Effects of fatty acids on myocardial calcium control during hypothermic perfusion

Although hypothermia is regarded as providing protection of the myocardium during cardiac operations, rapid cooling of the myocardium in the nonarrested state may have detrimental effects on the function of the myocardial cell membrane as a permeability barrier. We therefore measured total cellular calcium in isolated working rat hearts, receiving either glucose (11.1 mmol/L) or glucose plus palmitate (1.2 mmol/L), before, during, and after a 40-minute hypothermic arrest (10° C, Langendorff perfusion). In both groups a rise in total cellular calcium, measured by $^{45}\text{Ca}^{2+}$ technique, was observed during hypothermia, followed by a decline on rewarming. However, the rise in total cellular calcium during hypothermia was significantly ($p < 0.05$) higher in hearts perfused with palmitate (from 1.0 ± 0.2 to 3.5 ± 0.2 nmol/mg dry weight) compared with that in glucose-perfused hearts (from 1.1 ± 0.13 to 2.6 ± 0.2 nmol/mg dry weight). Palmitate-perfused, but not glucose-perfused, hearts showed arrhythmias and delayed pressure development 1 to 2 minutes after rewarming. In addition cardiac output of these hearts was significantly lower ($p < 0.025$) than that of glucose-perfused hearts 5 to 10 minutes after rewarming. These data show that hypothermia per se causes a net calcium uptake in isolated rat hearts and that this effect is aggravated by high concentrations of fatty acids. Thus the impaired recovery of myocardial function in palmitate-perfused hearts can possibly be related to a distorted calcium handling. (J THORAC CARDIOVASC SURG 1994;107:233-41)

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Hypothermic crystalloid or blood-based cardioplegia is regarded as providing adequate myocardial protection during cardiac operations in adult patients.¹ Nevertheless, it has been associated with some disadvantages, such as distortion of enzymatic function and loss of myocardial cell integrity. Bull and associates² pointed to an apparent inadequate intraoperative myocardial protection during cardiac operations in children with congenital heart dis-

ease. Rebeyka and coworkers^{3,4} subsequently provided evidence suggesting that the period of unprotected cooling of the neonatal heart, before application of chemical cardioplegia, is particularly critical for induction of contracture and postoperative low output failure. These authors have described this contracture as a clinical parallel to the phenomenon of rapid cooling contracture observed in isolated heart preparations⁵ and proposed that the period of unprotected rapid cooling leads to intracellular calcium accumulation, which is not completely reversed on rewarming. In support of this proposal Williams and associates⁶ reported that a bolus injection of warm (37° C) cardioplegic solution before cooling was correlated with a reduced mortality in pediatric patients.

Rapid cooling (to less than 2° C in 2.5 seconds) increases cytoplasmic free calcium,⁷ possibly through a rapid release of calcium from sarcoplasmic reticulum⁸ or through an increased sarcolemmal calcium influx proposed to occur through $\text{Na}^+/\text{Ca}^{2+}$ exchange,⁹ or through both mechanisms. Whether myocardial calcium uptake is increased during more moderate cooling, though often stated as a matter of fact, is not unequivocally proven.

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Fig. 1. Perfusion protocol showing temperature profile, perfusion conditions, and duration of various stages of heart perfusions. Arrows indicate end points at which hearts were switched to calcium-free buffer, that is, 3 minutes before they were frozen between Wollenberger clamps cooled to temperature of liquid nitrogen.

Recently, Kusuoka and associates¹⁰ proposed the positive inotropic effect of hypothermia to be caused by an increased myofilament calcium responsiveness. In contrast, Harrison and Bers¹¹ have shown that generated tension in skinned cardiac muscle declines at constant ionized calcium levels when temperature is lowered. These authors suggested that the inotropic effect of hypothermia is presumably related to elevated levels of free cytosolic calcium in the region of the myofibrils. This correlates well with the proposition by Kato and associates¹² that the inotropic effect of hypothermia can be ascribed to a modulated calcium transient.

In a recent paper, Mjøs and associates¹³ found high concentrations (1.2 mmol/L) of fatty acids in the perfusate to hamper mechanical recovery after unprotected hypothermic perfusion (10° C) of isolated rat hearts. This study further indicated that loss of mechanical function in fatty acid-perfused hearts was mediated through an altered calcium homeostasis; lowering of calcium in the perfusate from 2.5 mmol/L to 1.25 mmol/L or addition of verapamil to the perfusion buffer improved mechanical recovery in the rewarming phase in hearts perfused with fatty acids. In the present study we used the same model of unprotected hypothermia to address the following questions: (1) is there an increased myocardial uptake of calcium during hypothermia or the following rewarming phase and (2) will addition of fatty acids influence the myocardial calcium uptake under these conditions? Sev-

eral studies in the past have addressed myocardial calcium control during hypothermia, but their interpretations have been complicated by additional components of ischemia^{14, 15} or the calcium paradox,^{16, 17} or both. To our knowledge, therefore, the present study for the first time examines the impact of hypothermia per se on myocardial calcium uptake in isolated rat hearts.

Methods

Perfusion protocol. Hearts were obtained from adult male Sprague-Dawley rats (320 to 390 gm, Charles River, Germany, specific-pathogen-free quality) that had free access to food (EWOS, Södertälje, Sweden) and water. All animals were treated according to the guidelines on accommodation and care of animals formulated by the European Convention for the protection of vertebrate animals used for experimental and other scientific purposes. The rats were killed by a blow to the head and subsequent decapitation before the heart was rapidly excised and mounted on the perfusion apparatus by cannulation of the aorta. No anticoagulant was used. The hearts were used as spontaneously beating preparations, because electrical stimulation prompts norepinephrine release from endogenous stores,¹⁸ which could affect substrate metabolism and calcium homeostasis.

The perfusion protocol is outlined in Fig. 1. The hearts were initially perfusion-washed by the Langendorff technique (73.5 mm Hg aortic pressure) for 15 minutes with Krebs-Henseleit bicarbonate buffer (37° C) containing 11.1 mmol/L glucose.¹⁹ The washout buffer was discarded. This was followed by 20 minutes of recirculating perfusion by the working-heart technique with a left atrial filling pressure of 12.5 mm Hg and an outflow pressure of 73.5 mm Hg.²⁰ The hearts were then sub-

jected to 40 minutes of hypothermic arrest by switching to recirculating Langendorff perfusion (73.5 mm Hg aortic pressure) at 10° C.

Recovery of ventricular function was followed for 15 minutes after reestablishment of the prehypothermic working-heart conditions (the hearts were switched directly from hypothermic arrest at 10° C to the working-heart mode at 37° C). Except for the medium used during the initial washout period, the perfusion medium was Krebs-Henseleit bicarbonate buffer (NaCl 118.5 mmol/L, NaHCO₃ 25.0 mmol/L, KCl 4.7 mmol/L, MgSO₄ 1.2 mmol/L, KH₂PO₄ 1.2 mmol/L, CaCl₂ 1.5 mmol/L) containing bovine serum albumin (2%) and glucose (11.1 mmol/L). Where indicated 1.2 mmol/L palmitate complexed to albumin was added in addition to glucose. The buffer was continuously gassed with a 95% oxygen/5% carbon dioxide mixture by means of oxygenating drums (surface oxygenation) and filtered through an on-line filter (5 µm, Millipore Corporation, Bedford, Mass.). The concentration of ionized calcium (Ca²⁺) in the buffer was 1.1 mmol/L after addition of albumin (glucose-perfused hearts) or the albumin-fatty acid complex (fatty acid-perfused hearts). All recirculating buffers were supplemented with tracer quantities (0.1 µCi · ml⁻¹) of ⁴⁵Ca²⁺ (NEN research products; Du Pont Diagnostic Imaging Division, Wilmington, Del.). For each experiment 300 ml of recirculating buffer was made as one homogenous solution and divided in two parts: 200 ml in the working-heart system and 100 ml in the Langendorff system.

Aortic pressure and heart rate were monitored on a Beckman Dynograph recorder (Beckman Instruments, Inc., Fullerton, Calif.) via a pressure transducer (Gould P23 ID; Statham, Los Angeles, Calif.) connected to a side arm on the aortic cannula. Aortic and coronary flows were measured by timed collections in graduated cylinders of the aortic and right ventricular output, respectively.

Measurement of calcium content in myocardial tissue. Total cellular calcium ([Ca²⁺]_{total}) was measured in groups of hearts that were frozen with Wollenberger clamps cooled in liquid nitrogen (freeze clamped) at different end points during the perfusion protocol, as indicated in the results. Three minutes before every end point we switched from the perfusion medium containing ⁴⁵Ca²⁺ to an identical perfusion medium (composition and temperature) without ⁴⁵Ca²⁺ to wash out any radioactivity trapped in the heart chambers, vasculature, and interstitial space. This procedure should eliminate the possibility of calcium being trapped in the extracellular compartments, because according to Tani and Neely²¹ the half-life for ⁴⁵Ca²⁺ washout is 4 seconds. The ventricles were then homogenized and stored in liquid nitrogen for later measurement of myocardial calcium content (method previously described by Tani and Neely²²) and tissue metabolites. For calcium measurements approximately 100 mg homogenate was extracted with 1 ml ice cold (0° C) 0.42 mmol/L perchloric acid. After centrifugation 0.6 ml of the supernatant was counted in 5 ml scintillation fluid (Instagel II; Packard Instruments B.V., Chemical Operations, Groningen, The Netherlands) on a Packard Tri-Carb liquid scintillation spectrometer (Hewlett-Packard Co., Palo Alto, Calif.). The myocardial calcium content was determined on the basis of the radioactivity in the tissue extracts and the specific activity of the perfusion buffer.

Chemical measurements. Fatty acids and triacylglycerol were extracted from myocardial homogenate (approximately 40 mg) in 4 ml chloroform:methanol (2:1 with 0.05 vol% buty-

lated hydroxytoluene) by the method of Folch. Phase separation was achieved by addition of 600 µl H₂O and 30 minutes of centrifugation (500 g, 4° C). The organic phase was transferred to new glass tubes and evaporated under a stream of nitrogen. The dried lipids were redissolved in 500 µl chloroform and triacylglycerol was separated from other lipid components using Bond Elut aminopropyl columns (Analytichem Int., Harbor City, Calif.) as described by Tracy.²³ Triacylglycerol was then hydrolyzed and the fatty acids (both triacylglycerol fatty acids and free fatty acids) derivatized to their phenacyl esters according to the method of Durst and associates.²⁴ The phenacyl esters were finally separated and quantified by a high-performance liquid chromatography (HPLC) technique (Waters Chromatography Div., Millipore, Milford, Mass.) as described by Halgunset and associates²⁵ with tripentadecanoin and heptadecanoic acid (free fatty acids) used as internal standards.

Tissue contents of adenosine triphosphate (ATP) and creatine phosphate (CP) were analyzed in neutralized perchloric acid extracts of the tissue homogenate according to the method of Sellevold and associates,²⁶ with a Waters HPLC unit. Aliquots of these extracts were also used for measurement of lactate, which was done according to the method of Passoneau.²⁷ Glycogen content was measured according to the method of Passoneau and Lauderale²⁸ after extraction of tissue homogenate in trichloroacetic acid.

Materials. The following biochemicals were obtained from Sigma Chemical Company (St. Louis, Mo.): fraction V bovine serum albumin (Sigma No. A-8022), l-lactic dehydrogenase (l-lactate: nicotinamide-adenine dinucleotide oxidoreductase, EC 1.1.1.27), tripentadecanoin, and heptadecanoic acid. HPLC-grade methanol and acetonitrile were obtained from Rathburn Chemicals Ltd. (Walkenburn, Scotland). Radioactively labeled calcium (⁴⁵CaCl₂) was obtained from NEN research products. Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: nicotinamide-adenine dinucleotide phosphate 1-oxidoreductase, EC 1.1.1.49) and hexokinase (ATP: D-hexose 6-phosphotransferase, EC 2.7.1.1) were obtained from Boehringer Mannheim GmbH, Mannheim, Germany. All other chemicals used were from Sigma Chemical Company or from Merck a/s, Oslo, Norway.

Statistical analysis. Data are presented as means plus or minus the standard error. Differences were considered to be significant at the 95% confidence level. Analysis of within-group variations in mechanical performance was made by analysis of variance (ANOVA) followed by a paired two-tailed Student's *t* test applying Bonferroni's method for simultaneous multiple comparisons when *F* values indicated statistical difference. Analysis of within-group variation of calcium and metabolic parameters was done by ANOVA followed by an unpaired two-tailed Student's *t* test applying Bonferroni's method for simultaneous multiple comparisons when *F* values indicated statistical difference. Analysis of between-group variation was done by a two-tailed unpaired Student's *t* test.

Results

Hemodynamic measurements. Heart rate, peak systolic pressure, aortic flow (AF), and coronary flow (CF) were measured every 5 minutes during perfusion before and after hypothermia. In addition, CF was measured during the hypothermic (Langendorff) perfusion.

Fig. 2. Cardiac output in normothermic (37° C) working rat hearts before and after 40 minutes of hypothermic (10° C) perfusion. Two groups of rat hearts were studied: one group perfused with Krebs-Henseleit bicarbonate buffer containing 11.1 mmol/L glucose (open symbols) and another with buffer containing 11.1 mmol/L glucose plus 1.2 mmol/L albumin-bound palmitate (filled symbols). Cardiac output was calculated on basis of timed collections of AF and CF every 5 minutes. Symbols represent mean plus or minus standard error of eight to nine hearts in each group. # $p < 0.025$ versus prehypothermic value in same group; * $p < 0.05$ versus corresponding value in glucose-perfused hearts: WH, working-heart perfusion; L, Langendorff perfusion.

Pilot studies revealed a fall in AF during the initial phase of the prehypothermic (stabilization) period in palmitate-perfused, but not in glucose-perfused, hearts. To determine the duration of the stabilization period, therefore, five separate hearts were perfused for 30 minutes with buffer containing 11.1 mmol/L glucose and 1.2 mmol/L palmitate. In these hearts the fall in AF was accompanied by an increase in CF (not shown). However, there were no significant differences either between the AF or the CF values obtained after 20 and 30 minutes of perfusion. The duration of the stabilization period was therefore decided to be 20 minutes.

Fig. 2 shows values before and after hypothermia for cardiac output (CO; AF + CF) of glucose- and palmitate-perfused hearts while they were perfused in the working mode. Both palmitate- and glucose-perfused hearts showed significantly lower CO values 5 minutes after rewarming, as compared with the control values measured immediately before cooling (31% and 16% reduction, respectively; $p < 0.025$). Interestingly, the CO value obtained for palmitate-perfused hearts at this time ($38 \pm 3 \text{ ml} \cdot \text{min}^{-1}$) turned out to be significantly lower than that of glucose-perfused hearts ($52 \pm 2 \text{ ml} \cdot \text{min}^{-1}$; $p < 0.05$). However, 15 minutes after rewarming the difference in CO between the two groups was no longer statistically significant, both groups showing CO values that were approximately 80% of their prehypothermic control values. One cannot exclude, however, that the palmitate

group may have had significant deterioration of function, which could have been revealed had the reperfusion been monitored for 30 or 60 minutes. However, because the major object of this study was to examine the effect of lowered temperature on myocardial calcium, we decided to limit the reperfusion period to 15 minutes. It should be emphasized that marked disturbances in the heart rhythm and impaired pressure development were seen in palmitate-perfused hearts during the first 1 to 2 minutes after rewarming (Fig. 3). Glucose-perfused hearts, on the other hand, recovered hemodynamic function immediately on rewarming.

On introduction of the hypothermic Langendorff perfusion (hypothermic cardiac arrest) CF fell from prehypothermic control values of $25 \pm 1 \text{ ml} \cdot \text{min}^{-1}$ (palmitate perfused) and $16 \pm 1 \text{ ml} \cdot \text{min}^{-1}$ (glucose perfused) to approximately $10 \text{ ml} \cdot \text{min}^{-1}$ (both groups). This value was maintained throughout the entire hypothermic perfusion period, which is in contrast to results of Mjøs and associates,¹³ who reported a decrease in CF during hypothermia.

Myocardial content of calcium. Total cellular calcium ($[\text{Ca}^{2+}]_{\text{total}}$) extracted from myocardial tissue of glucose- or palmitate-perfused hearts at different end points is shown in Fig. 4. At the end of the prehypothermic stabilization period the average $[\text{Ca}^{2+}]_{\text{total}}$ (both groups) was $1.0 \mu\text{mol} \cdot \text{gm dry weight}^{-1}$. Hearts that were clamped during the following hypothermic phase showed

Fig. 3. Registrations of aortic pressure during first minutes after rewarming in glucose-perfused (A) and palmitate-perfused (B) hearts. Arrows indicate switch from hypothermic Langendorff perfusion to normothermic working heart perfusion. Note delayed recovery of aortic pressure in palmitate-perfused hearts.

Fig. 4. $[Ca^{2+}]_{total}$ in glucose-perfused and fatty acid-perfused hearts at different end points throughout experimental period, as indicated along horizontal axis. Calcium content was determined on basis of uptake of $^{45}Ca^{2+}$. Values represent mean plus or minus standard error of five to eight hearts. # $p < 0.0125$ versus prehypothermic value in same perfusion group; * $p < 0.05$ versus corresponding value in glucose group; WH, working-heart perfusion; L, Langendorff perfusion.

significantly increased $[Ca^{2+}]_{total}$, compared with the values in their prehypothermic controls. This response was more pronounced in palmitate-perfused hearts than in glucose-perfused hearts, so that at the end of the 40 minute hypothermic perfusion $[Ca^{2+}]_{total}$ of palmitate-perfused hearts ($3.5 \pm 0.2 \mu\text{mol} \cdot \text{gm dry weight}^{-1}$) was significantly ($p < 0.01$) higher than that of glucose-per-

fused hearts ($2.6 \pm 0.3 \mu\text{mol} \cdot \text{gm dry weight}^{-1}$). This finding shows that hypothermia causes increased calcium uptake and that this uptake is enhanced by high concentrations of fatty acids. The level of total cellular calcium in rewarmed hearts (both groups) was lower than the maximum value measured during hypothermia, and after 15 minutes (end of perfusion) control values of $[Ca^{2+}]_{total}$

Table I. Myocardial content of ATP and CP in glucose- and palmitate-perfused hearts at different times during the various perfusion periods (same hearts as those in Fig. 4)

	Prehypothermia (20 min)	Hypothermia		Posthypothermia	
		5 min	40 min	2 min	15 min
ATP ($\mu\text{mol/gm dry wt}$)					
Glucose	22.9 $\pm$ 1.0	23.6 $\pm$ 1.1	22.3 $\pm$ 1.5	19.7 $\pm$ 2.6	22.4 $\pm$ 2.3
Glucose + palmitate	21.6 $\pm$ 1.6	23.7 $\pm$ 1.2	19.5 $\pm$ 1.5	17.5 $\pm$ 2.2	20.4 $\pm$ 2.4
CP ($\mu\text{mol/gm dry wt}$)					
Glucose	54.4 $\pm$ 2.1	66.0 $\pm$ 2.5	66.7 $\pm$ 4.4	45.2 $\pm$ 8.3	68.9 $\pm$ 6.1
Glucose + palmitate	47.6 $\pm$ 7.6	67.8 $\pm$ 4.7	58.6 $\pm$ 5.6	38.7 $\pm$ 4.7	47.7 $\pm$ 6.1

The high-energy phosphates were analyzed in neutralized perchloric acid extracts of myocardial homogenate by the HPLC technique. The values are mean plus or minus the standard error.

were restored in glucose-perfused hearts. Palmitate-perfused hearts still expressed elevated $[\text{Ca}^{2+}]_{\text{total}}$ levels compared with those in glucose-perfused hearts ($p < 0.05$) at this time.

Metabolic measurements. Table I shows myocardial content of high-energy phosphates (ATP and CP) of the same hearts as those referred to in Fig. 4. There were no statistically significant changes in ATP or CP content neither within nor between the two perfusion groups.

Table II presents myocardial content of lactate, glycogen, free fatty acids, and triacylglycerol in the hearts included in Fig. 4. The content of lactate was low both in glucose- and palmitate-perfused hearts throughout the whole perfusion period, with no significant differences within or between the perfusion groups. Likewise, hearts clamped at the different end points showed similar contents of glycogen, indicating that myocardial energy homeostasis was maintained without substantial contribution of anaerobic consumption of endogenous glycogen.

The myocardial content of free fatty acids in palmitate-perfused hearts was approximately twice as high as that of glucose-perfused hearts ($p < 0.05$). Palmitate contributed 60% to the total free fatty acids in palmitate-perfused hearts in contrast to 30% in glucose-perfused hearts (not shown). However, within the two perfusion groups there was no difference in free fatty acid content between hearts clamped at the various end points.

Myocardial triacylglycerol content ranged between 18 and 26 $\mu\text{mol} \cdot \text{gm dry weight}^{-1}$ in glucose-perfused hearts and 25 and 29 $\mu\text{mol} \cdot \text{gm dry weight}^{-1}$ in palmitate-perfused hearts. However, there were no significant differences in triacylglycerol content between hearts clamped at the various end points. The HPLC chromatograms showed that the triacylglycerol fatty acids of palmitate-perfused hearts contained 35% palmitate (in contrast to 25% in glucose-perfused hearts), indicating substantial incorporation of palmitate into the triacylglycerol depot of these hearts.

Discussion

Effects of hypothermia on myocardial calcium content. Since the pioneering work by Bigelow, Lindsay, and Greenwood²⁹ hypothermia has been a fundamental component of all techniques developed for myocardial protection and used clinically. However, both experimental and clinical studies indicate that hypothermia may have unfavorable effects on myocardial calcium control, especially when hypothermia is applied without foregoing chemical arrest of the heart.^{3, 4, 30, 31} Except for "rapid cooling contracture" in isolated muscle preparations,⁷ our results for the first time demonstrate directly an increase in myocardial calcium content of isolated rat hearts during unprotected hypothermia. In contrast to the vast majority of experimental studies on isolated rat hearts in the past, the present study included albumin-bound fatty acids in the perfusion media. Although fatty acids are regarded to be the preferred substrate for aerobic energy metabolism in the normothermic heart, their presence during hypothermia produced an even higher accumulation of calcium than was found in hearts receiving glucose as the sole energy substrate. The presence of fatty acids did not influence myocardial calcium content in normothermic hearts, inasmuch as the same calcium levels were obtained both in glucose- and palmitate-perfused hearts at the end of the prehypothermic perfusion.

Total cellular calcium content in our prehypothermic hearts was about 1 $\mu\text{mol/gm dry weight}$, which corresponds well to the baseline values obtained by Tani and Neely,²² who used the same method to measure changes in myocardial calcium during global ischemia and reperfusion in Langendorff-perfused rat hearts. In their study myocardial calcium content increased 16-fold during ischemia, whereas in our hypothermic model a 3- to 4-fold increase was observed. This may indicate that the loss of calcium control of myocardial cell membranes during normoxic hypothermia (present study) is considerably less than that of an ischemic period of similar duration.

Table II. Myocardial content of lactate, glycogen, free fatty acids, and triacylglycerol (same hearts as those in Fig. 4)

	Prehypothermia (20 min)	Hypothermia		Posthypothermia	
		5 min	40 min	2 min	15 min
Lactate					
Glucose	5.5 ± 1.0	3.2 ± 0.9	2.1 ± 0.3	3.2 ± 0.4	3.0 ± 0.8
Glucose + palmitate	8.2 ± 2.6	3.1 ± 0.5	3.6 ± 0.7	5.9 ± 1.0	2.0 ± 0.2
Glycogen					
Glucose	170.1 ± 14.0	150.0 ± 13.5	137.8 ± 4.0	143.5 ± 9.1	172.0 ± 8.7
Glucose + palmitate	172.2 ± 27.9	160.4 ± 7.6	166.2 ± 10.2	140.5 ± 7.5	153.4 ± 7.7
FFA					
Glucose	5.5 ± 0.8	5.4 ± 0.9	3.6 ± 0.6	3.3 ± 0.3	3.6 ± 0.2
Glucose + palmitate	9.8 ± 1.1*	10.0 ± 1.4*	8.7 ± 1.5*	9.3 ± 1.2*	4.5 ± 1.0
TG					
Glucose	18.0 ± 5.0	21.6 ± 4.2	26.1 ± 6.1	20.3 ± 4.6	23.6 ± 5.2
Glucose + palmitate	27.1 ± 1.7	28.6 ± 4.6	25.6 ± 8.2	25.1 ± 4.7	26.1 ± 2.8

*Values given as mean plus or minus standard error. FFA, Free fatty acids; TG, triacylglycerol.

$p < 0.05$ versus corresponding value in glucose-perfused hearts.

Another factor that may be related to the lower calcium content in our hypothermic hearts is the presence of colloids (2% albumin) in the perfusates.

The delayed recovery of mechanical function after rewarming in fatty acid, compared with that in glucose-perfused hearts, is in accordance with the study by Mjøs and associates.¹³ In our experiments this finding was correlated to significantly higher values of $[Ca^{2+}]_{total}$ during hypothermia, as well as a slower decline in the calcium level on rewarming, in fatty acid-perfused than in glucose-perfused hearts. It is important to notice, however, that the method for determination of myocardial calcium represents $[Ca^{2+}]_{total}$, including not only cytosolic, sarcoplasmic reticulum, and mitochondrial stores, but probably also calcium bound to the sarcolemma. These cellular compartments are suggested to buffer cytoplasmic calcium,³² but on the basis of our measurements of mechanical function of the heart during rewarming, as well as previous reports of elevated inotropy in hypothermic states,^{9, 33} there is reason to believe that the observed rise in $[Ca^{2+}]_{total}$ also implies a rise in the calcium concentration in the region of the contractile apparatus. Interestingly, $[Ca^{2+}]_{total}$ did not increase in the rewarming phase after hypothermia, as has been suggested for isolated myocytes.¹⁴

Possible mechanisms of the calcium load. One obvious mechanism that would explain the observed increase in $[Ca^{2+}]_{total}$ during hypothermia is cold inhibition of the sarcolemmal Ca^{2+} pumps, which would otherwise extrude Ca^{2+} from the cytoplasm. The lowered myocardial temperature will in addition decelerate the sarcolemmal Na^+/K^+ ATPase, resulting in increased intracellular Na^+ levels. This in turn will lead to a negative shift in

the reversal potential of the Na^+/Ca^{2+} exchange mechanism, resulting in an increased influx of calcium.^{14, 22} The fact that the Na^+/Ca^{2+} exchanger is less affected by temperature than the Na^+/K^+ ATPase (Q_{10} coefficients of 1.35 and 3.0, respectively⁹) makes this possibility very likely.

Changes in membrane structure termed *thermotropic lateral phase separations* have been reported in cases in which rat cardiac membrane preparations have been cooled below their physiologic temperature.³⁴ This process involves so-called phase transitions, in which the membrane phospholipids change from a liquid state to a rigid crystalline or gel state, forming solid "islands" or domains of lipid within the membrane that will exclude intramembranous proteins.³⁵ Such a change in membrane structure would be expected to influence permeability of the myocardial cell membrane,³⁶ including changes in the chemical environment of ion channels and of the ordered structure of the lipid bilayer itself. Moreover, the enhancement of the hypothermia-induced rise in myocardial calcium by fatty acids could be explained in terms of increased membrane permeability because of their detergent properties. However, unpublished results from our own laboratory show that cellular calcium content of isolated rat myocardial cells, incubated for 40 minutes at 10° C in the presence of 11.1 mmol/L glucose plus 1.2 mmol/L palmitate, was not different from that of cells incubated with 11.1 mmol/L glucose alone. Furthermore, neither group showed different calcium levels from those of normothermic control cells. This fact argues against the view that increased myocardial calcium can be ascribed to phase separations of membrane lipids or detergent effects on the sarcolemma by fatty acids. On the

other hand, because isolated cardiomyocytes are quiescent, the rise in $[Ca^{2+}]_{total}$ in the intact rat heart apparently is intimately related to the inherent electromechanical activity in these hearts (although it is markedly depressed in the hypothermic state).

Another possibility is that the effect of fatty acids on the calcium homeostasis is mediated via inhibition of intracellular enzyme systems involved in cellular energy production, either directly or via their acyl coenzyme A or acylcarnitine derivatives.³⁷ Thus the level of intracellular free fatty acids in the present study was twice as high in palmitate-perfused hearts as in glucose-perfused hearts throughout the entire incubation period. Moreover, Mjøs and associates¹³ reported several-fold increases in intracellular acyl coenzyme A and acylcarnitine under identical incubation conditions. The myocardial ATP content obtained in hypothermic (or rewarmed) hearts was, however, not different from that of normothermic control hearts, indicating that the loss of calcium control was not related to ATP shortage.

On the other hand, several authors have focused on the importance of glycolytically produced ATP in the maintenance of membrane integrity³⁸ and thus of low intracellular calcium levels.^{39,40} Furthermore, in a recent study by Saddik and Lopaschuk⁴¹ fatty acids were found to inhibit glucose oxidation to a greater extent than glycolysis. Therefore it cannot be excluded that the glycolytic production of ATP in our fatty acid-perfused hearts was inadequate to preserve the myocardial calcium homeostasis, despite normal tissue ATP values.

In conclusion, the present study has shown that rapid cooling of nonarrested isolated rat hearts leads to a marked rise in intracellular calcium levels. This effect is aggravated in the presence of fatty acids and may under such conditions result in significant myocardial damage. Although results obtained from isolated rat hearts perfused with a crystalloid perfusate may not be directly applicable to hearts perfused with blood, the data obtained in this study may be particularly useful in the discussion of warm versus cold cardioplegia. Moreover, because high concentrations of fatty acids are seen during and after clinical hypothermia, more attention should be aimed at controlling the plasma concentration of free fatty acids during cardiac operations. Another suggestion would be to minimize the hypothermic perfusion of the nonarrested heart.

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