Alpha Adrenergic-mediated Accumulation of Calcium in Reperfused Myocardium

ARJUN D. SHARMA, JEFFREY E. SAFFITZ, BENJAMIN I. LEE, BURTON E. SOBEL, and PETER B. CORR, *Cardiovascular Division, Washington University School of Medicine, St. Louis, Missouri 63110*

ABSTRACT Reperfusion of ischemic myocardium is associated with increases in total myocardial calcium (Ca^{+2}), which may influence the ultimate extent of ischemic damage as well as the development of arrhythmias. Since reperfusion is also associated with enhanced α-adrenergic responsivity, this study was performed to determine the potential interactions between α-adrenergic receptors and myocardial calcium during reperfusion. Cats were subjected to 35 min of left anterior descending coronary artery occlusion and 10 min of reperfusion. Total myocardial calcium was measured by atomic absorption spectrometry. Intracellular calcium was calculated from measurements of extracellular space ([^{3}H]inulin). In control animals with reperfusion, total calcium increased from 0.32±0.03 to 0.65±0.05 mmol/100 g dry tissue ($P < 0.0001$), while intracellular calcium increased from 0.15±0.03 to 0.40±0.05 mmol/100 g dry tissue ($P < 0.001$). Pretreatment with the α-adrenergic blocking agents phentolamine or prazosin prevented the increase in total and intracellular calcium. Phentolamine and the aqueous soluble α_1-adrenergic antagonist BE-2254 administered as late as 2 min before reperfusion similarly attenuated the increase in tissue calcium. Although administration of BE-2254 2 min before reperfusion failed to block the reperfusion-induced increase in extracellular space, the increase in calculated intracellular calcium was prevented. β-Adrenergic blockade with propranolol partially attenuated but did not prevent an increase in total tissue calcium. Labetalol, a combined α- and β-adrenergic blocking agent completely blocked the increase in tissue calcium during reperfusion. Additional experiments performed after 70 min of ischemia with reperfusion demonstrated a 49% attenuation of the increase in tissue calcium with α-adrenergic blockade. Electron microscopy with pyroantimonate and x-ray microprobe analysis demonstrated a large increase in calcium precipitate in mitochondria after reperfusion in untreated animals. Though α-adrenergic blockade prevented the calcium deposition in mitochondria, other criteria of ischemia persisted. Thus, α-adrenergic blockade specifically prevents the increase in intracellular calcium during reperfusion in reversibly injured tissue, independent of alterations in extracellular space and tissue water.

INTRODUCTION

Reperfusion of myocardium after ischemia elicits malignant ventricular dysrhythmia (1), sarcolemmal disruption (2), and accumulation of intracellular calcium (3), primarily in mitochondria (4), which may contribute to irreversible injury (5). Reperfusion also is associated with enhanced α-adrenergic responsiveness manifested electrophysiologically (6). Furthermore, α-adrenergic blockade is antiarrhythmic and antifibrillatory in reperfused myocardium, independent of altered regional perfusion or hemodynamics (6). The enhanced α-adrenergic responsiveness is associated with a twofold reversible increase in α_1-adrenergic receptors (7). In other tissues, such as vascular smooth muscle (8) and liver (9), α-adrenergic stimulation may evoke calcium accumulation. However, the potential role of α-adrenergic stimulation on calcium accumulation in myocardium has not been elucidated. Several observations suggest its possible importance during ischemia and reperfusion including: (*a*) myocardial contractility is augmented (10) and the calcium-mediated slow inward current (I_{si})[1] is potentiated (11) by

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[1] *Abbreviation used in this paper:* I_{si}, slow inward current; LAD, left anterior descending.

α- rather than β-adrenergic stimulation in depressed myocardium; and (*b*) the incidence of ventricular tachycardia and ventricular fibrillation associated with reperfusion is attenuated by verapamil (12, 13), an agent with α-adrenergic, as well as voltage-dependent calcium channel blocking properties (14, 15). For these reasons, we undertook this study to assess effects of α- compared with β-adrenergic blockade on the accumulation of calcium associated with reperfusion.

METHODS

Animal preparation. Adult cats were anesthetized with intramuscular ketamine (12.5 mg/kg) and intravenous α-chloralose (75 mg/kg) administered via a femoral vein catheter. After endotracheal intubation, the animals were ventilated with a 90% O_2/10% room-air mixture with a Harvard ventilator (Harvard Apparatus Co., Inc., The Ealing Corp., Millis, MA), which maintained arterial P_{O_2}, P_{CO_2}, and pH at physiological levels. Adequate muscular relaxation was obtained with a single injection of decamethonium bromide (0.25 mg/kg, i.v.). Body temperature was maintained at 37°C with an esophageal thermistor probe connected to a thermostatically controlled heating lamp. Femoral arterial pressure was monitored with a Statham p23dB transducer (Statham Instruments, Inc., Oxnard, CA) and Gould Brush transducer amplifier (Gould Inc., Instruments Div., Cleveland, OH). The arterial pressure and surface electrocardiogram were displayed with a Gould model 260 recorder (Gould Inc.) The heart was exposed by a left thoracotomy with excision of the second to fourth ribs, and suspended in a pericardial cradle. The left anterior descending (LAD) coronary artery was dissected free at its bifurcation from the left main coronary artery and a snare placed around the coronary artery proximal to all branches. Coronary occlusion was accomplished by tightening the snare for 35 min, and reperfusion was induced by removal of the snare for 10 min. In previous studies, the adequacy of the reperfusion and the presence of hyperemia with this procedure was verified by measurement of regional myocardial blood flow with radiolabeled microspheres (6).

Measurement of tissue calcium. Calcium is present both in extracellular and intracellular compartments. Thus, relative changes in tissue spaces may, by themselves, alter total tissue calcium content. Intracellular calcium content was measured by determining total tissue calcium and subtracting the amount attributable to extracellular calcium. Extracellular calcium encompasses both intravascular and interstitial calcium. Intravascular calcium concentration and content were determined by measurement of plasma calcium and intravascular volume. However, most of the extracellular calcium is in the interstitial space and can be estimated based on the plasma calcium concentration and the interstitial fluid volume. Thus, intracellular calcium content is derived from

$$[Ca]_{ic} = [Ca]_{tot} - ([Ca]_{iv} + [Ca]_{is}), \quad (1)$$

where ic is intracellular fluid compartment, tot is total tissue compartment, iv is intravascular compartment, and is is interstitial fluid compartment.

Each of the compartmental concentrations was expressed as millimoles per 100 gram dry tissue. Dry weight was chosen, since wet weight would be affected by variability of tissue water content and could alter the values of total tissue calcium content. However, total tissue water only increased up to 2% in the reperfused zone (Results). Either before coronary occlusion or after 35 min of coronary occlusion plus 10 min of reperfusion, a sample of arterial blood was taken with a 1-ml aliquot placed in a preweighed vial. The heart was excised and arrested for 10 s in saline cooled with dry ice. A small (0.5 g) transmural segment at the center of the ischemic zone and a corresponding segment of normal, posterior left ventricular free wall were removed. The epicardium and endocardium were trimmed rapidly, the midmyocardial tissue was blotted to remove surface blood and saline, and the sample was placed in a preweighed vial and minced. The blood and tissue samples were weighed and lyophilized to determine dry weight (~100 mg). The percentage of water was calculated by subtraction. The dried samples were completely digested with concentrated nitric acid followed by perchloric acid at 100°C. Lanthanum chloride was added to prevent interference in the determination of calcium by atomic absorption spectrometry (IL 251 Atomic Absorption Spectrometer, Instrumentation Laboratory, Inc., Lexington, MA). The total calcium per sample was determined and corrected for sample weight to yield millimoles per 100 g dry weight.

The intravascular volume was determined with autologous erythrocytes labeled with ^{51}chromate (n = 12). An 8-ml venous sample of blood was incubated with sodium ^{51}chromate (2 μCi) (Mallinckrodt Inc., St. Louis, MO) for 20 min. The cells were centrifuged, washed three times, and resuspended in saline to remove free chromate. They were then injected intravenously. After a 10-min equilibration, an 8-ml venous sample was obtained. The heart was excised simultaneously, and a midmyocardial segment obtained as previously described. The radioactivity in the samples was determined with a well gamma counter (Picker-Pace, Picker Corp., Cleveland, OH) and expressed as disintegrations per minute. The specific gravity of tissue and plasma were assumed to be identical. The percentage of intravascular space (IVS) was determined from the equation

$$\text{IVS (\%)} = \frac{\text{Tissue dpm}}{\text{Tissue weight (g)}} \times \frac{1}{\text{Plasma dpm (ml)}} \times 100. \quad (2)$$

Total intravascular calcium (iv) was calculated and expressed per 100 g dry wt:

$$[Ca]_{iv} = \frac{\text{Blood calcium (mmol/ml)} \times \text{IVS\%}}{1 - H_2O_T}, \quad (3)$$

where H_2O_T is the fraction of the tissue that is water.

Interstitial calcium content was calculated after total extracellular space and plasma calcium had been measured. Extracellular space was determined with [^{3}H]inulin (sp act = 5.6 Ci/mmol, Amersham Corp., Arlington Heights, IL). Although calcium binds to cell membranes, this factor was assumed to be constant. Thus, extracellular calcium space was equated to [^{3}H]inulin extracellular space. [^{3}H]Inulin (50 μCi) was injected intravenously just before reperfusion. After 10 min of reperfusion, 8 ml venous blood were withdrawn and the heart excised. Because a 10-min [^{3}H]inulin equilibration period may not be adequate to attain steady state, in an additional three animals, [^{3}H]inulin was injected at the time of reperfusion and the hearts excised 30 min later. The blood was centrifuged at 1,500 rpm for 10 min in a Beckman TJ-6 centrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, CA), and aliquots of the plasma were placed in scintillation vials. Simultaneously, the myocardial tissue from the reperfused and the normal region of the left lateral ventricular wall was separately trimmed, minced, weighed,

and lyophilized. Wet weight, dry weight, and percentage of tissue water were obtained. The dry tissue samples were digested in 1 ml KOH (1 N) with the aid of a sonicator (S/50 American Electrical Heater Co., Detroit, MI). The alkali was neutralized with 1 ml HCl (1 N) and aliquots of digested tissue were placed in scintillation vials. Aquasol (10 ml) (New England Nuclear, Boston, MA) was added to each scintillation vial and disintegrations per minute determined by scintillation spectrometry (Delto 300, Searle Analytical Inc., Des Plaine, IL). The percentage of extracellular space (ECS), as assayed with [^{3}H]inulin was calculated from the equation

$$\%ECS = \frac{\text{dpm/g tissue} \times \text{fraction plasma } H_2O \times DF}{\text{dpm/ml plasma}} \times 100, \quad (4)$$

where DF is the Donnan factor (1.0 for inulin). For determination of serum calcium, arterial blood samples were obtained before coronary occlusion and immediately before reperfusion. Each was placed in a sealed vacuum tube. After clotting, the samples were centrifuged at 1,500 rpm for 10 min (Beckman TJ-6), and the serum aspirated into a sealed tube and cooled to 4°C before calcium determination. Total serum calcium was determined by atomic absorption spectrometry (model 503, Perkin-Elmer Corp., Norwalk, CT), and ionized calcium was determined with the use of an ion-specific electrode (SS20 ionized calcium analyzer, Orion Research Inc., Cambridge, MA). Total interstitial calcium content was calculated from the equation

$$[Ca]_{is} = \frac{\{ECS - [IVS \times (1 - HCt)]\} \times \text{serum calcium (mmol/ml)} \times 0.714}{1 - H_2O_T} \quad (5)$$

The extracellular value was corrected for plasma volume (1 − Hct), where Hct is hematocrit.

The distribution of electrolytes across a semipermeable membrane is sometimes related to concentration by the Donnan factor. However, calcium is highly protein bound, and thus the Donnan factor does not directly or precisely determine the calcium content in the interstitial space. Accordingly, an experimentally derived constant (0.714) was used that related total serum calcium to total interstitial calcium (16). Intracellular calcium concentration, $[Ca]_{ic}$, was then calculated as shown in Eq. 1.

Ultrastructural localization of calcium. Subcellular localization of calcium was assessed with the pyroantimonate technique, modified from Legato and Langer (17). Potassium pyroantimonate was dissolved in water (2% wt/vol) and the pH adjusted to 7.6 with the addition of dilute acetic acid. Crystalline osmium tetroxide was added to a final concentration of 1% and sucrose was added to 2.8%, the pH adjusted to 7.5 with dilute potassium hydroxide, and the solution filtered through a 1.2-μm membrane filter.

At the conclusion of each experiment, the heart was excised rapidly and the aorta cannulated with polyethylene tubing. The heart was immediately fixed by perfusion with 100 ml of the pyroantimonate-osmium tetroxide solution at 4°C. Tissue from control and ischemic regions was minced into blocks (~1 mm^3) and fixed for an additional 2 h in the pyroantimonate-osmium tetroxide solution at 4°C. The tissue was dehydrated in a graded series of ethanol and embedded in Spurr's low viscosity resin. In each experiment, five randomly selected blocks from normal and ischemic regions were sectioned with a Sorvall Porter-Blum ultramicrotome and diamond knife (Du Pont de Nemours, E. I. & Co., Inc./Sorvall Instruments Div., Newton, CT). Approximately 15 sections from each block were collected on copper mesh grids, stained with uranyl acetate and lead citrate, coated with carbon, and examined in a JEOL 100 C electron microscope at 60 KeV (JEOL USA, Electron Optics Div., Peabody, MA).

Two approaches were taken to identify calcium in the precipitate found in pyroantimonate-perfused control and ischemic myocardium. First, hearts were perfused with 100 ml of a solution composed of (in millimolars) NaCl (130), $MgCl_2$ (1), NaH_2PO_4 (0.435), $NaHCO_3$ (14), glucose (5.5), and EGTA (5) at pH = 7.4, before perfusion with the standard pyroantimonate solution (17). Tissue was processed for electron microscopy. The absence of the precipitate in tissue by perfusion with EGTA indicated that calcium was a constituent in the pyroantimonate reaction product in the non-EGTA-perfused tissue.

With the second approach, elemental composition of precipitate in control and ischemic tissue was determined by x-ray microprobe analysis. Microprobe analysis was performed on 100-nm unstained sections mounted on carbon mesh grids. Selected regions were scanned for 200 s at 40 KeV in a JEOL 100 CX electron microscope and analyzed by a PGT-1000 energy dispersive x-ray microanalyzer. To determine the presence of calcium as a constituent in the precipitate, x-ray spectra were obtained from the region of the junction of the A-band and I-band in 15 individual sarcomeres in normal myocardium. In ischemic tissue, x-ray spectra were obtained by scanning individual particles of electron-dense material in mitochondria (five spectra from separate sections of ischemic tissue with and without α-adrenergic blockade).

Because of the close proximity of the K(α) peak of calcium and the L(α) peak of antimony, the presence of calcium in spectra from tissue precipitate was confirmed by subtracting the spectral contribution of antimony. A pure antimony spectrum was obtained by scanning submicron particles of sodium pyroantimonate prepared by adding an excess of sodium chloride to 2% potassium pyroantimonate, washing the resultant precipitate extensively, and applying the finely ground material to a carbon-coated grid. The L(α) peak of the pure antimony spectrum was adjusted to the same level as the combined calcium and antimony peak of the tissue precipitate spectrum and the pure antimony contribution was subtracted.

Statistics. Differences between treatment groups were tested for significance with a one-way analysis of variance yielding an F statistic. With a general linear models procedure the least-squares means from this model were computed to allow performance of a t test between the treatment groups.

Protocols. 13 groups of animals were studied. In group 1, sham-operated animals were used, on whom coronary occlusion was not performed. In group 2, LAD coronary artery occlusion was maintained for 35 min and reperfusion was not performed. In group 3, LAD coronary artery occlusion was maintained for 35 min, followed by 10 min of reperfusion. Groups 4 to 9 underwent the same protocol as group 3, but selected drugs were administered. In group 4, phentolamine (5 mg/kg) was administered intravenously 20 min before coronary occlusion. In group 5, α_1-adrenergic blockade was induced with prazosin (0.1 mg/kg) administered intravenously 20 min before coronary occlusion. In group 6, animals received D,L-propranolol (0.75 mg/kg) 20 min before coronary occlusion and were paced from the right atria at 200 beats/min corresponding to the sinus rate in control, untreated animals, and animals treated with α-adrenergic blockade. In group 7, animals received labetalol (5 mg/kg), a combined α- and β-adrenergic blocking agent

20 min before coronary occlusion. In group 8, animals received phentolamine (5 mg/kg) 33 min after ischemia and 2 min before reperfusion. In group 9, animals received BE-2254 (0.4 mg/kg), a selective aqueous soluble α_1-adrenergic antagonist 33 min after ischemia and 2 min before reperfusion. In group 10, animals underwent LAD coronary artery occlusion for 35 min, followed by 30 min of reperfusion. In group 11, animals underwent 35 min of LAD coronary artery occlusion with 30 min of reperfusion, but BE-2254 (0.4 mg/kg) was administered 2 min before reperfusion. In group 12, the LAD coronary artery was occluded for 70 min, followed by 10 min of reperfusion. Group 13 received phentolamine (5 mg/kg) 20 min before occlusion of the LAD coronary artery for 70 min, followed by 10 min of reperfusion. All doses of antagonists were selected on the basis of the relative log dose ratio minus one (DR_{10}) for each drug. Adequate α- or β-adrenergic blockade was sustained throughout the coronary occlusion and reperfusion interval, as demonstrated by persistent attenuation of phenylephrine-induced increases in systemic arterial pressure (α) or isoproterenol-induced increase in sinus rate (β).

RESULTS

Adrenergic influences on tissue calcium in reperfused myocardium. In group 1, sham-operated animals without coronary occlusion, there was no significant difference in total tissue calcium between anterior (0.377±0.04 mmol/100 g dry wt) and posterior (0.380±0.06 mmol/100 g dry wt) (±SEM) left ventricle.

After 35 min of ischemia without reperfusion (group 2) (n = 4), there was no significant difference between the anterior (ischemic) (0.370±0.084 mmol/100 g dry wt) and posterior (normal) zones (0.329±0.049 mmol/100 g dry wt). In control untreated animals (group 3), reperfusion for 10 min beginning 35 min after coronary occlusion resulted in a greater than twofold increase (mean = 104%) of total tissue calcium in the reperfused tissue with no alteration of tissue calcium in adjacent normal zones (Fig. 1). Reperfusion results also in a reproducible incidence of ventricular dysrhythmia in this preparation, characterized in detail previously (1, 6). Reperfusion for 30 min after 35 min ischemia also resulted in a significant increase in tissue calcium in the ischemic zone (0.760±0.171) compared with the normal zone (0.341±0.049, Table I). Thus, reperfusion for either 10 or 30 min after 35 min of

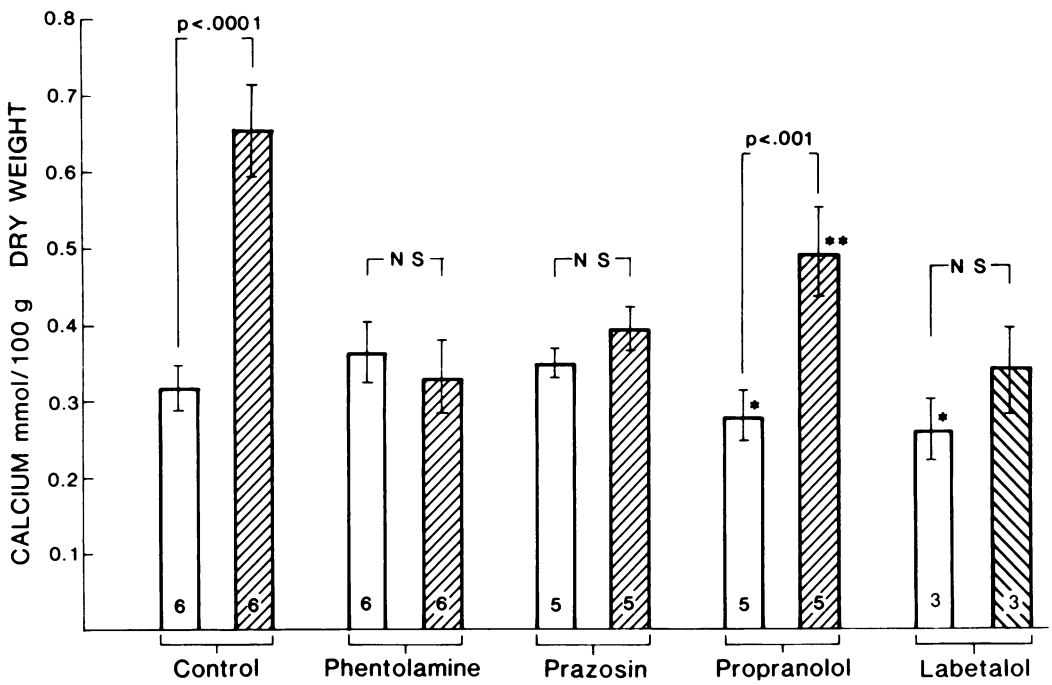


FIGURE 1 Influence of pretreatment with phentolamine (α_1- and α_2-adrenergic blocking agent), prazosin (α_1-adrenergic blocking agent), D,L-propranolol (β-adrenergic blocking agent), and labetalol (a combined α- and β-adrenergic blocking agent) on total tissue calcium in both normal (□) and reperfused regions subjected to 35 min of coronary occlusion and 10 min of reperfusion (▨). The values are means±SEM. Numbers within histograms indicate number of animals in each group. The only other significant differences are: *, significantly less ($P < 0.03$) compared with normal regions in phentolamine-treated animals; **, significantly less than control untreated animals ($P < 0.04$) and significantly greater than phentolamine-treated animals ($P < 0.03$).

TABLE I
Effect of Prolonged Ischemia and Reperfusion on Total Tissue Calcium

	n	Normal region	Ischemic region
		mmol/100 g	
35 min ischemia + 30 min reperfusion			
Untreated, group 10	5	0.345±0.049	0.760±0.171*
BE 2254, group 11	4	0.291±0.025	0.415±0.063*
70 min ischemia + 10 min reperfusion			
Untreated, group 12	6	0.331±0.027	1.291±0.275*
Phentolamine, group 13	5	0.309±0.030	0.652±0.163*

Data represent mean±SEM.
* $P < 0.05$ compared with corresponding control region.

ischemia produced similar effects on the total tissue calcium, indicating that recovery of the increase did not occur over time. Pretreatment with either phentolamine (group 4) or the specific α_1-antagonist prazosin (group 5) completely attenuated the increase in total tissue calcium induced by reperfusion (Fig. 1). In contrast, pretreatment with a β-adrenergic blocking agent, D,L-propranolol (group 6), attenuated the increase only partially (74% increase). Thus, although propranolol reduced total tissue calcium in normal myocardium compared with values in phentolamine-treated animals, a significant and comparable percentage increase in total tissue calcium was evident with reperfusion (Fig. 1). In group 7 animals, labetalol, a combined α- and β-adrenergic blocking agent, not only reduced total tissue calcium in normal myocardium (similar to the effect of β-adrenergic blockade), but also blocked the increase in reperfused zones (similar to the effect of α-adrenergic blockade).

The effect of prolonged ischemia was examined in groups 12 and 13. In untreated cats rendered ischemic for 70 min followed by reperfusion for 10 min, the highest increase in total tissue calcium was seen (Table I). Phentolamine attenuated significantly but did not prevent an increase in tissue calcium with this duration of ischemia (Table I). Light microscopy of tissue from these animals demonstrated subendocardial hemorrhage and contraction banding suggestive of areas of irreversible cell damage. Thus, α-blockade is still partially effective when the duration of ischemia is sufficient to produce some irreversible damage.

The concentration of free, nonprotein-bound calcium in the extracellular space exceeds that in the intracellular compartment. Increases of total tissue calcium after reperfusion could therefore reflect either an increase in the intracellular calcium concentration or an increase in the size of the extracellular space without an increase in the concentration of intracellular calcium. Thus, the total extracellular space was

TABLE II
Serum Calcium, Myocardial Tissue Water, and Plasma Water Content

	n	Serum calcium	H_2O_{tissue}	H_2O_{plasma}
		mg/dl	%	
Sham-operated, group 1				
Posterior zone	5	8.04±0.19	78.24±0.81	92.0±0.4
Anterior zone			78.23±0.67	
Control, group 3				
Normal zone	5	7.72±0.26	78.50±0.37	92.1±0.5
Reperfused zone			80.37±0.50	
Phentolamine, group 4				
Normal zone	5	7.90±0.36	77.09±0.44	92.4±0.2
Reperfused zone			79.61±0.35	
Prazosin, group 5				
Normal zone	5	8.44±0.65	78.03±0.55	93.5±0.2
Reperfused zone			78.84±0.41	
BE 2254, group 9				
Normal zone	5	8.20±0.42	78.05±0.28	93.5±0.2
Ischemic zone			79.61±0.45	

In the sham-operated animals, the anterior zone refers to the area that would have been ischemic.

measured with [^{3}H]inulin. Measurements of serum calcium, tissue water, and plasma water used to calculate extracellular and intracellular calcium concentrations are shown in Table II. Under control conditions, the extracellular space contained 25±3% of total tissue volume. After 35 min of ischemia and 10 min of reperfusion there was a significant increase in the measured extracellular space in the reperfused region (35±4%), with no significant change in the [^{3}H]inulin space in the adjacent normal zone (Fig. 2). Likewise, in three additional experiments, reperfusion for 30 min resulted in similar changes in extracellular space (32±3%), indicating that the 10-min equilibration period for [^{3}H]inulin was sufficient to attain steady state. However, the 40% increase in the extracellular space does not account fully for the observed increase in total tissue calcium shown in Fig. 1. Thus, the calculated intracellular calcium increased nearly 160% after reperfusion (Fig. 3). On the basis of Eq. 5, the increase in extracellular space from 25 to 35% of tissue volume would account for only a 19% increase in total tissue calcium, rather than the measured 107% increase of

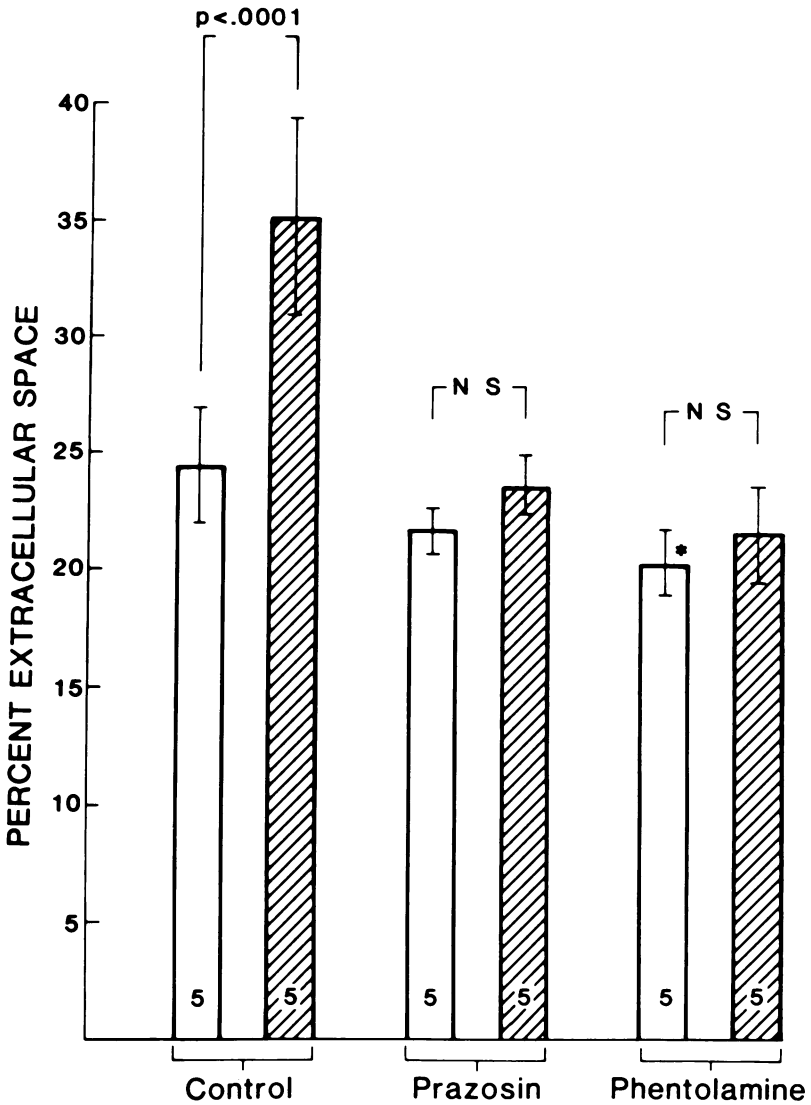


FIGURE 2 Influence of pretreatment with prazosin or phentolamine on the percentage of extracellular space determined by [^{3}H]inulin in normal (□) and reperfused regions subjected to 35 min of coronary occlusion and 10 min of reperfusion (▨). The values are means±SEM, and numbers within histograms indicate number of animals in each group. *, not significantly different ($P > 0.05$) from normal region of control or prazosin-pretreated animals.

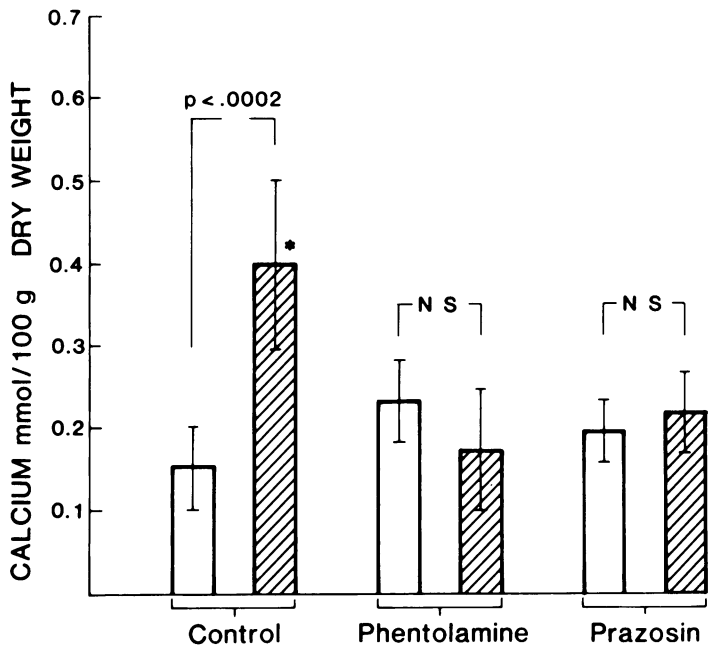


FIGURE 3 The calculated changes in intracellular calcium in control (□), phentolamine, and prazosin-pretreated animals (n = 5 in each group) subjected to 35 min of coronary occlusion and 10 min of reperfusion (▨). The values in the normal zone were not significantly different in each group. The values are means±SEM. *, significantly ($P < 0.002$) increased over corresponding values in the phentolamine- or prazosin-treated animals.

total tissue calcium. The marked increase in total tissue calcium reflects increases in intracellular calcium. The increase in intracellular calcium of 160% was completely attenuated by pretreatment with either phentolamine or prazosin (Fig. 3). Pretreatment with either prazosin or phentolamine also prevented the increase in the extracellular space with reperfusion (Fig. 2).

Some have found that verapamil protects against reperfusion-induced increases in tissue calcium, but only when it is administered before ischemia (18). Therefore, we evaluated effects of α-adrenergic blocking agents administered intravenously 33 min after coronary occlusion but 2 min before reperfusion. Total tissue calcium was measured in each region 10 min after reperfusion. Phentolamine significantly attenuated the increase of total tissue calcium otherwise induced by reperfusion (Fig. 4). Because prazosin is relatively insoluble in neutral aqueous media and must be administered over several minutes to avoid alterations of plasma pH, its effects were not assessed in this particular protocol. However, an investigational, water soluble α_1-adrenergic blocking agent, BE-2254 (Sandoz Pharmaceutical, Hanover, NJ) (19) given 33 min after occlusion and 2 min before reperfusion also attenuated the increase in total tissue calcium seen with reperfusion. However, a small but significant increase was seen (Fig. 4).

Since α_1-adrenergic blockade with BE-2254 2 min before reperfusion greatly attenuated but did not completely prevent the increase in total tissue calcium, the effect of this intervention on calculated intracellular calcium was assessed with [^{3}H]inulin to measure the extracellular space (Fig. 5). Administration of BE-2254 2 min before reperfusion failed to alter the increase in extracellular space induced by reperfusion but completely blocked the increase in intracellular calcium. Thus, the small but significant increase in total tissue calcium in animals treated with BE-2254 2 min before reperfusion was due entirely to the expansion in extracellular space rather than an increase in intracellular calcium content (Fig. 5). This result also indicates that α_1-adrenergic blockade can attenuate myocyte calcium uptake during reperfusion independent of any effect on expansion of the extracellular compartment.

Subcellular localization of calcium. To further assess the influence of α-adrenergic blockade on intracellular calcium accumulation induced by reperfusion, the subcellular distribution of calcium was analyzed by the pyroantimonate method. Normal nonischemic tissue was obtained from the posterolateral left ventricle after 35 min of ischemia and 10 min of reperfusion of the anterior left ventricle. In normal tissue (Fig. 6 *A*, *B*) electron dense granules of antimonate precipitate were concentrated in regions of calcium

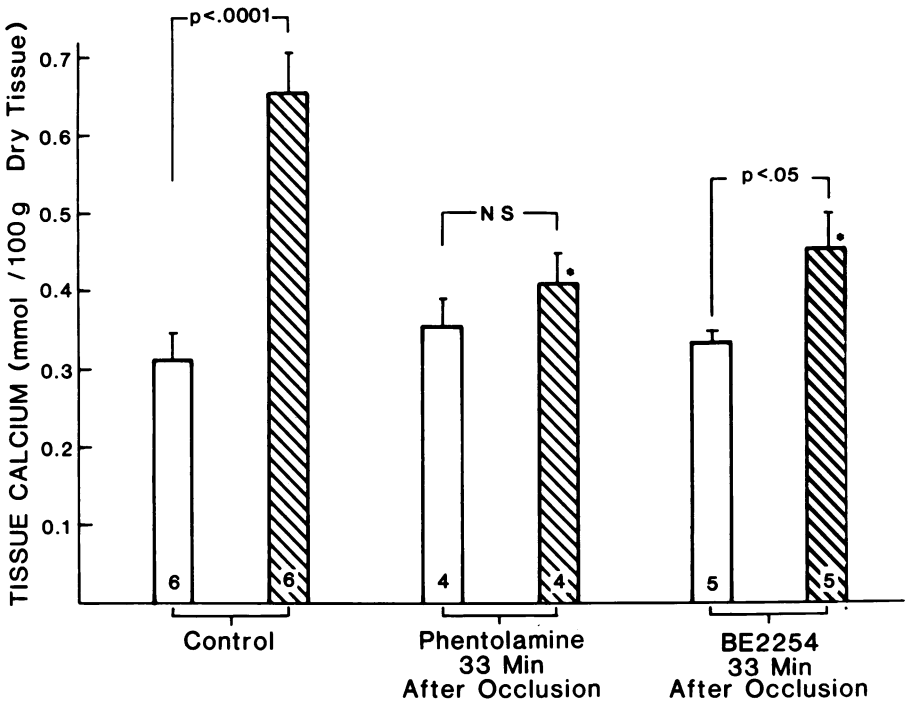


FIGURE 4 Influence of phentolamine or BE-2254 administered intravenously 2 min before reperfusion on total tissue calcium in normal (□) and reperfused regions. Occlusion was for 35 min followed by 10 min of reperfusion (▨). The values are means±SEM, and numbers within histograms indicate the number of animals in each group. °, significantly less ($P < 0.001$) than the corresponding value in untreated, control animals.

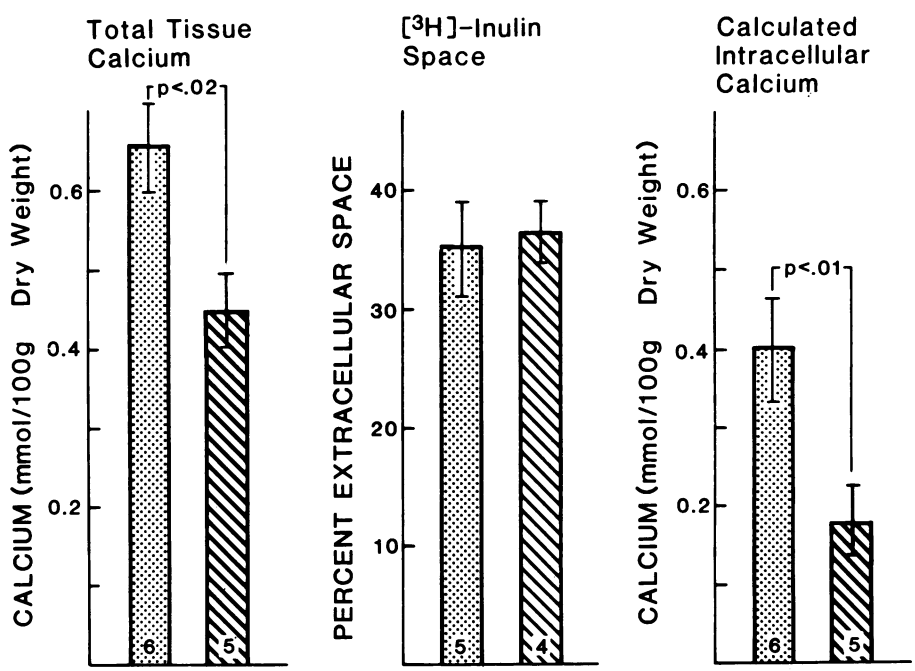
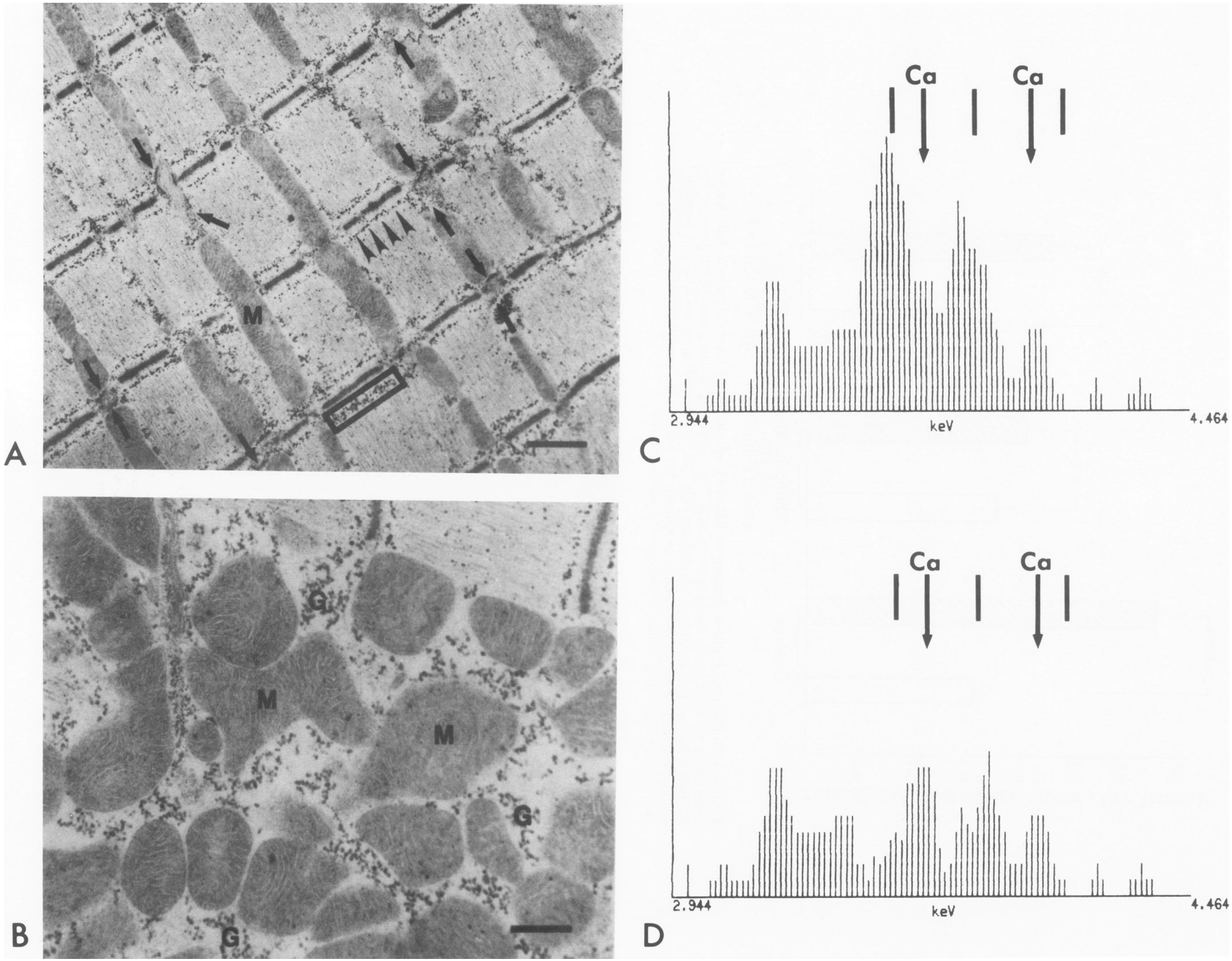


FIGURE 5 Measurements of total tissue calcium (*left*), percent extracellular space (*center*) and calculated intracellular calcium (*right*) in reperfused regions of control untreated animals (□) and animals treated with BE-2254 (0.4 mg/kg) 2 min before reperfusion (▨). The numbers within the histograms indicate number of animals in each group. Values are means±SEM.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sequestration: at the site of calcium-dependent myofibrillar ATPase (A band-I band junction) and in the lateral sacs of sarcoplasmic reticulum adjacent to transverse tubules. X-ray microprobe analysis of precipitate at the A-I junction confirmed the presence of calcium (Fig. 6 *C* and *D*). Mitochondria, however, were uniformly devoid of precipitate. Ultrastructurally, the myocardium appeared normal. Identical patterns of the distribution of precipitate have been observed by others in normal hearts (17, 20). The abundance of glycogen indicated normal aerobic metabolism at the time of tissue fixation (Fig. 6 *B*).

Tissue from reperfused regions of the same heart as that shown in Fig. 6 demonstrated significant differences in the distribution of the antimonate precipitate (Fig. 7 *A*, *B*). In contrast to the case in normal tissue, mitochondria contained numerous coarse granules of calcium antimonate, verified by x-ray microprobe analysis. The tissue was virtually devoid of glycogen, indicative of a shift to anaerobic glycolytic metabolism resulting from the antecedent ischemia. The myocardium did not, however, exhibit ultrastructural features of irreversible damage, such as disruption of the sarcolemma, mitochondrial inclusions, or contraction bands. Thus, despite the accumulation of calcium in mitochondria, only morphological features of reversible ischemic injury were observed.

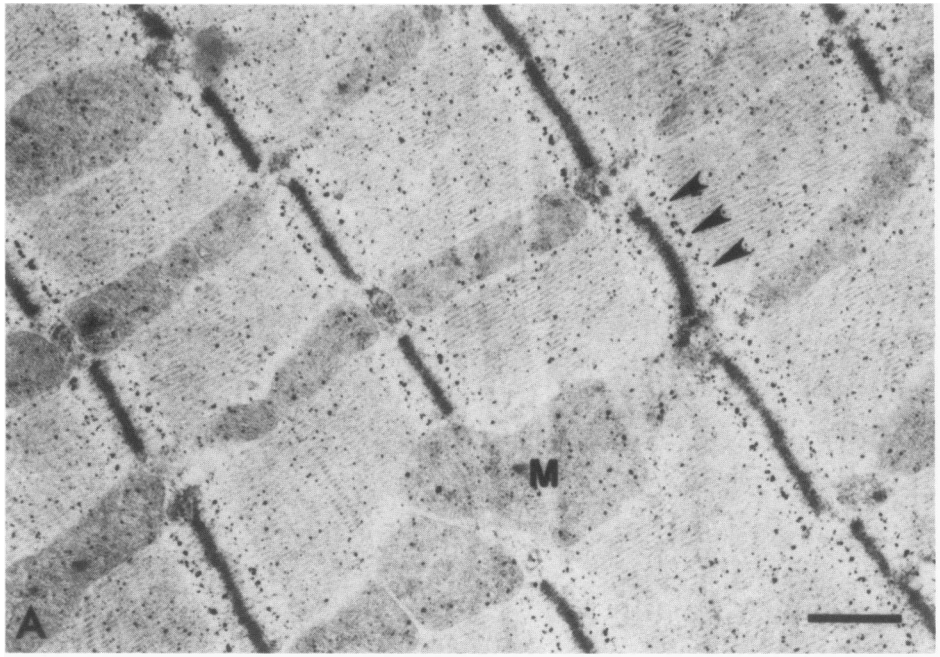
Reperfused myocardium from animals pretreated with phentolamine was ultrastructurally similar to reperfused tissue in cats unexposed to α-adrenergic blockade (Fig. 8). Glycogen was depleted, again indicative of a shift to anaerobic metabolism induced by the antecedent ischemia. Ultrastructural features of irreversible ischemic injury were absent. However, no significant deposition of antimonate precipitate was observed in mitochondria. Spectra obtained by x-ray microprobe analysis of occasional minute electron-dense particles in mitochondria failed to identify calcium or antimony (Fig. 8). Thus, despite manifestations indicative of comparably severe antecedent ischemia, such as glycogen depletion, no calcium accumulation occurred in mitochondria when animals were protected with α-adrenergic blockade.

Virtually all of the electron-dense precipitate at the A-I junction and sarcoplasmic reticulum in normal myocardium was absent in tissue perfused with EGTA before pyroantimonate-osmium tetroxide perfusion (Fig. 9). Electron-dense precipitate located in vascular spaces contained sodium and antimony only. The absence of myocardial precipitate after EGTA perfusion supports the x-ray microprobe analysis indication that the cellular precipitate contained calcium.

DISCUSSION

Reperfusion of ischemic myocardium provides substrate and removes potentially deleterious metabolites that accumulate during the period of ischemia (21). However, reperfusion also is accompanied by arrhythmia and marked electrolyte shifts. Calcium accumulation is prominent (3, 22) and has been implicated in subsequent cell death (5) as well as in arrhythmogenesis (23). The present results confirm an increase in myocardial tissue calcium content with reperfusion. The extent of calcium accumulation increases with the duration of preceding ischemia and length of reperfusion (22). These findings were also demonstrated in our feline model of ischemia. In studies of prolonged ischemia with associated irreversible damage, pharmacologic prevention of calcium influx has been unsuccessful (24). This is likely a reflection of irreversible membrane damage induced by prolonged intervals of ischemia. Alpha adrenergic blockade with phentolamine was only partially effective in preventing tissue calcium uptake when a prolonged duration of ischemia was studied. In contrast, after shorter periods of ischemia, which did not produce ultrastructural evidence of irreversible ischemic injury, α-adrenergic blockade

FIGURE 6 (*A*) An electron micrograph of normal myocardium fixed by perfusion with pyroantimonate-osmium tetroxide. Calcium antimonate precipitate is present at the junctions of the A and I bands (arrowheads) and in the lateral sacs of sarcoplasmic reticulum adjacent to transverse tubules (arrows). Mitochondria (M) are devoid of pyroantimonate precipitate. Glycogen (G) is present. Bar = 1.0 μm. (*B*) An electron micrograph of the perinuclear zone of a normal cardiac myocyte fixed by perfusion with pyroantimonate-osmium tetroxide. Mitochondria (M) exhibit no pyroantimonate precipitate. Abundant glycogen (G) is indicative of normal aerobic metabolism at the time of tissue fixation. Bar = 0.5 μm. (*C*, *D*) X-ray microprobe spectra of pyroantimonate precipitate. The region indicated by a rectangle in *A* was scanned. Peaks at energy levels characteristic of antimony (short lines) and calcium (Ca-labeled arrows) are present (*C*). A pure antimony spectrum, obtained by scanning submicron particles of sodium pyroantimonate, was adjusted to a maximum peak height equivalent to that antimony energy levels in *C*. After subtraction of the pure antimony spectrum, peaks characteristic of calcium persist (*D*). Low levels of energy present in *D* at the antimony peak positions represent background.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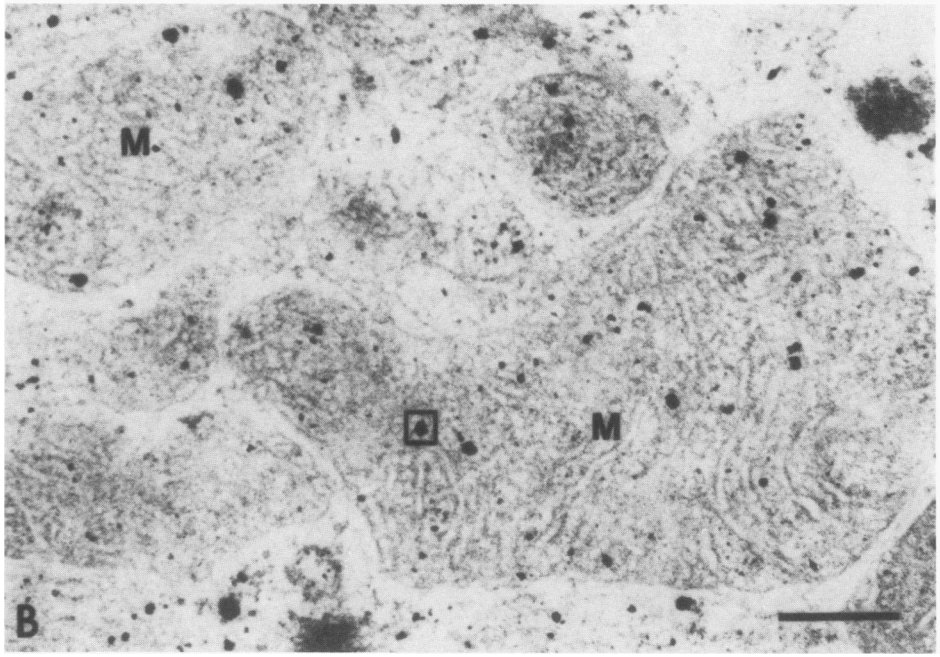
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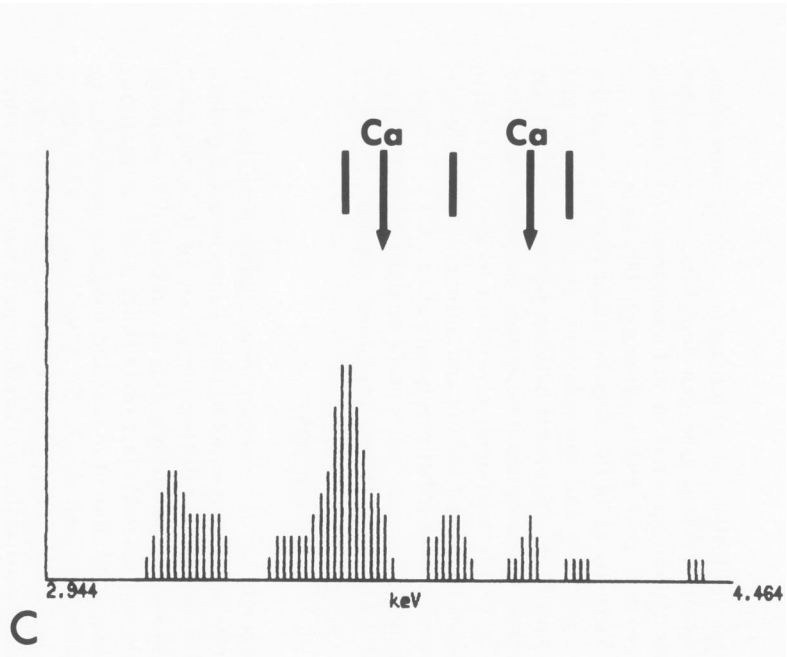
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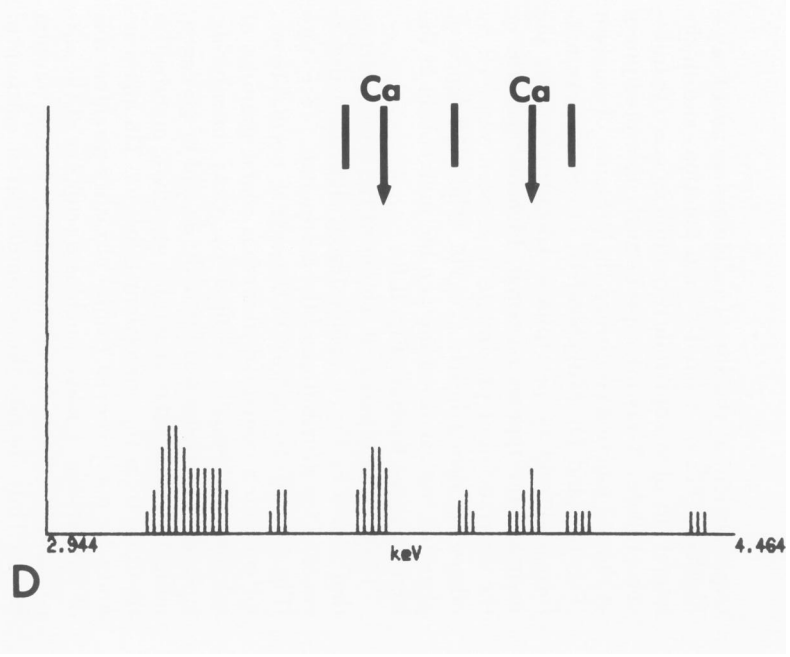
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completely prevented the increase in tissue calcium. The site of action of α-adrenergic blockade was further examined by determining that tissue calcium increases in untreated animals were due to increases in extracellular space and intracellular calcium. Alpha adrenergic blockade prevented calcium accumulation at both these sites when administered before ischemia. Beta adrenergic blockade was ineffective in preventing an increase in tissue calcium.

Electron microscopy was used to evaluate the ultrastructural appearance of normal and reperfused ischemic myocardium and to assess the intracellular distribution of calcium after simultaneous calcium precipitation with pyroantimonate and tissue fixation with osmium tetroxide. Normal mitochondria were devoid of antimonate precipitate. Mitochondria in reperfused ischemic myocardium, however, contained abundant coarse particles of antimonate precipitate, demonstrated to contain calcium by x-ray microprobe analysis. Ultrastructurally, the reperfused myocardium revealed features of reversible ischemic injury only. Reperfused ischemic myocardium from animals treated with α-adrenergic blocking agents manifested similar ultrastructural features of reversible ischemic injury, but not calcium accumulation in mitochondria. The identification of calcium in mitochondrial precipitate after pyroantimonate perfusion and x-ray microprobe analysis is purely qualitative. However, the presence of large particles of mitochondrial calcium antimonate precipitate in reperfused ischemic myocardium and their absence in tissue treated with α-adrenergic blocking agents imply a quantitative difference in mitochondrial calcium content. The ultrastructural studies thus support the conclusion that α-adrenergic blockade prevented intracellular accumulation of calcium despite ischemia. Furthermore, the results indicate that despite a marked increase in intracellular calcium, the reversibly injured myocardium maintained a near normal ultrastructural appearance. Thus, calcium accumulation per se is not tantamount to cell death nor is it secondary only to irreversible ischemic injury. Irreversible injury may depend, in part, on the magnitude of the calcium influx itself, rather than simply the membrane damage that facilitates it.

Alpha adrenergic effects on intracellular calcium accumulation induced by reperfusion are unlikely to be nonspecific manifestations of reduced injury during the antecedent ischemic interval for several reasons. First, calcium accumulation was attenuated when either phentolamine or the aqueous soluble α_1-adrenergic blocking agent BE-2254 was administered intravenously only 2 min before reperfusion, at a time when most of the ischemic damage would have occurred already. Second, the modest increase in total tissue calcium when BE-2254 was administered 2 min before reperfusion was due to continued expansion of the extracellular space, but intracellular increases were completely blocked. Third, the β-adrenergic blocking agent propranolol did not prevent a doubling of total tissue calcium induced by reperfusion. Fourth, reduced calcium accumulation resulted from chemically dissimilar α-adrenergic blocking agents including phentolamine, prazosin, labetalol, and BE-2254. Fifth, no significant differences in heart rate, left ventricular end diastolic pressure, cardiac output, or stroke work are demonstrable with reperfusion in α-adrenergically blocked animals compared with control, untreated animals in our laboratory (6), suggesting that secondary effects on cardiac and peripheral hemodynamics were not responsible. Sixth, myocardial blood flow augmentation after reperfusion in the center of the previously ischemic region is not attenuated by α-adrenergic blockade (6). Seventh, electron microscopic criteria of cellular responses to ischemia including glycogen depletion occur despite blockade of the increase of intracellular calcium. Thus, α-adrenergic blockade blocks intracellular calcium accumulation specifically.

Alpha adrenergic stimulation augments calcium uptake in blood vessels (8), nerves (25), and liver (9). In myocardium, evaluation of calcium uptake is complicated by a multiplicity of calcium transport mechanisms and binding (26). The extent to which the I_{si} of

FIGURE 7 (*A*) An electron micrograph of control ischemic myocardium (without α-adrenergic blockade) fixed by perfusion with pyroantimonate-osmium tetroxide. Calcium antimonate precipitate is seen at the A band-I band junction (arrowheads). In contrast to normal tissue, mitochondria (M) exhibit antimonate precipitate. Glycogen is absent. Bar = 1.0 μm.(*B*) An electron micrograph of the perinuclear zone of an ischemic myocyte. Mitochondria (M) contain abundant antimonate precipitate. The precipitate contains calcium as demonstrated by x-ray microanalysis. Glycogen is absent from the perinuclear zone indicating a recent interval of anaerobic metabolism. Bar = 0.5 μm. (*C, D*) X-ray microprobe spectra of pyroantimonate precipitate. A single particle of mitochondrial precipitate (indicated in *B*) was scanned. The particle contained antimony (short lines) and calcium (Ca-labeled arrows) as shown in *C*. Peaks corresponding to the characteristic energies of calcium remain after subtraction of the antimony spectrum (*D*).


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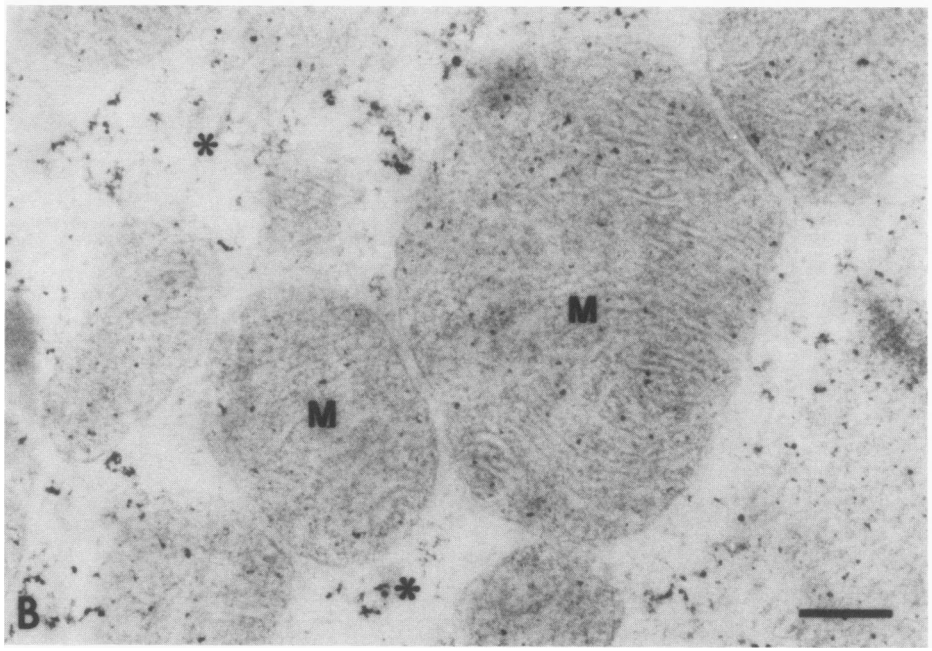
A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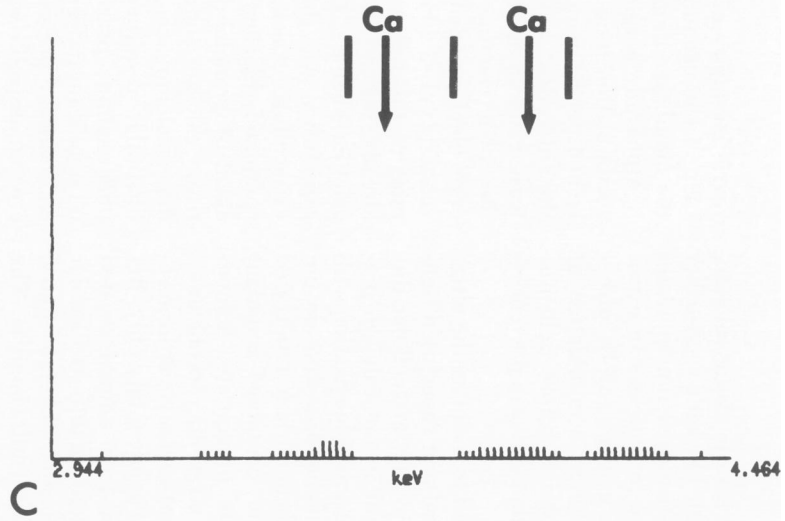
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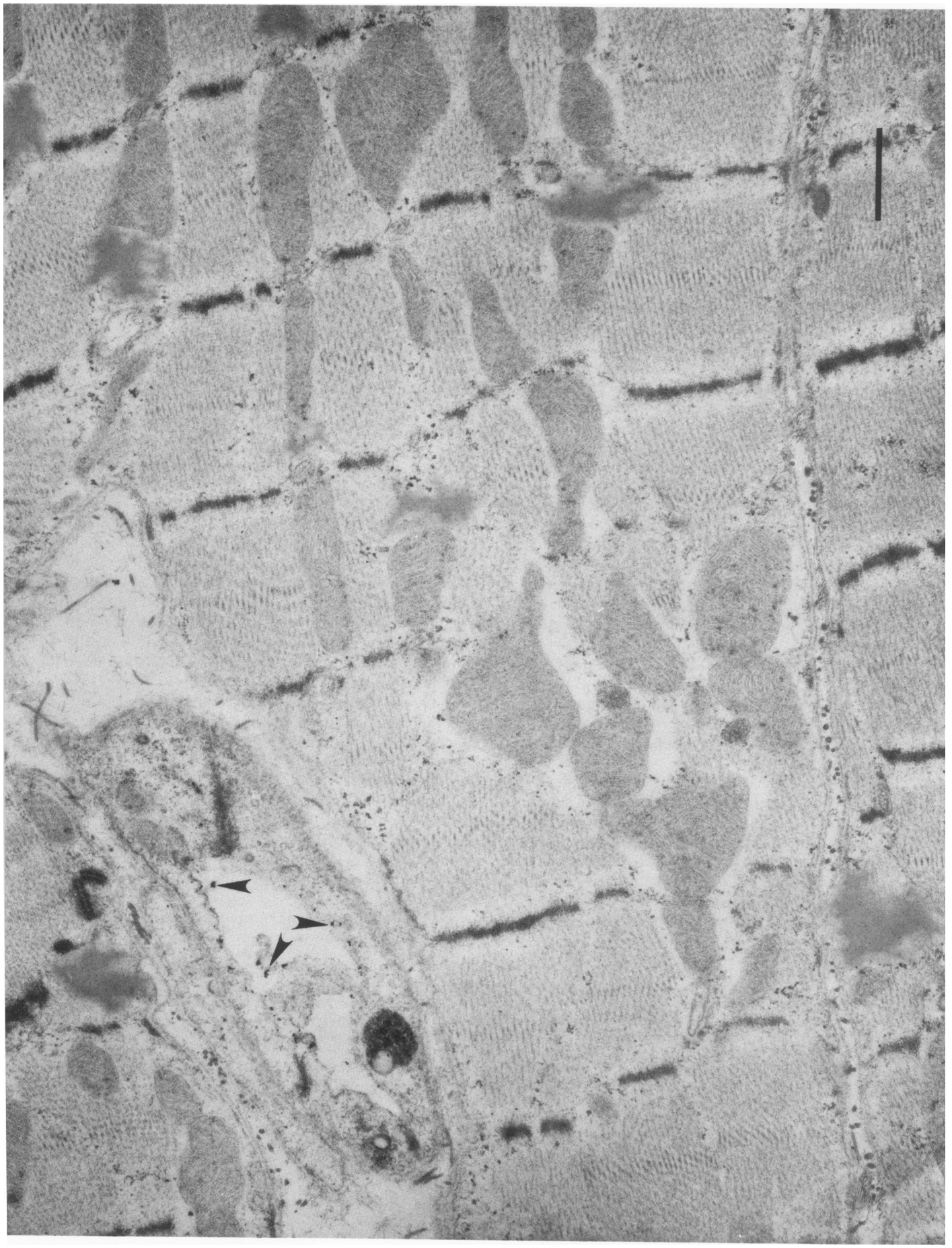
the cardiac action potential contributes to total tissue calcium transport is limited and estimated to be <1 pmol/beat per cm^2 (27). Although the I_{si} may be augmented by β-adrenergic stimulation in normal tissue (28), disparate results have been obtained with impaired myocardium. In rabbit papillary muscle (11) and canine myocardial fibers (29) depressed by a high concentration of potassium, action potentials that appear to be calcium dependent are augmented by α-adrenergic but not by β-adrenergic stimulation. Nevertheless, the marked effects of α-adrenergic blockade on the large increase in total myocardial calcium content after reperfusion are likely to depend on mechanisms other than the I_{si}. In cultured myocytes, sodium-calcium exchange appears to be a major source of cellular calcium (30). This mechanism may be particularly active with reperfusion, since cellular sodium increases (3, 22). Thus, sodium may be partly removed from the cell in exchange for calcium particularly if Na^+-K^+ ATPase is inhibited. The more modest magnitudes of calcium transport associated with endogenous ionophores, passive diffusion, or potassium-calcium exchange are not likely to account for the extensive changes in intracellular calcium observed with reperfusion (26). Reduced active, calcium efflux has not been demonstrated directly in the isolated perfused rabbit septum (31), but in vivo this mechanism could potentially result in increased tissue calcium. Any of these potential mechanisms, if responsive to α_1-adrenergic stimulation, may be amplified in reperfused tissue since α_1-adrenergic receptor density is increased during ischemia in both the cat (7) and dog (32). Thus, even though α-adrenergic stimulation may not influence calcium transport in normal myocardium, the effect may be augmented in ischemic and reperfused tissue.

It is not necessarily surprising that voltage-dependent calcium channel blockers diminish calcium accumulation in reperfused myocardium (33) and reduce the likelihood of malignant arrhythmias (34, 35). However, this effect may be attributable to the α-adrenergic blocking properties of these agents. This is very likely the case with verapamil, since the concentration achieved in blood very closely parallels the K_i for inhibition of the α_1-adrenergic receptor (14, 15, 36, 37) and in vivo tissue levels of verapamil in the heart far exceed the plasma levels (38). However, the potency of agents that block the voltage-dependent calcium channel (39) does not parallel their potency as α_1-adrenergic blocking agents. Nifedipine exhibits a much lower affinity for α_1-adrenergic receptors compared with verapamil (15, 37), despite the far greater potency of nifedipine as a blocker of the voltage-dependent calcium channel (39). This finding further suggests that the large increase in intracellular calcium during reperfusion is likely to result from α-adrenergic influences on sites other than the voltage-dependent calcium channel or I_{si}.

Although verapamil administered before ischemia attenuates calcium uptake and induction of arrhythmias by reperfusion (35), calcium antagonists administered at the onset of reperfusion do not (18, 34, 40). The beneficial effects of verapamil given before ischemia have been attributed, in part, to a reduction of myocardial work with secondarily reduced calcium transport across the sarcolemma (18). Likewise, hypoxia in vitro followed by reoxygenation augments mitochondrial calcium independent of sarcolemmal changes (41). However, in studies leading to these findings (18, 41), experiments were performed at 32°C with perfusion with Krebs buffer associated with a large extracellular space, presumably owing to increased capillary permeability. Thus, conditions may not be analogous to ischemia in vivo. In addition, the observations reflect, in part, effects of hypoxia rather than ischemia. However, ischemia in vivo differs markedly from hypoxia alone as accumulation of extracellular ions and metabolites may markedly affect sarcolemmal calcium transport in this setting (21). This conclusion is supported by Shine et al. (42), who observed altered sarcolemmal permeability to calcium with ischemia in blood-perfused tissue at 37°C. Our results are consistent with protection against increased sarcolemmal permeability to calcium ions possibly mediated by α_1-adrenergic blocking properties, independent of any change in regional blood flow in the ischemic region (43).

Previous studies of cats in vivo from our laboratory

FIGURE 8 (*A*) An electron micrograph of ischemic tissue treated with phentolamine. Calcium antimonate precipitate is present at the A band-I band junction but not in mitochondria (M). Bar = 1.0 μm. (*B*) An electron micrograph of the perinuclear zone of an ischemic myocyte treated with phentolamine. Mitochondria (M) contain occasional minute electron-dense particles but are devoid of the coarse granules of calcium pyroantimonate precipitate observed in mitochondria in ischemic zones from untreated animals. The absence of glycogen, normally abundant in the perinuclear zone. (*), reflects a recent period of anaerobic metabolism. Bar = 0.5 μm. (*C*) X-ray microprobe spectrum obtained by scanning minute electron-dense particles in mitochondria from ischemic tissue in an animal treated with phentolamine. Antimony (short lines) and calcium (arrows) are not detected.



(6) have delineated the antiarrhythmic influences of α-adrenergic blockade with reperfusion. The antiarrhythmic effects of low doses of alpha adrenergic blockers has been confirmed in cats (44) and dogs (45, 46). In contrast, massive doses of alpha adrenergic blockers had deleterious effects in an ischemic canine model (47). Thus, α-adrenergic-mediated calcium movement during reperfusion may be related to the development of not only cellular injury, but also malignant ventricular arrhythmias.

In concert, the present findings indicate that the increased intracellular calcium induced by reperfusion can be attenuated specifically by α-adrenergic blockade in reversibly injured tissue. Furthermore, they suggest that α-adrenergic mechanisms may contribute to arrhythmogenesis and cellular injury mediated by calcium in this setting.

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FIGURE 9 An electron micrograph of normal tissue perfused with EGTA before perfusion with pyroantimonate-osmium tetroxide. Electron-dense precipitate is present in vascular spaces (arrowheads) but is virtually absent in cardiac myocytes. Bar = 1.0 μm.

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