Kinetics of Calcium Accumulation in Acute Myocardial Ischemic Injury

Anthony C. Shen, PhD, MD and Robert B. Jennings, MD

The effect of ischemic injury on calcium uptake by dog myocardial cells was investigated in tissue damaged by transient or permanent occlusion of the circumflex branch of the left coronary artery. Tracer doses of $^{45}CaCl_2$ were given at selected intervals before or after occlusion, and tissue uptake was measured in damaged and control left ventricular myocardium. No significant uptake of ^{45}Ca occurred after 60 minutes of ischemia produced by permanent occlusion of a coronary artery. However, 40 minutes of ischemia followed by 10 minutes of arterial reflow resulted in an 18-fold increase in Ca uptake in the injured tissue. Tissue ^{45}Ca increased linearly up through 10 minutes of arterial reflow but did not increase further with an additional 10 minutes of reflow. Myocardium reversibly injured by 10 minutes of ischemia followed by 20 minutes of arterial reflow did not accumulate excess ^{45}Ca. Calcium uptake is assumed to be an active process associated with mitochondrial accumulation of calcium into dense intramitochondrial granules of calcium phosphate. The uptake is a feature of irreversible cellular injury, but occurs only when arterial blood flow is present. The mechanism of the uptake has not been established. It appears to be related to defects in cellular permeability or mitochondrial function (Am J Pathol 67:441–452, 1972).

MYOCARDIAL CELLS mortally injured by a transient episode of ischemia show an acute marked increase in Ca^{++}.[1] Most of this increased Ca^{++} is found in mitochondria in the form of calcium phosphate. Moreover, microincineration studies indicate that much of the new Ca^{++} is restricted to newly formed intramitochondrial dense bodies.[1] These dense bodies are a characteristic feature of the injured cells and are not found in either control or reversibly injured myocardial cells. Although mitochondria containing these dense bodies show marked functional defects, we have assumed in this experiment that the initial accumulation of Ca^{++} in the mitochondria of the damaged cells is an active process, probably analogous to the active cation accumulation exhibited by

From the Department of Pathology, Northwestern University Medical School, Chicago, Ill.

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Address reprint requests to Dr. Robert B. Jennings, Department of Pathology, The Medical School, Northwestern University, Ward Memorial Building, 303 E. Chicago Ave, Chicago, Ill 60611.

cardiac and other mitochondria *in vitro*.[2-5] Data presented in this paper is consistent with this assumption and conclusively demonstrates that the source of the Ca^{++} is the arterial blood perfusing the damaged tissue.

Methods

General

Healthy mongrel dogs of either sex were used. They were housed in air-conditioned quarters, allowed free access to Purina dog meal and water, and were fasted overnight prior to experiments.

Infarcts were produced in the posterior papillary muscle of the left ventricle by occluding the circumflex branch of the left coronary artery. The dogs were anesthetized with sufficient sodium pentobarbital to abolish the corneal reflex (60 mg 5 lbs body weight). Respiration was maintained at a rate of 130 ml room air 10 lbs body weight minute through a 1063 Harvard model respiration pump. Results of earlier experiments have shown that 60 minutes of ischemia without restoration of the arterial circulation or 40 minutes of ischemia and 20 minutes of arterial reflow result in a significant proportion of damaged cells in the posterior papillary muscle (PP).[6] Tissue samples of this readily identifiable site are easily obtained. After analysis the results are compared to those obtained in nonischemic control myocardium from the anterior superior left ventricle and septum (LV) of the same dog.

Determination of the Source of Myocardial Calcium

Permanent Ischemia

Two groups of dogs were used. The circumflex artery of 4 dogs was occluded near the origin with a Goldblatt clamp. Immediately following occlusion 50 μCi $^{45}CaCl_2$, 50μg total, in 5.0 ml N Saline (radiometric purity of 99%, New England Nuclear Corporation) was rapidly injected via a catheter in the femoral artery and the catheter was flushed with normal saline. After 60 minutes of ischemia the heart was excised. In 3 dogs, 50 μCi $^{45}CaCl_2$ was injected 30 to 48 minutes prior to permanent occlusion of the circumflex artery for 60 minutes. Again, the heart was excised and cooled.

Transient Ischemia

Two groups of 4 dogs were used. In the first group, the circumflex artery was occluded near its origin with a Goldblatt clamp for 10 minutes; the clamp was opened and arterial blood was allowed to reperfuse the ischemic tissue for the next 20 minutes. The heart was then excised. The procedure in the second group of 4 dogs was identical except that the period of ischemia was 40 minutes and the period of reflow was 10 minutes. In both transient ischemia groups, 50 μCi of $^{45}CaCl_2$ was rapidly injected intravenously immediately after occlusion by the technic employed in the permanent ischemia group.

Determination of the Kinetics of ^{45}Ca Uptake

Twenty-four healthy adult mongrel dogs of both sexes were anesthetized and injected with a tracer dose of $^{45}CaCl_2$ using a femoral catheter as described above. We opened the chest 15 minutes later, isolated the circumflex artery and placed a Goldblatt clamp within 15 mm of the origin of the vessel. After the ^{45}Ca serum level had become stable (30 to 48 minutes after injection) the clamp was

tightened in order to occlude the artery. Exactly 40 minutes later, we opened the clamp and allowed arterial blood to reperfuse the ischemic tissue. Groups of 4 to 8 dogs were killed by excision of the heart 2, 5, 10 or 20 minutes after restoration of the circulation, and tissue ^{45}Ca was measured.

Tissue ^{45}Ca Activity

The heart was excised rapidly. The left ventricle was incised and the heart was plunged into 500–700 ml of 0.25 M Sucrose (Mallinckrodt) at 0 C in a 1 liter beaker surrounded by crushed ice; ice cubes made of isotonic sucrose also were employed for cooling. Approximately 5 minutes of constant stirring was required for the surface of the heart to reach 2 C.

The PP and the LV were trimmed free of endocardium on filter paper and duplicate pieces, each weighing about 100 mg, were rinsed sequentially in 2 beakers, each containing 50 ml of cold (4 C) 0.25 M sucrose, blotted dry and weighed. Only pale damaged tissue was used for the PP samples. Large vessels and fat were trimmed from the samples prior to rinsing and blotting. Weighing was done with a precision balance (Roller-Smith Co, Newark, NJ). The weighed tissue samples were solubilized in 1.0 ml of soluene (Packard Instrument Co). The radioactivity of the vials of serum and tissue was determined with 5.0 ml of ethylene glycol monoethyl ether (Fisher Scientific Co) and 10.0 ml of scintillation fluid which consists of 5.5% 2,5-phenyloxazole and 0.1% 4-bis [2-(4 methyl-5 phenyloxazole)] (Packard Instrument Co). There was no difference between the efficiency of counting in serum and in tissue in the range of 0.5 to 1.5 times the proportions employed here, according to internal standardization experiments. Accordingly, only counts min are employed in calculations.

Serum ^{45}Ca Activity

Arterial blood samples were taken at timed intervals from a polyethylene catheter (ID 0.066 inch, Intramedic, Clay-Adams) from the femoral artery. This site was chosen for its convenience, since both temporal displacement and downward displacement of the ^{45}Ca disappearance curve will not affect results since the time scale is in minutes rather than seconds. Sheppard,[7] for example, found a time lag of only 3 seconds between the femoral artery and the common carotid artery in the dog. Similarly, the shearing effect of laminar flow in large catheters, which will obscure small changes in serum activity during rapid events, does not present a problem in these experiments. The blood samples, ranging from 2 to 5 ml, were collected into clean centrifuge tubes and allowed to clot by sitting at room temperature for 2 hours. The clot was separated from the wall of the tube with a wooden applicator stick. The tube was capped with parafilm and stored at 4 C for 18 to 24 hours. The tubes were centrifuged at 1000 rpm for 30 minutes at 2 C in model PR2 International centrifuge. Two hundred μl of serum were pipetted in duplicate into 17 ml scintillation vials (Packard Instrument Co) containing 1.0 ml of soluene (Packard Instrument Co) each. A mechanical pipetter (Eppendorf Brinkman Instruments, Chicago) was used.

Rationale of ^{45}Ca Uptake Calculations

If we assume that all of the increase in tissue calcium comes from the serum, myocardial uptake of ^{45}Ca is a function of the specific activity of this ion in the serum. The specific activity of the serum varies from dog to dog, a phenomenon which makes comparison of individual dogs difficult, unless we express the myocardial uptake of Ca as the activity of the tissue relative to the counts min of ^{45}Ca

per ml of serum of the same animal. This is a satisfactory system as long as the ^{45}Ca level of the serum is stable. The work of Mulryan[8] in the rat suggested that the rate of ^{45}Ca disappearance from the blood is stable and we found this to be true in dogs as well (Figure 1).

The ^{45}Ca disappearance curve can be resolved into early and late periodic components, as in the case for ^{42}K in the dog[7] and ^{24}Na and ^{42}K in the rabbit.[9] We found that the rate of change in ^{45}Ca concentration in the late periodic part of the ^{45}Ca disappearance curve is relatively stable and reproducible from dog to dog, averaging 3.67 ± SE 0.03% decrease in activity min. The serum level of ^{45}Ca, by 30 minutes postinjection, remains stable during the entire 60 minute period required to produce the ischemic injury and to permit adequate reflow of arterial blood into the damaged tissue. Thus, in the kinetic experiments we occluded the circumflex artery sometime between 30 minutes and 48 minutes after injection of ^{45}Ca. The occlusion lasted for 40 minutes and then the blood was permitted to reflow for periods up to 20 minutes. The total duration of every experiment from injection to termination was always 90 minutes. Thus, the entire surgical procedure, including arterial occlusion and subsequent reperfusion with arterial blood, was done on the stable flat portion of the ^{45}Ca disappearance curve. This part of the curve is determined in every dog and extrapolated to zero time. The ^{45}Ca activity at 45 minutes postinjection, the midpoint of all experiments, is taken to be the mean serum ^{45}Ca activity.

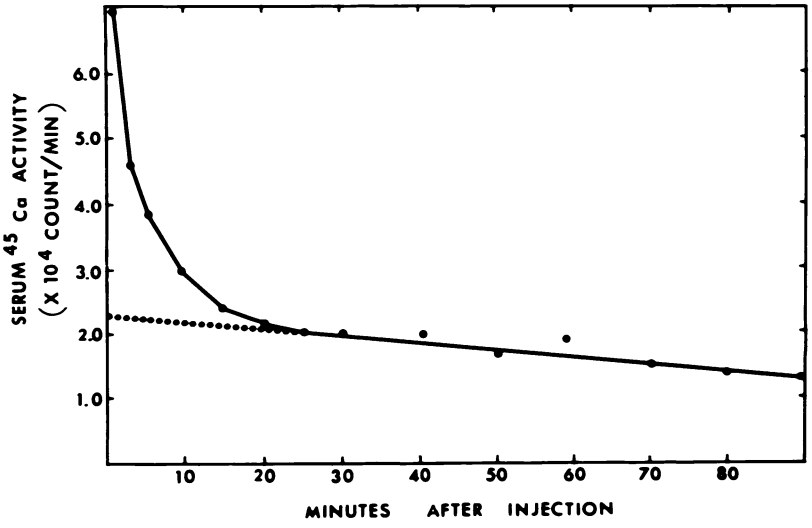
Full equilibration between serum ^{45}Ca and the heart requires less than 40 minutes in the rat.[8] It is probable that a similar situation exists for the dog and that full equilibration between the PP and LV tissue calcium, and the serum ^{45}Ca, has occurred by the time occlusion was performed. Thus, both the tissue activity for LV and the ischemic PP probably are overestimated slightly by using the 45 minute serum ^{45}Ca activity as the mean serum activity in the denominator. The difference between tissue activities for LV and PP, however, represents the calcium uptake by the ischemic PP following reflow of blood. This difference could not be due to loss of ^{45}Ca from the LV during the period of PP ischemia since the LV tissue ^{45}Ca activity remains constant during this period (Figure 2).

Results

Figure 1 shows a representative curve of the disappearance of ^{45}Ca from the vascular compartment of the dog. The rate of disappearance is exponential. Results from 5 dogs demonstrate that the rate of change in serum ^{45}Ca concentration following a single tracer dose was constant, 3.67 ± 0.03% min. The t-½ was about 10 minutes. At this rate of disappearance from the circulation, within 30 minutes the serum ^{45}Ca had become quite stable. This period of fairly constant serum ^{45}Ca activity was employed during several subsequent investigations of myocardial uptake of calcium.

Myocardial Uptake of ^{45}Ca in Permanent Ischemia

The ^{45}Ca content of the ischemic and nonischemic myocardium of dogs given 60 minutes of ischemia are presented in Table 1. In these experiments, the ^{45}Ca was injected immediately after occlusion of the circumflex artery. The nonischemic interventricular septal myocardial



TEXT-FIG 1—A typical $^{45}Ca^{++}$ disappearance curve following intravenous injection of a tracer dose of $^{45}CaCl_2$ in the dog. Only the late or aperiodic part of the dilution curve is shown. Note that the serum activity of ^{45}Ca is relatively stable from 25 to 90 minutes after injection.

samples showed a mean uptake of 2115 counts/min ^{45}Ca/g of wet weight while the posterior papillary muscle, an area of maximum ischemia in this preparation, showed less than 10% as much uptake as the non-ischemic tissue. Thus, if the tracer was administered after the onset of ischemia, the ischemic tissue took up essentially no ^{45}Ca.

Table 1—Calcium Uptake in Ischemic Myocardium after Permanent Arterial Occlusion (Sixty Minutes Ischemia)*

	Tissue ^{45}Ca Activity $\left(\frac{\text{counts/min/g wet weight}}{10^4 \text{ counts/min/ml serum}}\right)$	
Dog No.	LV†	PP†
1280	2078	437
1280	2219	142
1309	2166	98
1309	1988	133
Mean ± SE	2115 ± 42	203 ± 68‡

* 50 μCi $^{45}CaCl_2$ injected one minute after occlusion of the circumflex branch of the left coronary artery. The artery occluded for 60 minutes and samples were obtained as described in Methods.

† LV = anterior superior left ventricle and septum; PP = posterior papillary muscle.

‡ The probability that the difference between the means is significant is <0.001. The results were analyzed by a two-tailed paired t test.

Alternatively, injection of ^{45}Ca 30 to 48 minutes prior to occlusion of the circumflex branch of the left coronary artery for 60 minutes in three dogs resulted in no additional uptake of ^{45}Ca over that which occurred prior to the onset of ischemia. The ischemic posterior papillary muscle showed 1802 ± 138 and the nonischemic left ventricle showed 1860 ± 67 counts/min per g wet weight per 10^4 counts/min per ml plasma. These experiments tell nothing of possible redistribution of ^{45}Ca within the damaged tissue but confirm that damaged myocardium takes up no ^{45}Ca at this time interval in the absence of normal arterial flow.

Myocardial Uptake of ^{45}Ca in Transient Ischemia

The results of injection of a tracer dose of ^{45}Ca in animals subjected to occlusion of the circumflex artery for 40 minutes followed by 10 minutes of arterial reflow are presented in Table 2. In this instance, the ^{45}Ca was injected immediately after the vessel was occluded. A period of ischemia of 40 minutes duration is known to be sufficient to kill an average of 55% of the cells in the posterior papillary muscle.[6] Samples of pale gray damaged myocardium from the PP showed an enormous uptake of ^{45}Ca. Almost 18 times more ^{45}Ca was observed in the PP than was found in the nonischemic control left ventricular tissue, thus clearly establishing that these damaged cells not only accumulated extraordinary quantities of calcium but that this calcium originated in the arterial blood reperfusing through the damaged tissue.

The effect of 10 minutes of ischemia, which is a time period known to

Table 2—Calcium Uptake in Myocardium Damaged by Transient Episodes of Ischemia*

	Tissue ^{45}Ca Activity $\left(\frac{\text{counts min/g wet weight}}{10^4 \text{ counts min ml serum}}\right)$	
	LV†	PP†
Reversible Injury (10 min followed by 20 min of reflow)‡	2,092 ± 78	2,207 ± 58 NS
Irreversible Injury (40 min followed by 10 min of reflow)	2,087 ± 129	38,358 ± 1268 $P < 0.001$

* Values are means ± standard error of the mean (N = 4). The probability of the means being identical was assessed by a paired t test. 50 μCi of $^{45}CaCl_2$ was injected immediately after occlusion of the circumflex artery.

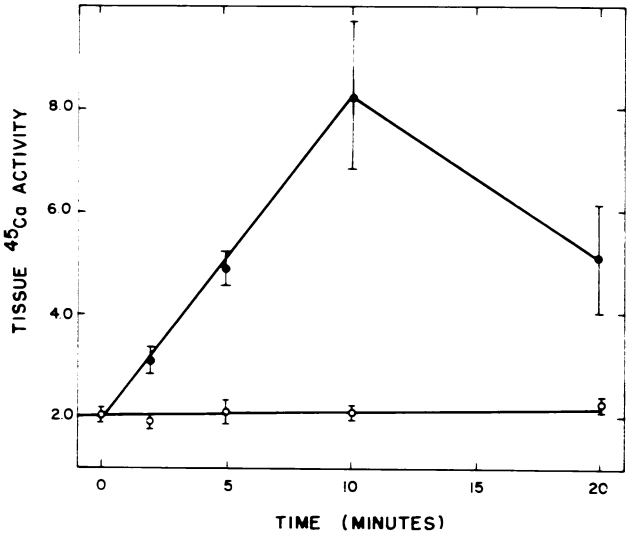
† LV = nonischemic anterior superior left ventricle and septum; PP = posterior papillary muscle.

‡ A 20 minute period of reflow was used instead of the 10 minutes employed in the irreversible injury experiment because the total duration of the experiment (10 plus 10) otherwise would be on the steep part of the ^{45}Ca disappearance shown in Text-figure 1.

be insufficient to irreversibly injure myocardial cells of the PP,[6] on ^{45}Ca uptake was tested after 20 minutes reperfusion of the affected tissue by arterial blood, in order to determine if the irreversibility of cell damage was related to the ^{45}Ca uptake. Again, the ^{45}Ca was injected immediately after occlusion of the artery. The results of these experiments are presented in Table 2. No increased uptake of ^{45}Ca was noted in the damaged tissue. Thus, ^{45}Ca uptake was dependent upon the presence of irreversibly damaged cells in the tissue samples.

Kinetics of ^{45}Ca Uptake during Transient Ischemia

In this set of experiments the circumflex artery was occluded between 30 to 48 minutes after injection of the ^{45}Ca. After 40 minutes of ischemia, the Goldblatt clamp was opened and blood was permitted to reflow for 2, 5, 10 or 20 minutes. The total duration of every experiment from injection to termination was always 90 minutes or less, hence the entire procedure, including coronary occlusion and subsequent reperfusion of the affected tissue with arterial blood, was done on the stable flat portion of the ^{45}Ca disappearance curve (Text-figure 1).



TEXT-FIG 2—Graph illustrating calcium accumulation in control and damaged dog myocardium as a function of time. ^{45}Ca was injected 30 minutes prior to occlusion of the circumflex branch of the left coronary artery for 40 minutes followed by the periods of arterial reflow noted on the abscissa. Tissue ^{45}Ca activity has been adjusted for the level of serum ^{45}Ca activity found 45 minutes after intravenous injection of the ^{45}Ca. The units of tissue ^{45}Ca activity are identical to those used in Tables 1 and 2, except that they have been divided by 1000. The damaged tissue (*PP*) is indicated by solid circles and the control tissue (*LV*) is indicated by open circles. The magnitude of the standard error is represented by the brackets.

The results are plotted in Text-figure 2. The PP ^{45}Ca concentration prior to reflow was the same in LV and PP. However, with progressive increase in the duration of reflow of arterial blood, the ^{45}Ca activity of the PP increased linearly and finally peaked at 10 minutes. After 20 minutes of reflow, the uptake appeared to be slightly decreased, but the 10- and 20-minute reflow values were not significantly different. The nonischemic left ventricle showed a stable level of radioactivity throughout the experiment.

The amount of Ca^{--} taken up by the injured tissue from the blood can be estimated if the specific activity of the serum calcium is calculated on the assumption that the serum calcium of the dog is within the normal range. Using a value of 9 mg% the amount of calcium taken up by the injured tissue from the blood is 4.31 mmoles/100 g dry weight.* The total Ca^{--} of the PP measured by flame atomic absorption spectroscopy in 4 other dogs subjected to the same period of ischemia and arterial reflow was 4.21 mmoles per 100 grams dry weight.[1] Thus, it seems clear that the arterial blood calcium can account for all of the increase in tissue calcium in myocardium injured by temporary ischemia.

Discussion

The results of these experiments showed that all of the increase in tissue calcium in myocardial cells irreversibly injured by a 40 minute episode of transient ischemia could be accounted for by Ca^{++} uptake from the serum. Furthermore, the uptake of Ca^{++} occurred quickly; the tissue Ca^{--} increased linearly with time for about the first 5 and probably 10 minutes of reperfusion of coronary arterial blood, while the subsequent 10 minutes of reperfusion was not associated with the high rate of accumulation noted earlier. Calcium accumulation proved to be a feature of irreversible injury because reversibly injured myocardial cells—*ie*, cells exposed to 10 minutes of ischemia and 20 minutes of coronary arterial reflow, failed to accumulate this ion.

Earlier studies on the distribution of Ca^{--} in myocardial cells mortally injured by an episode of transient ischemia and a brief period of arterial reflow had shown that the Ca^{--} of the tissue was about 10 times greater than control myocardium of the same animal.[1] More-

* Assuming serum calcium to be 9 mg/100 ml, and myocardial tissue water to be 80% of tissue wet weight,[1] then:

$$\frac{3.8 \times 10^4 \text{ counts/min tissue } ^{45}Ca/\text{g tissue}}{1 \times 10^4 \text{ counts/min/ml serum } ^{45}Ca} \cdot \frac{100}{20\% \times \text{mol wt } Ca^{--}} = \frac{4.3 \text{ mmoles } Ca^{--}}{100 \text{ g dry wgt}}$$

over, much of this calcium was localized in mitochondria which contained markedly increased quantities of calcium and phosphate.[1] The mitochondria with high Ca^{++} contained dense bodies which have not been isolated but almost certainly contain the calcium phosphate. The strongest evidence for this conclusion is the fact that they leave an ash after microincineration,[1] while other parts of the cell do not leave an ash. These data, taken together, strongly suggest that much of the ^{45}Ca which has accumulated in the tissue is located within the intramitochondrial dense bodies. Thus, the calcium from the intravascular space must have crossed the cell and mitochondrial membranes before being precipitated as calcium phosphate within the intramitochondrial dense bodies. It seems likely that the accumulation of this ion must be an active process requiring the expenditure of energy. Furthermore, the kinetic data shows that a maximal rate is reached during the first 5 to 10 minutes of arterial reflow and also suggests that the uptake mechanism may be saturated at the onset.

Two additional observations provide indirect evidence supporting the idea that calcium uptake is an active process. Most important are the findings of numerous investigators that isolated myocardial mitochondria *in vitro* accumulate calcium phosphate in an energy-dependent fashion.[2-5,10] The accumulation will occur in the presence of a substrate such as succinate or β-hydroxybutyrate as long as ATP, certain electrolytes and inorganic phosphate are present. The most striking feature of this process is that the calcium is localized in intramitochondrial dense bodies that are virtually indistinguishable from those noted *in vivo* in transient ischemia.[1] Secondary, and perhaps more direct evidence that active metabolism is occurring in the ischemic tissue during the first 4 to 5 minutes of reflow of arterial blood, is the fact that the temperature of this tissue rises remarkably during this period of time.[11] The most likely explanation for this transient temperature increase is increased local heat production associated with increased metabolism and perhaps uncoupling of oxidative phosphorylation.

A critical problem, unsolved by these experiments and of considerable importance to understanding this system, involves the question, "What causes calcium to accumulate in the mitochondria of the damaged cells?" The likely answers involve postulation of a defect in mitochondrial function or in the permeability of the cell membrane to calcium. For example, if the principal effect of ischemia was to produce an increase in cellular permeability, then abundant plasma Ca^{++} would be available for accumulation by damaged mitochondria still capable of accumulating

this ion or perhaps even "normal", mitochondria in the damaged cells. No data currently is available to assess this attractive possibility. On the other hand, ischemia could induce a primary mitochondrial defect, a feature of which would be accumulation of Ca^{++} occurring simultaneously with the return of arterial blood and an available supply of exogenous Ca^{++}. Mitochondria of myocardial cells irreversibly injured by 60 minutes of ischemia show a greatly reduced capacity to perform integrated functions [12] and it seems likely that a primary defect in mitochondrial function may be present at the time the blood flow is restored. However, it is not known whether this defect is associated with Ca^{++} accumulation. The fact that reversibly injured myocardial cells and the mitochondria within them do not accumulate Ca^{++} does not differentiate between the alternatives except to suggest that reversibly injured cells are less permeable to Ca^{++} or do not have a mitochondrial defect. *In vivo*, the temperature of reversibly injured myocardium decreases when arterial flow is restored to the affected tissue.[13]

The relationship of the intramitochondrial amorphous densities of permanent ischemia and the calcium phosphate-type densities of transient ischemia remains to be established. It seems likely that they are unrelated phenomena. Also unanswered is the question: Is Ca^{++} accumulation lethal in this system? On the one hand, Ca^{++} is known to uncouple oxidative phosphorylation and, on the other hand, intramitochondrial calcium phosphate precipitates occur in mitochondria of some types of undamaged cells.[10]

The difference in tissue ^{45}Ca activity between 10 and 20 minutes of reflow is not significant. It is possible, however, that there has been either a decrease in the rate of uptake or a loss of calcium from the damaged cells and mitochondria during this interval. This may be due to a decreased ability of the plasma membranes to maintain a high level of intracellular calcium. Supporting the idea that the 20-minute value is truly lower are the observations[5] that the capacity of normal liver mitochondria to accumulate Ca^{++} *in vitro* diminishes greatly or disappears after 10 to 15 minutes of incubation and the fact that mitochondria which have accumulated calcium *in vitro* extrude it either in the absence of ATP or under anaerobic conditions.[14]

Comparison of myofibers injured by 10 minutes of transient ischemia with those injured by 40 minutes of transient ischemia shows striking differences. After 10 minutes of ischemia followed by 20 minutes of blood reflow, no structural changes can be found with the use of either the light microscope or the electron microscope. In contrast,

myofibers injured by 40 minutes of ischemia followed by 20 minutes of reflow of arterial blood show extensive morphological changes, including striking contraction bands in addition to the mitochondrial changes described earlier.[15] Also, transient ischemia of 40 minutes duration produces irreversible injury whereas 10 minutes of ischemia does not.[6] This report documents a third difference: The severely injured cells of 40 minutes of transient ischemia accumulate large quantities of calcium from the blood whereas the morphologically unaffected cells of 10 minutes of transient ischemia do not. Whether the calcium uptake is causally related to the development of irreversibility remains to be investigated.

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