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Protective Effect of Pretreatment With the Calcium Antagonist Anipamil on the Ischemic-Reperfused Rat Myocardium: A Phosphorus-31 Nuclear Magnetic Resonance Study

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To assess whether the prophylactic administration of anipamil, a new calcium antagonist, protects the heart against the effects of ischemia and reperfusion, rats were injected intraperitoneally twice daily for 5 days with 5 mg/kg body weight of this drug. The heart was then isolated and perfused by the Langendorff technique. Phosphorus-31 nuclear magnetic resonance spectroscopy was used to monitor myocardial energy metabolism and intracellular pH during control perfusion and 30 min of total ischemia (37°C), followed by 30 min of reperfusion.

Pretreatment with anipamil altered neither left ventricular developed pressure under normoxic conditions nor the rate and extent of depletion of adenosine triphosphate (ATP) and creatine phosphate during ischemia. Intracellular acidification, however, was attenuated.

On reperfusion, hearts from anipamil-pretreated animals recovered significantly better than untreated hearts with respect to replenishment of ATP and creatine phosphate stores, restitution of low levels of intracellular inorganic phosphate and recovery of left ventricular function and coronary flow. Intracellular pH recovered rapidly to preischemic levels, whereas in untreated hearts a complex intracellular inorganic phosphate peak indicated the existence of areas of different pH within the myocardium.

It is concluded that anipamil pretreatment protects the heart against some of the deleterious effects of ischemia and reperfusion. Because this protection occurred in the absence of a negative inotropic effect during normoxia, it cannot be attributed to an energy-sparing effect during ischemia. Therefore, alternative mechanisms of action are to be considered.

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Severe myocardial ischemia results in a number of events, including depletion of high energy phosphate stores (1,2), intracellular acidification (3,4), loss of ionic homeostasis and mitochondrial and membrane damage (5), ultimately leading to cell death. To date, the exact sequence of events and the relative importance of several contributing mechanisms are still unclear. The only way to stop this process, however, is by timely reestablishment of coronary flow (6), although after a critical period of ischemia this may paradoxically extend or accelerate ischemic cell damage (5,7,8). Reperfusion before this critical period will cause either uncomplicated recovery or delayed restoration of metabolism and function ("stunned myocardium") (9).

Because ischemia-reperfusion damage in myocardial cells is associated with the accumulation of calcium (Ca^{2+}) ions in the cytoplasm and mitochondrial matrix (6,10,11), calcium antagonists have been used to protect the myocardium during ischemia (2,12,13) and subsequent reperfusion (14,15). Several mechanisms for their effectiveness have been proposed (16), including afterload reduction by peripheral arterial vasodilation, negative inotropic and chronotropic effects reducing myocardial oxygen consumption and coronary vasodilation and enhancement of collateral flow improving blood supply to the jeopardized tissue (17). The combination of these effects would help to maintain the balance of energy expenditure and supply under circumstances of impaired oxygen and substrate availability. More recently, additional direct effects of calcium antagonists

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have been postulated on myocardial metabolism (18), the myocardial cell membrane (19) or cellular viability (20,21) during ischemia and reperfusion.

The aim of this study was to determine whether the prophylactic treatment of rats with anipamil, a new calcium antagonist, protects the isolated heart during ischemia and subsequent reperfusion with regard to high energy phosphate metabolism, intracellular pH and cardiac function. Anipamil is a highly lipophilic verapamil-type calcium antagonist intended for long-lasting antihypertensive and cardioprotective action (22).

The potential of phosphorus-31 nuclear magnetic resonance (^{31}P-NMR) spectroscopy to evaluate the protective effect of a calcium antagonist was first demonstrated by Nunnally and Bottomley (23). We have used this technique to nondestructively follow the time course in phosphorus-containing metabolites and intracellular pH (2,4,24) with a simultaneous determination of left ventricular function.

Methods

Animal preparation. Male Wistar rats, weighing 300 to 350 g, received twice daily intraperitoneal injections containing either 5 mg/kg body weight anipamil (Knoll AG) dissolved in 5% glucose solution or the solvent without the drug. On the 5th day of this regimen, 2 h after the last injection, the rats were anesthetized with ether and heparinized; the heart was then rapidly excised. Perfusion was started by the Langendorff technique at a constant pressure of 75 mm Hg as previously described (2). The heart was stimulated throughout the experiment at 300 beats/min by two sodium chloride wick electrodes sutured to the right ventricle. Left ventricular pressure was measured by way of a perfusate-filled open catheter inserted through the apex (25,26). The difference between peak systolic and end-diastolic pressure was considered to be the left ventricular developed pressure. The heart was placed in a 20 mm NMR tube with a capillary containing methylene diphosphonate for spectral reference. The glass tube with the submerged heart was then lowered into the magnet. The effluent was collected in 5 min fractions for determination of coronary flow. Myocardial temperature was carefully maintained at 37°C.

NMR methods. ^{31}P-NMR spectra were obtained on a Bruker MSL 200 spectrometer equipped with a 4.7 tesla vertical bore magnet. No field frequency lock was used. Five minute spectra were obtained from 128 accumulated free induction decays after 90° pulses repeated at 2.3 s intervals. The data were accumulated using a 2k timetable and 5,000 Hz spectral width. Exponential multiplication resulting in 10 Hz line broadening was applied and baseline correction was performed on the spectra. Figure 1 shows typical examples of spectra obtained during preischemic control perfusion of a heart from an untreated (A) and an anipamil-pretreated rat

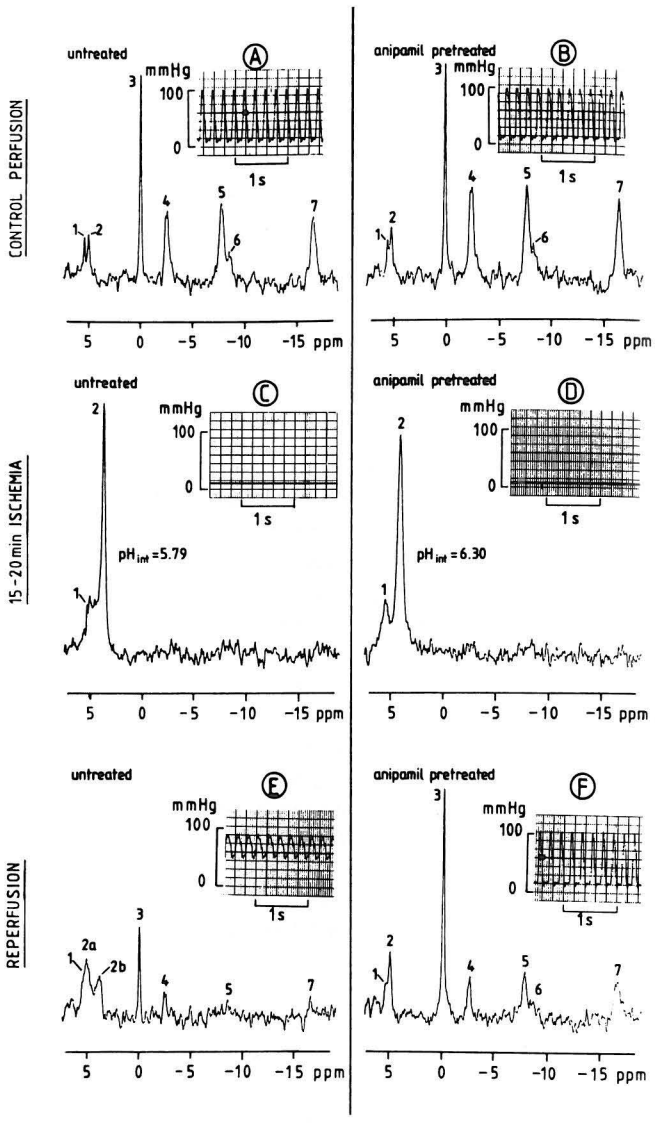

Figure 1. ^{31}P-NMR spectra and simultaneous left ventricular pressure recordings from an untreated (**A,C,E**) and an anipamil-pretreated (**B,D,F**) rat heart during control perfusion (**A,B**), between 15 and 20 min of ischemia (**C,D**) and between 25 and 30 min of reperfusion (**E,F**). Numbered peaks are: 1, extracellular inorganic phosphate (P_i); 2, intracellular P_i; 3, creatine phosphate; 4,5,7, γ-, α- and β-phosphate group of adenosine triphosphate; and 6, nicotinamide adenine dinucleotide.

(B). Intra- and extracellular pH were calculated from the chemical shift of the respective inorganic phosphate peaks relative to methylene diphosphonate. Zero parts per million was assigned to creatine phosphate (2,4). Quantitation of metabolites was achieved by integrating the areas under individual peaks of interest in each spectrum, with the beta-phosphate peak of adenosine triphosphate (ATP) representing ATP levels. Data on high energy phosphate metabolites are presented as changes relative to the average of

Table 1. Effects of Pretreatment With Anipamil on Myocardial Function and Coronary Flow of Isolated Rat Hearts During Control Perfusion and After 30 min of Reperfusion Following 30 min of Total Ischemia

	Preischemic			Reperfusion		
	LVDP (mm Hg)	CF (ml/min)	LVEDP (mm Hg)	LVDP (mm Hg)	CF (ml/min)	LVEDP (mm Hg)
Untreated (n = 13)	85.4 ± 3.0	13.2 ± 3.2	3.4 ± 0.9	32.4 ± 24.8	8.2 ± 2.2	32.3 ± 24.3
Anipamil-pretreated (n = 25)	82.4 ± 6.8	12.6 ± 2.0	3.3 ± 0.8	79.8 ± 10.9*	14.6 ± 2.1*	7.6 ± 6.0*

*$p < 0.0001$ vs. untreated; CF = coronary flow; LVDP = left ventricular developed pressure; LVEDP = left ventricular end-diastolic pressure.

three successive control measurements preceding ischemia. Data were processed in a blinded fashion. Levels of intracellular inorganic phosphate (P_i) are expressed as a percentage of the total amount of phosphate groups from creatine phosphate (CP), ATP and intracellular P_i during preischemic control perfusion:

$$P_i/[CP + 3ATP + P_i]_{\text{control perfusion}} \times 100\%.$$

Creatine phosphate and intracellular P_i were corrected for partial saturation; the saturation factors 1.5 and 1.1, respectively, were determined using a 10 s recycle time.

Experimental protocol. After 30 to 35 min of control perfusion, all hearts were made totally ischemic for 30 min by cross-clamping the perfusion line; this period was followed by 30 min of reperfusion. During the control period the hearts were allowed to stabilize for about 15 min. Subsequently, three spectra were recorded during control perfusion, six during ischemia and six during reperfusion.

Statistical analysis. Results are presented as mean ± standard deviation (SD) of 25 anipamil and 13 control experiments. Because consecutive measurements were performed on each heart to establish the time course in phosphorus-containing metabolites and pH, differences between treated and untreated hearts were statistically evaluated by "analysis of variance with repeated measurements" (27,28). A test result with a p value <0.05 was considered significant. Data on ischemia and reperfusion were treated separately. In addition, data on creatine phosphate, ATP and intracellular P_i during reperfusion were analyzed for the occurrence of a steady state, as were pH data during ischemia.

Results

Myocardial function and coronary flow. Typical examples of left ventricular pressure recordings obtained during control perfusion, ischemia and reperfusion from an untreated and an anipamil-pretreated heart are shown in Figure 1. Table 1 summarizes values for left ventricular developed pressure and end-diastolic pressure during preischemic control perfusion and at the end of reperfusion. During control perfusion there was no significant difference in contractile performance between anipamil-pretreated and untreated hearts, whereas functional recovery after ischemia and reperfusion was significantly better in the anipamil group. Moreover, contracture, as indicated by the increase in end-diastolic pressure, was less pronounced in treated hearts. During ischemia, left ventricular developed pressure decreased rapidly to zero in both groups and the period of still detectable contractile activity was equal (2.5 to 3 min). On reperfusion, contraction usually began with a period of ventricular fibrillation or chaotic ventricular arrhythmia, which gradually gave way to stable contractile function. As this process showed large variations, only values for left ventricular developed pressure at the end of reperfusion are presented.

Coronary flow during preischemic control perfusion in treated hearts was not significantly different from flow in untreated hearts (Table 1), indicating that there was no vasodilation due to anipamil pretreatment. Like recovery of mechanical function, restoration of coronary flow on reperfusion was more complete in the anipamil group and even exceeded flow rates during control perfusion. In untreated hearts a considerable reduction in coronary flow was observed after ischemia.

NMR spectroscopy: creatine phosphate, ATP and inorganic phosphate (P_i). Figure 1 shows examples of ^{31}P-NMR spectra with simultaneous left ventricular pressure recordings during control perfusion (A and B), between 15 and 20 min of ischemia (C and D) and at the end of reperfusion (E and F), obtained from an untreated heart (A,C,E) and a heart from an anipamil-pretreated rat (B,D,F). They illustrate the similarity of untreated and anipamil-pretreated hearts in phosphorus-containing metabolites during control perfusion and ischemia, but not during reperfusion. Figure 2 summarizes creatine phosphate, ATP and intracellular P_i levels in hearts from untreated and anipamil-pretreated rats during ischemia and reperfusion. The depletion of high energy phosphates during ischemia, which was balanced by a simultaneous accumulation of intracellular P_i, was similar in both groups.

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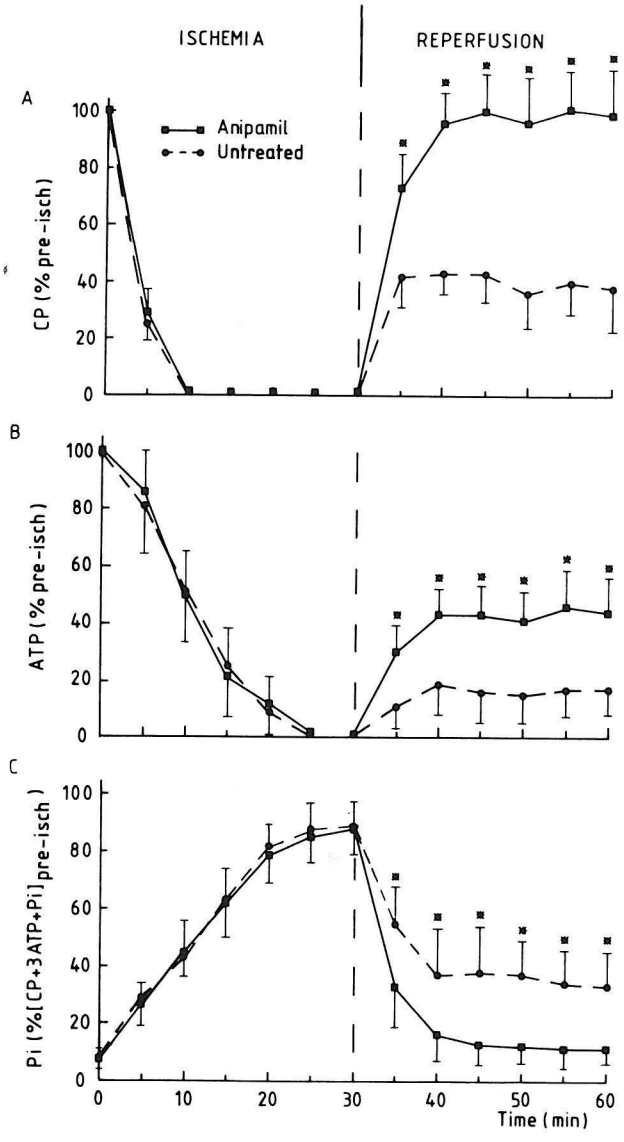

Figure 2. Effect of pretreatment of rats with anipamil on the time course in levels of (**A**) creatine phosphate (CP); (**B**) adenosine triphosphate (ATP); and (**C**) intracellular inorganic phosphate (P_i) as measured with ^{31}P-NMR in isolated perfused hearts submitted to 30 min of normothermic global ischemia followed by 30 min of reperfusion. CP and ATP levels are expressed as a percent of their respective preischemic values. Intracellular P_i is expressed as a percent of the amount of phosphate groups from CP, ATP and intracellular P_i during preischemic control perfusion: $P_i/[CP + 3ATP + P_i]_{preischemic} \times 100\%$. Measurements were obtained from consecutive 5 min ^{31}P-NMR spectra. Each point represents the mean ± SD of 25 anipamil-pretreated or 13 untreated hearts. During ischemia, treated and untreated hearts were not different, whereas during reperfusion, recovery of CP and ATP was significantly better in treated hearts and intracellular P_i levels were lower. *$p < 0.0001$ versus untreated.

Reperfusion induced a rapid and complete restoration of creatine phosphate in anipamil-pretreated hearts, whereas in untreated hearts only about 40% of preischemic levels were replenished, the difference between treated and untreated hearts being significant ($p < 0.0001$). Recovery of ATP in anipamil-pretreated hearts was also better than in untreated hearts ($p < 0.0001$), but no complete restoration of ATP was found. Statistical analysis indicated that after 10 min of reperfusion, no further improvement of high energy phosphate metabolites occurred. Pretreatment with anipamil also helped to restore low levels of intracellular P_i; in untreated hearts, intracellular P_i levels remained elevated even after 30 min of reperfusion and were significantly higher than in treated hearts ($p < 0.0001$). The major changes in intracellular P_i levels took place during the first 10 min of reperfusion, but no real steady state was reached.

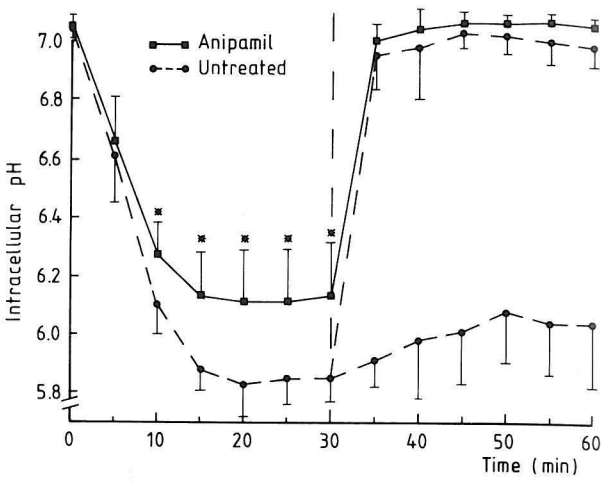

Figure 3. Effect of pretreatment of rats with anipamil on intracellular pH of their isolated perfused heart during 30 min of global ischemia followed by 30 min of reperfusion, as assessed with ^{31}P-NMR spectroscopy. During reperfusion of the untreated heart, a complex pattern in the intracellular P_i region was observed, corresponding with a range of intracellular pH values (see Fig. 1E). The highest and lowest values are indicated by the **upper** and **lower dashed curves**, respectively. Each point represents the mean ± SD of 25 pretreated or 13 untreated hearts. After 10 min of ischemia, pH in treated hearts was significantly higher than in untreated hearts. *$p < 0.0001$ versus untreated.

Intracellular pH. During preischemic control perfusion intracellular pH was not affected by pretreatment with anipamil (Fig. 3), but after 10 min of ischemia acidification of the cytosol was attenuated as compared with that in untreated hearts ($p < 0.0001$). Unlike depletion of high energy phosphates during ischemia, which was unaffected by anipamil pretreatment, pH values stabilized at a higher level in the heart of the anipamil-pretreated rat than in the untreated heart.

During reperfusion, intracellular pH rapidly recovered to preischemic values in treated hearts, whereas in untreated hearts the ^{31}P-NMR spectra were often characterized by the presence of multiple intracellular P_i peaks (Fig. 1E) corresponding with different pH values within the myocar-

dium (29). Despite this incomplete or inhomogeneous reperfusion, the main P_i peak corresponded with a pH of 7.00 to 7.10.

Discussion

These results demonstrate that the isolated perfused heart of the rat pretreated with the new calcium antagonist anipamil is protected against some of the effects of total ischemia and reperfusion. Intracellular acidosis during ischemia was attenuated. On reperfusion, the heart in the anipamil-pretreated animals showed a significantly better recovery of biochemical and functional variables than did the untreated heart.

Previous studies. It is generally accepted that, for optimal protection against ischemia-reperfusion–induced damage, calcium antagonists should be present before or, at the latest, during the ischemic event (30,31). The protective effect of calcium antagonists is commonly attributed to the energy-sparing effect during ischemia due to their negative inotropic properties (2,7,11,13,15,32–35). In that way, more energy would remain available for the maintenance of cellular ionic homeostasis, particularly with respect to sodium (Na^+) and calcium (Ca^{2+}) ions.

On the other hand, there is evidence that mechanisms unrelated to reduction of cardiac work may play a role in the protection exerted by calcium antagonists. Henry and Wahl (36) demonstrated that hypoxic contracture in electrically and mechanically quiescent myocardium was suppressed by diltiazem and nifedipine, thereby excluding a protective effect in terms of energy conservation. Others (18,20) reported protection of isolated rat hearts in the presence of diltiazem, nifedipine and verapamil at such a low concentration that no negative inotropic effect could be observed. They suggested a direct preservation of cellular viability (20) and a direct beneficial effect on the energy metabolism of the ischemic heart (18).

To date, only a few studies on the cardioprotective action of anipamil are available. Raschack and Kirchengast (37) demonstrated that ST elevation and potassium release after coronary artery occlusion in pigs were attenuated by anipamil. Brezinski et al. (21), who ligated the left anterior descending coronary artery of cats in vivo, found cardioprotection without reduced oxygen demand in the presence of anipamil. They proposed a direct cytoprotective action of anipamil during ischemia. Curtis et al. (38) studied the effects of anipamil in rats in vivo after coronary artery ligation and observed an antiarrhythmic effect during ischemia without changes of heart rate and blood pressure during the preischemic period.

Biochemical and functional effects of anipamil. In our study, no negative inotropic effect was observed in the isolated heart of anipamil-pretreated rats. It is in accordance with this finding that neither the rate nor the extent of depletion of adenosine triphosphate (ATP) and creatine phosphate during ischemia was attenuated. Despite the lack of an energy-sparing effect during ischemia, recovery of high energy phosphates and cardiac function during reperfusion was better than in untreated hearts and contracture was prevented.

Anipamil pretreatment also improved restoration of coronary flow of the isolated heart during reperfusion as compared with the untreated heart. It is questionable whether this is a direct consequence of vasodilation by anipamil, because coronary flow under preischemic conditions was not different in treated and untreated hearts. In our experimental model, impaired reperfusion after transient ischemia (no reflow phenomenon) may be due to endothelial cell swelling and vasoconstriction and myocardial cell swelling and contracture of myocytes (31,39). Although prevention of contracture by anipamil (Table 1) may contribute to a better reperfusion, it may not be a complete explanation because nifedipine was found to improve coronary flow during reperfusion without reduction of contracture (31). We propose that either direct cytoprotective effects (20,21) or a rapid recovery of cardiac metabolism enables a better control of transmembrane ionic currents and cytosolic osmolarity in both vascular cells and myocytes, thereby preventing the formation of edema and contracture. This in turn will enhance tissue perfusion and may further facilitate aerobic metabolism.

Intracellular pH. In our experiments, the major difference during ischemia between treated and untreated hearts appeared to be the intracellular pH, which stabilized at a significantly higher level in treated hearts. A limitation of the amount of hydrogen ions generated during ischemia may reduce the cellular uptake of Ca^{2+} at the onset of reperfusion by way of the sarcolemmal H^+/Na^+ and Na^+/Ca^{2+} exchange mechanisms (40). Because uncontrolled influx of Ca^{2+} into the cells is one of the crucial steps in the development of reperfusion damage (6,10,11), limitation of the production of hydrogen ions during ischemia would eventually facilitate recovery of the myocardium.

There are several possible explanations for the attenuation of intracellular acidosis during ischemia in the anipamil-treated hearts. Because Ca^{2+} is known to stimulate the conversion of phosphorylase b into phosphorylase a, which is necessary for the breakdown of glycogen (41), limitation of Ca^{2+} influx during ischemia would contribute to a reduction of glycogen degradation and a concomitant attenuation of intracellular acidosis (42). It has been shown for several calcium antagonists (12,20) that a limitation of Ca^{2+} influx during ischemia can occur even in the absence of energy-sparing effects. It is conceivable that such a limitation of Ca^{2+} influx is not necessarily the result of a direct interaction between calcium antagonists and the slow channels, but may also be mediated by a reduced release of catecholamines (43), which activate the slow channels and augment

Ca^{2+} influx. It is also possible that catecholamine stores were already depleted because of the pretreatment of the animals, as has been shown for other verapamil-type calcium antagonists (30,44,45). A decreased availability of catecholamines during ischemia will also reduce the cyclic adenosine monophosphate- (cAMP)-mediated degradation of glycogen.

Alternative mechanism. It is uncertain whether the influx of Ca^{2+} early during reperfusion is mediated by the slow channels because these may be inactivated by acidosis and dephosphorylation (46). In most studies (31–33) the presence of calcium antagonists only during reperfusion failed to reduce reperfusion damage. However, Weishaar and Bing (35) suggested a limitation of the massive Ca^{2+} influx during reperfusion by diltiazem. More likely routes for Ca^{2+} entry are the Na^+/Ca^{2+} exchange mechanism (40), as mentioned before, and an increased membrane permeability for Ca^{2+} (6,20,47).

Therefore, the protection exerted by anipamil may also be related to effects on the cell membrane. In particular, the phenylalkylamines (like verapamil and anipamil) are supposed to exert their effects by initially dissolving into the phospholipid bilayer and subsequently migrating toward the slow channels (48). Anipamil would be a perfect candidate for such a mode of action because of its highly lipophilic properties. In addition, its presence in the phospholipid bilayer could make the sarcolemma less sensitive to Ca^{2+}-induced conformational changes during ischemia and reperfusion (49). By maintaining sarcolemmal integrity (19) during ischemia and reperfusion, excessive entry of Ca^{2+} and irreversible cell damage would then be prevented. Additional experiments will be needed to elucidate the exact mechanism of myocardial protection by anipamil.

Conclusions. Pretreatment of rats with anipamil protected their isolated heart against ischemia-reperfusion–induced damage. In contrast to most in vitro studies with calcium antagonists, in which protection can be attributed to a negative inotropic effect that in the in vivo situation might be overcome by compensatory mechanisms, in our experiments pretreatment with anipamil did not impair contractile performance of the heart. Because anipamil appeared to be an effective drug to prevent the aggravation or acceleration of ischemic damage by reperfusion, it may be a promising tool for clinical practice in which reperfusion is of increasing importance.

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