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H Kusuoka, … , M L Weisfeldt, E Marban

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Research Article

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Pathophysiology and Pathogenesis of Stunned Myocardium

Depressed Ca^{2+} Activation of Contraction as a Consequence of Reperfusion-induced Cellular Calcium Overload in Ferret Hearts

Hideo Kusuoka, James K. Porterfield, Harlan F. Weisman, Myron L. Weisfeldt, and Eduardo Marban
Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205

Abstract

Contractile dysfunction in stunned myocardium could result from a decrease in the intracellular free $[Ca^{2+}]$ transient during each beat, a decrease in maximal Ca^{2+}-activated force, or a shift in myofilament Ca^{2+} sensitivity. We measured developed pressure (DP) at several $[Ca]_o$ (0.5–7.5 mM) in isovolumic Langendorff-perfused ferret hearts at 37°C after 15 min of global ischemia (stunned group, $n = 13$) or in a nonischemic control group ($n = 6$). At all $[Ca]_o$, DP was depressed in the stunned group ($P < 0.001$). Maximal Ca^{2+}-activated pressure (MCAP), measured from tetani after exposure to ryanodine, was decreased after stunning ($P < 0.05$). Normalization of the DP–$[Ca]_o$ relationship by corresponding MCAP (Ca_o sensitivity) revealed a shift to higher $[Ca]_o$ in stunned hearts. To test whether cellular Ca overload initiates stunning, we reperfused with low-$[Ca]_o$ solution (0.1–0.5 mM; $n = 8$). DP and MCAP in the low-$[Ca]_o$ group were comparable to control ($P > 0.05$), and higher than in the stunned group ($P < 0.05$). Myocardial [ATP] observed by phosphorus NMR failed to correlate with functional recovery. In conclusion, contractile dysfunction in stunned myocardium is due to a decline in maximal force, and a shift in Ca_o sensitivity (which may reflect either decreased myofilament Ca^{2+} sensitivity or a decrease in the $[Ca^{2+}]$ transient). Our results also indicate that calcium entry upon reperfusion plays a major role in the pathogenesis of myocardial stunning.

Introduction

Reperfusion after myocardial ischemia of brief duration does not induce necrosis, but results in prolonged contractile dysfunction (1–3). This phenomenon, which has come to be known as myocardial stunning (4), is manifested clinically in the sluggish recovery of pump function after coronary revascularization after brief periods of ischemia (3). Despite its clinical importance, the physiological features of stunned myocardium are not well characterized, and the mechanism of stunning remains unresolved (5). What does seem clear is that stunned myocardium remains responsive to paired pacing (6) or catecholamines (6, 7), emphasizing the dynamic nature of the impairment in force generation.

Address correspondence and reprint requests to Dr. Marban, Division of Cardiology, Department of Medicine, Carnegie 584, The Johns Hopkins Hospital, Baltimore, MD 21205.

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The contractility lesion in stunned myocardium might affect either or both of two major links in force generation. The first of these is the intracellular free Ca^{2+} concentration, $[Ca^{2+}]_i$, during each cardiac cycle (the $[Ca^{2+}]$ transient). The $[Ca^{2+}]$ transient triggers the sequence of biochemical events leading to force generation by the contractile proteins. The second major link is the responsiveness of the contractile machinery to Ca^{2+}. Myofilament Ca^{2+} responsiveness itself consists of two terms (8): the myofilament sensitivity to Ca^{2+}, which describes the range of $[Ca^{2+}]_i$ that activates contraction, and maximal Ca^{2+}-activated force, which determines the amplitude of the contractile response. Therefore, any of the following three basic steps could account for the contractile dysfunction of stunned myocardium: (*a*) a decrease of $[Ca^{2+}]$ transients, (*b*) a shift in myofilament sensitivity to Ca^{2+}, or (*c*) a decrease in maximal Ca^{2+}-activated force.

Beyond the characterization of excitation–contraction coupling in stunned myocardium remains the question of the underlying mechanism of cellular injury. Two major hypotheses have been put forward: cellular ATP depletion, or "abnormalities in calcium flux" (4). Whereas a close correlation has been found between the recovery of ATP and the recovery of systolic function after regional ischemia in the dog heart (9), a cause-and-effect relationship between ATP depletion and depressed contractile force has been questioned (10–13). The possibility of abnormalities in Ca metabolism has received little attention in stunned myocardium, despite extensive evidence implicating cellular Ca overload as an etiologic factor in irreversible reperfusion injury (13, 14).

It has previously been impossible to characterize the pathophysiologic basis of stunned myocardium, because of lack of suitable methods in intact heart. Recently, a new approach has been described that enables the determination of maximal Ca^{2+}-activated force as reflected by isovolumic pressure in the isolated perfused heart (15), using tetani elicited by rapid pacing after exposure to ryanodine. We have used this approach to determine whether maximal Ca^{2+}-activated force is decreased in stunned myocardium induced by reperfusion after 15 min of global ischemia. We have also characterized the responsiveness of twitch force to changes in extracellular Ca (Ca_o responsiveness) before and after stunning. Ca_o responsiveness during twitches reports the net resultant of the $[Ca^{2+}]$ transient and myofilament Ca^{2+} sensitivity, as scaled by maximal Ca^{2+}-activated force. Finally, we have examined the role of cellular Ca overload in the pathogenesis of stunned myocardium by reperfusing with solutions of varying Ca concentration.

Methods

The experimental preparation has been described previously (8, 15). Briefly, male ferrets (10–14 wk of age) were anesthetized with sodium pentobarbital (80 mg/kg i.p.; Harvey Laboratories, Inc., Philadelphia, PA) and heparinized. After rapid excision of the heart, the aorta was

cannulated and retrogradely perfused with standard Tyrode solution (see below) at 37°C. The heart rate was maintained at 170–190 bpm by right ventricular pacing with an agar wick soaked in saturated KCl, encased in polyethylene tubing, and connected to a Grass S44 stimulator. A latex balloon tied to the end of a polyethylene tube was passed into the left ventricle through the mitral valve and connected to a Statham P23DB pressure transducer. The balloon was filled with aqueous solution to an end-diastolic pressure (EDP)[1] of 8–12 mmHg, then kept isovolumic throughout the experiment. Perfusion pressure was monitored at the cannulation point of the aorta. Left ventricular pressure and perfusion pressure were recorded with a direct-writing recorder and with an FM instrumentation tape recorder (model HP 3964A; Brush Machine Inc., Molalla, CA). After 20–30 min of stabilization, the coronary flow rate (CFR), controlled by a peristaltic pump, was adjusted such that perfusion pressure equalled 90 mmHg. Once adjusted, the CFR was kept constant throughout the experiment (except during the ischemic period; see below).

To create stunned myocardium, hearts were subjected to 15 min of global ischemia at 37°C. This temperature was chosen, and was maintained during ischemia, to mimic as closely as possible the conditions during myocardial stunning in situ. Aortic inflow was totally interrupted by shutting off the peristaltic pump and by cross-clamping the perfusion line. After the period of global ischemia, hearts were reperfused until developed pressure (DP) recovered to a new steady state (20–30 min). Pacing was discontinued during ischemia and for the first 20 min of reperfusion; the spontaneous heart rate during early reperfusion (2.5–3.0 Hz) approximated that during pacing. To facilitate explicit comparison with the control period, pacing was restarted before any new experimental measurements. The balloon in the left ventricle was not deflated during the ischemic period.

Solutions. The perfusate was a phosphate-free Hepes-buffered Tyrode solution, which has the advantage that the perfusate calcium concentration, $[Ca]_o$, can be increased at high levels without Ca carbonate or Ca phosphate precipitation. The standard medium contained (in millimolars): NaCl 108, KCl 5, $MgCl_2$ 1, Hepes 5, $CaCl_2$ 2, glucose 10, and sodium acetate 20. $[Ca]_o$ was changed by using 1 M $CaCl_2$ as necessary. The pH of the solution was adjusted to 7.4 at 37°C by addition of HCl or NaOH as required, and the solution was bubbled continuously with 100% O_2.

Ca_o responsiveness and maximal Ca^{2+}-activated pressure (MCAP). The responsiveness of myocardium to Ca was determined by measuring the isovolumic DP during twitch contractions as a function of varying levels of $[Ca]_o$ (0.5, 1, 2, 5 and 7.5 mM), as Fig. 1 shows. Fig. 1 *A* shows a continuous record of left ventricular pressure at a slow time base, as $[Ca]_o$ was varied from the control level of 2 mM to the other levels indicated above the record. As illustrated here, all measurements were bracketed by exposure to 2 mM $[Ca]_o$ solution to verify the stability of the preparation. To avoid calcium paradox or calcium overload, $[Ca]_o$ was restored to 2 mM as soon as DP reached steady state at each $[Ca]_o$. Fig. 1 *B* shows twitch contractions at steady state at each $[Ca]_o$, displayed at a faster time base. DP was measured from such records.

MCAP was determined from the DP during tetani as $[Ca]_o$ was increased, in an approach described previously (15). Briefly, hearts were exposed for 10–20 min to ryanodine (3 μM; Penick Corp., Lyndhurst, NJ), a plant-derived alkaloid believed to inhibit the release of calcium from the sarcoplasmic reticulum (SR) (16). Tetani were then elicited by high-frequency electrical stimulation (10–12 Hz) with rectangular pulses of 60–80 ms at 1.5–2.0 times the threshold. Pacing was discontinued in the intervals between tetani and the heart was allowed to beat spontaneously.

MCAP was determined from the saturation of DP during tetani as $[Ca]_o$ was increased from 2 to 20 mM. The greatest value of DP achieved during each tetanus was measured, and saturation was inferred if the values at two or more distinct $[Ca]_o$ agreed within 5%. Fig. 2 illustrates such an experiment. The upper row (*A*) shows records of left ventricular pressure during tetani at various $[Ca]_o$, at 37°C. As $[Ca]_o$ is raised from 2 to 10 mM, tetanic pressure increases, but there is no further increase beyond 10 mM $[Ca]_o$. DP during tetani is plotted in Fig. 2 *B* (*solid squares*), along with the DP during twitches (*solid circles*) measured in the same heart before exposure to ryanodine. Each point represents the average of three or more consecutive records of DP during twitches or tetani at each $[Ca]_o$. Throughout the range of $[Ca]_o$ examined, twitch force was lower than tetanic force, consistent with the idea that tetanic force in elevated $[Ca]_o$ indeed represents the maximal force of which the heart is capable. Elevation of $[Ca]_o$ beyond 7.5 mM generally fails to augment the twitch in ferret myocardium, and may even lead to a decrease in force (17).

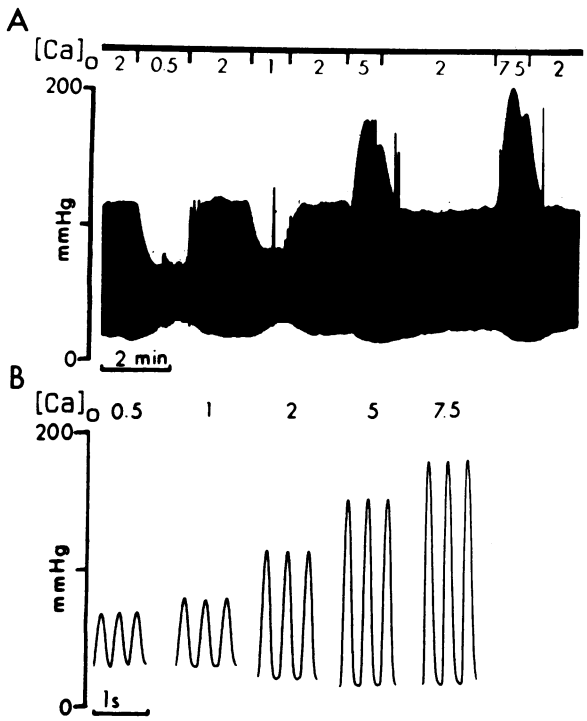

Figure 1. Responsiveness of myocardium to Ca_o. The myocardial responsiveness to Ca_o was determined from the response of left ventricular DP to varying $[Ca]_o$. (A) Continuous pressure record during the determination of Ca_o responsiveness. $[Ca]_o$ changes are indicated above the record. (B) Pressure recordings on an expanded time base from the experiment shown in A at 0.5, 1, 2, 5, and 7.5 mM $[Ca]_o$ (as indicated above the individual records).

In some cases (e.g., after stunning), clear-cut saturation was not produced by increasing $[Ca]_o$ alone. To confirm the saturation of DP in such cases, Bay K 8644 (300 nM; Miles Institute for Preclinical Pharmacology, New Haven, CT), a Ca channel agonist, was added to perfusate containing 20 mM $[Ca]_o$. Bay K 8644 increases $[Ca^{2+}]_i$ during twitches and tetani at any given $[Ca]_o$ (15, 18); saturation can be confirmed by an increase in twitch, but not tetanic, pressure after the addition of Bay K 8644.

Terminology. Experiments in which twitch pressure is measured as a function of $[Ca]_o$ (Fig. 1) yield Ca_o responsiveness. This can be expressed by a relationship between $[Ca]_o$ and DP such as that plotted in Fig. 2 (*solid circles*). The link between responsiveness and sensitivity is one of normalization: the sensitivity of myocardium to Ca_o is defined as the relationship between $[Ca]_o$ and DP normalized by the corresponding MCAP. In the example Fig. 2 shows, MCAP is 274 mmHg. Each of the twitch pressures would be divided by this value to yield sensitivity, which is therefore in units of percentage of maximal force.

1. *Abbreviations used in this paper:* CFR, coronary flow rate; CPP, coronary perfusion rate; DP, developed pressure; EDP, end-diastolic pressure; MCAP, maximal Ca^{2+}-activated pressure; NMR, nuclear magnetic resonance; PCr, phosphocreatine; Pi, inorganic phosphate; SR, sarcoplasmic reticulum.

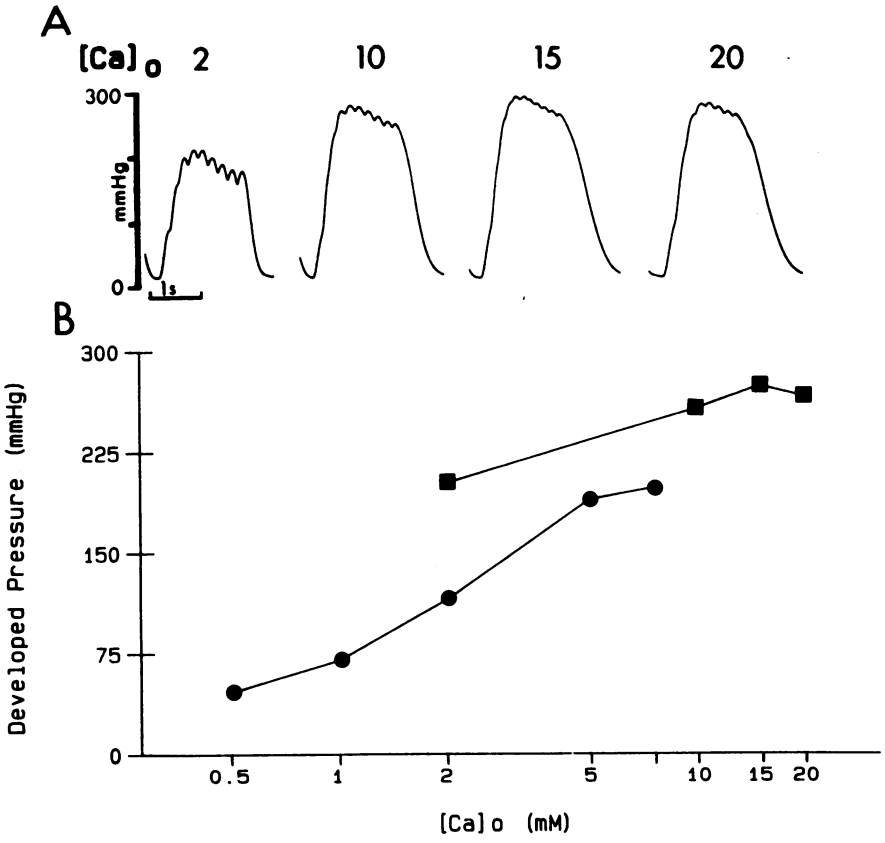

Figure 2. Saturation of DP during tetani with respect to $[Ca]_0$. (A) Pressure recordings of tetani from a typical experiment at 2, 10, 15, and 20 mM $[Ca]_0$. (B) DP during tetani (*solid squares*) and the Ca_0 responsiveness evaluated in the same preparation before exposure to ryanodine (*solid circles*), plotted as a function of $[Ca]_0$.

These terms are analogous but not identical to the corresponding nomenclature for $[Ca^{2+}]_i$–tension relations (8). It must be emphasized that $[Ca^{2+}]_i$ is not determined in these experiments. This does not affect the interpretation of MCAP (15), but it does qualify the meaning of Ca_0 responsiveness and Ca_0 sensitivity. The contractile force of twitches as a function of $[Ca]_0$ is determined by both the $[Ca^{2+}]$ transient and the myofilament properties. Therefore, Ca_0 responsiveness and sensitivity are not solely reflections of myofilament responsiveness and sensitivity, unless $[Ca^{2+}]$ transients remain unchanged. On the other hand, MCAP directly reflects myofilament properties, because $[Ca^{2+}]_i$ reaches saturating levels.

Morphological assessment of stunned myocardium. At the end of experimental protocols, the perfusate was switched to cold 30 mM KCl saline solution to achieve rapid diastolic arrest. Hearts were then perfused with cold 3% gluteraldehyde in 0.1 M phosphate buffer (pH 7.4). A transmural wedge (~ 1 cm) located midway between the apex and the base of the left ventricle was cut between the anterior and posterior papillary muscles and further divided into inner (endocardial) and outer (epicardial) halves. Each half was then cut into 1-mm^3 samples. After fixation, samples were washed with several changes of 0.1 M phosphate buffer, postfixed for 1 h with 1% osmium tetroxide in phosphate buffer, dehydrated in a graded series of alcohols and propylene oxide, embedded in epoxy resin, and cut into semithin (1-μm thick) sections. These sections were stained with toluidine blue and examined with the light microscope. Ultrathin sections (75 nm) were stained with lead citrate and uranyl acetate and examined with a JEOL 100S electron microscope.

Electron micrographs were evaluated qualitatively using the presence and severity of mitochondrial injury (swelling, disruption, and presence of amorphous densities), the extent of contraction and disorganization of sarcomeres, and the clumping of nuclear chromatin as signs of ischemic injury. All slides and micrographs were read blindly.

Phosphorus nuclear magnetic resonance (NMR) measurements. The phosphorus-31 NMR methods have been described previously (8). Briefly, the preparation was lowered into a 25-mm diameter NMR tube and placed into the wide-bore superconducting magnet of a spectrometer (4.2 Tesla, 72.39 MHz for ^{31}P; model WH-180, Bruker Instruments, Inc., Billerica, MA). P-NMR spectra were obtained at a spectral width of 3 kHz using 45° pulses delivered at 2-s intervals. Proton decoupling was not used. Exponential multiplication equivalent to 10-Hz line broadening was used to smooth the spectrum. In NMR experiments, the balloon in the left ventricle was filled with a 15-mM solution of magnesium trimetaphosphate as a standard.

The amounts of inorganic phosphate (Pi), phosphocreatine (PCr), and ATP in the myocardium were obtained by planimetric measurement of the areas under individual peaks using a digitizer (model 9810A; Hewlett-Packard Co., Palo Alto, CA). The tissue contents of Pi, PCr, and ATP were normalized by the peak for the magnesium trimetaphosphate standard in the left ventricular balloon. The calculated amounts of Pi, PCr, and ATP were divided by the measured weight of each heart to yield concentrations ([Pi], [PCr], and [ATP]) in units of micromole per gram wet weight. The saturation in spectra was corrected (19) using the following T_1 relaxation times: 1.9 s for Pi, 2.3 s for PCr, 0.81 s for beta-ATP, and 4.2 s for magnesium trimetaphosphate. Intramyocardial pH was usually estimated from the chemical shift of the Pi peak measured relative to the resonance of PCr (20). When the peak of PCr could not be observed during ischemia, the chemical shift of Pi was measured relative to the peak of magnesium trimetaphosphate.

Experimental design. Experiments were performed in a total of 27 hearts. Of these, 13 were subjected to stunning, 6 were nonischemic controls, and 8 were used to study the effects of reperfusion with low-$[Ca]_0$ solution.

The design of the experiments in stunned myocardium will be discussed first. During an initial control phase, i.e., before global ischemia, the Ca_0 responsiveness was measured as in Fig. 1. The heart was then subjected to 15 min of global ischemia. After 20 min of reperfusion with the standard solution ($[Ca]_0$ = 2 mM), Ca_0 responsiveness was once again measured, this time in stunned myocardium. Ryanodine was added to the perfusate after the second evaluation of Ca_0 responsiveness, and

Table I. Left Ventricular Pressure Parameters in the Various Experimental Groups

	$[Ca^{2+}]_o$	Initial control (mean±SEM)	After experimental intervention (mean±SEM)
		mmHg	*mmHg*
Nonischemic control group (n = 6)			
EDP	0.5	21.3±1.5	23.5±2.7
	1	19.7±1.1	23.0±3.0
	2	17.3±0.8	19.2±2.0
	5	17.2±1.3	17.2±1.9
	7.5	17.3±1.7	17.0±2.0
DP	0.5	27.5±4.3	33.7±3.4
	1	58.8±7.4	54.2±4.0
	2	103.2±4.3	102.5±5.7
	5	165.5±10.3	161.2±9.0
	7.5	176.0±10.5	173.7±10.7
Stunned group (n = 13)			
EDP*	0.5	23.5±2.5	46.4±6.3
	1	16.0±2.1	39.9±6.6
	2	13.5±1.5	34.9±6.3
	5	11.2±1.4	29.6±6.1
	7.5	11.2±1.4	25.7±4.1
DP*	0.5	28.9±3.4	17.0±1.7
	1	70.6±5.3	40.5±3.2
	2	103.6±5.5	67.4±5.5
	5	140.9±6.4	111.5±8.0
	7.5	151.8±7.0	126.7±7.1
Low $[Ca]_o$ reperfusion group (n = 8)			
EDP‡	0.5	17.3±2.4	29.6±5.2
	1	13.9±1.9	26.3±4.7
	2	11.3±1.5	19.8±3.9
	5	9.9±1.5	16.0±3.6
	7.5	10.6±1.4	16.8±3.6
DP	0.5	41.1±6.9	23.9±3.5
	1	75.0±8.5	52.2±6.4
	2	115.8±6.0	92.6±4.5
	5	145.8±5.6	123.1±5.6
	7.5	149.6±6.4	125.8±6.3
Pooled initial control (n = 27)			
EDP	0.5	21.2±1.4	
	1	16.0±1.2	
	2	13.7±0.9	
	5	12.2±1.0	
	7.5	12.4±1.0	
DP	0.5	33.5±3.1	
	1	69.8±4.4	
	2	107.1±3.4	
	5	143.5±6.7	
	7.5	152.2±7.2	

Significance levels (*$P < 0.001$, ‡ $P < 0.05$) refer to comparisons between each individual experimental intervention and the pooled initial control data.

MCAP was then measured. In 5 of 13 hearts devoted to this protocol, myocardial high-energy phosphate compounds and pH were measured simultaneously with phosphorus NMR.

In the nonischemic control group, the objective was to account for any time-dependent deterioration of function that might occur even in the absence of ischemia and reperfusion. After an initial control phase identical to that in the stunned group, hearts were perfused with the standard perfusate ($[Ca]_o$ = 2 mM) for 35 min without ischemia (instead of 15 min of global ischemia and 20 min of reperfusion). Ca_o responsiveness was then determined for a second time, followed by measurement of MCAP after exposure to ryanodine.

In the third protocol, the initial control and ischemic periods were identical to those described for stunned hearts. The only difference was in the $[Ca]_o$ of the reperfusion solution. Hearts were reperfused stepwise with low-$[Ca]_o$ solutions, first 0.1 mM $[Ca]_o$, then 0.2 and 0.5 mM $[Ca]_o$, and finally with 2 mM $[Ca]_o$ solution. The duration of perfusion was 7 min in each solution. After equilibration in 2 mM $[Ca]_o$ reperfusate, the responsiveness to Ca_o was evaluated and tetanic pressure was measured as in the other two protocols. In three of eight hearts devoted to this protocol, metabolic changes in the myocardium were measured simultaneously using P-NMR.

Statistical analysis. Data are presented as mean±SEM. Statistical analysis was performed using paired or nonpaired t test and the analysis of variance, including multivariate analysis of variance to compare Ca_o sensitivity and responsiveness in different experimental groups (21, 22). Data from the initial control phase of all hearts (n = 27) was pooled to yield control data for statistical tests (Table I).

Results

Stability of ventricular function in the nonischemic control group. Fig. 3 shows typical records of left ventricular pressure obtained in a nonischemic control heart (Fig. 3 *A*) and in a stunned heart (Fig. 3 *B*). In the nonischemic control, there was little change in Ca_o responsiveness between the first and second evaluations (compare left and center panels, Fig. 3 *A*). In contrast, DP was decreased at all $[Ca]_o$ after ischemia and reperfusion (center panel, Fig. 3 *B*). The right-hand panels show records of tetani at MCAP in control (Fig. 3 *A*) and in stunned (Fig. 3 *B*) hearts. MCAP was also depressed in the stunned heart compared with that in the nonischemic control.

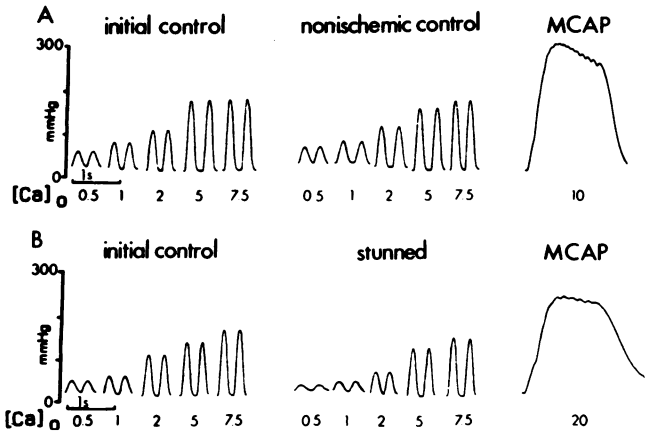

Figure 3. Ca_o responsiveness and MCAP in nonischemic control (A) and in a stunned heart (B). Records of pressure at various $[Ca]_o$ are shown during the initial control phase, after a 35-min nonischemic period (i.e., 35 min of perfusion without ischemia; A, nonischemic control) or after stunning (20-min reperfusion after 15 min of global ischemia; B, stunned). The right panels show tetani demonstrating MCAP in nonischemic control (A) and after stunning (B).

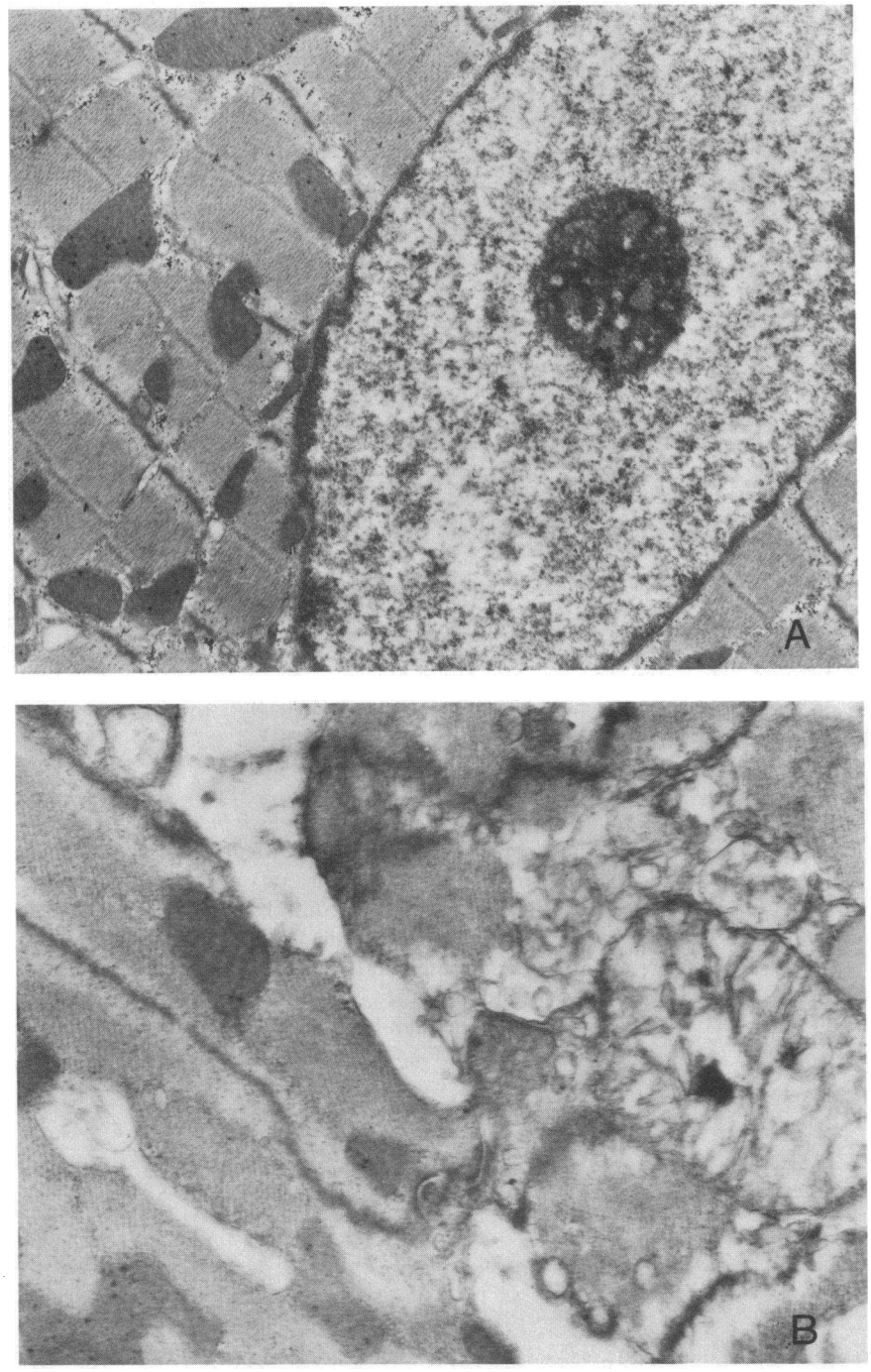

Figure 4. Histopathology of stunned myocardium. Both electron micrographs were taken from the same heart. (A) The vast majority of myocytes showed normal ultrastructure; mitochondria with tightly packed cristae, abundant glycogen stores, and sarcomeres arrested in diastole with prominent I bands. (× 8,000) (B) Rare, isolated myocytes (< 1%) were abnormal. The cell on the right shows swollen mitochondria with disrupted criatae and dense inclusions and disrupted sarcomeres. This cell adjoins a normal myocyte on the left. An intact intercalated disk can be seen between the two cells (× 15,000).

The stability of ventricular function Fig. 3 *A* exemplifies was a consistent finding in all nonischemic control experiments. Table I shows the results from the initial control stage and from the second evaluation of Ca_0 responsiveness in the nonischemic group; EDP and DP in the nonischemic control phase were not significantly different from the values in the initial control ($P > 0.60$ for EDP and $P > 0.20$ for DP). This result confirms that cardiac function in the nonischemic group was quite stable. Therefore, the average of MCAP in the nonischemic group (270 ± 15.3 mmHg) was used to normalize DP in the initial control when normalization was required. As expected, ventricular function was comparable in all groups during the initial control phase (Table I; $P > 0.10$ for EDP and $P > 0.15$ for DP), justifying the pooling of initial control data from all hearts for statistical tests (see Methods).

Morphological analysis in myocardium after brief global ischemia. Morphological damage of myocardium in stunned hearts was evaluated with light and electron microscopy. Four hearts were chosen randomly from the stunned group, and tissue from both the subendocardial and the subepicardial regions was examined. By light microscopy, stunned myocardium appeared normal. In particular, there was no contraction band injury indicative of reperfusion damage. Electron microscopy revealed that all four hearts exhibited predominantly normal ultrastructure, as Fig. 4 *A* illustrates. Two hearts showed isolated myocytes with mild mitochondrial swelling (figure not shown), which

comprised < 1% of total myocytes. One heart also showed rare cells with more severe injury; again almost all myocytes were normal, but a few isolated cells had contraction bands with disrupted sarcomeres and markedly swollen mitochondria with disrupted cristae and abnormal inclusion densities (Fig. 4 *B*). These abnormal myocytes were always isolated and surrounded by normal myocytes. The overall paucity of histologic damage is consistent with the morphological criteria for stunned myocardium (4).

Ca_0 responsiveness and MCAP in stunned myocardium. The experiment Fig. 3 illustrates suggests that Ca_0 responsiveness and MCAP are both depressed in stunned hearts. Fig. 5 summarizes the results from the initial control and stunned group. Fig. 5 *A* shows Ca_0-responsiveness curves in control (*solid squares*) and in stunned (*solid triangles*) hearts. The force of contraction of the twitch was significantly depressed at all $[Ca]_0$ after stunning ($P < 0.001$).

DP during tetani is also depressed after stunning, as Fig. 5 *B* illustrates. Tetanic pressure in the nonischemic control hearts (*solid squares*) and in the stunned hearts (*solid triangles*) increases as $[Ca]_0$ is increased. Tetanic pressure was depressed at all $[Ca]_0$ in the stunned hearts compared with the nonischemic control group ($P < 0.001$). As is evident from Fig. 5 *B*, the curves of tetanic pressure vs. $[Ca]_0$ also reach saturation at different $[Ca]_0$ in the control and stunned groups. DP during tetani in the nonischemic control group saturates at $[Ca]_0$ = 10 mM, in agreement with previous results (14). In the stunned group, higher $[Ca]_0$ (15–20 mM) was required for DP to reach saturation. We confirmed that saturation had indeed been reached in stunned hearts by adding Bay K 8644 (300 nM), a Ca channel agonist, to 20 mM $[Ca]_0$ perfusate. The rightmost point for the stunned group shows that tetanic pressure did not increase further after the addition of Bay K 8644, although twitch pressure did increase (Fig. 5 *B*, inset). The saturating pressure (i.e., MCAP) was different in the two groups: in stunned myocardium, MCAP was significantly decreased compared with that in nonischemic control (216 ± 10.2 mmHg in stunned heart vs. 270 ± 15.3 mmHg in control, $P < 0.05$).

Does the observed decrease in MCAP account fully for the decrease in responsiveness to $[Ca]_0$ in stunned myocardium? If so, the Ca_0 responsiveness curves in Fig. 5 *A* should differ only by a constant scaling factor determined by the ratio of the MCAPs measured in Fig. 5 *B*. To answer this question, we plotted Ca_0 sensitivity (i.e., Ca_0 responsiveness normalized by corresponding MCAP) in the control (*solid squares*) and stunned (*solid triangles*) hearts (Fig. 5 *C*). The Ca_0 sensitivity of stunned myocardium is significantly decreased, i.e. shifted to higher $[Ca]_0$, after stunning ($P < 0.01$ vs. control). We also checked the Ca_0 sensitivity as inferred from tetani, for the following reason. Ca_0 sensitivity determined from twitch contractions might, in principle, reflect not only active tension generation but also relaxation processes; tetanic force, which reaches steady state, requires no such consideration. When the relationship between tetanic pressure and $[Ca]_0$ (Fig. 5 *B*) was normalized by corresponding MCAP, a shift to higher $[Ca]_0$ was again evident for the stunned group ($P < 0.01$). Therefore, these results indicate that the contractile depression in stunned myocardium is characterized by decreased responsiveness to $[Ca]_0$: this change in Ca_0 responsiveness is due to both a decrease in MCAP and decreased sensitivity to $[Ca]_0$.

Coronary resistance after stunning. What is the underlying cause of the changes in MCAP and Ca_0 sensitivity in stunned myocardium? Coronary perfusion is known to be a major primary determinant of DP in normal hearts (23). In reperfused myocardium, it has been suggested that occlusion of small vessels by postischemic cell swelling or by microthrombi may cause stunning (no-reflow phenomenon; 5). If 20% or more of terminal

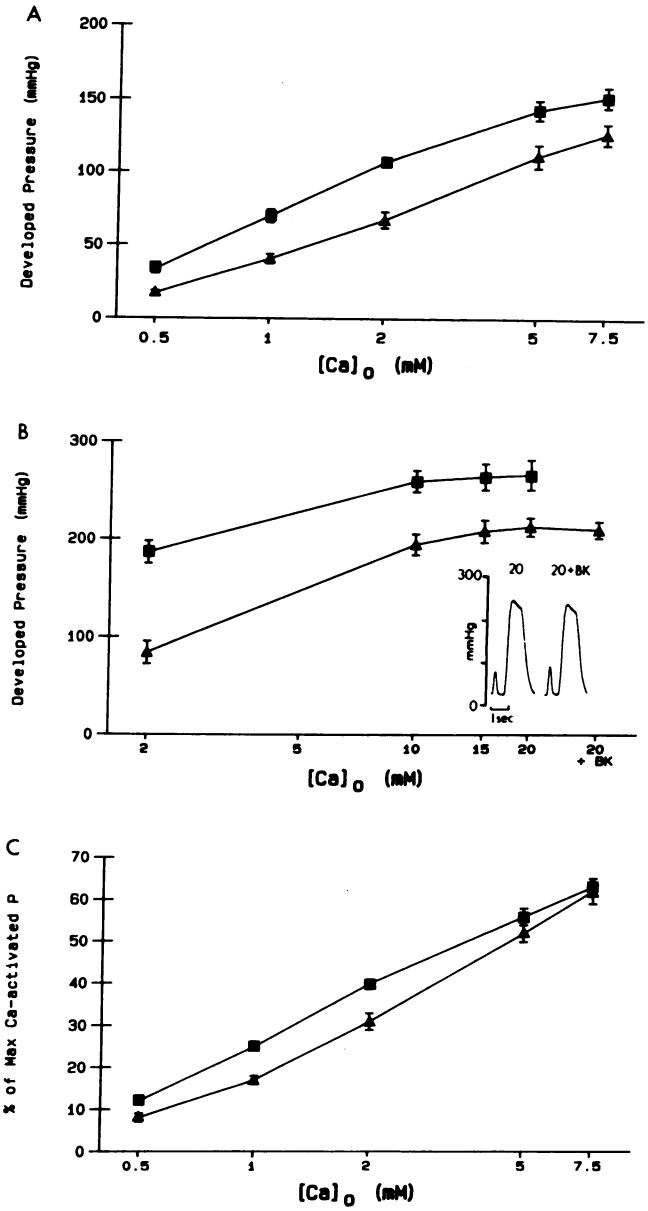

Figure 5. Characterization of contractile dysfunction in stunned myocardium. (A) Ca_0 responsiveness during initial control (*solid squares*) pooled from all experiments (n = 27) and after stunning (*solid triangles*) in 13 hearts. (B) The DP during tetani measured in 6 nonischemic control hearts (*solid squares*) and in 13 stunned hearts (*solid triangles*). Inset shows the saturation of DP during tetani confirmed by adding Bay K 8644 (300 nM) to 20 mM $[Ca]_0$ perfusate. Note that the addition of Bay K 8644 increased the DP during twitches but not during tetani. *C*, Ca_0 sensitivity in control (*solid squares*, the Ca_0 responsiveness during initial control [i.e., *solid squares* in A] normalized by the average MCAP in nonischemic hearts) and in stunned myocardium (*solid triangles*, the Ca_0 responsiveness in stunned hearts normalized by the corresponding MCAP). All data are shown as mean±SEM.

arterioles were affected, recent results with quantitative coronary microembolization (24) predict a measurable increase in overall coronary resistance. We checked to see whether a change in coronary resistance accompanies myocardial stunning. Coronary resistance was defined as coronary perfusion pressure (CPP)/CFR, and CPP was determined from the difference between the perfusion pressure and the EDP. As Table II shows, coronary resistance measured in seven hearts did not change significantly after stunning compared with control ($P > 0.60$). This lack of change in coronary resistance argues against a major degree of microvessel occlusion in our preparation, although a relatively small effect cannot be excluded.

Myocardial metabolites as potential causes of a change in Ca_0 responsiveness. An increase of Pi in myocardium has been reported to decrease maximal Ca^{2+}-activated force and myofilament Ca^{2+} sensitivity in skinned papillary muscle (25). Acidosis has qualitatively similar effects (26, 27). An increase of either [Pi] or [H^+] can also depress MCAP in intact heart (8). Are [Pi] or [H^+] increased in stunned myocardium, so that one or the other might account for the depressed Ca^{2+} responsiveness? Both [Pi] and pH are known to return to normal levels very rapidly in regional models of stunned myocardium (9), but we have re-examined this question in our preparation using ^{31}P-NMR. Fig. 6 shows typical ^{31}P-NMR spectra obtained during the initial control phase (Fig. 6 *A*), global ischemia (Fig. 6 *B*), and after reperfusion (Fig. 6 *C*) in the stunning protocol. During global ischemia, [Pi] increased markedly (from 1.33 μmol/g wet wt at control to 8.50 μmol/g wet wt), concomitant with the development of intracellular acidosis (from pH 7.06 to 6.27). [PCr] decreased from 7.33 μmol/g wet wt to an undetectably low level, and [ATP] decreased from 2.71 to 1.71 μmol/g wet wt. Upon reperfusion, the [Pi], [PCr], and pH quickly returned to control levels (or, indeed, overshot slightly), although [ATP] remained depressed.

The quick recovery of [Pi], [PCr], and pH evident in the NMR spectra of Fig. 6 was observed consistenty. Fig. 7 shows pooled data for the concentrations of energy-related phosphorus compounds in myocardium and intracellular pH (pH_i) from five hearts. Intramyocardial levels of Pi, PCr, and pH_i showed no significant change after stunning ($P > 0.05$ vs. control), whereas there was a persistent decrease in [ATP] ($P > 0.05$ vs. control). Therefore, neither [Pi] nor pH_i can be responsible for the persistent changes in Ca_0 sensitivity and MCAP in stunned myocardium. In this regard, the mechanism of stunning differs fundamentally from that of contractile dysfunction during hypoxia, in which the accumulation of Pi plays a major role (8). In stunned myocardium, the depletion of ATP remains as a possible causal factor for contractile depression. The role of [ATP] is re-examined later.

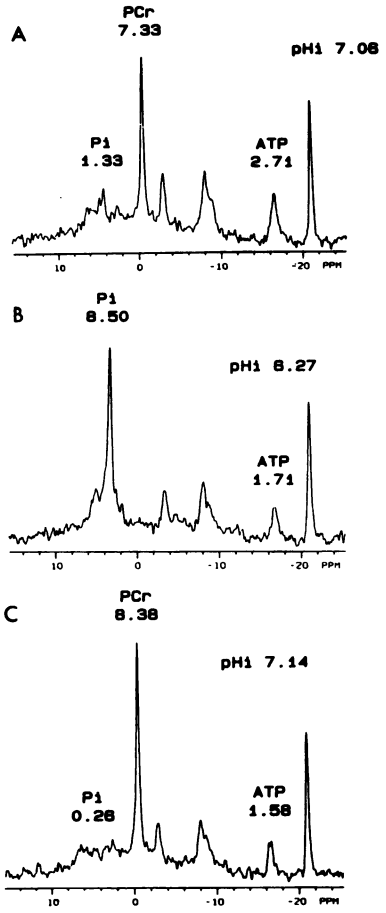

Figure 6. Phosphorus NMR spectra (5-min acquisition time) measured in stunning protocol. (A) During initial control phase. (B) During 10–15 min of global ischemia. (C) During 10–15 min of reperfusion. PCr is undetectable during global ischemia (B).

Table II. Coronary Resistance before and after Stunning

Hemodynamic parameters	Before ischemia (mean±SEM)	After ischemia (mean±SEM)
Systolic LVP*	118.9±8.4 (*mmHg*)	95.4±6.2
End-diastolic LVP‡	11.6±2.2 (*mmHg*)	22.6±5.3
Perfusion pressure	91.1±4.0 (*mmHg*)	98.7±7.3
Coronary resistance	2.41±0.08 (*mmHg/ml per min*)	2.31±0.16

Data before ischemia and after ischemia were determined before the measurement of Ca_0 responsiveness during the initial control phase and during steady state after reperfusion, respectively, from seven hearts in the stunning protocol.
* $P < 0.01$.
‡ $P < 0.05$; before vs. after ischemia.

Effect of reperfusion with low-$[Ca]_0$ solution on stunning. Irreversible reperfusion injury in myocardium is characterized histopathologically by contraction band necrosis, mitochondrial calcium phosphate deposition, and the disruption of the intercalated disks by hypercontraction (28), suggesting that catastrophic cellular Ca overload is the mechanism of injury. Cellular Ca overload will occur if Ca influx pathways that are inhibited during ischemia are suddenly re-activated upon reperfusion. One specific hypothesis (29, 30) involves Na–Ca exchange. This pathway normally extrudes Ca from the cell at the expense of the transsarcolemmal Na gradient. During ischemia, the Na–K ATPase is inhibited by the metabolic suppression, so that intracellular Na accumulates. At the same time, Na–Ca exchange is inhibited by the intracellular acidosis. Upon reperfusion, pH_i returns to normal very rapidly (Fig. 7), removing the inhibition of Na–Ca exchange. When the Na–Ca exchange is re-activated under these conditions, intracellular [Na] is still elevated, and consequently the Na gradient drives Ca into the cells. While Ca overload can be directly deleterious to cardiac function (31), indirect actions, such as activation of phospholipases or stimulation of free radical production (32), can unleash a cascade of injury that might persist even after the Ca overload itself has resolved.

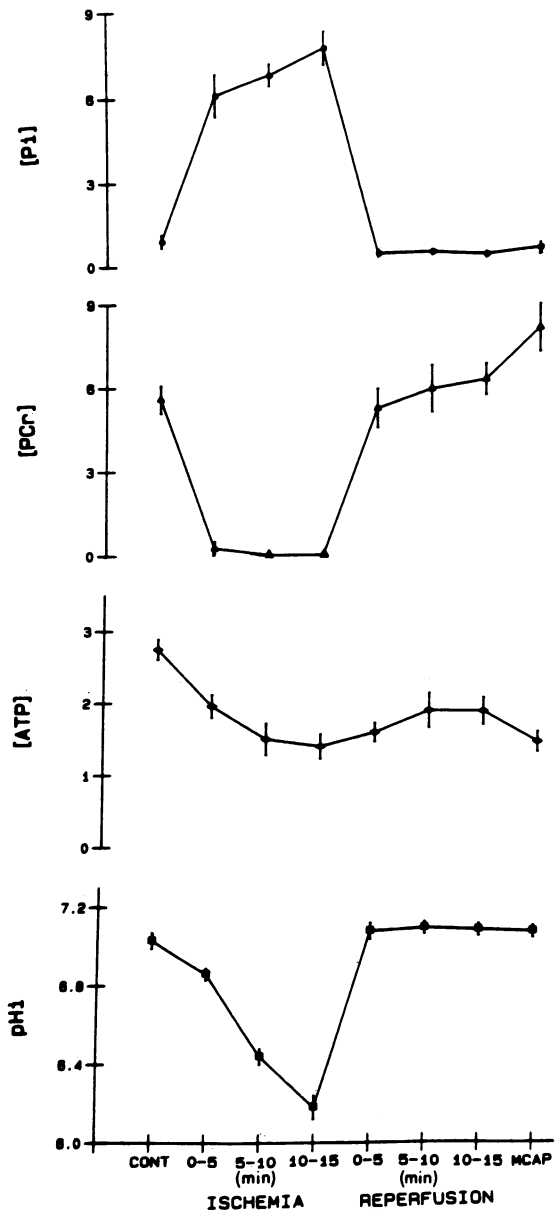

Figure 7. Intramyocardial levels of phosphate compounds and pH in stunned myocardium. Mean and SEM of *Pi, PCr,* ATP, and pH_i from five hearts are shown during initial control (*CONT*), at various times (in minutes) during global ischemia and reperfusion, and during the measurement of *MCAP.*

In stunned myocardium, the histologic changes are not pathognomonic for Ca-mediated injury, as they are in reperfusion after longer periods of ischemia. Nevertheless, stunning may share the same basic mechanism, with the major difference being one of degree of injury. To test the hypothesis that stunning involves overload of intracellular Ca^{2+}, we subjected hearts to the same ischemic protocol as in the conventional stunning group. The only variable was the $[Ca]_0$ of the reperfusate. We reasoned as follows: if massive Ca influx occurs upon reperfusion and is required for stunning, then reduction of $[Ca]_0$ in the reperfusate will decrease stunning by decreasing the driving force for Ca influx. A similar experimental strategy has been found to decrease the irreversible injury that occurs after much longer periods of ischemia (14).

Eight hearts were reperfused initially with 0.1 mM $[Ca]_0$ solution; $[Ca]_0$ was increased thereafter in several increments before returning to control level (2 mM; see Methods for details). Fig. 8 *A* compares pooled results for Ca_0 responsiveness during initial control (*solid squares*) and after low-$[Ca]_0$ reperfusion (*solid circles*). Unlike hearts reperfused with 2 mM $[Ca]_0$ solution (Fig. 5 *A*), there was no significant depression of Ca_0 responsiveness after low-$[Ca]_0$ reperfusion compared with initial control ($P > 0.05$). Recovery of twitch responsiveness was significantly higher than in routinely stunned hearts ($P < 0.05$). Similarly, MCAP (257±4.3 mmHG) was statistically indistinguishable from nonischemic controls ($P > 0.05$), but significantly higher than in stunned hearts ($P < 0.05$). This is not to say that low-$[Ca]_0$ reperfused hearts showed no evidence of stunning: saturation of DP during tetani occurred at a higher $[Ca]_0$ (15–20 mM) than in control, as was the case for stunned myocardium (Fig. 5 *B*). Likewise, hearts reperfused with low-$[Ca]_0$ solution showed a significant increase in EDP, as in stunned myocardium (Table I). Nevertheless, the excellent preservation of Ca_0 responsiveness and MCAP shows that recovery of function is virtually complete after low-$[Ca]_0$ reperfusion. The protective effect of low-$[Ca]_0$ reperfusion indicates a major, if not dominant role for cellular Ca overload in the pathogenesis of stunning.

Three hearts chosen randomly from the low-$[Ca]_0$ reperfusion group were examined by light and electron microscopy and showed comparable structure to that of the hearts in the stunned group. Although this would imply that the morphological changes did not correlate with the extent of functional recovery, histologic abnormalities were so unusual even in stunned hearts that a small improvement with low-$[Ca]_0$ reperfusion cannot be excluded.

Role of ATP depletion in contractile dysfunction after stunning. In our phosphorus NMR experiments, we found that depletion of ATP was the only sustained metabolic change in stunned myocardium (Figs. 6 and 7). Does the beneficial effect of reperfusion with low $[Ca]_0$ result from a reduction of ATP depletion under these conditions? The beneficial effects of pretreatment with Ca-channel blockers in reperfusion injury have been attributed to improved ATP levels (13). Alternatively, the lower level of ATP might be an epiphenomenon and not a causal factor in stunning, as suggested previously (10, 11). Fig. 9 shows pooled results for myocardial concentrations of phosphorus compounds and pH_i from three hearts reperfused with low-$[Ca]_0$ solution. All three hearts gave consistent results: the changes in [Pi], [PCr], [ATP], and pH_i during ischemia and reperfusion were quite similar to those observed in hearts reperfused with standard solution (compare Fig. 7). In particular, the only change observed after low-$[Ca]_0$ reperfusion was a decrease in [ATP] comparable to that in stunned hearts, despite the marked difference in functional recovery between the two groups.

The lack of a unique correlation between [ATP] and MCAP is readily apparent in Fig. 10. The control hearts (*solid square*) show high [ATP] (2.81±0.11 μmol/g wet wt) and high MCAP (270±15.3 mmHg). Stunned myocardium (*solid triangle*) shows low [ATP] (1.47±0.15 μmol/g wet wt) and low MCAP (216±10.2 mmHg). These two observations alone are consistent with a cause-and-effect relationship between [ATP] and contractile function. However, the hearts reperfused with low-$[Ca]_0$ solution (*solid circle*) also show a significantly lower [ATP] (1.63±0.17 μmol/g wet wt) than in control ($P < 0.05$), but much higher MCAP (257±4.3 mmHg) than in stunned hearts ($P < 0.05$). It is readily apparent that there is no unique relation between [ATP]

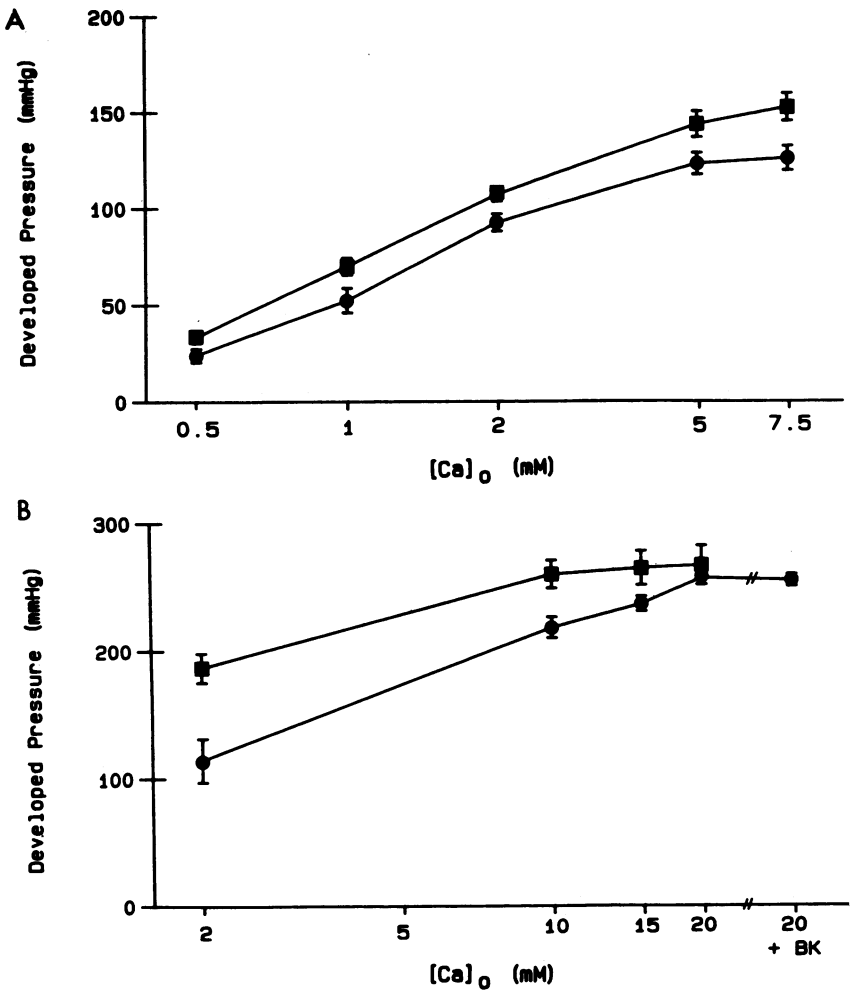

Figure 8. Contractile function in hearts reperfused with low-$[Ca]_0$ solution. (*A*) Ca_0 responsiveness during initial control (*solid squares; n* = 27) and in eight hearts reperfused with low-$[Ca]_0$ solution after 15 min global ischemia (*solid circles*). (*B*) DP during tetani measured in six nonischemic control hearts (*solid squares*) and in the hearts reperfused with low-$[Ca]_0$ solution (*solid circles; n* = 8). All data are shown as mean±SEM.

and contractile function, which indicates that ATP depletion is not the cause of myocardial stunning. This result is consistent with the observation in skinned ventricular muscle that a change in [ATP] of this magnitude has no effect on the sensitivity of the contractile machinery to Ca^{2+} (33–35), and with the observation that [ATP] does not correlate with the decrease of MCAP during the early phase of hypoxia in whole hearts (8).

Discussion

We have found that stunned myocardium is characterized by two distinctive features: (*a*) a decrease in MCAP and (*b*) a decrease in Ca_0 sensitivity. The physiological meaning of these alterations can be understood most easily by referring to Fig. 11, which illustrates the fundamental concepts that underlie our analysis. Fig. 11 *A*, which depicts the situation in a normal heart, shows a schematic $[Ca^{2+}]$ transient on the left, and a twitch contraction on the right. The $[Ca^{2+}]$ transient and force are linked by an arrow, which represents the $[Ca^{2+}]_i$–force relation (drawn above the arrow as it might appear in this normal tissue).

There are three distinct (but not mutually exclusive) ways in which the twitch can become depressed, as Fig. 11, *B–D* shows. A decrease in the amplitude of the $[Ca^{2+}]$ transient (Fig. 11 *B*, left panel) will, of course, decrease force, even if the Ca^{2+}–force relation remains unchanged. In contrast, Fig. 11 *C* illustrates the effect of keeping the $[Ca^{2+}]$ transient unchanged but decreasing myofilament Ca^{2+} sensitivity (as shown by the dashed $[Ca^{2+}]_i$–force relation, with its midpoint shifted to the right relative to control); in this case, less force is generated at any submaximal $[Ca^{2+}]_i$, but maximal force remains the same. Fig. 11 *D* shows yet a third possibility. Here, maximal force has been scaled down (as shown by the dashed $[Ca^{2+}]_i$–force relation above the arrow), but neither the $[Ca^{2+}]$ transient nor the midpoint of the $[Ca^{2+}]_i$–force relation has been changed. This or any of the other changes depicted in Fig. 11, *B–D* individually suffices to decrease twitch force; if two or more of these changes were present in combination, twitch force would be decreased even further.

Pathophysiologic basis of stunned myocardium. We can now interpret our results within this framework. First of all, our finding that MCAP is depressed in stunned hearts demonstrates unequivocally that the hypothesis in Fig. 11 *D* contributes to the contractile dysfunction.

We have also observed a decrease in Ca_0 sensitivity in stunned hearts. The underlying basis of a shift in Ca_0 sensitivity could be a decrease in the $[Ca^{2+}]$ transient (Fig. 11 *B*), a shift in myofilament Ca^{2+} sensitivity (Fig. 11 *C*), or a combination of these effects. Both possibilities are subsumed within our measured Ca_0 sensitivity. To distinguish explicitly whether the primary change is in the $[Ca^{2+}]$ transient or in myofilament Ca^{2+} sensitivity, reliable measurements of $[Ca^{2+}]_i$ in intact hearts will be required.

It is important to consider the strengths and limitations of

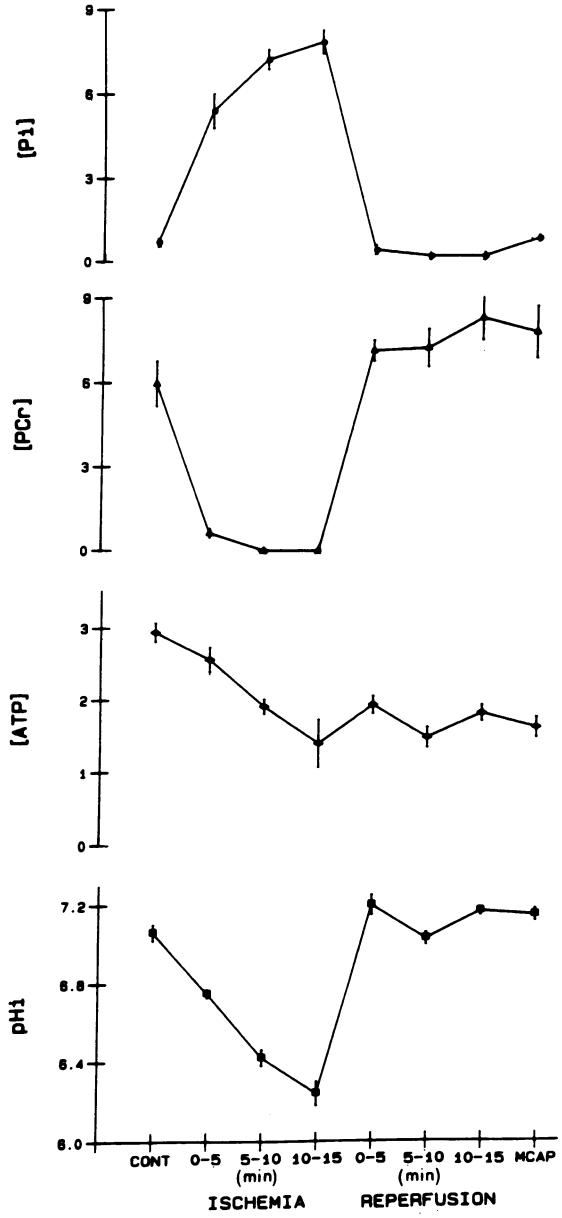

Figure 9. Intramyocardial levels of phosphate compounds and pH in hearts reperfused with low-$[Ca]_0$ solution. Mean and SEM of *Pi*, *PCr*, ATP, and pH_i from three hearts are shown during initial control (*CONT*), at various times (in minutes) during global ischemia and reperfusion, and during the measurement of *MCAP*.

our approach in terms of relating these parameters measured in whole hearts to the underlying cellular events. MCAP is directly comparable to maximal Ca^{2+}-activated force determined in papillary muscles (18) or in single-skinned cardiac cells (36). We have demonstrated previously that $[Ca^{2+}]_i$ and $[Ca]_0$ are directly correlated during tetani, and that $[Ca^{2+}]_i$ need not be known explicitly to determine MCAP (15).

The relationship between myofilament sensitivity and the Ca_0 sensitivity determined from Ca_0 responsiveness and MCAP in whole hearts does require consideration of possible changes in $[Ca^{2+}]$ transients. Ca_0 sensitivity reflects the net resultant of two cellular processes, the $[Ca^{2+}]$ transient and myofilament Ca^{2+} sensitivity. In the absence of changes in the $[Ca^{2+}]$ transient,

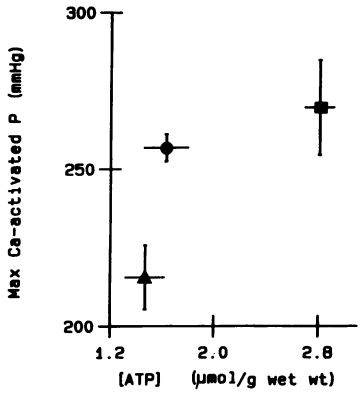

Figure 10. Relationship between intramyocardial ATP level and MCAP. Each point represents the mean±SEM of [ATP] and MCAP in the initial control (*solid square*), after stunning (*solid triangle*) or after reperfusion with low-$[Ca]_0$ solution (*solid circle*).

myofilament Ca^{2+} sensitivity and Ca_0 sensitivity are homologous. On the other hand, if the $[Ca^{2+}]$ transient were decreased after stunning, this could account for a decrease in Ca_0 sensitivity even if myofilament sensitivity were to remain unchanged. The only direct evidence available on this point comes from measurements of $[Ca^{2+}]$ transients during re-oxygenation after brief periods of hypoxia and metabolic inhibition in ferret papillary muscles (37). The $[Ca^{2+}]$ transients are similar or slightly greater in amplitude after reoxygenation in association with incomplete recovery of contractile force. If this is also true in postischemic, reperfused myocardium, then the decrease in Ca_0 sensitivity that we have measured would reflect an underlying decrease in myofilament Ca^{2+} sensitivity.

Contractile dysfunction during hypoxia and during reperfusion. We have previously used a similar approach to investigate the mechanism of early contractile failure in hypoxia (8). Unlike stunning, brief periods of hypoxia produce a dynamic, reversible

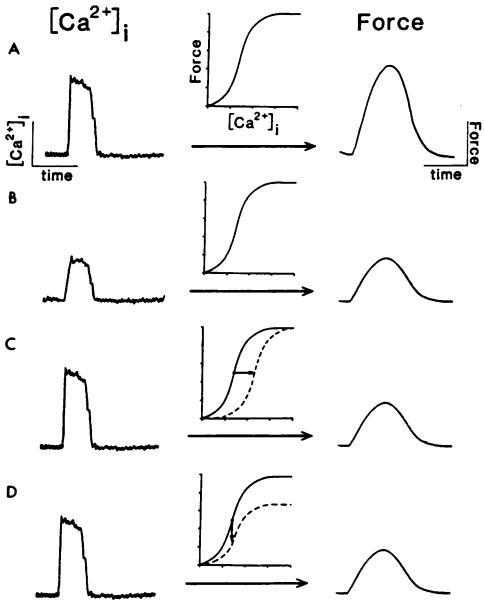

Figure 11. Schematic representation of $[Ca^{2+}]_i$ (left), force (right), and the $[Ca^{2+}]_i$–force relation. (A) Control. (B) Effect of decreasing the amplitude of the $[Ca^{2+}]$ transient. (C) Effect of a shift in myofilament Ca^{2+} sensitivity (compare control relation, drawn as a solid curve, to the shifted, dashed curve). (D) Effect of a decrease in maximal Ca^{2+}-activated force. See Discussion for details.

impairment of force generation, with concomitant clear-cut metabolic alterations. We found a strong correlation between the intracellular accumulation of Pi and the depression of MCAP during hypoxia. Pi is known to depress crossbridge cycling and force generation at the level of the myofibrils, making the observed correlation attractive as a possible causal link in the early contractile failure of hypoxia.

In contrast to hypoxia or ischemia, [Pi] is not appreciably altered in stunned myocardium. Indeed, the only metabolic deficit detectable by phosphorus NMR is a decrease in [ATP]. Although ATP depletion has profound consequences when extreme (i.e., when [ATP] < 5% of control), there is little evidence to support the idea that ATP depletion of 40–50% limits contractile function. Our results agree with previous observations that ATP depletion occurs in stunned myocardium, but is not the primary causal factor (10, 11).

Cellular mechanism of stunning. We have found that recovery of function after ischemia is markedly improved by reperfusion with low-$[Ca]_0$ solutions. These results are particularly striking in that the ischemic period in these hearts is not altered: pretreatment is not required for the preservation of function. This observation points out that the primary determinant of stunning is not ischemia, but rather reperfusion. This fundamental concept negates a number of simple theories of stunning that focus only on the ischemic period, such as the notion that ischemia-induced cell swelling may be sufficient to explain stunning (see reference 5 for references).

One way in which MCAP might be decreased by stunning would be if small scattered regions of myocardium undergo ischemic necrosis, so that there is a loss of contractile units. Such necrosis might conceivably be missed in a limited histologic analysis of stunned myocardium. Several observations argue against this idea. First of all, the improved functional recovery after low-$[Ca]_0$ reperfusion would necessarily restrict such a theory to reperfusion-induced necrosis, which is thought to occur only in cells irreversibly injured during the ischemic period (3). Secondly, patchy necrosis should be detectable by phosphorus NMR as an incomplete recovery of PCr and a persistent elevation of Pi, but we observed no such tendency (Figs. 6, 7, and 9). Finally, loss of contractile units could account for a decrease in MCAP, but not for the observed decrease in Ca_0 sensitivity.

Although the results with low-$[Ca]_0$ reperfusion implicate Ca overload in the pathogenesis of stunning, the exact mechanism of cellular injury is not clear. Ca-related injury after longer periods of ischemia is easier to understand, given the extensive histologic (28) and metabolic (14) damage. The histologic changes of stunning are mild, and metabolic recovery is nearly complete. Even in the absence of histologic damage, Ca overload is characterized by asynchronous oscillations of intracellular $[Ca^{2+}]$ throughout cells (38–40). These $[Ca^{2+}]_i$ oscillations cause subcellular inhomogeneity of contractile activation and consequently attenuate overall force generation (31). Nevertheless, $[Ca^{2+}]_i$ oscillations alone cannot explain the contractile dysfunction of stunned hearts: Ca-overloaded myocardium responds with a paradoxical negative inotropic effect as $[Ca]_0$ is increased (17, 41), unlike stunned hearts in which force increases as $[Ca]_0$ is elevated (Fig. 5 *A*).

Two other possible mechanisms of injury would invoke the activation of phospholipases and/or the production of free radicals by increased $[Ca^{2+}]_i$ (see reference 32 for review). Cellular Ca overload may act (alone or in synergy with other factors) as the trigger for a turbulent cascade of events which, once initiated, may not require a continued increase in $[Ca^{2+}]_i$. Several lines of evidence support a role for free radicals in stunning. Recent measurements using electron spin resonance have detected a considerable increase in myocardial-free radical concentrations upon reperfusion after only 10 min of global ischemia (42). In addition, superoxide dismutase, an inhibitor of oxygen-derived free radical production, has been found to decrease the degree of stunning (43), as has the oxygen-free radical scavenger 2-mercaptopropionylglycine (44). Phospholipid degradation products and free radicals attack cell membranes, including those of the SR. It is possible that Ca^{2+} release from the SR is impaired by stunning; one of the few electron microscopic hallmarks of stunned canine myocardium examined several days after reperfusion is a moth-eaten appearance thought to represent edema of the SR (3). More experimental work will be required to elucidate the relative contributions of $[Ca^{2+}]_i$, free radicals, and phospholipid degradation products to stunning.

Finally, it has not escaped our attention that reperfusion with low-$[Ca]_0$ solutions may be useful in improving the recovery of cardiac performance in clinical settings such as angioplasty or cardiac surgery, in which the reperfusate $[Ca^{2+}]$ can be modified selectively.

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