. Hearts with mechanical detachment of cells caused by hypocalcemic perfusion and isolated myocytes both allow unrestrained contracture of cells and permit direct optical measurements to quantitate the degree of cell contracture. With the use of the calcium paradox as a model of contraction band necrosis, it was found that cells with lengths of less than 37.3 μ could be considered as containing contraction bands. It was found that the mitochondrial inhibitors cyanide and amytal, as well as the uncoupler 2,4-dinitrophenol, allowed cell contracture but inhibited hypercontracture of sarcomeres into contraction bands during both the calcium paradox and caffeine injury to perfused hearts. However, when 2mM adenosine triphosphate (ATP) was included in the perfusion media, contraction band formation occurred despite the continued presence of cyanide or amytal. In isolated myocyte preparations the addition of the glycolytic inhibitor iodoacetate (IAA, 5 mM) and the mitochondrial inhibitor amytal (3 mM) caused relaxed rod-shaped cells (length/width ratio >3:1) to contract into a stable population of square-shaped forms (length/width ratio <3:1), indicating an abrupt and severe decline in cellular ATP levels. Removal of amytal from the incubation medium in the presence of IAA produced a significant conversion of square-shaped cells into round-shaped cells containing contraction bands. Either IAA alone or amytal alone resulted in a mixed population of square and round cells. The results indicate that ATP is required for the formation of contraction bands in intact hearts and for the rounding of isolated myocytes. Formation of contraction bands appears to be an energy-dependent process requiring ATP. (Am J Pathol 1986, 125:55-68)

CONTRACTION BANDS are zonal aggregates of densely hypercontracted sarcomere segments that may occur singly or in multiple groups in a myocyte. Contraction band necrosis is a characteristic lesion of reperfusion or reoxygenation injury to myocardium or of the calcium paradox.[1] Inhibition or uncoupling of mitochondrial respiration will prevent both contraction banding and oxygen-induced enzyme release when anoxic hearts are reoxygenated.[2] The role of mitochondria in mediating oxygen-induced hypercontracture of cells is, however, not fully understood. Oxygen could induce contraction banding by 1) allowing mitochondria to produce toxic free radicals,[3] 2) supporting rapid cytoplasmic acidification via mitochondrial hydrogen ion extrusion,[1] or 3) allowing mitochondria to resume oxidative phosphorylation to provide adenosine triphosphate (ATP) to energize contraction of sarcomeres.[4]

It has been difficult to study contraction band formation in intact hearts because degrees of hypercontracture are difficult to quantitate and there are no precise definitions of "contracture," "hypercontracture," and "contraction band." The term "contracture" indicates a physiologic degree of cell shortening that does not exceed that occurring in empty beating hearts or in beating isolated myocytes. This degree of contracture results in the disappearance of the I-band region of normal sarcomere structure and a Z-band spacing slightly longer than the normal A-band width of 1.6 μ. "Hypercontracture" is a nonphysiologic degree of sarcomere shortening that results in some degree of A-band compression or distortion and apparent thickening of Z-bands. Lesser degrees of hypercontracture occur in

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ischemic contracture or in rapidly energy-depleted isolated myocytes that shorten to a "square" configuration. Extreme degrees of hypercontracture result in marked disorganization of normal sarcomere structure and actin–myosin relationships and a Z-band spacing of considerably less than 1.5 μ. In intact hearts, such extreme hypercontractures can occur as contraction bands only in association with rupture or lysis of adjacent myofibrillar proteins. Isolated myocytes, in contrast, are capable of unrestrained contracture and therefore can shorten to form single, central contraction bands that are observed under light microscopy as "round cells."

In the present study we have utilized the calcium-free perfused heart to overcome some of these technical and semantic problems. Calcium-free perfused hearts develop weakening of intercalated disk junctions with subsequent mechanical uncoupling of cells.[2,5] If hearts are then stimulated to contract by the reintroduction of calcium or addition of caffeine, the cells separate at intercalated disk junctions with subsequent sarcolemmal membrane injury and loss of cytoplasmic enzymes.[2,5] The separated cells are then capable of unrestrained contracture and in addition have permeable sarcolemmal membranes that allow access of normally excluded extracellular materials to the intracellular space. Because the separated myocytes can shorten without physical constraints, the previously injured cells shorten further to form single, central, contraction bands consisting of the entire cellular complement of sarcomeres. The calcium paradox possesses the unique advantage that nearly all the cells in a given heart are uniformly and synchronously affected, allowing direct optical cell measurements that can be used to define a contraction band in terms of absolute cell length. The sarcolemmal membrane damage that occurs during the calcium paradox would allow study of the effects of exogenously added ATP on contracture development. Therefore, in the present study, the effects of caffeine, mitochondrial inhibitors, and exogenously supplied ATP on contraction band formation in calcium paradox and caffeine-injured perfused hearts were studied. In addition, isolated adult rat heart myocytes were used as a model to test critically the hypothesis that intracellular ATP production can support hypercontracture. The results suggest that high-energy phosphates, especially mitochondrial produced ATP, play a critical role in contraction band formation.

Materials and Methods

Perfused Rat Hearts

Male Sprague–Dawley rats weighing 200–300 g were anesthetized by intraperitoneal injection of sodium pentobarbital (Diabutal, Diamond Laboratories Inc., Des Moines, Iowa). After intravenous injection of 1000 IU sodium heparin (Elkins-Sinn, Cherry Hill, NJ), hearts were removed and immersed in ice-cold Krebs-Henseleit bicarbonate (KHB) solution. Hearts were then mounted for Langendorff perfusion on a triple reservoir, nonrecirculating apparatus and were perfused at 85 mm Hg pressure. Temperature was maintained at 37 C with a thermister probe placed in the left ventricle of the heart.

Control perfusion solution was a modified Krebs-Henseleit bicarbonate medium that contained 126 mM NaCl, 25 mM $NaHCO_3$, 1.2 mM KH_2PO_4, 4.8 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, and 11 mM glucose. Calcium-free media were prepared by exclusion of $CaCl_2$ without osmotic substitution and addition of 0.1 mM EDTA to ensure removal of calcium contamination. Analytic reagent grade caffeine, dinitrophenol (DNP), potassium cyanide (KCN), and ATP disodium salt obtained from Sigma Chemical Co. (St. Louis, Mo) and were added in the appropriate concentrations to solutions as required. All solutions were continuously gassed with 95% O_2–5% CO_2 and were maintained at a pH between 7.3 and 7.4.

Isolated Adult Rat Heart Myocytes

Isolated adult rat heart cells were prepared with the use of a modification of the technique described by Altschuld et al.[6] Male Sprague–Dawley rats weighing 275–325 g were individually anesthetized by intraperitoneal injection of 55 mg/kg sodium pentobarbital (Diabutal, Diamond Laboratories). One minute after intravenous injection of 2000 IU of sodium heparin (Elkins-Sinn), the hearts were removed and rapidly immersed in an ice-cold solution. After removal of the extraneous tissue, the aorta was cannulated on a 22-gauge stainless steel needle connected to a 1-ml syringe filled with ice-cold calcium-free KHB. The heart was flushed with 1 ml of the solution and mounted on the perfusion apparatus. Hearts were perfused on a modified Langendorff apparatus at 37 C in a nonrecirculating mode for 5 minutes with nominally calcium-free ($Ca^{2+} < 25$ μM by atomic absorption spectrophotometry) perfusion buffer containing NaCl (90 mM), KCl (30 mM), $NaHCO_3$ (25 mM), KH_2PO_4 (1.2 mM), bovine serum albumin (BSA) (1 mg/ml) (Pentex Fraction V), $MgCl_2$ (1.2 mM), glucose (11 mM), taurine (60 mM), creatine (20 mM), and a complete amino acid mixture (GIBCO basal medium, Eagle, (BME) amino acid solution and modified Eagle's medium, nonessential amino acid solution diluted 1:100 with KHB). After the 5-minute washout period to remove blood and extracellular calcium, collagenase (Class II, Cooper Biomedical, Malvern, Pa) dissolved in the nominally calcium-free perfusion buffer was added to 1.25 mg/ml, and the hearts

were perfused in a recirculating fashion until they became soft (45–60 minutes). The buffer was equilibrated with 95% O_2–5% CO_2, and the pH and temperature were maintained at 7.2 and 37 C, respectively. The flow rate was increased from 7 ml/min (during the calcium-free perfusion) to 25 ml/min over the course of the collagenase digestion. After softening, the hearts were transferred to 5 ml of perfusion buffer containing 4% wt/vol BSA and were minced with scissors. At this point, 10 ml of the perfusion buffer containing the collagenase was added to the mince, and the cells were gently dispersed with a large-bore Nalgene pipette. The cells were then transferred to a Nalgene flask and incubated in a shaking water bath for 10 minutes at 30 C under an O_2 atmosphere. After incubation, cells underwent a final dispersion using a Nalgene pipette and were filtered through a nylon mesh into a Nalgene beaker. The cells were then washed once in low K^+ (5 mM) buffer containing 5 mg/ml BSA and once more in low K^+ buffer containing 20 mg/ml BSA by sedimentation for 1 minute at 75 g in a tabletop clinical centrifuge. All wash solutions were a variation of a nominally calcium-free, low K^+ (5 mM) Krebs-Henseleit buffer containing 10 mM bicarbonate, 10 mM HEPES, 60 mM taurine, 20 mM creatine, 11 mM glucose, the amino acid mixtures, and a vitamin supplement (GIBCO BME vitamin solution, diluted 1:100). After the final wash the cells were resuspended in 10 ml of nominally calcium-free wash buffer containing 20 mg/ml (2%) BSA. The cells were then incubated for 30 minutes in a shaking water bath at 30 C under an O_2 atmosphere so that they could reestablish normal ionic gradients.[6] At the end of the 30-minute incubation, calcium chloride was added to the cells to a final concentration of 1.12 mM. Calcium-tolerant cells were harvested by centrifugation at low speed (20*g*) for 150 seconds in the tabletop centrifuge. The supernatant was discarded, and the cells were washed twice with basic wash buffer supplemented with 1.12 mM Ca^{2+} and 2% BSA with the use of slow spin centrifugation and discarding of the supernatant after each wash. After purification, the cells were resuspended in 10 ml of the 2% BSA, 1.12 mM Ca^{2+}-supplemented wash solution. All further incubations were carried out in this suspension buffer. Reagent grade iodoacetic acid was obtained from Sigma, and amobarbital was obtained from Eli Lilly and Company (Indianapolis, Ind).

Experimental Design

Perfused Rat Hearts

Four basic groups of experiments were performed as outlined below. All hearts were initially equilibrated with oxygenated control KHB, and all experiments were conducted at 37 C.

Group 1: Control

To determine the average cell length in control and calcium-free perfused hearts, we perfused two hearts for 5 minutes with control KHB and then fixed them, whereas two others were perfused with control KHB and then for an additional 5 minutes with calcium-free KHB prior to fixation.

Group 2: 2,4-Dinitrophenol (DNP)-Induced Injury

Six hearts were initially perfused with calcium-free KHB for 5 minutes and then for 5 minutes with calcium-free KHB containing 1 mM DNP. These experiments were conducted for determination of the average cell length of injured myocytes exhibiting the maximal amount of cell shortening possible in the absence of significant ATP production.

Group 3: Caffeine Experiments

For determination of the average cell length of myocytes following caffeine-induced injury, five hearts were perfused for 5 minutes with calcium-free KHB followed by calcium-free KHB containing 10 mM caffeine for 5 minutes. A separate group of experiments was conducted to determine the effect of mitochondrial inhibition on cell length following caffeine-induced injury. This group of six hearts was similarly treated, except that 3 mM amytal was included in the caffeine-containing calcium-free solution.

Group 4: Calcium Paradox Experiments

To objectively quantify the average length of a cell containing a contraction band, we used the calcium paradox as a model of severe, uniform contraction band necrosis. In this group, six hearts were perfused with calcium-free KHB for 5 minutes followed by 5 minutes reperfusion with control KHB. To determine the effect of metabolic inhibition on average cell length in the calcium paradox, four to six hearts were perfused for 5 minutes with calcium-free KHB followed by 5 minutes of calcium repletion with control KHB containing the metabolic inhibitors amytal (3 mM) or KCN (5 mM). (To ensure complete inhibition of respiration by KCN, the KCN was also included during the calcium-free perfusion period.) A final series of experiments was conducted to determine whether an exogenously added energy source (ATP) could influence the degree of cell shortening observed in the calcium paradox under conditions of metabolic inhibition. The heart perfusion protocol for this set of experiments was identical to that of the metabolic inhibitor series described above except

that 2 mM ATP was included in the reperfusion media in the continued presence of the metabolic inhibitors.

Isolated Adult Rat Heart Myocytes

Group 1: Control Studies

Following isolation and purification, each of three separate cell preparations was divided into four separate groups, each group containing 2.5 ml of cells in the suspension buffer: 1) control cells, 2) cells with 3 mM amytal added, 3) cells with 5 mM IAA added, and 4) cells with both 3 mM amytal and 5 mM IAA added. The cells were placed in 50-ml Nalgene beakers, and the final volume of each group was brought to 5 ml with suspension buffer. The four beakers containing the calcium-tolerant myocytes and the appropriate concentration of inhibitors were placed in a Dubnoff shaking water bath and incubated for 1 hour at 30 C under room air conditions. The cells were sampled and fixed at the various times indicated in Table 2 for both light and electron-microscopic analysis.

Group 2: Experimental studies

The experimental group consisted of four experiments studying the effect of selective inhibition and resumption of mitochondrial respiration on the morphology of calcium-tolerant rat myocytes. Two separate groups of myocytes were placed in suspension buffer containing 5 mM IAA and 3 mM amytal and incubated for 30 minutes in a shaking water bath at 30 C in room air. After 30 minutes, control cells were washed twice at 75*g* with the suspension buffer containing 5 mM IAA and 3 mM amytal, whereas the experimental group was washed twice with buffer containing only 5 mM IAA, omitting the amytal.

Morphologic Studies

Perfused Rat Hearts

At the end of each experiment perfused hearts were fixed by infusion of 50 ml of 1% glutaraldehyde in a modified Tyrode's buffer at a rate equal to the last recorded coronary flow rate prior to initiation of fixation. The fixative excluded calcium when the final experimental perfusion medium was calcium-free. Hearts were then immersed in fixative for 24–48 hours prior to transfer into 10% neutral buffered formalin. Four equally spaced short axis sections were taken and embedded in paraffin, sectioned, and stained with hemotoxylin and eosin (H&E).

Slides were coded with a number and were evaluated blindly. Cells were sequentially examined for longitudinal orientation, and care was taken that only cells with both ends clearly defined or with clusters of mitochondria on either end were measured. Tangentially sectioned cells were excluded from study. Because morphologic changes appeared uniform throughout all sections, an initial evaluation of five sections was made to determine the minimum number of cells to be counted from each section. Measurements of 100, 200, and 500 cells from each of the five sample sections were made. It was found that there was no significant difference in the means or the standard deviations between 200 and 500 counted cells. A minimum of 200 cells were then measured from which an average cell length was determined for each heart. An average value for each group of hearts (n = 4–6) was then determined and compared with that of other groups. Measurements were made with the use of a high dry objective on a microscope fitted with a vernier scale micrometer ocular that was calibrated against a ruled hemocytometer glass slide.

Isolated Cells

For cell counts and light-microscopic analysis myocytes were mixed with an equal volume of 0.3% trypan blue–1% glutaraldehyde (wt/vol) solution, mounted under a coverslip, and viewed with a light microscope. To determine cell yields, we counted four fields in a hemocytometer. The average yield was approximately 8–12 million cells/g heart. To estimate the percentage of the cell population that was rod-shaped, square-shaped, or round-shaped, and to determine which cells excluded trypan blue, we mixed the cells with the trypan blue–glutaraldehyde solution and counted 400–700 cells using a laboratory counter (Clay Adams, Parsippany, NJ). Both the relative frequency of each cell type and the viability of the preparation were expressed as a percentage of the total cells counted. Square-shaped cells were defined by light microscopy as those cells that exhibited length/width ratios of less than 3:1. Round cells were easily recognizable. Cells exhibiting length/width ratios of greater then 3:1 were defined as rod-shaped cells. For electron-microscopic analysis, approximately 1×10^5 cells in a 1-ml volume were sedimented by low-speed centrifugation and fixed in an equal volume of 1% glutaraldehyde for 24 hours. Tissue was postfixed with 1% osmium tetroxide, stained *en bloc* with aqueous uranyl acetate, dehydrated, centrifuged into the tip of a conical capsule, and embedded in Epon. Semithin (1-μ) sections of plastic blocks were cut and stained with toluidine blue for light microscopy. Representative areas from these sections were selected for thin sections, stained with lead citrate, and studied with a JEOL 100CX electron microscope.

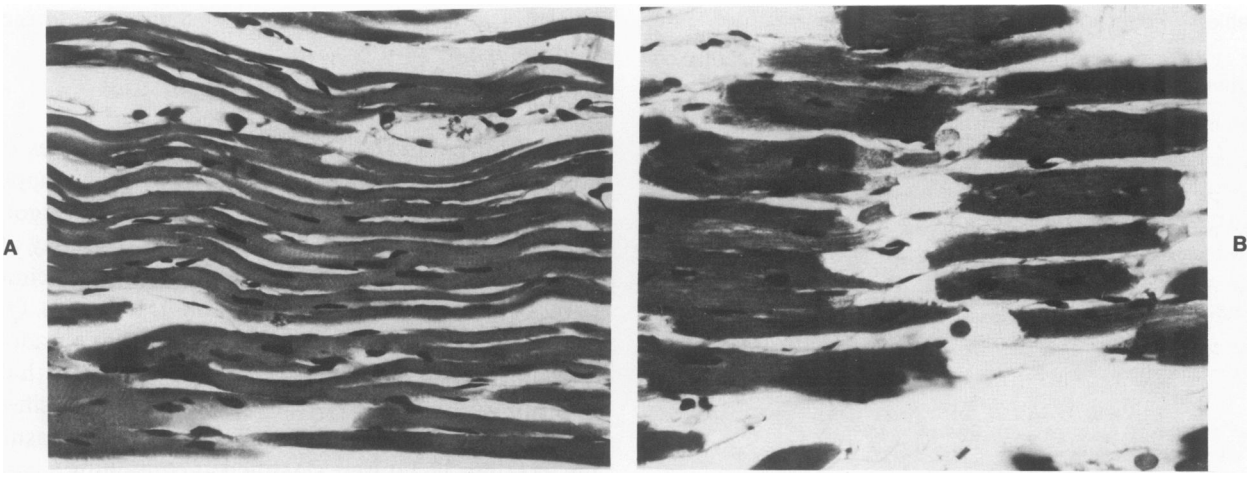

Figure 1A—Light micrograph of a control calcium-free perfused heart showing relaxed cells and minimal or no separations of intercalated disk junctions. **B**—A calcium-free heart was treated with 1 mM DNP to produce severe ATP depletion (rigor) contracture. All cells have shortened with wide separations of intercalated disk junctions. (H&E, ×300)

Statistical Analysis

The PROPHET computer system was used to aid in the calculations of mean cell lengths and data analysis. The results are expressed as the mean ± standard error of the mean (SEM) unless otherwise indicated. The Student unpaired *t* test was used for comparisons of two samples. Normality was demonstrated with the Wilk-Shapiro test, and homogeneity of variance was established with Levene's test. Analysis of variance followed by a Dunnett's test or a Newman–Keuls test was used for statistical comparisons for multiple samples. Pooled data from each experimental group were used to construct frequency distribution curves for determination of the percentage of cells containing contraction bands.

Results

Perfused Hearts

Cells from control hearts or 5 minute calcium-free hearts appeared relaxed at the time of fixation (Figure 1A). Sarcomeres were evenly spaced, and the average length of calcium-free myocytes (150 ± 0.6 μ) was only slightly greater than that of the calcium-perfused control cells (144.4 ± 0.4 μ; Figure 2; Table 1).

DNP Injury

In calcium-free hearts, 1 mM DNP caused a cellular contracture but no apparent contraction banding. Contracted cells were separated from neighboring cells at intercalated disks. Fragments of cytoplasm or cytoplasmic blebs could be seen protruding from the intercalated disk membrane faces (Figure 1B). Dinitrophenol-treated hearts had an average cell length of 68.2 ± 4.3 μ.

Caffeine Injury

Calcium-free perfused hearts exposed to 10 mM caffeine develop rapid contracture with contraction band necrosis nearly identical to but less severe than that occurring after the calcium paradox (Figure 3C). The average cell length from caffeine-treated hearts was

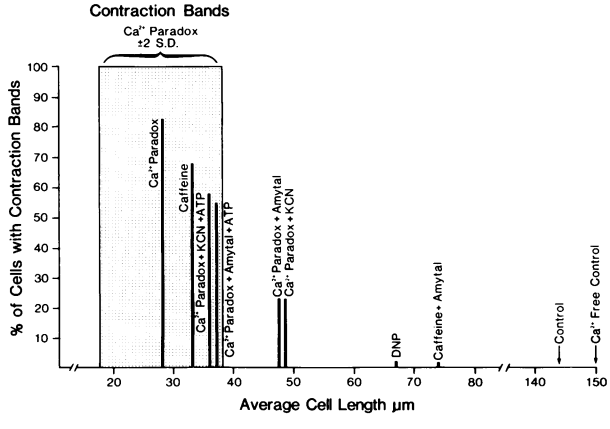

Figure 2—Composite graph of mean cell lengths in hearts treated as indicated. The height of the bars indicate the percent of cells in each group which measured less than 37.3 μ, and which are considered to contain contraction bands. The *shaded area* represents the mean cell lengths in calcium paradox hearts ± 2 SD from the mean. This area represent the lengths of cells defined as having contraction bands. Caffeine caused severe contraction band necrosis, which was inhibited by amytal. Both amytal and KCN inhibited contraction band formation in calcium paradox hearts. Addition of 2 mM ATP to the calcium repletion reperfusate containing amytal or KCN overcame the inhibition of hypercontracture by amytal or KCN, with a significant reduction in mean cell length.

Table 1—Perfused Rat Hearts*

Experiments		Mean length (μ)	SD	% of contradiction bands
Ca^{2+} control	2	150.2	0.6	0
O_2 control	2	144.8	0.4	0
Ca^{2+} paradox	6	27.3	5.0	82.3
Ca^{2+} paradox with amytal	6	47.9	5.9	22.6
Ca^{2+} paradox with KCN	4	48.7	6.8	23
Ca^{2+} paradox with amytal + ATP	6	36.5	2.4	54.5
Ca^{2+} paradox with KCN + ATP	4	36.1	1.6	58
Caffeine	5	3.28	4.5	66.9
Caffeine with amytal	6	74.1	5.7	0.8
DNP	6	68.2	4.3	1.5

* Mean cell lengths and the percentage of cells containing contraction bands in perfused rat hearts for each experimental group. The cell lengths were initially calculated as a mean for each individual heart, and then a group mean and SD was calculated. The percentage of contraction bands was determined from a frequency distribution curve of individual cell lengths for the entire group of hearts. Cells measuring 37.3 μ or less in length were classified as containing contraction bands.

not significantly longer than that of those subjected to the calcium paradox, and 66.9% of cells were contracted enough to be classified as containing contraction bands. Amytal produced near total inhibition of contraction banding, but cells were still contracted, with wide cell separations (Figure 3D). Figure 2 and Table 2 indicate that average cell length of amytal-inhibited hearts was 74.1 μ, and less than 1% of cells contained contraction bands.

Calcium Paradox

Calcium paradox hearts showed severe degrees of contracture, many cells assuming a rectangular shape, the cell width being greater than the cell length (Figure 3A). There appeared to be severe injury to nearly all cells in a given heart. The mean cell length in calcium paradox hearts was 27.3 ± 5 μ (Table 1; Figure 2). On the basis of the measurements of the calcium paradox hearts, it was decided that a cell with a length less than 27.3 μ + 2 standard deviations (SD) would be classified as having a contraction band. Thus, all cells measuring less than 37.3 μ were classified as containing a contraction band. With this criterion, 82.3% of cells contained contraction bands in calcium paradox hearts; whereas only 1.5% of cells from DNP hearts could be so classified.

Respiratory Inhibition of Calcium Paradox

Both 3 mM amytal and 5 mM KCN inhibited contraction banding when added at the time of calcium repletion. Neither inhibitor prevented contracture, cell separation, or KCN inhibition (Figures 3B and 4A) (Figure 2). Table 1 indicates that in the hearts severe contracture developed. Both amytal and KCN, however, significantly decreased the percentage of cells with contraction bands from 82% to approximately 23% ($P < 0.01$).

Effect of Exogenous ATP on Cell Contracture in the Calcium Paradox

As compared with KCN and amytal-inhibited hearts,

Table 2—Isolated Adult Rat Heart Myocytes

	Experimental conditions											
	Control			3 mM amytal			5mM iodoacetic acid (IAA)			5mM IAA + 3 mM amytal		
Time	Rod	Square	Round	Rod	Square	Round	Rod	Square	Round	Rod	Square	Round
5 minutes	98.5%	0%	1.5%	91.0%	0%	8.5%	82.5%	2.6%	15.1%	8.6%	83.0%	9.0%
10 minutes	95.5%	0%	4.2%	90.0%	1.%	9.0%	46.8%	19.6%	33.6%	6.0%	89.4%	5.0%
15 minutes	93.1%	0%	6.9%	83.4%	8.6%	7.5%	20.1%	31.7%	48.3%	3.9%	93.1%	3.9%
20 minutes	93.9%	0%	6.0%	65.3%	19.2%	16.0%	6.5%	31.7%	61.2%	3.3%	90.0%	6.9%
25 minutes	87.9%	0%	11.5%	47.2%	31.7%	20.5%	1.4%	30.2%	68.3%	1.1%	95.5%	3.5%
30 minutes	77.9%	0%	22.0%	32.7%	37.3%	30.6%	0.3%	32.1%	67.3%	0.8%	96.1%	3.0%
40 minutes	64.9%	0%	34.7%	10.1%	55.4%	35.6%	0%	37.3%	62.7%	0.5%	95.0%	4.5%
60 minutes	48.3%	0%	50.7%†	1.0%	56.9%*†	41.6%†	0%	36.2%*†	63.3%†	0%	96.1%*	3.9%*

Relative proportions of rod, square, and round isolated myocytes present at indicated time intervals during 60 minutes of incubation in control buffer or buffer with metabolic inhibitors added. The presence of either IAA or amytal alone accelerated the change from rod to round cells and produced a new population of square cells. The addition of both IAA and amytal together rapidly converted nearly all of the rod cells into a stable population of square cells. Round cell values reflect the subtraction of the level of calcium intolerant round cells contaminating the control preparation at time zero (t = 0: 66.7% ± 1.9% rod, 33.7% ± 1.7% round, n = 3). Each value is the mean of three experiments. In each experiment (control, amytal, IAA, or IAA + amytal), 1100–1600 individual cells were counted for each time point. All incubations carried out in the final suspension buffer containing 1.12 mM Ca^{2+}, 2% BSA, and 11 mM glucose (see Materials and Methods).

* $P < 0.01$, compared with the corresponding control value.

† $P < 0.01$, compared with the corresponding IAA + amytal value.

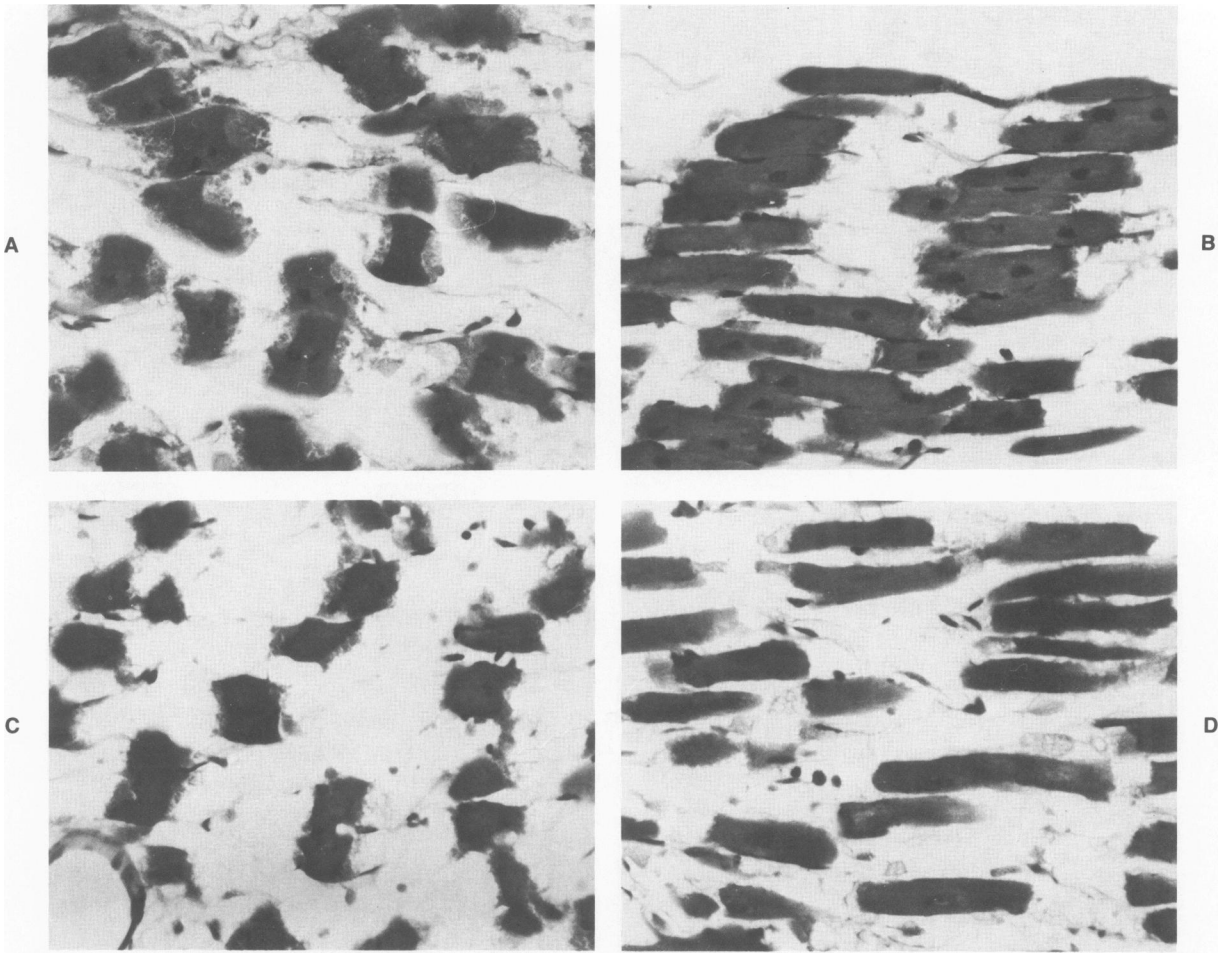

Figure 3A—A heart after calcium repletion (calcium paradox) shows nearly all cells in severe hypercontracture. Most cells in such hearts are considered to contain contraction bands. **B**—Hearts subjected to the calcium paradox in the presence of 5 mM KCN showed contracture, but hypercontracture and contraction bands were largely inhibited. **C**—Calcium-free heart following 10 mM caffeine shows severe contraction band necrosis with wide separation of cells. **D**—Calcium-free hearts exposed to 10 mM caffeine and 3 mM amytal developed contracture, but hypercontracture and contraction-band formation was largely inhibited. (H&E, ×300)

the addition of 2 mM ATP to the calcium repletion perfusate significantly increased the degree of contracture of cells (Figure 4A–D, Figure 2). In the presence of amytal and ATP, average cell length decreased from 47.9 ± 5.9 μ to 36.5 ± 2.4 μ ($P < 0.01$), and the percentage of cells with contraction bands increased from 23% to 55% ($P < 0.01$), whereas in the presence of KCN and ATP, average cell length decreased from 48.7 ± 3.4 to 36.1 ± 0.78 μ, and percentage of contraction bands increased from 23% to 58% ($P < 0.01$).

Isolated Myocyte Studies

Morphology of Isolated Myocytes

Initial cell preparations averaged 79% ± 5% rod cell morphology and were 85% ± 5% viable by the trypan blue exclusion test in the absence of calcium. When 1.12 mM calcium was added to the cells, the final preparations averaged 71% ± 3% rod and 87% ± 5% viable by trypan blue exclusion. Figure 5 shows the light-microscopic appearance of a rod-shaped cell, a square-shaped cell, and a round-shaped cell. The morphologic appearance of the rod cell is nearly identical to that of normal cells in an intact heart. The length/width ratio of this cell is approximately 6.5:1. The sarcomeres of the square-shaped cell appear to be in a state of near maximal contracture. The length/width ratio of this cell is approximately 1:1. The round-shaped cell appears as a ball, and the myofibrils appear hypercontracted into a disorganized mass.

Electron micrographs of a rod cell, a square cell, and a round cell are shown in Figures 6–8. The round cell appears identical by electron microscopy to a contraction band in the intact heart.[2,5] The sarcolemmal membrane of this cell is ruptured, but many round cells have intact membranes. Mitochondria appear to be extruded

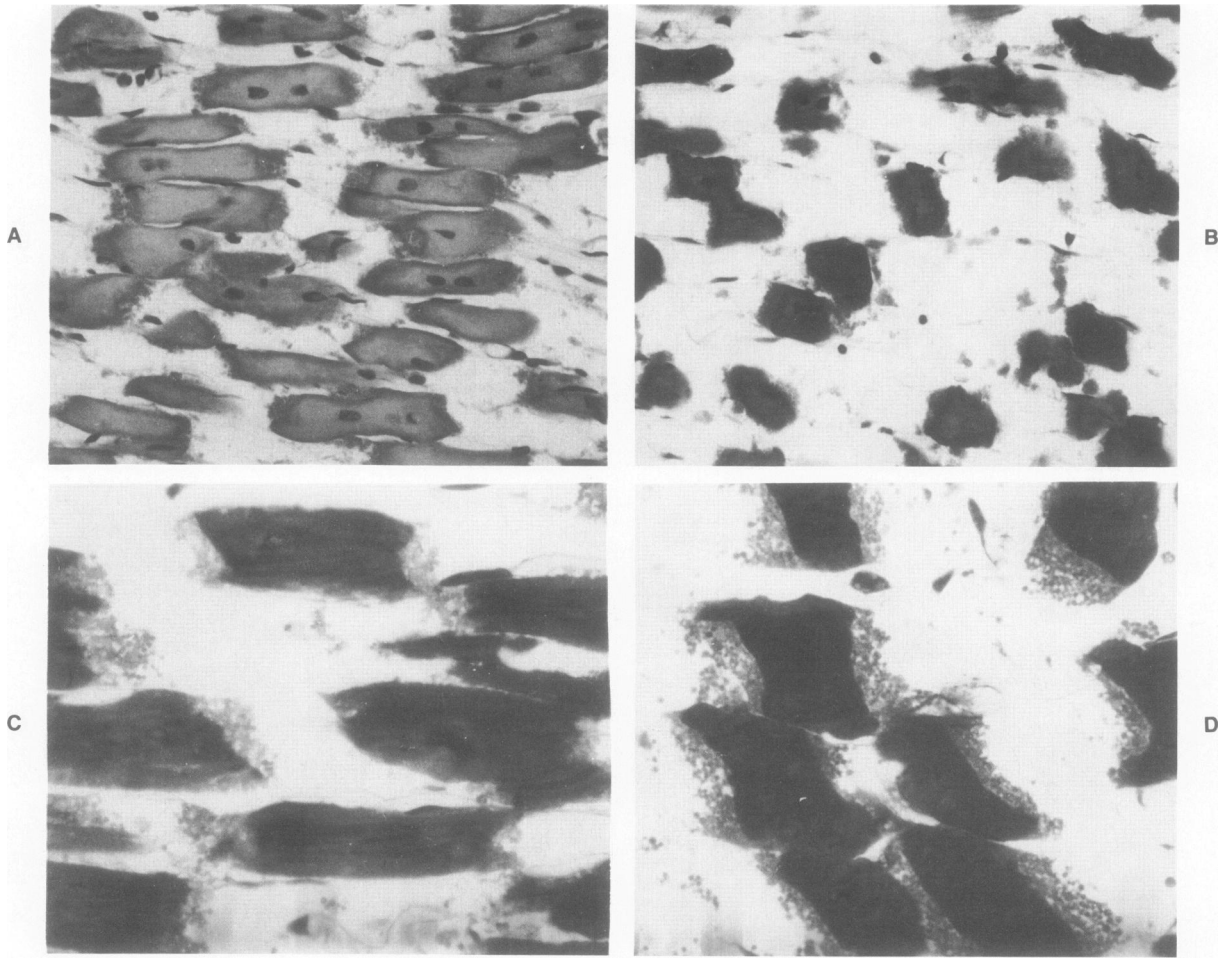

Figure 4A—A calcium paradox heart inhibited with amytal is shown. **B**—When 2 mM ATP was infused along with 3 mM amytal, the inhibition of hypercontracture was overcome, and most cells were hypercontracted or contained contraction bands. **C**—A calcium paradox heart inhibited with KCN is shown. **D**—When 2 mM ATP was added with 5 mM KCN, most cells were hypercontracted or contained contraction bands. (**A** and **B**, H&E, ×300; **C** and **D**, toluidine blue, ×480)

to the periphery of the cell, and the myofibrils are hypercontracted into an amorphous mass in the center of the cell. Electron-microscopic analysis revealed that the sarcolemmal membrane of the square cell appears intact, whereas the myofibrils are in a maximally contracted state. Rod cells possessed intact sarcolemmal membranes and were relaxed, with wide sarcomere spacings. These myocytes appeared identical to cells in intact hearts.

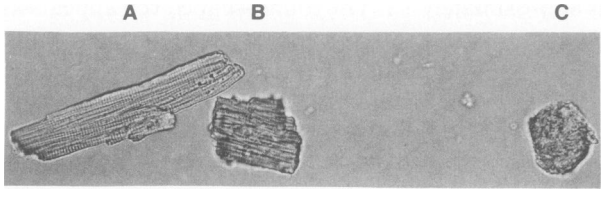

Figure 5—Light micrograph showing the three types of isolated myocytes discussed in the text. **A**—Rod cell. **B**—Square cell. **C**—Round cell. (×300)

Inhibitor Studies

Calcium-tolerant mycoytes were incubated for 60 minutes at 30 C under the various conditions indicated in Table 2. At the indicated time intervals the cells were characterized under light microscopy as to whether they possessed rod-shaped, square-shaped, or round-shaped cells. Values were then corrected by subtraction of the initial numbers of round cells present. Myocytes incubated for 60 minutes in control suspension buffer containing 2% BSA, 1.12 mM Ca^{2+}, and 11 mM glucose showed a decline in the proportion of rod-shaped cells from an initial (5-minute) value of 98.5% to 48.3%, whereas round-shaped cells increased from 1.5% to 50% at 60 minutes. Rod-shaped myocytes incubated 60 minutes in the presence of 3 mM amytal declined from initial values of 98.5% to 1.0%, whereas round-shaped myocytes increased from an initial value of 8.5% to a final value of 41.6%. Square-shaped myocytes, defined

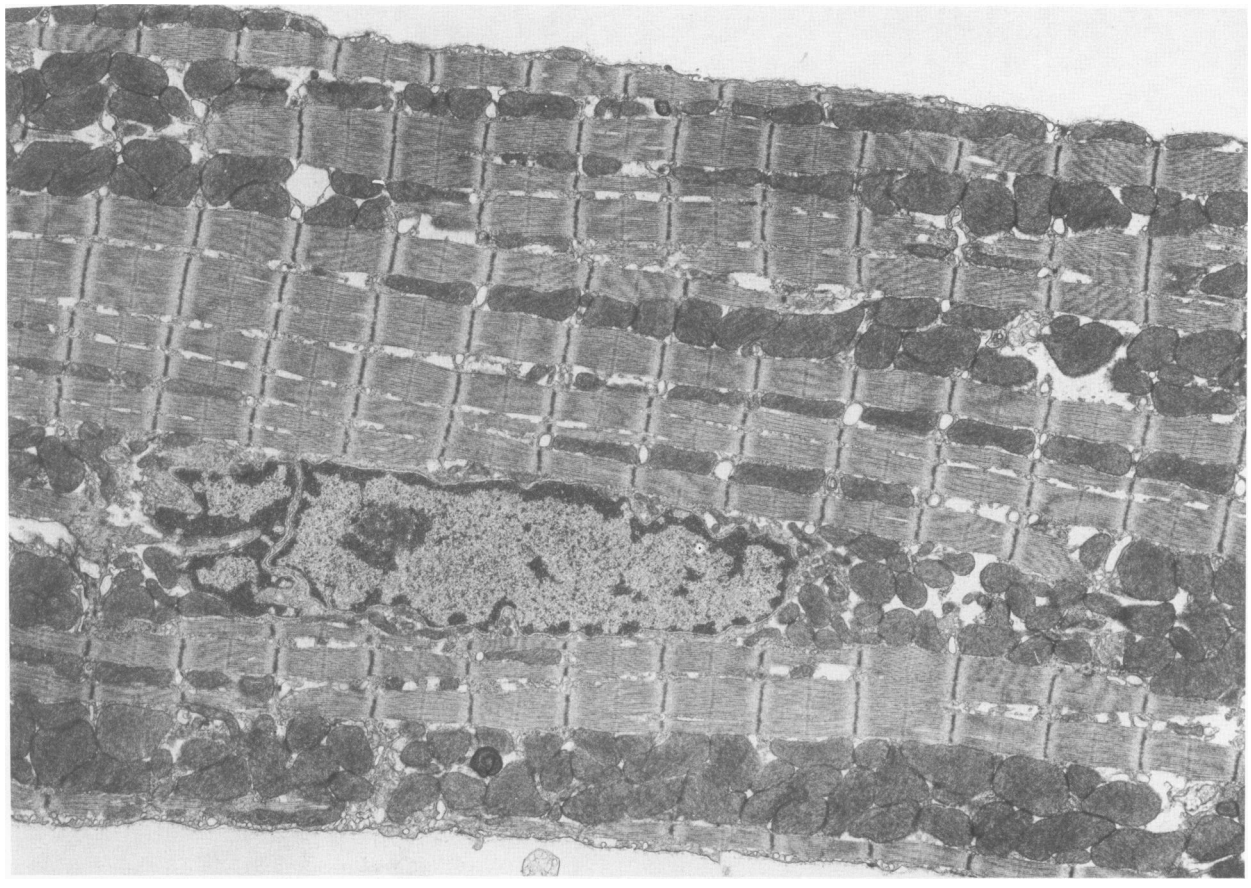

Figure 6—Electron micrograph of a control calcium-tolerant adult rat heart myocyte. The sarcolemmal membrane is intact, and the cell appears relaxed, with wide sarcomere spacings and prominent I-bands. (×5900)

as cells exhibiting length/width ratios of less than 3:1, were not present initially. However, by 20 minutes of incubation square-shaped cells accounted for 19.2% of the cells present in the cell preparation, and by 60 minutes square-shaped myocytes accounted for 56.9% of the total myocytes present (Table 2).

Cells incubated in the presence of 5 mM IAA showed a decline in the percentage of rod-shaped cells, whereas the percentage of round and square-shaped cells increased. By 60 minutes no rod-shaped cells remained, and the myocyte population consisted of a mixture of square and round cells (Table 2).

Within 5–10 minutes of addition of both 5 mM IAA and 3 mM amytal, the majority of the myocytes exhibited square shapes (Table 2). By 60 minutes 96.1% of the cells were square, and only 3.9% were round. No rod-shaped cells were present after 60 minutes of incubation (Table 2).

Washout of Amytal

To determine whether the resumption of mitochondrial respiration could influence the morphologic features of myocytes which had assumed a square configuration due to inhibition of both glycolytic and mitochondrial energy pathways, cells were split into two groups and incubated with 5 mM IAA and 3 mM amytal for 30 minutes for production of a stable population of square cells. After 30 minutes control cells were washed twice with suspension buffer containing 5 mM IAA and 3 mM amytal, and experimental cells were washed twice with buffer containing only 5 mM IAA for washing out the amytal and thereby allowing resumption of mitochondrial respiration. When amytal was washed out from cells initially inhibited with both IAA and amytal, the percentage of square cells significantly decreased from 92.8% ± 4.1% (n = 4; two separate preparations) to 57.0% ± 4.2% ($P < 0.01$), whereas the proportion of round-shaped cells significantly increased 43% from −0.8% ± 2.8% to 42.3% ± 4.8% ($P < 0.01$). Control cells were washed in parallel with experimental cells except that the buffer contained both IAA and amytal. The percentage of square cells in the control preparation after washing showed a small increase (1.5% ± 3.2%) at time zero,

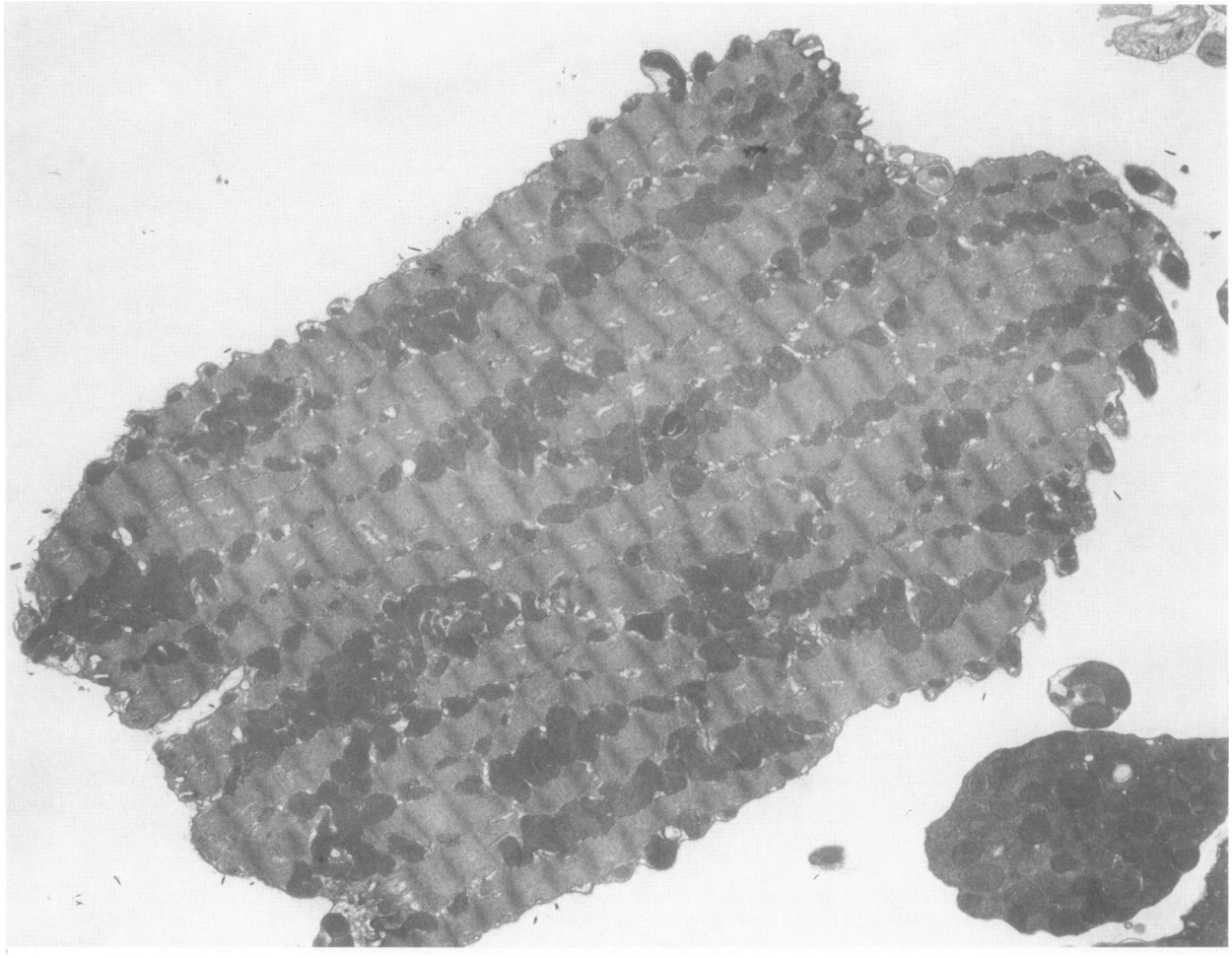

Figure 7—Isolated calcium-tolerant myocyte exposed to iodoacetic acid and amytal for 30 minutes. The sarcomeres lack prominent I-bands, which indicates that the myofibrils are maximally contracted. The sarcolemmal membrane appears intact. (×5400)

which remained relatively stable (decreased approximately 10%) throughout an additional hour of incubation. Round cells showed a small decrease (−2.5% ± 3.2%) immediately after washing, which also remained constant throughout an additional hour of incubation (increased approximately 10%).

Discussion

Cardiac contraction is believed to be mediated by controlled fluctuations of cytosolic calcium concentrations in the presence of excess ATP.[7] In a normal contraction, sarcomeric shortening can occur from a fully relaxed length of 2.4 μ to between 1.75 and 1.6 μ in hearts with an empty ventricle.[8] In intact hearts, limitation of the extent of sarcomere shortening is probably determined by several factors, including the availability of calcium, the internal sarcomere structure, and the overall geometry of the heart. At the end of systole, when the ventricular cavity is obliterated, further shortening of myocytes would be mechanically restricted. The importance of internal sarcomere structure as a limitation to cell shortening is suggested by observations of contracting isolated myocytes, which shorten to a limited degree and then spontaneously relax.[6] In a state of full contracture I-bands become obliterated at 1.6 μ (the width of an A-band). Further shortening can only occur with distortion of sarcomeres and compression, folding, or overlapping of sarcomeric elements.

Normal restrictions of the degree of sarcomere shortening are overcome under pathologic conditions. In intact hearts, contraction band necrosis can occur under a variety of conditions. Following severe anoxic or ischemic injury, cells are depleted of ATP, and a rigor contracture develops, with obliteration of I-bands but no apparent distortion of sarcomeric elements.[9] In intact hearts, cells are attached to one another at intercalated disks, and once the ventricular cavity is obliterated by the rigor contracture, cell shortening is resisted by the

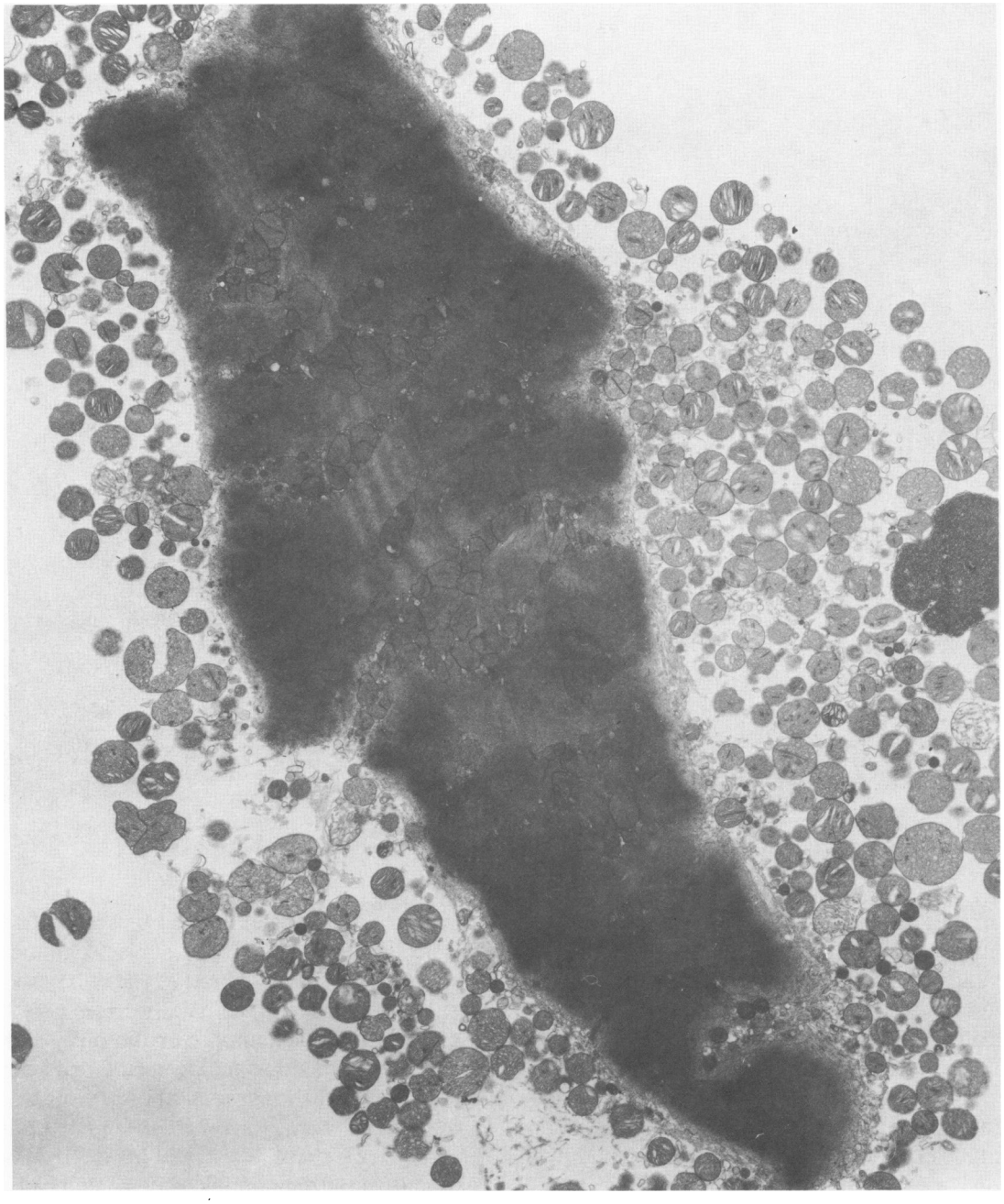

Figure 8—Electron micrograph of a round isolated myocyte. The sarcolemmal membrane is ruptured. The sarcomeres have hypercontracted into an amorphous mass in the center of the cell. (×4800)

ventricular geometry. With reoxygenation, further sarcomere shortening must occur only at the expense of adjacent sarcomeres with formation of alternating bands of hypercontracted and stretched or broken sarcomeres.[10,11] The result is the characteristic zebra stripe-like banding of cells seen in contraction band necrosis (Figure 9). Isolated myocytes, in contrast, are not mechanically restrained from shortening, and upon reoxygenation of anoxically injured myocytes the cells round up, forming a single contraction band.[12,13] Similarly, during the calcium paradox or caffeine-induced injury, neighboring myocytes separate at intercalated disks, causing sarcolemmal membrane rupture and allowing both loss of cytoplasmic enzymes and metabolites from the cells and free exchange of normally excluded compounds such as ATP across the membrane into the cell interior. The injured myocytes, free of mechanical constraints and in the presence of a permeable sarcolemmal membrane, hypercontract to form single, centrally located contraction bands.

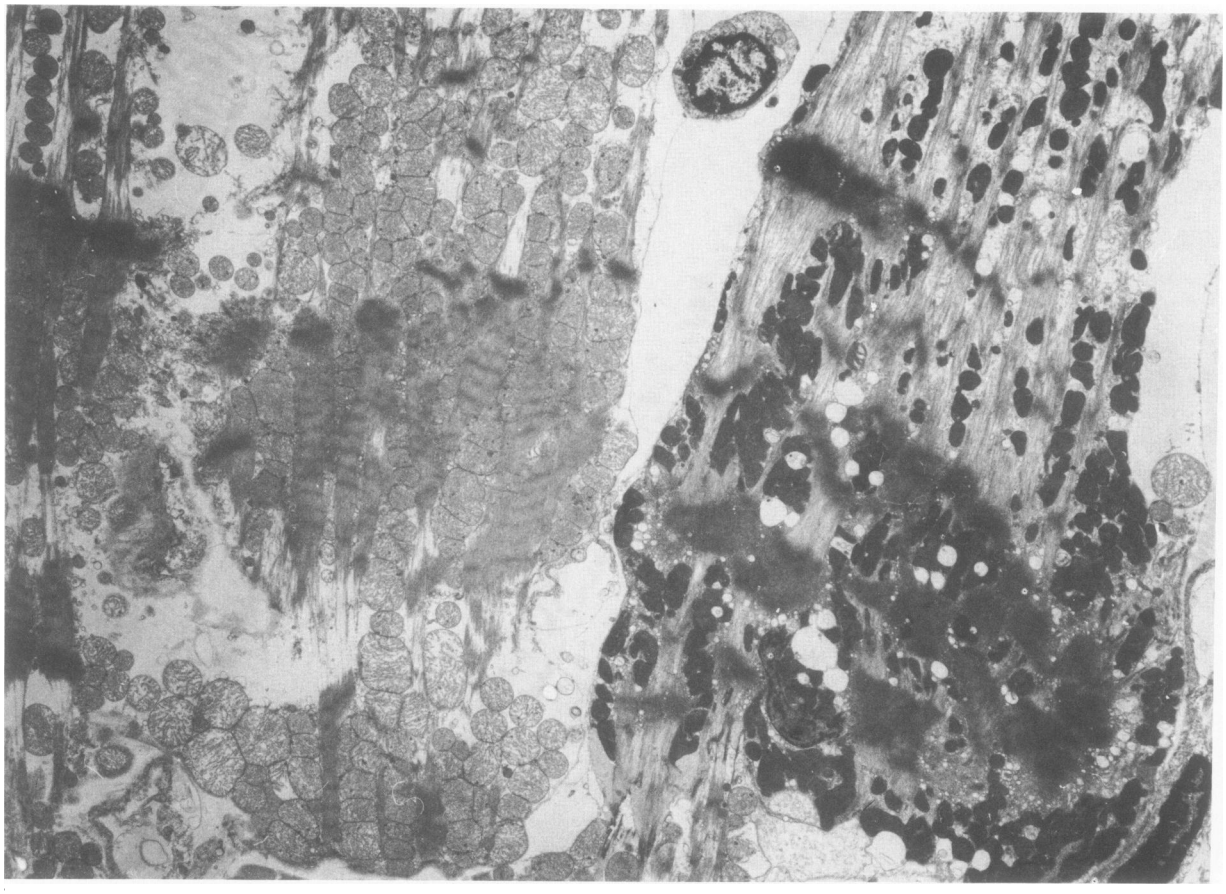

Figure 9—Electron micrograph of cells found in a heart after a period of hypoxia and reoxygenation. Myocytes are severely damaged and contain multiple, irregular contraction bands, swollen mitochondria, and disrupted sarcolemmal membranes. (×3000)

The results of the present study utilizing the perfused rat heart model demonstrate that mitochondrial inhibitors and uncouplers will allow or induce contracture of myocytes but will prevent the formation of contraction bands. In DNP-induced injury, the cells were contracted but were significantly longer than cells from hearts containing contraction bands. Indeed, nearly all the cells in hearts subjected to the calcium paradox or caffeine-induced injury were classified as containing contraction bands. However, when mitochondrial respiration was inhibited by the addition of cyanide or amytal to calcium paradox or caffeine-injured hearts, the injured cells were significantly longer than those of the corresponding injured hearts (Figure 2, Table 1). These results confirm our previous observations that mitochondrial inhibitors and uncouplers prevent the formation of contraction bands in caffeine-induced injury[5] and the oxygen paradox[4] as well as the injury following reperfusion of ischemic myocardium.[14] This inhibition is sustained over long periods of reperfusion, and we have observed no contraction band formation for periods of up to 3 hours in the continued presence of mitochondrial inhibitors. However, the addition of exogenous ATP in the continued presence of the mitochondrial inhibitors, to hearts subjected to the calcium paradox, significantly shortened the cells, as compared with those in calcium paradox hearts exposed only to the inhibitors. The ability of exogenously added ATP to induce contraction banding in perfused hearts in the presence of mitochondrial inhibition provides indirect evidence that energy is required to support contraction banding while also effectively ruling out the possibility that mitochondrial inhibitors are acting via direct nonspecific effects on contractile proteins.

It is our hypothesis that whole heart contraction bands result from an increase in the concentration of free Ca^{2+} available to interact with the myofibrils or from the presence of an abnormally low but continuously regenerated concentration of $MgATP^{2-}$. In the classical calcium paradox, the primary event leading to the development of contraction bands is the physical disruption of portions of the sarcolemma adjacent to the gap or nexus junctions when conditions allowing for excitation–contraction coupling are restored. Calcium enters the cell through these discontinuities in the sarcolemma, and cytosolic components are released. How-

ever, the efflux of cytosolic $MgATP^{2-}$ is not instantaneous, and residual adenine nucleotides are capable of supporting hypercontracture, especially when the ADP produced by myosin ATPase activity can be rephosphorylated by the mitochondria. When a respiratory inhibitor is present, residual ATP is degraded too rapidly to permit the full development of contraction bands, but exogenous ATP reverses this effect (Figure 2).

To test directly the hypothesis that ATP production is required to support hypercontracture of sarcomeres into contraction bands, we used the isolated adult rat heart myocyte model. It has been shown that the high energy stores of myocytes suspended in a buffer containing inhibitors of both glycolytic (IAA) and mitochondrial (amytal) energy production rapidly decline to near zero, as indicated by an abrupt transition from the normal rod-shaped configuration to a square or rectangular appearance.[12,13,15,16] When cells were suspended in a calcium-containing buffer with IAA and amytal, they were observed to undergo a reproducible morphological change from rod to square-shaped cells. This transformation occurred very rapidly, because near complete squaring of cells occurred within 5 minutes of addition of the inhibitors. Square cells maintained their morphologic appearance for at least 3 hours (data not shown) as long as the presence of the inhibitors was maintained in the suspending buffer.

If the hypothesis that high energy stores, specifically mitochondrial ATP production, are necessary to support hypercontracture is correct, then the resumption of mitochondrial ATP production in the presence of continued inhibition of glycolytic energy production should result in hypercontracture (rounding up) of isolated myocytes. Because amytal is known to be a fully reversible mitochondrial inhibitor, the washing and resuspension of myocytes in a buffer containing only the glycolytic inhibitor IAA should wash out the amytal and allow the resumption of mitochondrial energy production. Indeed, preliminary data have shown that upon washout of amytal from cells suspended in both IAA and amytal, the level of ATP in the cell suspension rebounds from 0.2 nmol/mg to 1 nmol/mg protein. Similar studies have shown that the ATP contained in anoxic, IAA-poisoned cells is retained after digitonin lysis, which suggests that cytosolic ATP falls essentially to zero[17] and upon resumption of oxidative phosphorylation, rebounds into the range known to produce hypercontracture in chemically skinned myocytes.[18] As a result, when cells were washed with buffer containing only IAA (amytal washout), the percentage of square-shaped cells decreased 36%, whereas the percentage of round cells increased by 43%. Control cells, washed and resuspended in buffer containing both inhibitors, did not show appreciable changes in the proportion of square and round cells even after 1 hour of additional incubation.

It is possible that the washing procedure itself may selectively wash out or retain a certain population of cells and thereby alter the relative proportions of cells in the preparation. Indeed, small changes in the proportions of rod and square cells were observed immediately upon washing of control cells. However, the percentage of round cells in the control preparations actually *decreased* 2.5% after washing, whereas the percentage of round cells in the amytal washout preparation *increased* by 43% after washing. We and other investigators have observed that washing cell suspensions in BSA-containing buffers enriches the proportion of rod-shaped cells in the preparation by selectively leaving round cells in the supernate. It therefore seems unlikely that a selective washing effect could account for the observed increase in round cells, because the washing step by itself should cause a *decrease* in the percentage of round cells present.

It may be argued that the mechanical forces involved in the centrifugation and resuspension of the cells may rupture the sarcolemmal membrane of square cells, causing them to round up in the presence of external calcium. However, because both the control and the experimental cells were subjected to the same protocol, and therefore the same mechanical forces, and the percentage of round cells in the control preparation did not increase, this explanation does not seem likely.

It may also be argued that IAA or amytal at the concentrations used may have a toxic or nonspecific effect on the cells' ability to undergo contracture. However, the amytal washout experiments demonstrate that in the continued presence of 5 mM IAA the cells are capable of undergoing hypercontracture. In addition, the control experiments demonstrate that cells exposed to both IAA and amytal squared up almost immediately and remained in the square configuration for an additional 1 hour of incubation.

These results indicate that the increase in the percentage of round cells observed upon washout of amytal is not a result of mechanical fragility, a cell-selective washing procedure, or a nonspecific toxic effect, but is the result of the resumption of mitochondrial respiration. The possibility that mitochondrial superoxide production may support hypercontracture[1,3] seems unlikely, because our results indicate that significant cell rounding occurs in isolated myocytes when the mitochondrial inhibitor amytal alone is present, and this rounding is totally prevented by addition of IAA. This result also indicates that glycolytically produced ATP can support hypercontracture under certain experimental conditions. Thus, as the studies in intact hearts show, ATP from any source, whether produced by glycolysis

or by mitochondria, or added exogenously, can permit contraction band formation.

Further support for the hypothesis that ATP plays a crucial role in contraction band formation comes from experiments with digitonin-permeabilized myocytes, which recently established that hypercontracture of the myofibrils into a single amorphous mass exhibits an absolute requirement for ATP.[18] When free Ca^{2+} is held at normal resting cytosolic concentration of 10^{-7} M, hypercontracture occurs only when $MgATP^{2-}$ is less than 1 mM, whereas the 10 mM present in the cytosol of aerobic heart cells leaves the myofibrils in a fully relaxed configuration. When free Ca^{2+} exceeds 10^{-6} M, however, the cells remain elongated only if $MgATP^{2-}$ is completely absent from the suspending medium. A similar response is observed in myocytes with intact cell membranes. At a cytosolic free Ca^{2+} concentration of 10^{-7} M, as reported by Quin 2 fluorescence spectroscopy, the cells are normally elongated, but when free Ca^{2+} is increased to 10^{-6} M by loading the cells with Na^+ to activate Na^+/Ca^{2+} exchange and reduce the membrane potential, the cells all hypercontract.[17]

The present results emphasize the critical role that ATP may play in the formation of contraction bands. However, it should be emphasized that contraction bands form only in pathologic states (such as ischemia–reperfusion, anoxia–reoxygenation, or the calcium paradox) where sarcolemmal or structural cellular damage may be the necessary predisposing condition or in severely altered physiologic states such as catecholamine injury, which is thought to be the result of severe calcium overload. It would seem, therefore, that contraction band formation is morphologic evidence of underlying structural or biochemical defects in myocardial cells.

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