EFFECTS OF ISCHEMIC PRECONDITIONING ON ARRHYTHMOGENESIS IN THE ISOLATED RABBIT HEART: POSSIBLE ROLE OF PROTEIN KINASE C

A Thesis Presented to the University of Manitoba

In Partial Fulfillment of the Requirement

For the Degree of:

MASTER OF SCIENCE IN PHYSIOLOGY

By

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April, 1997

THE UNIVERSITY OF MANITOBA

FACULTY OF GRADUATE STUDIES

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A Thesis/Practicum submitted to the Faculty of Graduate Studies of The University of Manitoba in partial fulfillment of the requirements of the degree of

MASTER OF SCIENCE

ACKNOWLEDGMENTS

Firstly, I would like to thank my supervisor Dr. Anton Lukas for his time, support and guidance throughout my study. I would also like to thank the members of my advisory committee, Dr. P. K. Singal and Dr. V. Panagia for their time and invaluable input into completion of this thesis. I am also thankful to Dr. N.S. Dhalla for providing opportunities that ultimately lead to this course of study. My sincere appreciation goes to all the people at the Institute of Cardiovascular Sciences for always providing a friendly environment in which to work and learn.

Last but by no means least I would like to thank my wife Stephanie for her support, patience and understanding during this busy time in our lives and my two sons Brayden and Connor whose smiles were always there after many long days.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

I. ABSTRACT

Prolonged ischemia has detrimental effects on ultrastructure, metabolism and electrophysiology of the myocardium that include necrosis, contractile dysfunction as well as arrhythmias. However, short intermittent periods of ischemia prior to a prolonged ischemia may protect the myocardium from these forms of ischemia-induced damage. This protective phenomenon has been termed **ischemic preconditioning**.

Ischemic preconditioning protects the heart against infarction in all species tested. In contrast, its protection against contractile dysfunction and arrhythmias (ventricular tachycardia, ventricular fibrillation) is not well characterized. Also, the *mechanisms* involved in ischemic preconditioning are not known. The present study investigated the effects of multiple preconditioning cycles on arrhythmogenesis and contractile dysfunction. The possible role of protein kinase C (PKC) in ischemic preconditioning was investigated using the specific PKC inhibitor chelerythrine administered during various stages of the protocol.

Langendorff-perfused rabbit hearts were placed in a bath fitted with leads forming an Einthoven triangle to record a volume-conducted 3-lead electrocardiogram (ECG). Preconditioned hearts underwent 1-4 cycles of global ischemia (5 minutes each separated by 10 minutes of reperfusion) 30 minutes prior to a *test* ischemia (30 minutes) followed by 45 minutes of reperfusion. Control hearts received only the *test* ischemia/reperfusion. Chelerythrine was administered either during the preconditioning ischemia (protocol I), during the *test* ischemia/reperfusion (protocol II) or throughout the experiment (protocol III) in hearts exposed to one preconditioning cycle.

One period of ischemic preconditioning completely protected hearts against ischemia-induced ventricular fibrillation (0% versus 42% in non-preconditioned hearts) and to a lesser degree against reperfusion-induced ventricular fibrillation and ischemia-induced ventricular tachycardia. Additional preconditioning cycles offered minimal protection against arrhythmias (2 cycles) or actually increased their incidences (3 and 4 cycles). Hearts preconditioned with 2 or 3 cycles exhibited significantly less *ischemic* contracture compared to non-preconditioned hearts during the *test* ischemia, whereas the large increase in end diastolic pressure seen upon *reperfusion* was unaffected by the preconditioning protocols. Preconditioning with 1, 2, 3 or 4 cycles decreased the amount of monophasic action potential duration (MAPD) shortening in epicardium and endocardium (versus non-preconditioned hearts) during the first 10 minutes of the *test* ischemia. Hearts preconditioned with one cycle exhibited less MAPD dispersion between epicardium and endocardium. This decrease in MAPD dispersion correlated with the protection against ischemia-induced arrhythmias seen with 1 preconditioning cycle versus non-preconditioned hearts. However, a similar reduction in MAPD dispersion was seen in hearts exposed to 4 preconditioning cycles which did not show protection against arrhythmias.

Protein kinase C (PKC) inhibition had different effects on preconditioning against arrhythmias, depending on when the inhibitor (chelerythrine) was present. Exposure to chelerythrine during the preconditioning ischemia (protocol I) or during the *test* ischemia/reperfusion (protocol II) abolished the protective effect of preconditioning on ischemia-induced arrhythmias. In contrast, the protection afforded by preconditioning was

not affected if chelerythrine was present throughout the experiment (protocol III). PKC inhibition had little effect on contractile recovery in any of the preconditioning protocols (protocol I-III). Less shortening of MAPD was seen in epicardium and endocardium with PKC inhibition throughout the experiment (protocol III). As well, cheleryhtrine treatment throughout the experiment resulted in a biphasic MAPD profile in endocardium similar to that of non-preconditioned hearts. Thus, PKC effects on electrophysiologic parameters during the *test* ischemia did not correlate with its protection against arrhythmias.

These findings show that 1 or 2 preconditioning cycles protect the heart against ischemia-induced ventricular fibrillation, while 3 or 4 cycles potentiate this arrhythmia. Also, ischemic preconditioning is more effective at protecting against ischemia-induced versus reperfusion-induced arrhythmias. Preconditioning does not appear to protect against contractile dysfunction during reperfusion, however, 2 and 3 preconditioning cycles decreased the degree of ischemic contracture. This study also shows that PKC inhibition during either the preconditioning ischemia or *test* ischemia abolishes the protection conferred by 1 preconditioning cycle. In contrast, PKC inhibition throughout the protocol did not alter the preconditioning response against arrhythmias. However, hearts exposed to chelerythrine throughout the protocol exhibited less shortening of MAPD than non-preconditioned hearts or hearts exposed to 1 cycle during the *test* ischemia. These results suggest that the effects of PKC inhibition on MAPD changes during ischemia do not correlate with its effects on arrhythmogenesis.

II. INTRODUCTION

Ischemic preconditioning (PC) is the phenomenon whereby exposure of the heart to brief transient periods of ischemia (either regional or global in nature) confers protection against a later more prolonged period of ischemia. Murry et al. (1986) first described ischemic preconditioning in dogs using reduction in infarct size as the endpoint to assess preconditioning. Since then, investigators have shown that ischemia is not the only means by which to elicit preconditioning. Hypoxia, rapid pacing, stretch, and infusion of adenosine can also protect the heart against a subsequent prolonged ischemia.

Along with reduction in infarct size, ischemic preconditioning can also protect against other adverse effects of ischemia and reperfusion such as contractile dysfunction and arrhythmogenesis. Contractile dysfunction is manifest as an acceleration of ischemic contracture (increased end diastolic pressure, EDP) and/or stunning (prolonged but reversible depression in contractile function on reperfusion; Braunwald and Kloner, 1982). Arrhythmias such as premature ventricular complexes (PVCs), ventricular tachycardia (VT) and ventricular fibrillation (VF) appear after the onset of ischemia as a consequence of metabolic and electrophysiologic alterations. Very few studies have measured the effects of preconditioning on protection against arrhythmias. However, in the clinical setting a reduction in infarct size or improvement in contractility can only occur if the patient first survives the early phase of lethal arrhythmias. Therefore, a reduction in the incidence of arrhythmias represents a clinically relevant endpoint to assess whether protection has occurred as a result of preconditioning.

The endpoints used to assess preconditioning and the stimuli used to induce preconditioning are very species specific and protocol dependent. The duration and number of preconditioning cycles as well as the time between the preconditioning cycles and the subsequent prolonged ischemia are all capable of modifying the preconditioning response.

The first description of preconditioning ('classic' preconditioning) referred to a transient relatively short lived protection, lasting one to three hours after the initial preconditioning protocol. A 'second window' of protection that occurs 24 hours after the preconditioning protocol and can last 3 or more days has since been reported. As is the case for classic preconditioning, the appearance of this second window is also very species- and protocol-dependent.

The mechanisms involved in preconditioning remain unidentified and may differ based on species and end-point studied. Some mechanisms proposed for protection against infarction relate to conservation of high energy substrates such as ATP and phosphocreatine or reduction of the accumulation of catabolites such as lactate, H^+ ions, and inorganic phosphate. Another theory is that free radicals generated as a result of the brief episode of ischemia and reperfusion may precondition the heart by stunning and thus confer protection, or that preconditioning leads to a reduction of free radical generation. Activation of $I_{K\text{-}ATP}$ channels and phospholipases C and D, production of adenosine, nitric oxide (NO), and prostacyclin, activation of α_1 adrenergic, angiotensin AT_1, muscarinic M_2, endothelin ET_A, purinergic P_1 and bradykinin B_2 receptors, and the production of heat

shock proteins have also been proposed as mechanisms for preconditioning. Activation of many different receptors types (adenosine, bradykinin, NO and prostacyclin) and phopholipase D leads to activation of protein kinase C (PKC) via second messenger pathways. Thus, PKC is thought to be a pivotal player in the preconditioning story.

The objectives of the present study were: 1) To determine whether ischemic preconditioning can protect the rabbit heart against ischemia- and/or reperfusion-induced arrhythmias. 2) To determine whether preconditioning protocols that protect the heart against arrhythmogenesis are equally effective in protecting against contractile dysfunction. 3) To determine whether preconditioning affects the entire ventricular wall or specific layers (epicardium versus endocardium). 4) To elucidate the role of PKC in preconditioning-induced protection against arrhythmogenesis and/or contractile dysfunction in the rabbit heart.

III. LITERATURE REVIEW

A. General Background

In 1986, Reimer et al. investigated the rate of ATP breakdown during repeated coronary occlusions and unexpectedly found that further episodes did not produce a further depletion of intracellular ATP. In the same series of experiments, this group showed that four 10 minute ischemic episodes resulted in less myocardial necrosis than would be expected with 40 minutes of uninterrupted coronary occlusion. This suggested that short periods of ischemia may somehow protect the heart from subsequent injury. These observations formed the basis for the concept of **ischemic preconditioning**, which was first demonstrated by Murry et al. (1986). They demonstrated this protective phenomenon as a reduction in infarct size of the ischemic area at risk in the dog heart. In fact, preconditioning is presently the most powerful form of protection against myocardial necrosis described to date (Lawson and Downey, 1993). Since the publication of these two reports in 1986, other investigators have shown that this protection is not limited to this species nor to this endpoint (Table 1). Ischemic preconditioning can protect against contractile dysfunction in rat (Perchenet and Kreher, 1995; Kolocassides et al., 1996), rabbit (Omar et al., 1991; Adachi et al., 1994) and dog (Cohen and Downey, 1990; Shen and Vatner, 1996), but not pig (Shen and Vatner, 1996). With respect to arrhythmogenesis, ischemic preconditioning reduces the incidence of ischemia-induced arrhythmias in rat (Lawson et al., 1993; Li et al., 1992; Vegh et al., 1992), rabbit (Cohen et al., 1994) and dog (Vegh et al., 1995; Parratt and Vegh, 1994; Vegh et al., 1991; Vegh

TABLE 1. Effects of ischemic preconditioning on specific endpoints assessed in mammalian species.

Species	Infarct Size	Contractile Function		Arrhythmias
		Resting	Developed	
Rat	↓ 27%-35%[1-3]	↔ RT[4]	↑ DT[4]	↓ VT/VF (I)[3,9-12]
		↑ LVEDP[5]	↑ LVDP[5-8]	↓ VT/VF (R)[10-14]
Rabbit	↓ 23%-32%[15-19]	↓ LVEDP[20]	↑ DT[22]	↓ VT/VF (I)[16,20]
		↑ LVEDP[21]	↓ LVDP[20,21]	↓ VT/VF (R)[20]
Dog	↓ 20%-26%[23-26]	ND	↓ SS[27]	↓ VT/VF[12,28]
				↑ VT/VF [25,29]
Pig	↓ 26%-38%[30-32]	ND	↓ WT[33]	↔ VF[30]
				↓ time to VF[30]
Human	ND	ND	↑ DT[34]	ND

ND - no data, RT - resting tension (isolated tissues), LVEDP - left ventricular end diastolic pressure, LVDP - left ventricular developed pressure, VT - ventricular tachycardia, VF - ventricular fibrillation, I - ischemia, R - reperfusion, WT - wall thickening, DT - developed tension (isolated tissues), SS - segment shortening (isolated cardiomyocytes).

References from Table 1.

[1](Yellon et al., 1992), [2](Liu and Downey, 1992), [3](Li et al., 1992), [4](Perchenet and Kreher, 1995), [5](Kolocassides et al., 1996), [6](Asimakis et al., 1993), [7](Asimakis et al., 1992), [8](Cave et al., 1994), [9](Lawson et al., 1993), [10](Tosaki et al., 1994), [11](Shiki and Hearse, 1987), [12](Vegh et al., 1992), [13](Osada et al., 1991), [14](Hagar et al., 1991), [15](Miura et al., 1991), [16](Cohen et al., 1994), [17](Iwamoto et al., 1991), [181](Sandhu et al., 1993), [19](Thornton et al., 1993b), [20](Botsford and Lukas, 1996), [21](Bolling et al., 1994), [22](Omar et al., 1991), [23](Kuzuya et al., 1993), [24](Gross and Auchampach, 1992), [25](Murry et al., 1986), [26](Li et al., 1990), [27](Ovize et al., 1992), [28](Vegh et al., 1993), [29](Reimer et al., 1990), [30](Ovize et al., 1995), [31](Schott et al., 1990), [32](Schulz et al., 1995), [33](Miyamae et al., 1993), and [34](Walker et al., 1995).

et al., 1993; Vegh et al., 1992) and suppresses reperfusion-induced arrhythmias in rat (Shiki and Hearse, 1987; Tosaki et al., 1994) and dog (Vegh et al., 1992; Vegh et al., 1990). In contrast, preconditioning has no effect on the incidence of ischemia or reperfusion-induced arrhythmias in the pig and it actually decreases the time to ventricular fibrillation in this species (Ovize et al., 1995). In general, the ability of preconditioning to improve contractile recovery or protect against lethal arrhythmias is less consistent than its effects on infarct size and is highly dependent on the species and protocol used.

Studies now indicate that ischemia is not the only means by which to induce preconditioning. Infusion of adenosine (Downey et al., 1993; Toombs et al., 1992), hypoxic perfusion (Walsh et al., 1995; Moolman et al., 1994), rapid atrial or ventricular pacing (Walker et al., 1995) and myocardial stretch (Ovize et al., 1994) can also protect the heart against ischemia and reperfusion injury. Whether these stimuli work alone or in concert is currently the topic of considerable debate.

B. Endpoints Used to Assess Preconditioning

B.1. Infarct Size

The first endpoint used to assess the degree of protection induced by preconditioning was a reduction of infarct size. This assessment involves measuring the amount of necrotic tissue and expressing the size of the infarct as a percent of the area affected by the ischemic episode (area at risk). Such standardization is important in order to eliminate the effects of the collateral circulation which is not present in all mammalian species. The numerous studies performed to date clearly indicate that ischemic

preconditioning is universally effective as a cardioprotective agent against infarction. Preconditioning of the heart causes a marked reduction in myocardial infarct size in all species tested [e.g. rat (Li et al., 1992; Liu and Downey, 1992; Yellon et al., 1992), ferret (Gomoll, 1996) rabbit (Iwamoto et al., 1991; Cohen et al., 1991), dog (Murry et al., 1986; Li et al., 1990) and pig (Schott et al., 1990)].

B.2. Contractile Dysfunction

Contractile dysfunction is typically assessed during ischemia in isolated hearts as a rise in end diastolic pressure (EDP) or "ischemic contracture". Other contractile parameters, such as left ventricular developed pressure (LVDP), decline rapidly during ischemia and become unmeasurable. In isolated cardiac tissues, developed tension is measurable and this parameter as well as resting tension are often used to assess the effects of preconditioning in tissues. In isolated heart models, preconditioning accelerates the rate of ischemic contracture in rats (Kolocassides et al., 1996) but has no effect on contracture development in rabbits (Quantz et al., 1994).

Upon reperfusion, left ventricular developed pressure begins to recover and this parameter can be used to assess the degree of protection conferred by preconditioning. Some investigators refer to this postischemic phase of reversible contractile depression as 'stunning'. A beneficial effect of preconditioning against the contractile dysfunction upon reperfusion is seen in isolated rat hearts (Perchenet and Kreher, 1995; Kolocassides et al., 1996) or ventricular strips (Cleveland, Jr. et al., 1996b; Cleveland, Jr. et al., 1996a). In rabbit, some studies demonstrate an improvement in contractile function with

preconditioning (Omar et al., 1991; Cohen et al., 1991; Adachi et al., 1994), whereas others do not (Sandhu et al., 1993). Contradictory effects of preconditioning on postischemic contractile function are also seen in the dog heart (Yao et al., 1993; Shen and Vatner, 1996; Ovize et al., 1992). Whether these disparate effects of preconditioning on postischemic function in different experimental models are due to species differences, preparation differences, and/or the extent of irreversible injury remain to be determined (Lasley and Mentzer, Jr. 1995b). One possible explanation is that contractile dysfunction is only an indicator of myocyte necrosis and cell viability and not of stunning *per se*. Therefore, any assessment of preconditioning using this endpoint must be done with caution.

B.3. Arrhythmias

Inhomogeneous action potential characteristics presumably represent the electrophysiologic substrate for both ventricular tachycardia (VT) and ventricular fibrillation (VF) (Janse and Wit, 1989). These severe arrhythmias can occur during either ischemia (Janse and Wit, 1989) or reperfusion (Hearse and Bolli, 1992; Janse and Wit, 1989), although the mechanisms initiating these arrhythmias may differ during the two conditions. Prolonged ischemia can induce injury currents between ischemic and normal regions of myocardium which may initiate arrhythmias (Clusin et al., 1983). In contrast, arrhythmias occurring during reperfusion are likely to involve multiple, independently acting pathophysiologic factors such as free radicals (Bernier et al., 1986), calcium

imbalance (Hearse and Tosaki, 1988) and disturbances in potassium homeostasis (Tanaka and Hearse, 1988; Curtis, 1991).

Under optimal conditions, ischemic preconditioning exerts an antiarrhythmic effect that is as pronounced as that seen with classic antiarrhythmic drugs (Parratt and Vegh, 1994). The Cardiac Arrhythmia Suppression Trial (CAST 1, (Echt et al., 1991)), however, demonstrated that antiarrhythmic drugs can actually result in greater morbidity and mortality than placebo. Thus, the potential benefit of preconditioning as an intervention to protect against lethal arrhythmias is extremely significant. However, the exact mechanism underlying this protection remains unknown. Moreover, the mechanisms responsible for the protective effects of preconditioning on arrhythmias versus infarct size may be different (Vegh et al., 1993). Preconditioning has been shown to protect against ischemia-induced arrhythmias in rat (Hendrikx et al., 1993b; Li et al., 1992; Lawson et al., 1993; Vegh et al., 1992), rabbit (Cohen et al., 1994) and dog (Parratt and Vegh, 1994; Vegh et al., 1991; Vegh et al., 1993; Vegh et al., 1992). However, the study in rabbit (Cohen et al., 1994) did not focus specifically on arrhythmias, used only one preconditioning ischemia and tested low numbers (8) of animals. Moreover, contradictory studies showing no protective effects of preconditioning on arrhythmias also exist in dog (Murry et al., 1986; Reimer et al., 1990). Preconditioning also protects against reperfusion-induced arrhythmias in rat (Osada et al., 1991; Shiki and Hearse, 1987; Tosaki et al., 1994; Hagar et al., 1991) and dog (Vegh et al., 1992; Field et al., 1988). In contrast, preconditioning has no effect on the incidence of either ischemia or reperfusion-

induced VF in pig and it actually decreases the time to fibrillation in this species (Ovize et al., 1995).

In a clinical setting, lethal ventricular arrhythmias occur prior to any potential reduction in infarct size or improvement in contractility due to preconditioning. Thus, a true protection against other endpoints can only occur if the preconditioning protocol also reduces the incidence of severe ventricular arrhythmias.

C. Temporal Aspects of Preconditioning

In addition to the species tested, the particular preconditioning protocol used may also determine if preconditioning occurs. The protection afforded by preconditioning is dependent on the number and duration of the short preconditioning ischemic periods and also on the time that intervenes between the preconditioning ischemia and the subsequent prolonged ischemia.

C.1. Timing of the Preconditioning Protocol

C.1.1 Number and Duration of Preconditioning Cycles

In the initial study of preconditioning in dog (Murry et al., 1986), four 5 minute coronary occlusions were used to induce protection. However, one 15 minute or one, six or twelve preconditioning periods lasting five minutes are equally effective in preconditioning the canine heart (Murry et al., 1991; Li et al., 1990). In this species, five minutes appears to be the minimum time required to induce preconditioning. Van Winkle et al. (1991) demonstrated that the threshold for preconditioning in a rabbit heart model of

regional ischemia is between 2 and 5 minutes for the preconditioning ischemia. These investigators showed that two 2 minute periods of ischemic preconditioning were not adequate to induce preconditioning, whereas two 5 minute cycles protected the hearts against infarction. Additional studies in rabbit indicate that one 5 minute period of regional ischemia (Cohen et al., 1991; Miura et al., 1991) or global ischemia (Tracey et al., 1997) is sufficient to induce protection. One 5 minute period of regional ischemia also reduces infarct size in the rat (Yellon et al., 1992). Strasser et al. (1994) reported that significant reduction of infarct size requires an ischemic preconditioning period of longer than 10 minutes in pig. In humans, one 90 second coronary angioplasty balloon inflation may be sufficient to protect against ischemia during a second inflation (Deutsch et al., 1990). Thus, the current evidence indicates that one 5 minute preconditioning ischemia is the minimum needed to significantly reduce infarct size in rat, rabbit and dog. In contrast, a preconditioning ischemia exceeding 10 minutes is necessary to achieve protection in the pig.

C.1.2 Duration of the Intervening Reperfusion

The protection that ischemic preconditioning confers to the heart is not indefinite but begins to wane with time. Therefore, the duration of the intervening reperfusion period between the preconditioning cycles and prolonged ischemia is crucial in determining the degree of protection obtained. Murry et al. (1991) showed that extending the intervening reperfusion between the preconditioning period and sustained ischemia from 60 minutes to 120 minutes greatly reduced the degree of protection against infarction. A similar

reduction of the protection against infarction occurs if the duration of the intervening reperfusion period is increased in the rat (Li et al., 1992), rabbit (Van Winkle et al., 1991) and pig (Sack et al., 1993). This aspect of the preconditioning response also applies to protection against contractile dysfunction. In rabbit, intervening reperfusion periods of up to 30 minutes are not associated with loss of cardioprotection. However, an extension of the intervening reperfusion period to 120 minutes results in significantly less recovery of contractile function (Van Winkle et al., 1991).

Similarly, the protection by ischemic preconditioning against ischemia-induced arrhythmias is maximal when the intervening reperfusion period is less than 30 minutes in rats (Vegh et al., 1992) and less than 1 hour in dogs (Vegh et al., 1992). It is difficult to evaluate the effects of the intervening reperfusion duration on the occurrence of reperfusion arrhythmias following the prolonged ischemia. This difficulty arises because the incidence of reperfusion arrhythmias is closely related to the length of the ischemic insult. Preconditioning may simply delay the cell death or reperfusion arrhythmias that would normally occur with a longer ischemic insult (Walker and Yellon, 1992). Thus, it is imperative that variations in the intervening reperfusion period are tested only under conditions where the length of the ischemic insult remains fixed. Unfortunately, such data are currently unavailable. Moreover, the numerous protocol variations used to assess preconditioning make comparison of results from different laboratories or species extremely difficult. Thus, it is unknown whether the intervening duration between the preconditioning cycle and sustained ischemia also modifies reperfusion-induced arrhythmias.

C.2. Classic versus Late Phases of Preconditioning

Some exciting recent reports indicate that ischemic preconditioning elicits a biphasic pattern of cardioprotection that consists of both an acute phase ('classic' preconditioning) and a delayed phase of protection (Yang et al., 1996; Kuzuya et al., 1993). The 'first window' of protection is relatively short lived, lasting one to three hours after the initial preconditioning cycle(s) depending on species. The 'second window' or 'late' phase of protection develops gradually, appears about twelve hours after the preconditioning protocol, and increases in magnitude over the next 12-36 hours. To date, this second window of protection against infarction has been observed in rabbit (Yang et al., 1996) and dog (Kuzuya et al., 1993), but not in pig (Strasser et al., 1994). Other species currently remain untested. In rabbit, a 50% reduction in infarct size is seen 48 hours after preconditioning and this reduction remains for 72 hours following preconditioning (Yellon and Baxter, 1995). In dog, the early phase of protection with preconditioning on infarct size is lost after three hours. The second window begins to appear at 12 hours and a statistically significant reduction in infarct size is evident by 24 hours (Kuzuya et al., 1993).

Very little data exists concerning the late phase of preconditioning and arrhythmogenesis. Shiki and Hearse (1987) showed no significant antiarrhythmic effect of preconditioning in rat when the intervening interval between the preconditioning and prolonged ischemia was extended to 24 hours. More recently, a marked reduction in the incidence of ischemia-induced ventricular fibrillation was observed 20 hours after the preconditioning protocol in dogs (Vegh et al., 1994). Aside from species differences,

another possible explanation for the contradictory results is that that the preconditioning stimulus that evokes classical preconditioning may be insufficient to evoke a second window of protection. This explanation was first suggested by Yellon and Baxter (1995). Although the evidence suggests that the cellular mechanisms that confer protection against the different endpoints are not the same, the time course of the 'second window' protection against infarction and arrhythmogenesis is quite similar in both rabbit (Yang et al., 1996) and dog (Vegh et al., 1994; Kuzuya et al., 1993). The existence of a 'late' phase of protection is less well established in pig (Strasser et al., 1994; Sack et al., 1993). No data are available in this species with respect to arrhythmogenesis. However, when contractile dysfunction is the endpoint measured, protection is present 24 hours after preconditioning (Sun et al., 1995). This suggests that the effects of preconditioning may result from a combination of factors and the presence of a second window of protection depends on the species, the protocol and the specific endpoint assessed.

D. Alternative Preconditioning Stimuli

In addition to ischemia, other stimuli can mimic the effects of ischemic preconditioning. Whether a single stimulus or a combination of stimuli are responsible for protection is unknown. However, inconsistent results obtained with these stimuli suggest that a combination of factors is most likely. Further, these conditions are not mutually exclusive. Ischemia results in myocardial stretch and rapid pacing and hypoxia are similar to occlusion ischemia in that they upset the balance between myocardial supply and metabolic demand.

D.1. Infusion of Adenosine

Adenosine is postulated to be an important mediator of ischemic preconditioning. In fact, infusion of adenosine (in lieu of a preconditioning ischemia) mimics the protective effect of ischemic preconditioning on infarct size in rabbit (Liu et al., 1991; Toombs et al., 1992; Liu et al., 1994), dog (Yao and Gross, 1994) and pig (Van Winkle et al., 1994), but not in rat (Li and Kloner, 1993a; Li and Kloner, 1993b; Asimakis et al., 1993). Protection similar to that obtained with ischemic preconditioning on postischemic contractile recovery is also seen with prior infusion of adenosine in rabbit (Mosca et al., 1994), dog (Sekili et al., 1995), pig (Lasley and Mentzer, Jr. 1995a; Yokota et al., 1995), and humans (Kerensky et al., 1995; Lee et al., 1995; Cleveland, Jr. et al., 1997), but not in rat (Li and Kloner, 1993a; Asimakis et al., 1993). Moreover, agents that indirectly increase tissue adenosine levels, such as acadesine (Tsuchida et al., 1994b) or dipyridamole (Miura et al., 1992; Mosca et al., 1994), also confer protection. These results suggest that adenosine may play an integral role in the preconditioning process in all species with the exception of rat.

Evidence for a role of adenosine in the protective effect of preconditioning on arrhythmogenesis is limited at present. In rat, however, the protection afforded by preconditioning on ischemia-induced VT is not blocked by the *non-selective* adenosine receptor antagonist 8-(p-sulfophenyl)theophylline (Li and Kloner., 1993b). This antagonist also does not block the protective effect of preconditioning on reperfusion-induced VT and VF in the rat (Miura et al., 1995).

Recent studies have further characterized the adenosine receptor subtypes involved in the preconditioning response. Pretreatment with a *selective* adenosine A_1 agonist induces protection equivalent to that of ischemic preconditioning on infarct size in rat (Liu and Downey, 1992), rabbit (Liu et al., 1991; Tsuchida et al., 1993; Tsuchida et al., 1992; Hale et al., 1993; Sakamoto et al., 1995; Tracey et al., 1997), dog (Grover et al., 1992) and pig (Van Winkle et al., 1994). As well, protection against both ischemic contracture and postischemic contractile dysfunction is seen with *selective* A_1 agonists in rat (Lasley et al., 1990; Lasley and Mentzer, Jr. 1992). *Selective* A_3 agonists are also cardioprotective in rabbits (Liu et al., 1994; Armstrong and Ganote, 1994b), whereas *selective* A_2 agonists do not mimic preconditioning in any of the species tested (Armstrong and Ganote, 1994b).

The effects of adenosine receptor *antagonists* on preconditioning are less clear. Pretreatment with a *non-selective* adenosine receptor antagonist abolishes the protection afforded by ischemic preconditioning in rabbits (Tsuchida et al., 1992; Miura et al., 1992; Bunch et al., 1992; Thornton et al., 1993a; Liu et al., 1994) and dogs (Auchampach and Gross, 1993), while the effect of adenosine antagonism depends on the endpoint measured in rat (Li and Kloner, 1993b). If *selective* receptor antagonists are used, preconditioning in rabbits is abolished by A_3 antagonists (Liu et al., 1994; Armstrong and Ganote, 1994b) but not by A_1 antagonists (Liu et al., 1994; Armstrong and Ganote, 1994b). This contrasts the effects of *selective* A_1 or A_3 agonists to mimic preconditioning in rabbits. Thus, cardioprotection conferred by preconditioning on infarct size may involve *both* adenosine A_1 and A_3 receptors in rabbit heart. The mechanism by which adenosine acts is not known. However, beneficial metabolic effects of adenosine include slowing of the rate

of ATP catabolism during ischemia and improvement of the reperfusion myocardial phosphorylation potential (Lasley et al., 1990; Lasley and Mentzer, Jr. 1992), which may contribute to its preconditioning effects.

D.2. Hypoxia

A major component of ischemia is hypoxia. This has prompted several investigators to test whether hypoxia alone is able to induce preconditioning. In fact, the use of hypoxic perfusion instead of a preconditioning ischemia is effective at protecting the myocardium from ischemia/reperfusion injury. Hypoxic preconditioning reduces infarct size in rabbit (Cohen et al., 1995) and dog (Shizukuda et al., 1992). Hypoxia can also protect contractile processes in porcine myocytes (Zellner et al., 1996) and preserve contractile function in the rat (Neely and Grotyohann, 1984) and dog heart (Shizukuda et al., 1993). No studies to date have tested the effects of hypoxic preconditioning on arrhythmogenesis. Whether hypoxia is as effective as ischemia at invoking preconditioning is unclear, since many species and endpoints remain untested. However, the findings to date suggest that hypoxia is likely to contribute to preconditioning, but it is not solely responsible for the protective effects.

D.3. Myocardial Stunning

Stunning is a term used to describe the transient contractile dysfunction that accompanies reversible myocardial ischemia. The ability of the heart to utilize oxygen is depressed in stunning and therefore, less contractile work is developed per unit of oxygen

utilized. It has been proposed that myocardial stunning itself may account for the protection seen with preconditioning. Cohen et al. (1991) suggest that altered oxygen consumption during the ischemic phase of preconditioning may be important in preconditioning. Therefore, an initial coronary occlusion could result in myocardial stunning and the stunned but still viable tissue, which has diminished metabolic demands, could better withstand a subsequent prolonged ischemic insult than normally functioning tissue (Cohen et al., 1991).

A dissociation of the phenomena of stunning and preconditioning is suggested by studies in rat (Mitchell et al., 1993), dog (Murry et al., 1991) and pig (Sack et al., 1993), which show that the effects of preconditioning disappear before those of stunning. Miura et al. (1991) demonstrated that the infarct limiting effects of preconditioning were not different in two groups of rabbits that showed different degrees of myocardial stunning with different preconditioning protocols. The study by Schott et al. (1990) also argues against this hypothesis, since significant stunning was seen in the pig myocardium but oxygen consumption in the stunned myocardium was unchanged from control myocardium.

D.4. Rapid Cardiac Pacing

Rapid pacing also induces ischemia in the heart since it results in decreased supply (by increasing the length of time in systole) and increased demand (by increasing the amount of work per unit time). However, rapid pacing differs from ischemia induced by coronary artery occlusion since metabolite washout can still occur in the former condition.

This is an important difference, since metabolite accumulation can play a role in arrhythmogenesis, contractile dysfunction and/or infarction. In dog, two 2 minute periods of rapid ventricular pacing (300 beats per minute) provided protection against both VT and VF during a subsequent 25 minute coronary artery occlusion (Vegh et al., 1991). Protection against contractile dysfunction has also been reported with rapid pacing of human atrial tissue (Walker et al., 1995). In fact, rapid pacing may be as effective as ischemia in protecting the myocardium against subsequent ischemic injury (Vegh et al., 1991). Protection against contractile dysfunction in rabbit has been demonstrated with rapid ventricular pacing (Szilvassy et al., 1994), whereas protection against infarction in this species was not seen with rapid atrial pacing in a different study (Marber et al., 1993). However, the latter authors suggest that they did not reach the 'threshold' required to elicit a preconditioning response in their study. This method of preconditioning has not been investigated to the same extent as others, but it may have significant clinical applications since rapid pacing is a less invasive technique for inducing preconditioning than transient coronary artery occlusion.

E. Proposed Mediators of Preconditioning

E.1. Protein Kinase C (PKC)

The PKC enzymes belong to a family of serine/threonine kinases that phosphorylate serine and threonine bases on the target protein. To date, 12 isoforms of PKC have been identified, although not all isoforms are present in the heart (Puceat and Vassort, 1996). These isoforms are classified into three groups based on their different

activation properties and secondary structure. The *classical* isoforms are activated by Ca^{2+}, phosphatidylserine (PtdSer), and diacylglycerol (DG). They include PKC-α, PKC-β_1, PKC-β_2 and PKC-γ. The *novel* subfamily requires PtdSer and DG for activation, but not Ca^{2+}, and consist of PKC-δ, PKC-ε, PKC-η, PKC-θ and PKC-μ. The third *atypical* subfamily requires only PtdSer and not Ca^{2+} or DG for activity to occur (Sugden and Bogoyevitch, 1995). PKC-ζ, PKC-τ and PKC-λ are members of this group. The properties of the different PKC subfamilies may contribute to different preconditioning responses as will be discussed later.

During ischemia, numerous substances are released by the myocardium including adenosine, catecholamines, angiotensin II, bradykinin and endothelin (Cohen and Downey, 1996). The stimulation of adenosine (Kohl et al., 1990; Cohen and Downey, 1996), angiotensin (Sadoshima and Izumo, 1993), adrenergic (Sugden and Bogoyevitch, 1995), bradykinin (Minshall et al., 1995) and endothelin (Irons et al., 1993) receptors leads to activation of phospholipase C or D in many types of tissues, including myocardium. An increase in phospholipase C or D activity results in increased production of the second messenger DG. In turn, DG is a potent activator of PKC, which modifies a number of proteins in the cell by phosphorylation (Puceat and Vassort, 1996). PKC is present primarily in the cytosol of cardiac myocytes, where it is inactive. A rise in DG levels initiates translocation of the cytosolic PKC pool into the sarcolemmal membrane. DG also converts membrane-bound PKC into the active kinase (Sugden and Bogoyevitch, 1995).

In 1994, Liu et al. proposed the PKC translocation theory of preconditioning. This theory suggests that ischemic preconditioning induces the translocation of cytosolic PKC into the membrane where it is activated during the subsequent prolonged ischemia to phosphorylate an as yet unknown mediator of protection. The postulated sequence of events in the translocation theory of ischemic preconditioning is shown in Figure 1. These investigators propose that *translocation* of PKC is the sole effect of the preconditioning stimulus (Figure 1, pathway A). Inherent in this theory are several testable hypotheses. First, kinase activity is not necessary during the preconditioning ischemia and thus, blocking kinase activity during this period should have no effect on the protection. Second, translocation of PKC is accomplished by cytoskeletal microtubules. Therefore, disrupting these microtubules with an agent such as colchicine, should prevent preconditioning. Third, PKC activity during the prolonged ischemia is essential for protection to occur (Figure 1, pathway B).

In support of the translocation theory, recent studies indicate that PKC activity is indeed required during the sustained ischemia for preconditioning-induced protection to occur (Liu et al., 1994). In fact, administration of a PKC inhibitor (staurosporine, polymyxin B or chelerythrine) before the prolonged or *test* ischemia completely abolishes the protective effect of ischemic preconditioning on infarct size in rat (Speechly-Dick et al., 1994), rabbit (Ytrehus et al., 1994; Liu et al., 1994) and dog (Przyklenk et al., 1995). As well, the protective effects of ischemic preconditioning can be mimicked by phorbol

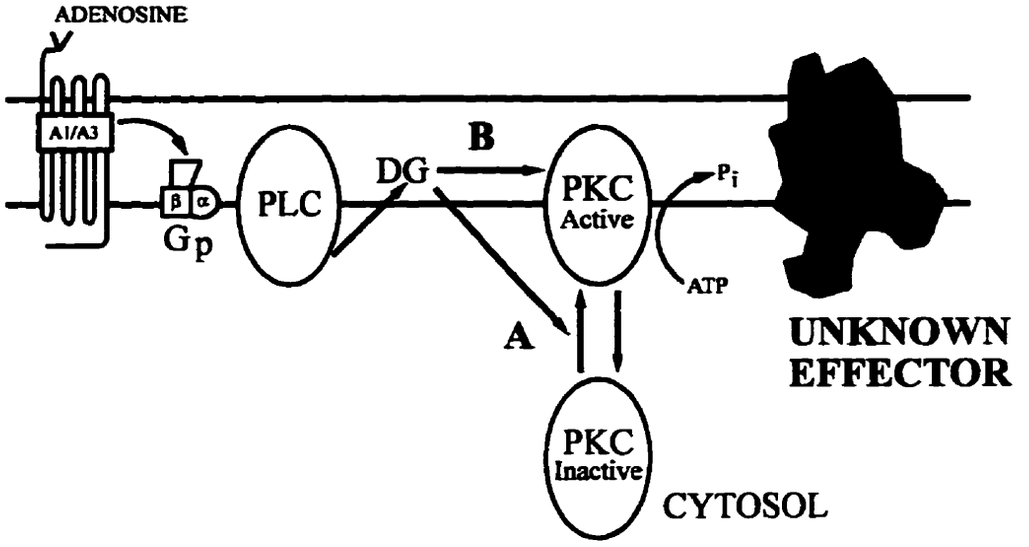

Figure 1. Diagram of proposed events in the 'translocation theory' of preconditioning. The short preconditioning ischemia activates receptors (see text) that increase PLC activity (via pertussis sensitive G-protein (Gp) coupling), which in turn stimulates the production of DG. DG stimulates the translocation of inactive cytosolic PKC to the membrane where it can be activated; however, this pathway is relatively slow (Pathway A). Once in the membrane, PKC can be more quickly activated during the *test* period to provide protection through an unknown effector. Direct activation of membrane bound PKC by DG is thought to provide protection during the subsequent prolonged ischemia (Pathway B). PLC - phospholipase C, DG - diacylglycerol, PKC - protein kinase C. Modified from Downey et al. (1994).

esters (phorbol 12-myristate 13-acetate, PMA; phorbol 12,13 dibutyrate, PDBu), adenosine receptor agonists (N6-(3-iodobenzyl)adenosine-5'-N-methyluronamide, IB-MECA; R-N^6-(2-phenylisopropyl)-adenosine, R-PIA) and by several other mediators that are capable of activating PKC either directly or indirectly (Liu et al., 1994; Ytrehus et al., 1994; Tsuchida et al., 1994a; Liu et al., 1995; Hendrikx et al., 1993a). PKC stimulation with the diacylglycerol analogue 1,2-dioctanoyl-*sn*-glycerol (DOG) results in the same degree of protection against infarction as seen with one 5 minute preconditioning ischemia in rat (Speechly-Dick et al., 1994). Moreover, this protection is blocked by pretreatment with the PKC inhibitor chelerythrine (Speechly-Dick et al., 1994).

Recently, several studies have questioned the validity of the translocation theory. Baxter et al. (1995) and Armstrong et al. (1996) have shown that the protective effects of preconditioning are abolished if a PKC inhibitor is present prior to preconditioning. The 'translocation theory' predicts that such treatment should not affect the preconditioning response, since the only purpose of the preconditioning ischemia is to translocate PKC to the sarcolemma so that it is available during the subsequent prolonged ischemia. Also, Simkhovich et al. (1996) showed that translocation of PKC did not occur in tissues from preconditioned versus control hearts. However, these authors looked only at total PKC distribution and not at specific PKC isoform profiles in the sarcolemmal versus cytosolic fractions. This may be significant since it has been suggested that different isoforms distribute differently within the cell (Wilson et al., 1996; Mitchell et al., 1995; Banerjee et al., 1996). Also, Banerjee et al. (1996) suggest that different preconditioning stimuli redistribute PKC isoforms in characteristic profiles that include algebraic sums of positive

and negative effects, and that preconditioning results in distribution of different PKC isoforms to distinct cellular compartments. Furthermore, they suggest that each preconditioning *stimulus* involves a characteristic mosaic of PKC isoforms. This reasoning may also explain the differences reported with respect to species and endpoints. Species differences may be due to the presence or absence of specific isoforms, since not all isoforms are present in all species. As well, different PKC isoforms may play different roles in preconditioning depending on which endpoint is measured. Not only are certain isoforms (PKC-ε) associated with contractile processes (Johnson et al., 1996), but recovery of contractile function after preconditioning is also associated with translocation of the specific isoforms PKC-α, PKC-δ and PKC-ζ (Meldrum et al., 1996). Finally, some PKC isoforms are localized to the nucleus during preconditioning (Banerjee et al., 1996) and this event may be responsible for induction of synthesis of stress proteins. There is evidence linking stress proteins to the second window of preconditioning protection (Karmazyn et al., 1990) (see section E.4.).

Until recently, the role of PKC in preconditioning against severe ventricular arrhythmias during ischemia and/or reperfusion was untested. Tosaki et al. (1996) have shown that calphostin C provides significant additional protection to preconditioning against reperfusion arrhythmias at moderate doses, but blocks the protective effect of preconditioning at high concentrations in the rat. These authors conclude that the dual effects of calphostin C appear to be strictly dose related and dependent on the degree of "enzyme inhibition". Inhibition of enzyme activity to various degrees may preferentially

inhibit the function of specific PKC isoforms involved in regulation and manifestation of arrhythmogenesis, or those involved in the regulation of cardiac function or infarct size (Banerjee et al., 1993; Hu and Nattel, 1995; Liu et al., 1996).

E.2. K_{ATP} Channels

Potassium channels are suggested to be the potential mediator of the preconditioning response since they are directly activated by PKC (Tohse et al., 1987; Tohse et al., 1990; Walsh and Kass, 1991; Murray et al., 1994; Hu et al., 1996). Adenosine increases potassium efflux by G protein-associated opening of specific potassium channels. These potassium channels (K_{ATP}) are sensitive to intracellular ATP levels, and are normally closed under physiological conditions. However, when ATP levels fall (i.e., during ischemia), these channels open and K^+ ions flow out of the cell to enhance repolarization. The resultant shortening of the action potential reduces the amount of calcium that enters the cell and decreases contractility. This decrease in contractility lowers ATP utilization and may thereby provide protection. The protection mediated by K_{ATP} channels is controversial, however, since exposure to the K_{ATP} blocker glibenclamide abolishes preconditioning in dogs (Gross and Auchampach, 1992), whereas the same blocker does not prevent preconditioning in rabbit (Thornton et al., 1993b). Speechly-Dick et al. (1995) showed that protection against contractile dysfunction following simulated ischemia was induced either by activation of PKC with DOG, or by the K_{ATP} channel opener cromakalim. Moreover, the protection induced by both PKC activation and preconditioning could be prevented by blocking the K_{ATP} channel with

glibenclamide. Thus, K_{ATP} channels may be involved in the preconditioning process, but their precise role remains to be defined.

E.3. Free Radicals

A feature common to rat and dog heart is the presence of high levels of the enzyme xanthine oxidase (Muxfeldt and Schaper, 1987). This enzyme catalyses the breakdown of hypoxanthine to xanthine, and xanthine to uric acid. Both reactions generate superoxide anions ($^{\bullet}O_2^-$) as a by-product. Free radicals have been implicated in the pathogenesis of cardiac dysfunction and arrhythmias during ischemia. In addition, reintroduction of oxygen upon reperfusion results in a burst of free radical generation, which may also contribute to the genesis of reperfusion-induced arrhythmias (Li et al., 1993; Bolli et al., 1989). In the rat and dog heart, xanthine oxidase is an important source of free radicals since the breakdown of ATP during ischemia produces large amounts of hypoxanthine. In fact, allopurinol (a specific inhibitor of xanthine oxidase) can decrease infarct size in dog (Hearse et al., 1986) and reduce the incidence of reperfusion-induced arrhythmias in rat (Hearse et al., 1986). In another study, preconditioning reduced the levels of free radicals (as assessed using malonaldehyde as an indirect marker) which correlated with a reduced incidence of reperfusion arrhythmias in the rat heart (Tosaki et al., 1994). Also, addition of free radical scavengers (superoxide dismutase, SOD and catalase) only during the reperfusion period reduced the incidence of reperfusion arrhythmias following preconditioning (Osada et al., 1991).

Unlike the rat and dog, however, the human, rabbit and pig are myocardial xanthine oxidase-deficient species (Muxfeldt and Schaper, 1987; Downey et al., 1987). Free radical damage induced by xanthine oxidase probably plays little role in the effects of ischemia in these species. In fact, Omar et al. (1991) reported that the free radical scavenger Mn-SOD had no effect on preconditioning against contractile dysfunction, even though it attenuated lactate dehydrogenase enzyme release in the isolated rabbit heart. Thus, ischemia-induced oxidative stress in xanthine oxidase containing species is probably not representative of that occurring in humans.

E.4. Stress Proteins

Stress proteins represent another potential mediator of preconditioning. Indeed, raising the body temperature 5°C for a 15 minute period protects the heart against an ischemic insult (Currie et al., 1988), presumably through the production of heat shock proteins (HSP). Both heat shock protein mRNA and HSP are increased in the heart after ischemia (Mehta et al., 1988), hypoxia (Howard and Geoghegan, 1986) and myocardial stretch (Knowlton et al., 1991b). Several groups have shown that 24 hours following heat stress, hearts from rats and rabbits display enhanced tolerance to a subsequent prolonged ischemia (Karmazyn et al., 1990; Walker et al., 1993). Sub-lethal myocardial ischemia can increase the expression of an inducible form of the 70 kDa heat stress protein (hsp70i) in the rabbit (Knowlton et al., 1991a). However, protection is still present in rabbits in which protein synthesis is inhibited prior to the preconditioning protocol (Thornton et al.,

1990). Thus, the mechanism by which HSPs afford cardioprotection in ischemia/reperfusion remains to be defined.

E.5. Metabolic Changes

Several metabolic features of preconditioned myocardium may play a role in the protection afforded by this phenomenon. Warner et al. (1989) reported that tissue acidosis during ischemia is attenuated following preconditioning. This was further investigated by Steenbergen et al. (1993) who hypothesized that decreased acidosis results in a smaller rise in intracellular sodium and calcium through Na^+-H^+ and Na^+-Ca^{2+} exchange, respectively. Thus, this reduction of the ionic derangement during ischemia may provide protection. As well, other groups have shown that the depletion of myocardial ATP levels during the prolonged ischemia is slowed and the accumulation of metabolites such as lactate is less in preconditioned myocardium (Murry et al., 1990; Reimer et al., 1986). Murry et al. (1990) reported that preconditioned cardiac tissue contains less glycogen than control hearts. These authors suggest that glycogen-depleted hearts develop less intracellular acidosis during prolonged ischemia, thus this glycogen depletion may be one means by which the heart protects itself. However, conflicting results on the role of glycogen depletion during ischemia exist in the literature (Wolfe et al., 1993; Armstrong and Ganote, 1994a).

Undoubtedly, metabolic changes play a role in preconditioning. However, the extent and mechanisms for these metabolic changes during preconditioning are not yet known.

F. Effects of Experimental Conditions on Preconditioning

The clinical implications of differences in perfusate composition are significant, since both crystalloid and blood perfusates are used during cardiac surgery. It remains to be established whether blood borne factors are required for ischemic preconditioning. In an effort to standardize the effects of the perfusate, Sandhu et al. (1993) tested the effects of preconditioning with global ischemia in blood versus buffer perfused hearts and found similar effects on recovery of function and necrosis between the two models. However, McHowat et al. (1993) found that blood perfused hearts had significantly greater recovery of left ventricular developed pressure than buffer perfused hearts upon reperfusion. As well, blood-perfused hearts were more responsive to cardioplegic protection (McHowat et al., 1993). These results are difficult to reconcile especially since neutrophils (a component of blood) are known to delay recovery of function in the rabbit heart (Kraemer and Mullane, 1989). Thus, the question of whether blood borne elements contribute to the preconditioning response still remains to be answered.

Preconditioning is a multifactorial phenomenon that is able to protect against infarction, contractile dysfunction and arrhythmias. As demonstrated from the preceding review of literature, preconditioning is very specific to the species, protocol and endpoint measured. The reasons for these differences are unknown, but are probably subtle (eg. differences in isoform expression/localization etc.). With this in mind, the current study was undertaken in order to better understand the processes involved in preconditioning.

We investigated the role of one of the most likely mediators of the preconditioning response, namely PKC.

IV. METHODS

A. Animals

Male New Zealand white rabbits (3.0 ± 0.5 kg) were housed at the animal holding facility of the St. Boniface General Hospital Research Centre. Animals received standard rabbit chow and water *ad libitum*

B. *In Vivo* Electrocardiograms (ECG)

Rabbits were anesthetized with isoflurane (5% in O_2 at a flow rate of 2 L/min) in an induction chamber and then maintained under anesthesia using an anesthetic mask. Animals were placed in a supine position and the chest, forelimbs and hindlimbs were shaved. ECG leads with alligator clips were attached directly to the skin according to placement of the standard limb leads I, II and III for the feline. Heart rate was calculated from the R-R interval and displayed on-line. If necessary, carotid massage was performed during acquisition of the three lead ECG to lower heart rate and obtain a stable rate of 120 beats/min. ECG signals were amplified using ECG 100 amplifiers (Biopac Systems Inc., Goleta, CA) and filtered to remove 60 Hz line interference. ECG signals were acquired at 2 kHz using an MP100WSW data acquisition system controlled by Acknowledge 3.0 software (Biopac System Inc., Goleta, CA).

C. Isolated Rabbit Heart Preparation

Rabbits (3.0 ± 0.5 kg) were anesthetized with isoflurane (5% in O_2 at a flow rate of 2 L/min) in an induction chamber and then maintained via an anesthetic mask. Animals were injected with 500 IU heparin and the heart was quickly removed via a median sternotomy and flushed with Tyrode's solution. The aorta was attached to a flared tube on a Langendorff apparatus (Figure 2) and perfusion was started immediately with a modified Tyrode's solution. The time from incision of the chest to the start of perfusion of the heart was typically < 90 seconds. The Tyrode's solution contained (mM): NaCl 115.0; NaH_2PO_4 0.5; $NaHCO_3$ 28.0; KCl 4.0; $CaCl_2$ 2.0; $MgCl_2$ 0.7 and d-glucose 20.0. The solution was gassed with 95%O_2-5%CO_2 and maintained at 37 ± 0.5°C. A roller pump was used to deliver this solution to the heart at a flow rate of 20 ml/minute (~3.5 ml/min/g wet wt., mean heart weight = 6.24 ± 0.13 g). Once the heart was mounted on the perfusion system, extraneous tissue was excised and the right atrium was removed. The atrioventricular node was destroyed by crushing the AV node with a Crile hemostat to slow the intrinsic heart rate and allow electrical pacing of the heart. Hearts were paced via bipolar hook electrodes inserted transmurally into the high right ventricle. Rectangular pulses of 5 ms duration were delivered from a Pulsar 6i digital stimulator (Frederick Haer Co., Brunswick, ME) at twice the diastolic threshold intensity. Hearts were paced at 2 Hz (120 beats/min) in all experiments.

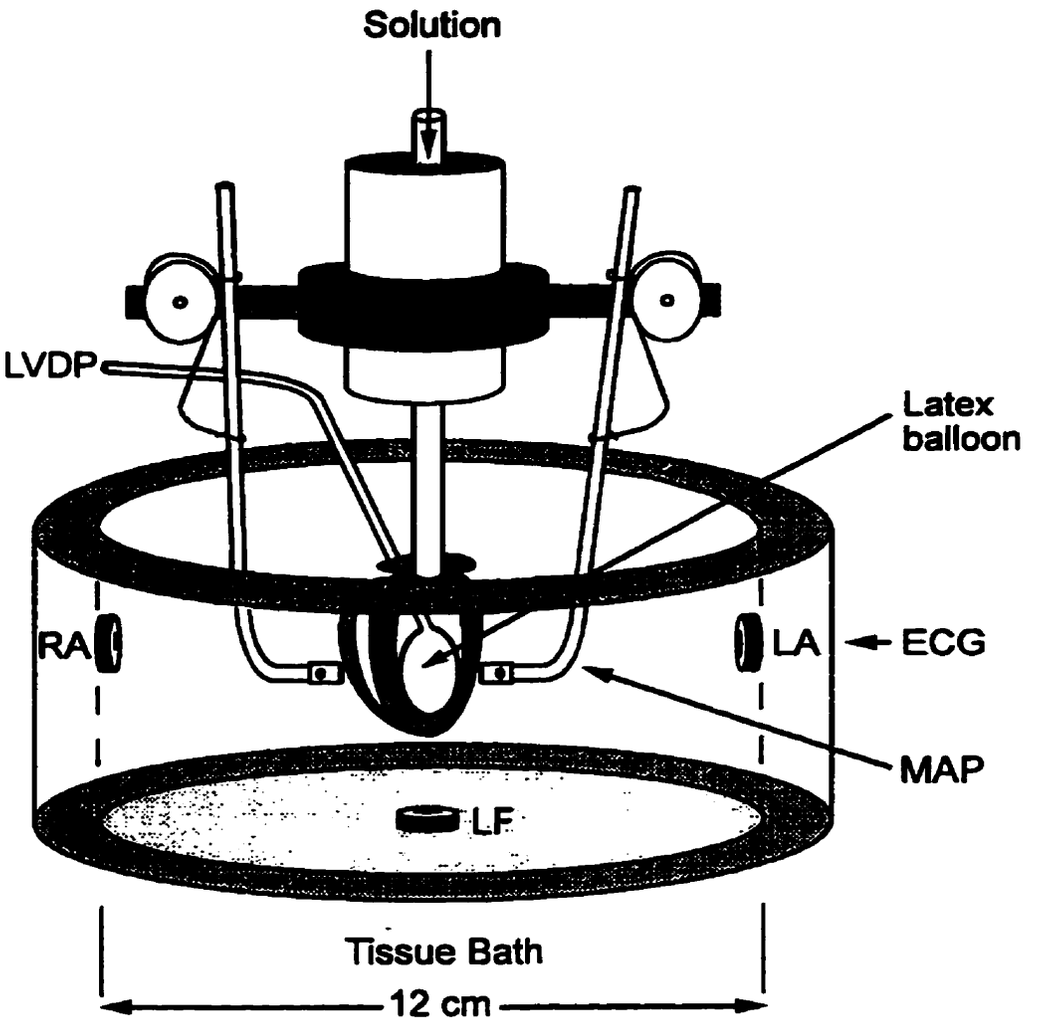

Figure 2. Diagramatic representation of the modified Langendorff apparatus used for isolated rabbit heart experiments. Hearts were suspended in the center of bath and contractile and electrophysiologic parameters were measured as detailed in the text. LVDP - left ventricular developed pressure, ECG - electrocardiogram, MAP - monophasic action potential electrode, LA, RA and LF - leads corresponding to left arm, right arm and left foot, respectively.

D. Recording of Left Ventricular Pressure, Monophasic Action Potentials and ECG

The left atrium was incised and the left ventricle (LV) was vented to prevent pressure build up via a polyethylene drain passed through the apex using a 24 gauge needle. A deflated #8 latex balloon was inserted through the left atrium and mitral valve into the LV. The intraventricular balloon was connected by a short cannula to a TSD104 pressure transducer (Biopac Systems Inc., Goleta, CA). A silk ligature tied around the remaining atrial tissue held the cannula and balloon in position. The pressure transducer allowed continuous monitoring of left ventricular developed pressure (LVDP) and end-diastolic pressure (EDP).

Monophasic action potentials (MAPs) were recorded simultaneously from the epicardial and endocardial surfaces of the left ventricular free wall using Ag-AgCl bipolar Franz MAP electrodes (model 225; EP Technologies Inc., Sunnyvale, CA). The epicardial MAP electrode was mounted on a cantilever arm with an adjustable tensioner that applied pressure against the epicardium, but allowed the heart to contract freely. A foam-lined, U-shaped cradle was positioned opposite to the epicardial electrode to keep the heart in a vertical position. An endocardial MAP electrode was placed in the LV near the apex, and held in place by the latex balloon.

To record a volume-conducted ECG, the heart was immersed in a circular acrylic bath filled with Tyrode's solution. This bath was similar in width to a rabbit's thorax (i.d. 12 cm). Three, 4 mm Ag-AgCl pellets were fixed in the bath to produce an inverted triangle with one electrode on the bottom and two on opposite side walls (Figure 2). The

electrodes were positioned to correspond to the right arm (RA), left arm (LA) and left foot (LF) limb lead placements. This simulated Einthoven configuration, with the heart in the center, generated limb leads I, II and III of the standard ECG (Franz et al., 1992; Zabel et al., 1995). The bath solution was constantly recirculated and its temperature monitored by a thermistor probe (model 421; Cole Parmer Instruments, Niles, Illinois) located at the bottom center of the bath to maintain 37 ± 0.5°C. In addition, a thermistor probe was inserted in the right ventricle to monitor the internal temperature of the heart and the temperature of the perfusing Tyrode's solution was adjusted accordingly to 37.0 ± 0.5°C. The differential between the heart temperature and bath temperature never exceeded 0.5°C. The Tyrode's solution surrounding the heart was bubbled with 95%O_2-5%CO_2 during control and reperfusion periods, and with 95%N_2-5%CO_2 during ischemia.

Time from excision of the heart to complete instrumentation was 5-6 minutes. Once the heart was instrumented, the LV balloon was inflated with water via a micrometer-controlled syringe to obtain an EDP of 10 mm Hg. The epicardial MAP electrode was positioned perpendicular to the ventricular surface and the tension on the MAP electrode was adjusted to the minimum needed to obtain a stable signal. This procedure was repeated for the endocardial MAP electrode. The heart was then allowed to stabilize for 15 minutes before starting the experimental protocols.

The ECG signals were amplified and filtered to remove 60 Hz interference using ECG100 amplifiers (Biopac Systems Inc., Goleta, CA). Signals from the pressure transducer and MAPs were amplified using DA100 amplifiers (Biopac Systems Inc.,

Goleta, CA). Signals were digitized at 2 kHz using an MP100WSW data acquisition system (Biopac Systems Inc., Goleta, CA) and acquired with AcKnowledge 3.0 software contolled via a 486 based PC computer.

E. Ischemic Preconditioning Experiments

Hearts were equilibrated for 15 minutes and then preconditioned with 1 to 4 preconditioning cycles. Preconditioning cycles consisted of 1, 2, 3 or 4 episodes of global ischemia (5 minutes each) separated by 10 minutes of reperfusion (Figure 3). This was followed 30 minutes later by the *test* ischemia/reperfusion, which consisted of 30 minutes of global ischemia and a subsequent 45 minutes of reperfusion. Non-preconditioned hearts were subjected only to the *test* ischemia/reperfusion. These hearts were equilibrated for either 15 minutes or 90 minutes prior to the *test* ischemia/reperfusion (Figure 3). Two durations of equilibration were used for the non-preconditioned hearts to eliminate possible time-dependent effects introduced by the much longer preconditioning protocols.

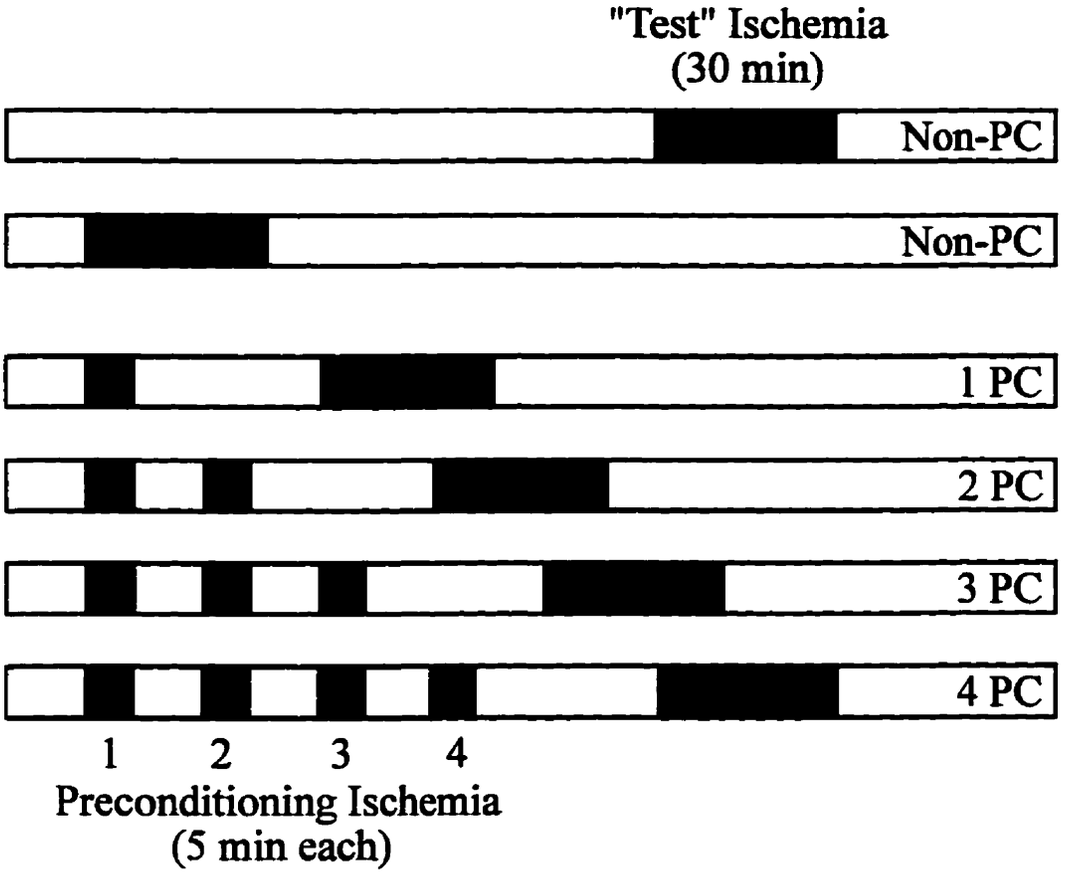

Figure 3. Schematic representation of the ischemic preconditioning (PC) protocols. All hearts were subjected to a *test* ischemia (30 min) and reperfusion (45 min). Non-preconditioned hearts (non-PC) were equilibrated for 15 or 90 minutes to preclude possible time-dependent effects. Preconditioned hearts received 1, 2, 3 or 4 periods of global ischemia (1-4 PC)(5 minutes each, separated by 10 minutes of reperfusion) 30 minutes prior to the *test* ischemia/reperfusion protocol. ■ - global ischemia; □ - normal flow (i.e., equilibration or reperfusion).

F. PKC Inhibition Experiments

The role of protein kinase C (PKC) in the ischemic preconditioning response was assessed using the specific PKC inhibitor, chelerythrine (Herbert et al., 1990). Chelerythrine chloride (Research Biochemicals International, Natick, MD) was prepared as a stock solution in double-distilled H_20 (5 mg/10 ml). Aliquots of the stock solution were frozen and stored at -20°C. Stock solutions were subjected to only one freeze-thaw cycle. Appropriate volumes of the stock solutions were added directly to the Tyrode's solution to obtain final concentrations of 2 μM, 5 μM or 20 μM.

Three experimental protocols were used to test the effects of chelerythrine on ischemic preconditioning. *Protocol 1* - In the first protocol, PKC activity was inhibited only during the preconditioning ischemia. Hearts were pretreated with 2 μM chelerythrine for 5 minutes prior to a single preconditioning cycle (Figure 4). Also, the drug remained in the bath during the 5 minute preconditioning ischemia. The perfusate was switched to normal Tyrode's solution without drug for the rest of the protocol. *Protocol 2* - In this protocol, PKC activity was inhibited only during the sustained *test* ischemia. Hearts were pretreated with 2 μM chelerythrine for 10 minutes prior to the *test* ischemia. Chelerythrine was present in the perfusate and bath solution during the entire *test* ischemia and subsequent reperfusion. *Protocol 3* - In the final protocol, PKC activity was inhibited throughout the protocol. Hearts were pretreated with 2 μM chelerythrine for 5 minutes prior to the single preconditioning cycle and the drug was present in the perfusate

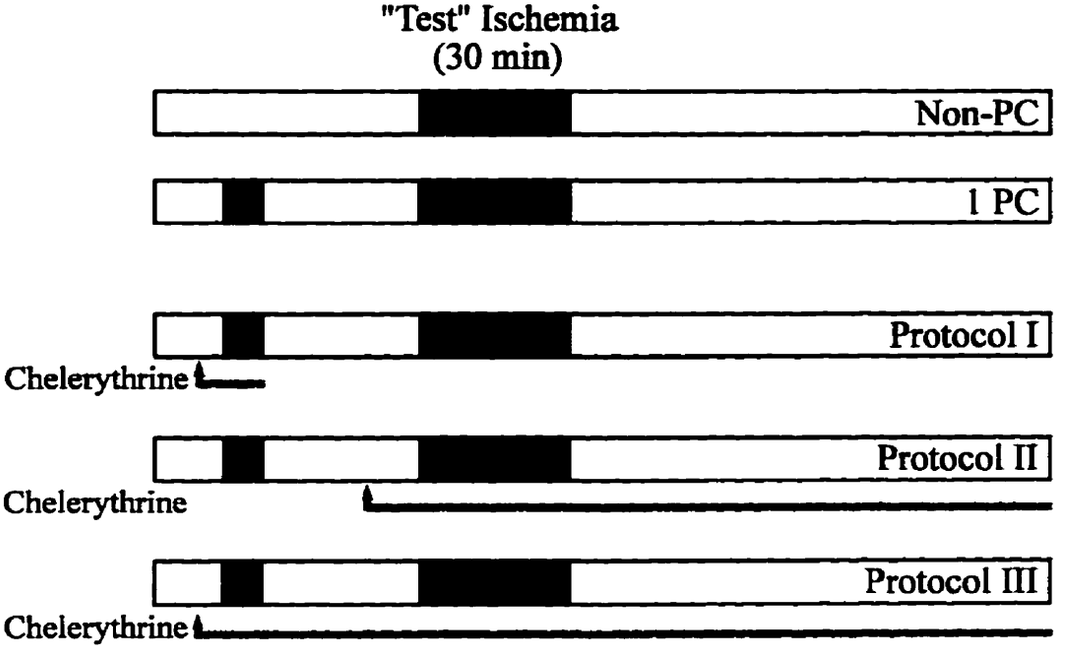

Figure 4. Schematic representation of the PKC inhibition protocols. All hearts were subjected to a *test* ischemia (30 min) and reperfusion (45 min). Preconditioned hearts received 1 period of global ischemia (5 min) 30 minutes prior to the *test* ischemia/reperfusion protocol. Arrows indicate when chelerythrine was introduced and the duration of the treatments during the various protocols (I, II, III; see text for description). ■ - global ischemia; □ - normal flow (i.e., equilibration or reperfusion).

and bath solution during the intervening reperfusion and subsequent *test* ischemia/reperfusion.

G. Data Analysis and Statistics

The criteria used to define ventricular tachycardia (VT) and ventricular fibrillation (VF) in this study were as follows: VT was any rhythmic, rapid rate lasting more than 30 seconds; VF was any rapid, non-synchronous rhythm lasting more than 30 seconds. Arrhythmias were classified as rhythmic versus chaotic based on post-acquisition examination of the ECG and MAP records. Hearts that did not attain a LVDP of > 15 mm Hg during the equilibration period or incurred VT or VF prior to the *test* ischemia (11 of 110 hearts) were not used for the subsequent data analysis.

Monophasic action potential duration at 90% repolarization ($MAPD_{90}$) was calculated post-acquisition from expanded records. $MAPD_{90}$ was measured from the beginning of the upstroke (excluding the stimulus artifact) to 90% of full repolarization.

All data are presented as means ± SE. Statistical analysis was performed using a Student's *t*-test (two-tailed). Incidences of arrhythmia were compared using a two-tailed Fisher's exact probability test. $P < 0.05$ was considered significant.

V. RESULTS

A. Comparison of ECGs Recorded *In Vivo* versus *In Vitro*

Figure 5 illustrates a typical 3-lead ECG recorded on the body surface of an adult rabbit (*in vivo*). The QRS complex is upright in leads II, III and inverted in lead I. A positive T-wave is evident in leads II and III. Figure 5 also shows records obtained with the same leads from an isolated heart in the ECG recording chamber (*in vitro*). With some exceptions, the polarity of the QRS complexes and T-waves in the *in vitro* ECGs duplicate those recorded *in vivo*. The morphology of the QRS complex in leads I-III in the isolated heart is typical of left axis shift. The apparent axis shift could reflect early activation of the right ventricle by the electrodes in the high right ventricular site used to pace these hearts. ECGs with similar characteristics to those shown in Figure 5 were recorded in 21 rabbits (*in vivo*) and 22 isolated hearts (*in vitro*) (Table 2).

B. Electrophysiologic and Contractile Responses of Isolated Rabbit Hearts

The isolated rabbit heart model employed in the present study allowed us to monitor several electrophysiologic and contractile parameters. Figure 6 depicts typical traces obtained for various parameters under control conditions. The top three traces are leads I-III of the volume-conducted ECG. The ECG chamber was rotated so that the recording electrodes were oriented in a manner similar to the lead placement used in intact animals.

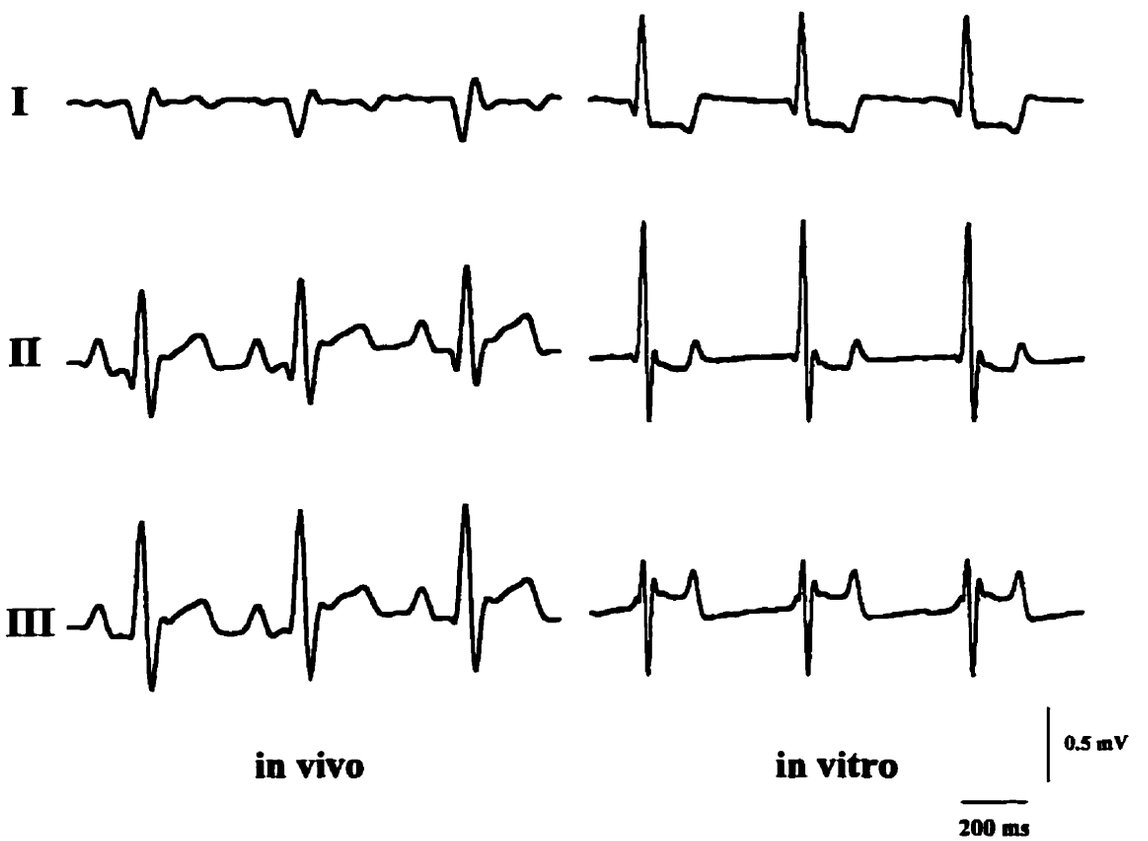

Figure 5. Comparison of typical 3 lead electrocardiograms (ECG) recorded *in vivo* and *in vitro*. *In vivo* traces show three beats recorded at a heart rate of 120 bpm. *In vitro* traces show three beats for hearts that were electrically stimulated at 120 bpm. The *in vitro* record was recorded after a 15 minute equilibration period. The absence of P-waves is due to removal of the atria for instrumentation of the heart.

Table 2. Comparison of *in vivo* and *in vitro* ECGs with respect to polarity of the major deflections of the QRS complex and T-wave.

Lead	Polarity*	*In Vivo*		*In Vitro*	
		QRS	**T-wave**	**QRS**	**T-wave**
I	+	4/21	19/21	19/22	4/22
	-	17/21	1/21	3/22	18/22
II	+	18/21	18/21	14/22	15/22
	-	3/21	1/21	8/22	6/22
III	+	21/21	15/21	3/22	20/22
	-	0/21	4/21	19/22	2/22

* polarity for QRS complex refers to the major deflection being upright (+) or inverted (-).

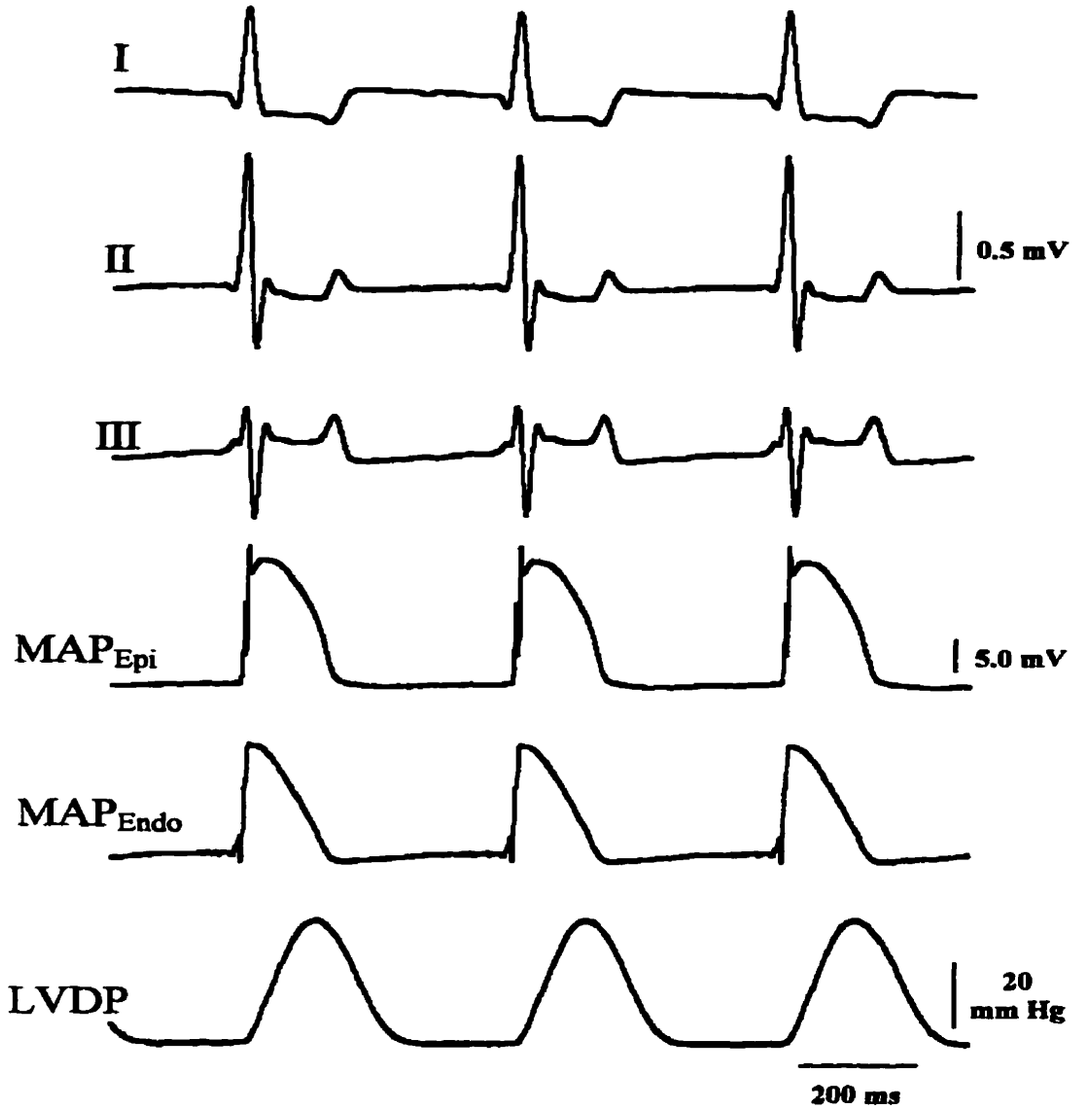

Figure 6. Typical records of electrophysiologic and contractile parameters recorded in an isolated rabbit heart paced at 120 bpm. The upper three traces are leads I-III of the ECG. Below this are monophasic action potentials recorded from the epicardial (MAP_{Epi}) and endocardial (MAP_{Endo}) surfaces of the left ventricle, respectively. The bottom trace is left ventricular developed pressure (LVDP). The record was obtained after a 15 minute equilibration period.

Below the ECG traces are monophasic action potentials (MAP) recorded from the epicardial and endocardial surfaces of the left ventricular free wall. MAP duration at 90% repolarization ($MAPD_{90}$) averaged 160.5 ± 2.6 ms in epicardium and 159.8 ± 2.8 ms in endocardium (n= 59). A notch during phase 1 is present in the epicardial action potential but is absent in the endocardial response. In 53 hearts tested, 28 epicardial MAPs exhibited a notch, whereas only 21 endocardial MAPs displayed a notch. The bottom trace in Figure 6 is left ventricular developed pressure (LVDP), which averaged 42.8 ± 1.9 mm Hg (n= 60). End diastolic pressure (EDP) averaged 9.3 ± 0.9 mm Hg in these hearts.

After equilibration for 15 or 90 min, all hearts were subjected to a *test* ischemia/reperfusion that consisted of 30 minutes of global ischemia, followed by 45 minutes of reperfusion. Two equilibration intervals were chosen to eliminate time-dependent factors introduced by the much longer ischemic preconditioning protocols used subsequently. There was no difference in the incidence of arrhythmias during the *test* ischemia or reperfusion in hearts equilibrated for 15 versus 90 min. Hearts were monitored for the presence of ventricular tachycardia (VT) or fibrillation (VF) during the *test* ischemia and reperfusion. VT and VF occurred during the *test* ischemia in 50% and 42% of non-preconditioned hearts, respectively. The multiple electrophysiologic parameters recorded in these hearts permitted us to clearly distinguish between the occurrence of VT (rapid but regular rhythm; Figure 7) and VF (rapid, chaotic rhythm; Figure 8) in these preparations.

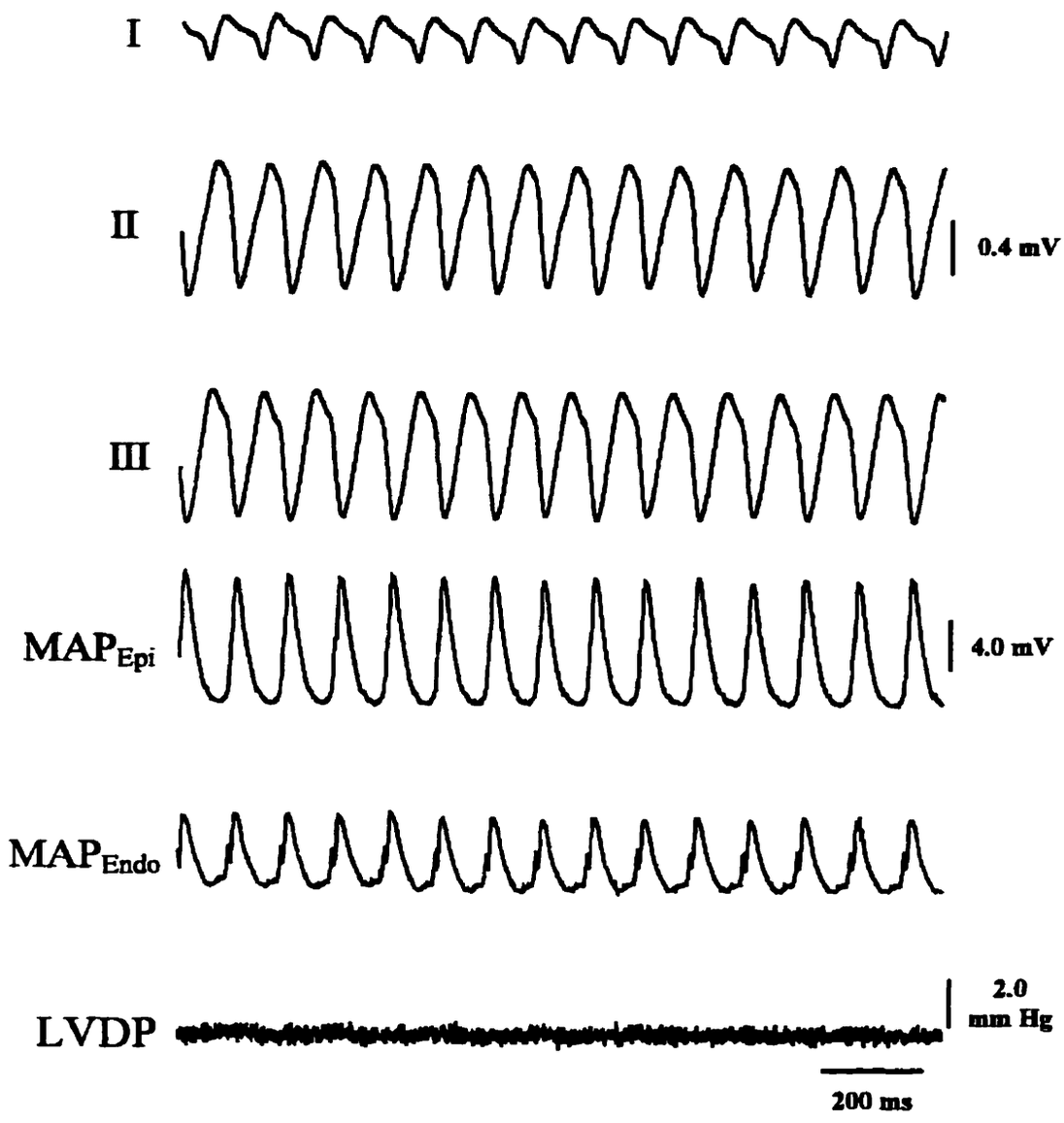

Figure 7. Typical trace of electrophysiologic and contractile parameters recorded during an episode of ischemia-induced ventricular tachycardia (VT). The criteria used to define this arrhythmia were a regular, rapid rate.

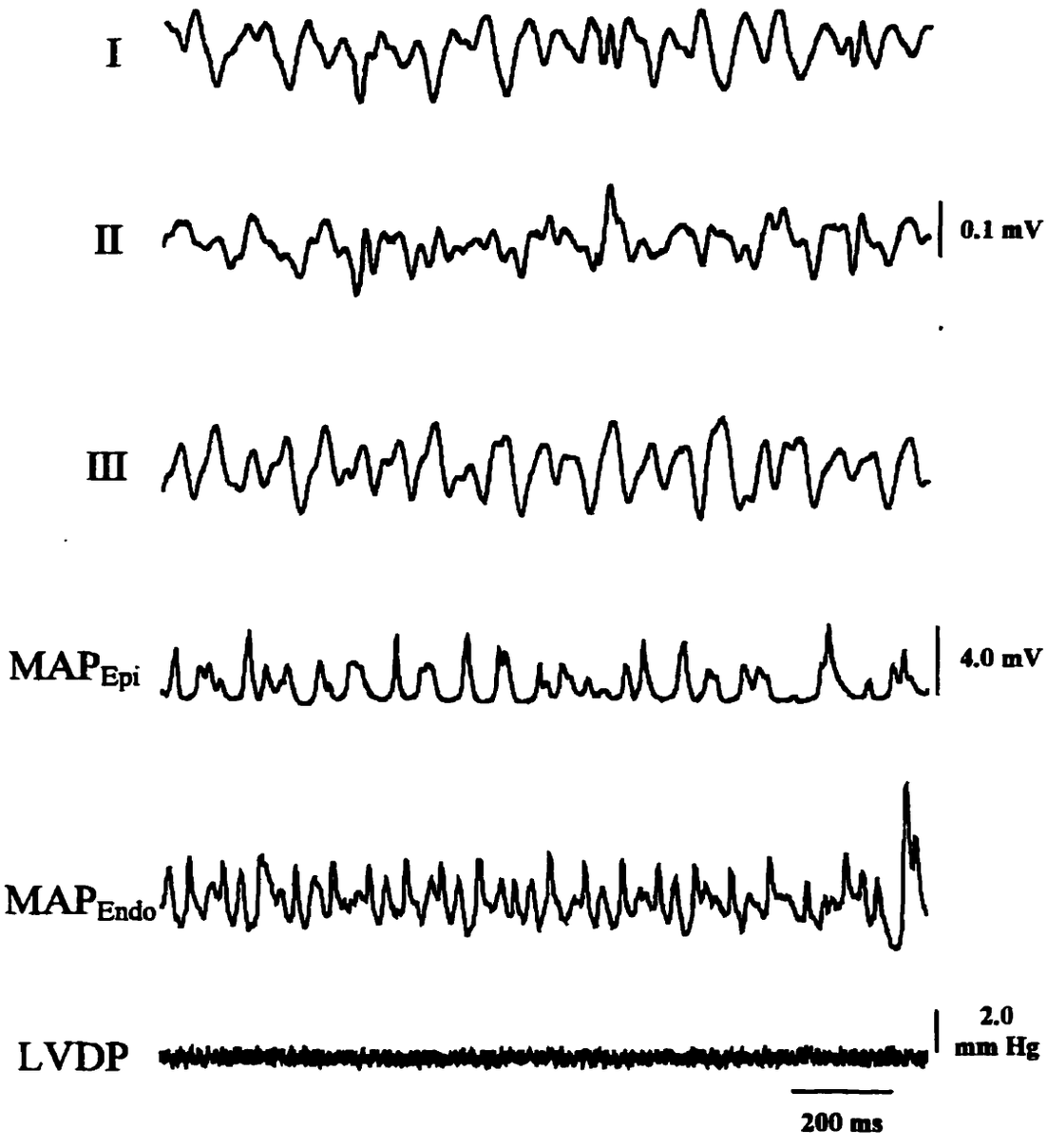

Figure 8. Typical trace of electrophysiologic and contractile parameters recorded during an episode of ischemia-induced ventricular fibrillation (VF). The electrical traces clearly show the random chaotic rhythm that was used to define this arrhythmia.

C. Effects of Multiple Preconditioning Periods on Ischemia and Reperfusion Arrhythmias

Preconditioned hearts received 1, 2, 3 or 4 preconditioning cycles (5 minutes of global ischemia separated by 10 minutes of reperfusion) followed 30 minutes later by the *test* ischemia/reperfusion. One preconditioning cycle reduced the incidence of VT to 30% from 50% in the non-preconditioned hearts (Figure 9, left panel). However, the mean VT duration during ischemia was similar in hearts preconditioned with 1 cycle (2.5 ± 0.9 minutes) and non-preconditioned hearts (3.3 ± 1.1 minutes; Table 3). In contrast, the incidence of VT was actually increased in hearts exposed to 2 preconditioning cycles (60%) or 3 preconditioning cycles (56%) as compared to non-preconditioned hearts or 1 preconditioning cycle. The mean duration of VT also was significantly longer in hearts preconditioned with 2 cycles (7.3 ± 0.8 min, $P< 0.05$) than in any other group (Table 3).

The effects of preconditioning on ischemia-induced VF were much more dramatic (Figure 9, right panel). One preconditioning cycle offered *complete protection* against VF during the *test* ischemia (0% versus 42% in non-preconditioned hearts, $P< 0.05$). Hearts that received 2 preconditioning cycles exhibited a partial protection against VF. The incidence of VF during ischemia was 30% in hearts exposed to 2 preconditioning cycles. Hearts exposed to 3 preconditioning cycles or 4 preconditioning cycles actually had a significantly higher incidence of VF (72% and 47%, respectively, $P< 0.05$) than hearts exposed to 1 preconditioning cycle. However, the time at which the first tachyarrhythmia (VT or VF) appeared during the *test* ischemia was similar in all groups of hearts (Table 3).

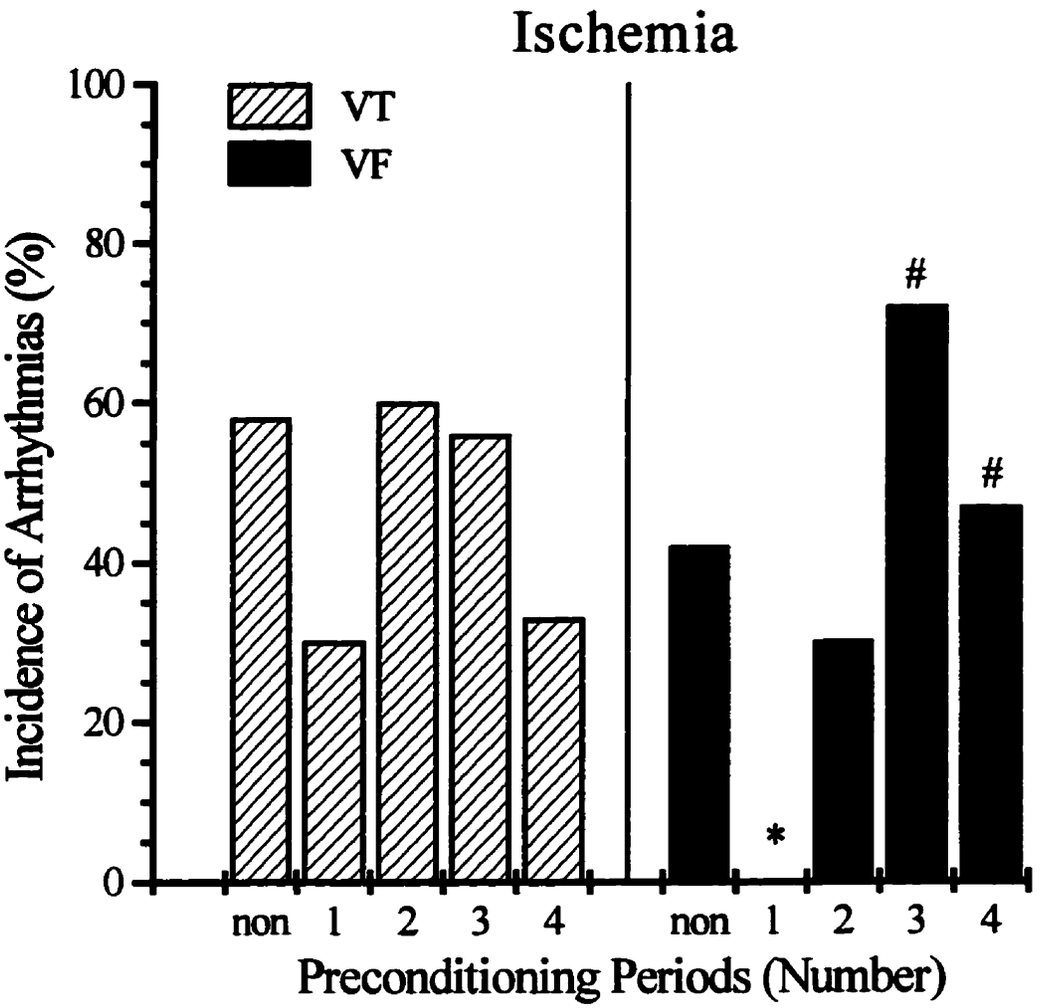

Figure 9. The effect of preconditioning on the incidence of arrhythmias during a subsequent 30 minute *test* ischemia. The incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF) are plotted for non-preconditioned hearts (non) and those receiving 1, 2, 3 or 4 preconditioning cycles prior to the *test* ischemia. Groups include the following number of hearts; non (n=12), 1 preconditioning cycle (n=10), 2 preconditioning cycles (n=10), 3 preconditioning cycles (n=18) and 4 preconditioning cycles (n=15). All hearts were paced at 120 bpm. $^{*}P < 0.05$ versus non-preconditioned hearts. $^{\#}P < 0.05$ versus hearts preconditioned with 1 PC.

TABLE 3. The effect of preconditioning on the onset and duration of sustained ischemia-induced or reperfusion-induced ventricular tachyarrhythmias in isolated rabbit hearts.

	Number of Preconditioning Cycles				
	None	**1 PC**	**2 PC**	**3 PC**	**4 PC**
ISCHEMIA	(n=12)	(n=11)	(n=10)	(n=18)	(n=15)
Time to first arrhythmia	23.8 ± 1.0	22.8 ± 1.1	19.3 ± 1.7	22.1 ± 2.0	20.1 ± 3.5
Mean VT duration (min)	3.3 ± 1.1	2.5 ± 0.9	7.3 ± 0.8*	1.8 ± 0.4	3.1 ± 1.5
Mean VF duration (min)	5.2 ± 1.3	0*	3.3 ± 0.3	7.5 ± 2.1	5.0 ± 1.3
REPERFUSION					
Time to first arrhythmia	0.2 ± 0.1	0.3 ± 0.1	0.8 ± 0.2*	0.3 ± 0.1	0.8 ± 0.1*
Mean VT duration (min)	2.7 ± 0.9	6.2 ± 5.5	1.0 ± 0.1	1.0 ± 0.2	7.4 ± 3.9
Mean VF duration (min)	19.3 ± 4.1	22.4 ± 7.9	17.2 ± 4.5	25.7 ± 3.3	19.5 ± 3.9

Time to first arrhythmia - time to onset of either ventricular tachycardia (VT) or fibrillation (VF) during the prolonged *test* ischemia, or during reperfusion following the *test* ischemia. Values are means ± S.E. * $P < 0.05$ versus non-preconditioned hearts.

Figure 10 summarizes the effects of ischemic preconditioning on the incidence of VT and VF during reperfusion following the *test* ischemia. None of the preconditioning protocols offered any protection against VT during the subsequent reperfusion. The incidence of VT was 40% in non-preconditioned hearts versus 60%, 80% ($P< 0.05$), 33% and 47% in hearts exposed to 1, 2, 3 and 4 preconditioning cycles, respectively. Moreover, the mean VT duration observed during reperfusion was not altered by any of the preconditioning protocols (Table 3).

Preconditioning also offered only partial protection against reperfusion-induced VF (Figure 10). Reperfusion following the *test* ischemia elicited VF in 75% of non-preconditioned hearts. Hearts exposed to 1 preconditioning cycle exhibited a 25% decrease in the incidence of VT (from 75% to 50%) but this decrease did not attain statistical significance. In contrast, exposure to additional preconditioning cycles actually increased the genesis of reperfusion arrhythmias. Reperfusion-induced VF occurred in 90%, 94% and 93% of hearts that received 2, 3 or 4 preconditioning cycles, respectively ($P< 0.05$ versus 1 preconditioning cycle). The mean duration of VF, however, was similar in all groups of hearts (Table 3).

The data presented in Figure 10 represent the total incidences of VT or VF seen during reperfusion. Data are not separated based on whether the arrhythmia started during reperfusion or was present during ischemia and continued into reperfusion. For non-preconditioned hearts, 6/12 hearts had a preexisting arrhythmias during the transition from ischemia to reperfusion (3 VT and 3 VF). Prior arrhythmias were present at the

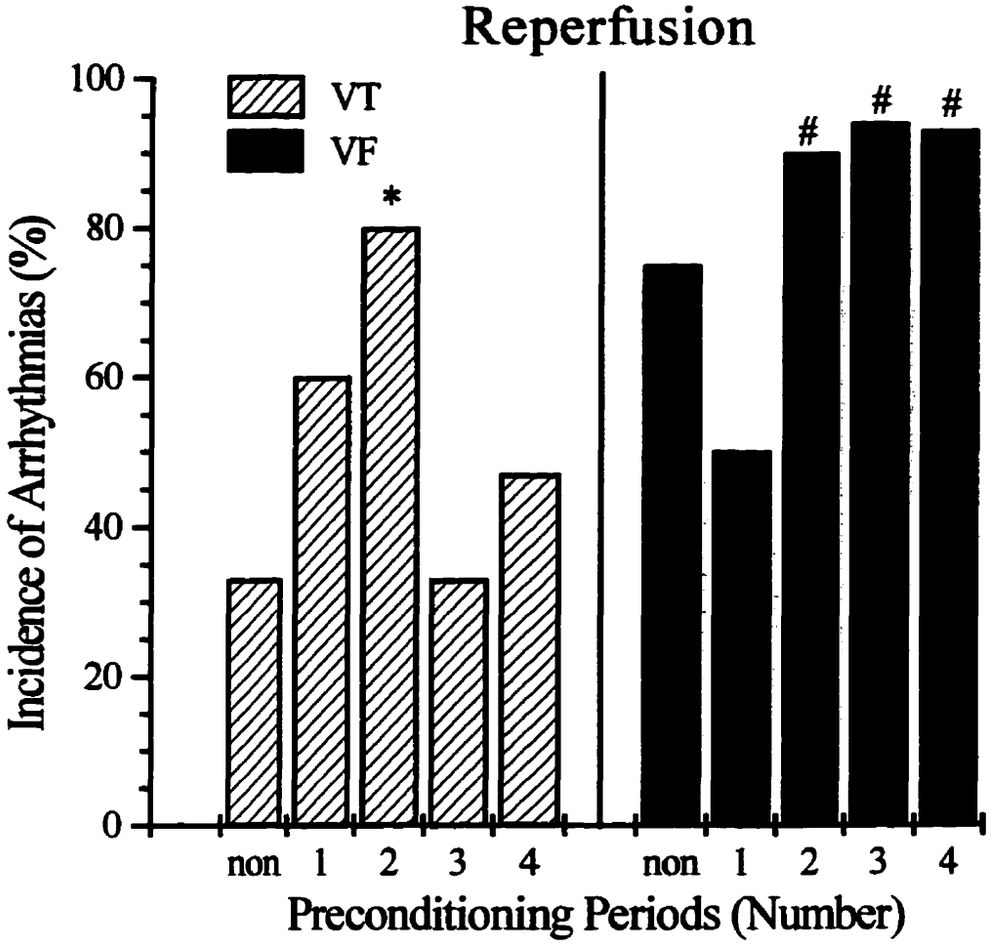

Figure 10. Effect of preconditioning on the incidence of arrhythmias during reperfusion following the 30 minute *test* ischemia. Incidences of ventricular tachycardia (VT) and ventricular fibrillation (VF) are plotted for non-preconditioned hearts and those receiving 1, 2, 3 or 4 preconditioning periods. Groups include the following number of hearts; non (n=12), 1 preconditioning cycle (n=10), 2 preconditioning cycles (n=10), 3 preconditioning cycles (n= 18) and 4 preconditioning cycles (n=15). All hearts were paced at 120 bpm. $^{*}P < 0.05$ versus non-preconditioned hearts. $^{\#}P < 0.05$ versus hearts preconditioned with 1 PC.

onset of reperfusion in 0/11 hearts exposed to 1 preconditioning cycle, 5/10 hearts after 2 preconditioning cycles (4 VT and 1 VF), 15/18 hearts after 3 preconditioning cycles (3 VT and 12 VF) and 2/15 hearts after 4 preconditioning cycles (0 VT and 2 VF). Thus, it is not possible to completely separate the effects of preconditioning on reperfusion versus ischemia-induced arrhythmias, except in the case of hearts exposed to 1 preconditioning cycle.

D. Effects of Preconditioning on Contractile Function

The number of cycles used to precondition the heart also affected contractile function during both the *test* ischemia and subsequent reperfusion. Figure 11 summarizes the changes in LVDP (upper panel) and EDP (lower panel) elicited during the *test* ischemia and subsequent reperfusion in non-preconditioned hearts and those receiving 1 to 4 preconditioning cycles. Ischemia produced a similar depression of LVDP (to ~3 mm Hg within 5 min) in all groups of hearts. Moreover, the rate and extent of recovery of LVDP during reperfusion was not different between non-preconditioned hearts and those receiving 1 to 4 preconditioning cycles. Full recovery of LVDP to pre-ischemic values did not occur during the 45 minute reperfusion period in any of the hearts tested (n=66) and LVDP for all hearts remained significantly depressed throughout the *test* reperfusion ($P<$ 0.05 versus pre-ischemic LVDP).

Preconditioning elicited significant effects on EDP changes in the different groups of hearts. Non-preconditioned hearts displayed a gradual but significant rise in EDP during

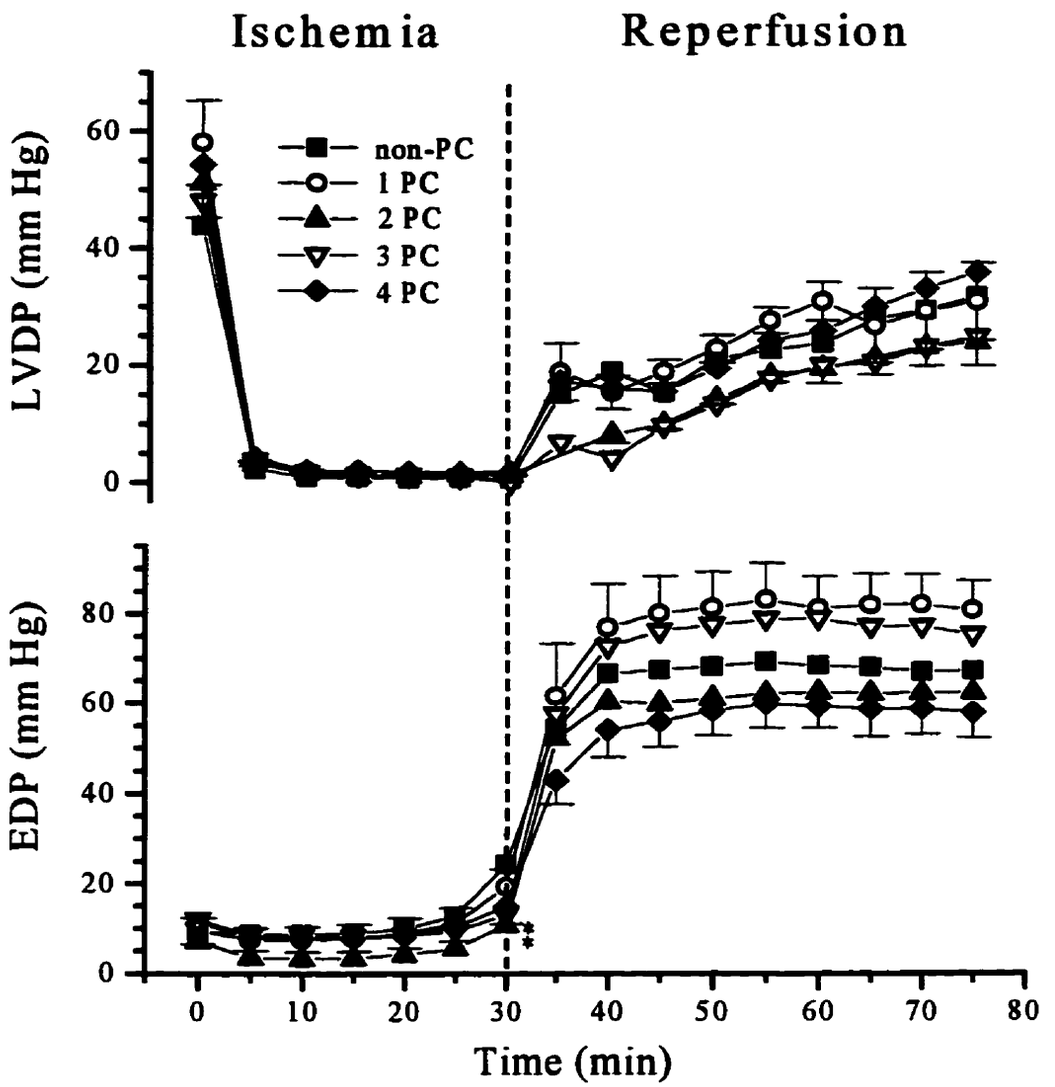

Figure 11. Effect of preconditioning on the changes in left ventricular developed pressure (LVDP) and end diastolic pressure (EDP) elicited during the subsequent *test* ischemia/reperfusion. Changes in LVDP (upper panel) and EDP (lower panel) are shown for non-preconditioned hearts (non-PC), and hearts receiving 1 to 4 preconditioning cycles (1-4 PC). Data are means ± SE for 6-11 hearts in each group. * $P < 0.05$ for 2 and 3 PC hearts versus non-preconditioned hearts.

the course of the 30 minute *test* ischemia (from 9.4 ± 0.7 mm Hg to 24.3 ± 2.5 mm Hg, $P< 0.05$). This was also the case in hearts exposed to 1 preconditioning cycle; EDP increased from 9.6 ± 2.7 mm Hg before the *test* ischemia to 19.3 ± 3.9 mm Hg at the end of the *test* ischemia ($P< 0.05$). In contrast, no significant increase in EDP was seen during the *test* ischemia in hearts exposed to 2, 3 or 4 preconditioning cycles. Hearts subjected to 2, 3 or 4 preconditioning cycles exhibited a rise in EDP of only 3.4 ± 2.1 mm Hg, 1.1 ± 1.9 mm Hg and 3.7 ± 3.9 mm Hg, respectively, versus 14.9 ± 1.6 mm Hg for non-preconditioned hearts ($P< 0.05$).

Reperfusion produced a further increase in EDP in non-preconditioned hearts (to 66.8 ± 7.5 mm Hg by 10 minutes of reperfusion, $P< 0.05$). However, changes in EDP obtained during reperfusion in hearts receiving 1, 2, 3 or 4 preconditioning cycles were not different from non-preconditioned hearts. None of the preconditioning protocols significantly attenuated the rise in EDP observed during reperfusion following the *test* ischemia.

E. Electrophysiologic and Contractile Responses During Ischemic Preconditioning

Successive ischemic preconditioning periods exerted similar effects on electrical and contractile changes in the heart as shown in Figure 12. This figure summarizes the changes in LVDP and $MAPD_{90}$ in epicardium and endocardium of 13 hearts subjected to 4 preconditioning cycles. Ischemia depressed LVDP during each of the 4 preconditioning

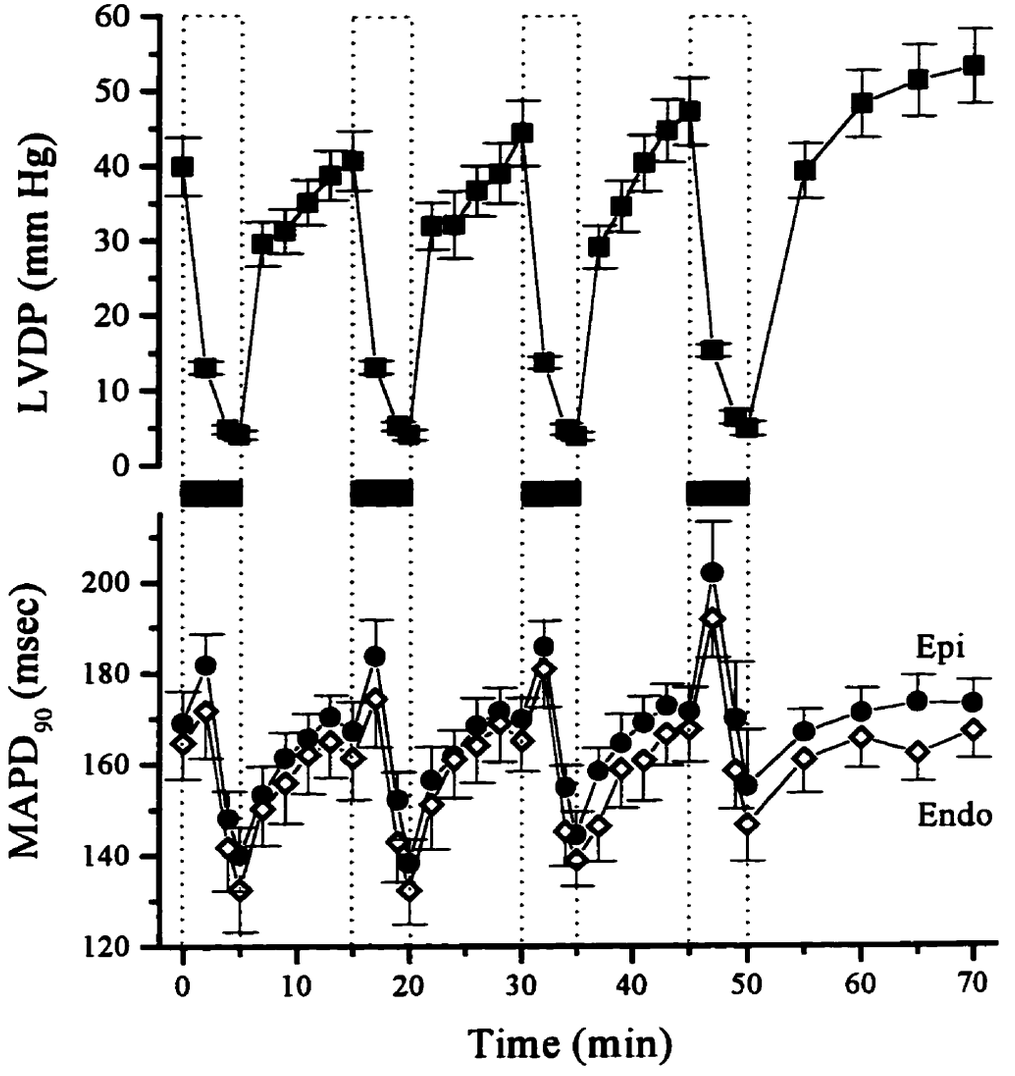

Figure 12. Contractile and electrophysiological changes elicited in hearts subjected to 4 successive periods of preconditioning. Preconditioning ischemia are indicated by heavy horizontal bars. The upper panel depicts the changes in left ventricular developed pressure (LVDP). Data are means ± SE for 13 hearts. The lower panel plots the corresponding changes in monophasic action potential duration at 90% repolarization ($MAPD_{90}$) in epicardium (●) and endocardium (◇). The final ischemic period is followed by a 30 minute reperfusion period.

cycles (to ~ 4.0 mm Hg, $P< 0.05$). The rate of decline of LVDP during ischemia was similar during each of the four preconditioning periods. However, the recovery of LVDP during each subsequent reperfusion gradually increased from 39.9 ± 3.9 mm Hg prior to the preconditioning ischemia to 54.3 ± 5.1 mm Hg after the 30 minute intervening reperfusion ($P< 0.05$).

The control $MAPD_{90}$ values for epicardium and endocardium were 169.0 ± 7.1 ms and 164.7 ± 14.0 ms, respectively. At all times during preconditioning, the epicardial $MAPD_{90}$ remained longer than the endocardial $MAPD_{90}$. Each preconditioning ischemia produced a biphasic change in $MAPD_{90}$ in both epicardium and endocardium. $MAPD_{90}$ increased rapidly over the first two minutes (from 169.0 ± 7.1 ms to 181.8 ± 6.8 ms in epicardium and from 164.7 ± 14.0 ms to 171.8 ± 10.4 ms in endocardium) and then shortened markedly in both the epicardium (to 140.1 ± 6.1 ms, $P< 0.05$ versus pre-ischemic value) and endocardium (to 132.5 ± 9.3 ms, $P< 0.05$ versus pre-ischemic value). The initial prolongation in $MAPD_{90}$ in both epicardium and endocardium became more pronounced with each successive preconditioning cycle, whereas the subsequent shortening became less pronounced. During each of the 4 preconditioning cycles, the $MAPD_{90}$ changes were qualitatively similar in both epicardium and endocardium.

F. Electrophysiologic Changes in Epicardium versus Endocardium During the *Test* Ischemia

The *test* ischemia produced similar effects on monophasic action potentials in both epicardium and endocardium regardless of the prior preconditioning protocol. $MAPD_{90}$ in both ventricular layers decreased significantly during the first 15 minutes of the *test* ischemia (Figure 13). The epicardial $MAPD_{90}$ decreased from ~165 ms to ~103 ms for all groups ($P< 0.05$). All groups of preconditioned hearts exhibited a longer $MAPD_{90}$ than the non-preconditioned hearts during the first 10 minutes of the *test* ischemia. However, only the $MAPD_{90}$ values in the non-preconditioned hearts at 5 and 10 minutes of ischemia were significantly shorter than the corresponding values in hearts preconditioned with 2 or 3 cycles. By 15 minutes, there were no differences in $MAPD_{90}$ between the preconditioned and non-preconditioned groups.

A similar trend was seen in endocardium during the first 15 minutes of the *test* ischemia. $MAPD_{90}$ decreased from ~160 ms to ~103 ms for both non-preconditioned hearts and those receiving 1-4 preconditioning cycles ($P< 0.05$). The $MAPD_{90}$ in endocardium for hearts preconditioned with 2, 3 and 4 cycles was significantly longer than non-preconditioned hearts at 5 minutes into the *test* ischemia, but by 10 minutes there were no differences between the groups.

In the final 15 minutes of the *test* ischemia, $MAPD_{90}$ in epicardium did not differ between groups. However, $MAPD_{90}$ in endocardium of non-preconditioned hearts exhibited prolongation in the last 15 minutes of the *test* ischemia.

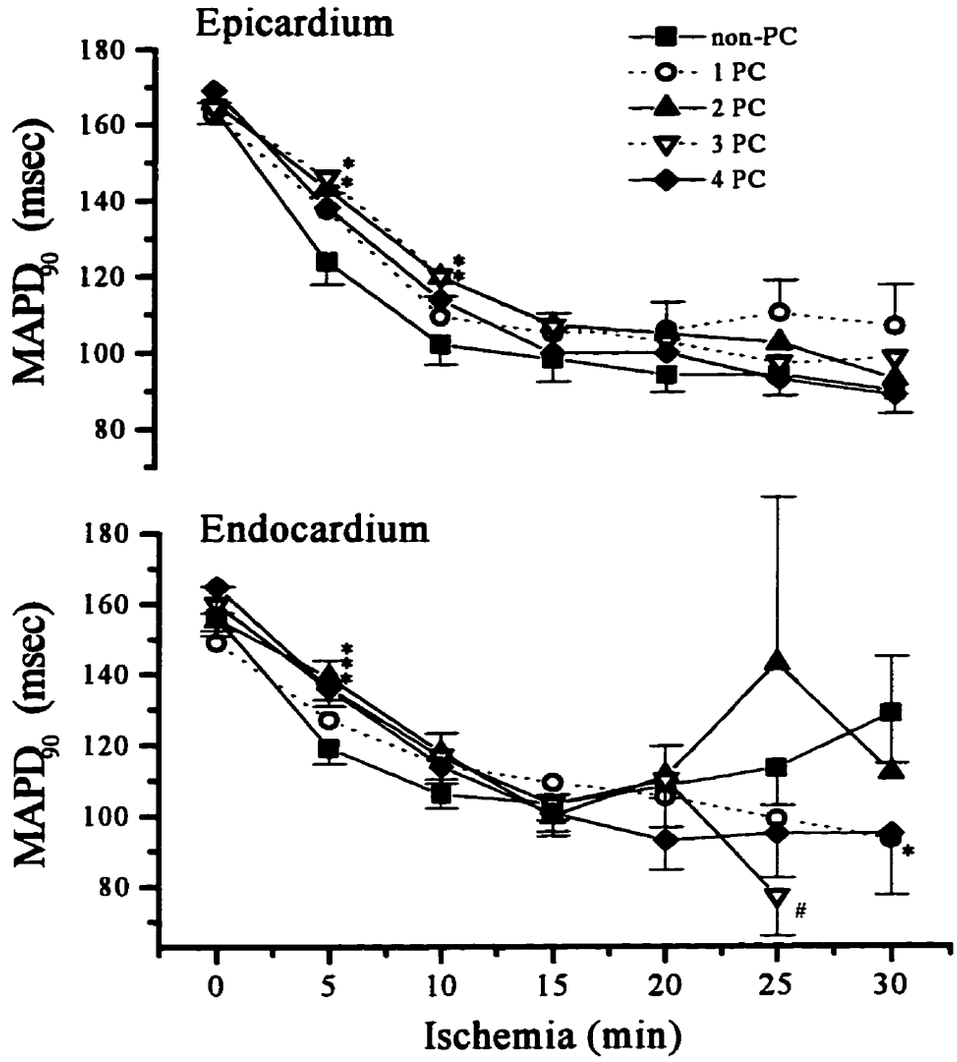

Figure 13. Electrophysiological changes occurring in epicardium and endocardium during the *test* ischemia in non-preconditioned hearts (non-PC) and hearts subjected to 1-4 preconditioning cycles (1-4 PC). Changes in monophasic action potential duration at 90% repolarization ($MAPD_{90}$) are shown for epicardium (upper panel) and endocardium (lower panel). Data represent means of 6-12 hearts in each group at the start of ischemia. $^{*}P< 0.05$ versus non-preconditioned hearts. $^{\#}P< 0.05$ versus 1 preconditioning cycle hearts.

The $MAPD_{90}$ in non-preconditioned hearts lengthened from 103.4 ± 4.6 ms to 128.8 ± 14.2 ms. In contrast, $MAPD_{90}$ in hearts exposed to 1 preconditioning cycle continued to shorten throughout the *test* ischemia ($P< 0.05$ versus non-preconditioned hearts). Changes in $MAPD_{90}$ of hearts exposed to 2 and 3 preconditioning cycles were difficult to interpret during the latter stages of ischemia due to the low numbers of hearts that did not exhibit VT or VF in these two groups (n=2).

G. Effects of PKC Inhibition on Ischemia and Reperfusion-induced Arrhythmias

The preceding studies examined whether ischemic preconditioning could confer protection against arrhythmogenesis and the conditions required for optimal protection. The subsequent sections address the mechanism underlying this protection and the role of PKC.

Figure 14 illustrates the effects of increasing concentrations of chelerythrine on the protection against arrhythmias conferred by 1 preconditioning cycle. Inhibition of PKC with 2 μM chelerythrine only during the preconditioning ischemia abolished the protective effects of one preconditioning cycle on ischemia-induced VT and VF. Chelerythrine treatment resulted in increased incidence of VT from 30% to 67% and the incidence of VF from 0% to 67% ($P< 0.05$ versus hearts exposed to one preconditioning cycle) during the *test* ischemia. In fact, the occurrence of VT and VF was actually higher in the presence of 2 μM chelerythrine as compared to non-preconditioned hearts (50% and 42%, respectively). Similarly, 5 μM and 20 μM chelerythrine also abolished the protective

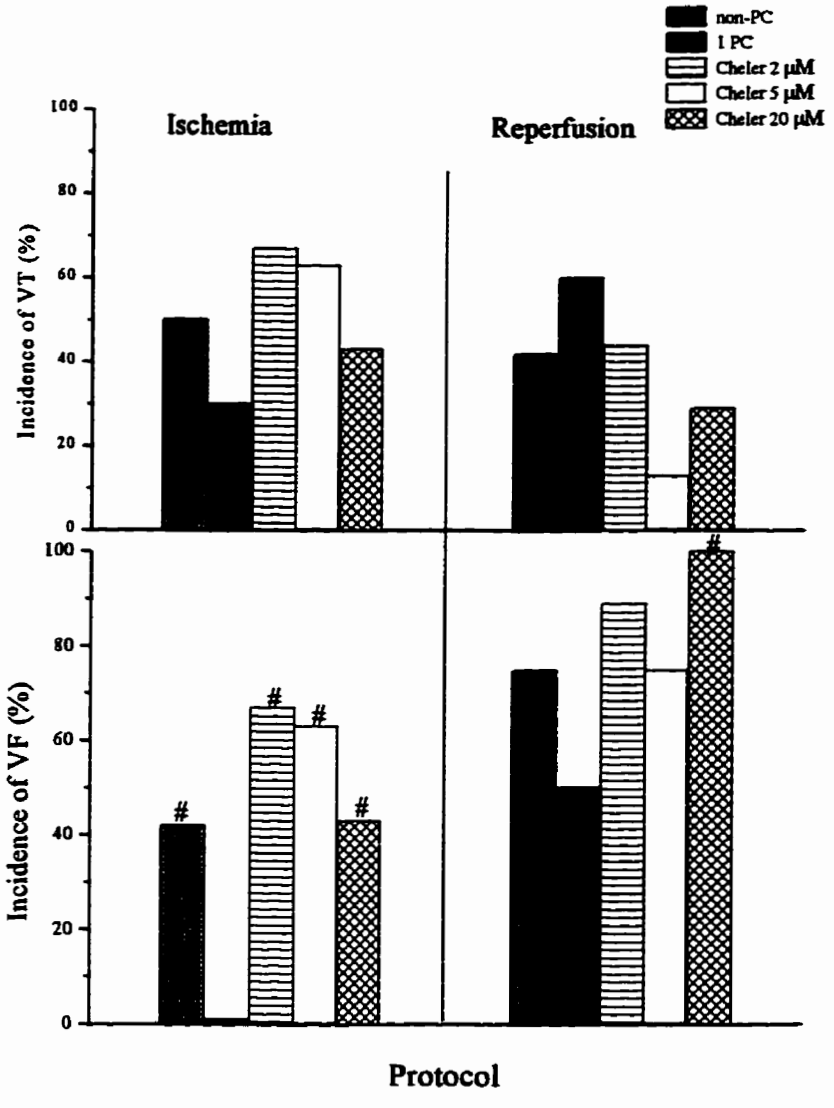

Figure 14. Effect of increasing concentrations of the PKC inhibitor chelerythrine on preconditioning against arrhythmias during the *test* ischemia/reperfusion. Incidences of ventricular tachycardia and ventricular fibrillation are plotted for non preconditioned and 1 preconditioning cycle hearts and those receiving 2 μM, 5 μM or 20 μM chelerythrine. Groups include the following number of hearts; non preconditioned (n=14), 1 preconditioning cycle (n=10), 2 μM (n=9), 5 μM (n=8) and 20 μM (n=7). Hearts were pretreated with drug for 5 minutes prior to the preconditioning episode. $^{\#}P < 0.05$ versus 1 preconditioning cycle hearts.

effect of one preconditioning cycle on VF. Pretreatment with 20 μM chelerythrine resulted in an incidence of VT (43%) and VF (43%) that were similar to non-preconditioned values (50% and 42%, respectively).

For reperfusion arrhythmias, VT and VF occurred in 42% and 75% of non-preconditioned hearts, respectively, and the incidences dropped to 60% and 50% in hearts exposed to 1 preconditioning cycle. As in the case of ischemia-induced arrhythmias, pretreatment with chelerythrine abolished the protection seen with 1 preconditioning cycle on reperfusion-induced arrhythmias. Interestingly, hearts exposed to 1 preconditioning cycle exhibited no protection against VT during reperfusion, yet 5 μM and 20 μM chelerythrine offered some protection. In non-preconditioned hearts, the incidence of VT during reperfusion was 42% which was decreased to 13% and 29% after pretreatment with 5 μM and 20 μM chelerythrine, respectively. However, the 20 μM chelerythrine group had a higher incidence of VF during reperfusion (100%), which may account for the lower incidence of VT during reperfusion in these hearts. In contrast, the protection against VF observed with 5 μM chelerythrine is more clear-cut, since the incidence of VF for both non-preconditioned and treated hearts was identical (75%). Also, the data for reperfusion arrhythmias does not distinguish between preexisting arrhythmias and those starting during reperfusion. Preexisting arrhythmias were present in 6 of 9 hearts pretreated with 2 μM chelerythrine, in 4 of 8 hearts pretreated with 5 μM chelerythrine and in 2 of 7 hearts pretreated with 20 μM chelerythrine.

H. Effects of PKC Inhibition During Different Stages of Preconditioning

PKC inhibition with 2 μM chelerythrine during the single preconditioning ischemia not only blocked the protection seen with 1 preconditioning cycle, but actually increased the incidences of VT and VF during ischemia and VF during reperfusion above that of the non-preconditioned hearts. The protective effect of preconditioning was also abolished when 2 μM chelerythrine was administered only during the *test* ischemia (Protocol II, Figure 15). Chelerythrine abolished both the protection against VT and VF during ischemia and VF during reperfusion. Qualitatively, the results were similar whether chelerythrine was administered only during the single preconditioning ischemia or the *test* ischemia. This was not the case when 2 μM chelerythrine was present throughout the experiment (Protocol III). Under these conditions, the protective effect of 1 preconditioning cycle against arrhythmogenesis was not abolished. The incidence of VT during ischemia in chelerythrine-treated hearts (40%) is similar to that seen in 1 preconditioning cycle hearts (30%). Moreover, the complete protection seen with 1 preconditioning cycle (0% incidence) was not changed if 2 μM chelerythrine was present throughout the experimental protocol. Similarly, the incidence of VF during reperfusion in hearts where chelerythrine was present throughout the experiment (60%) was similar to that seen with 1 preconditioning cycle (50%).

Chelerythrine alone did not affect any of the parameters in the isolated rabbit heart. The contractile and electrophysiologic effects of chelerythrine exerted during a 5 minute

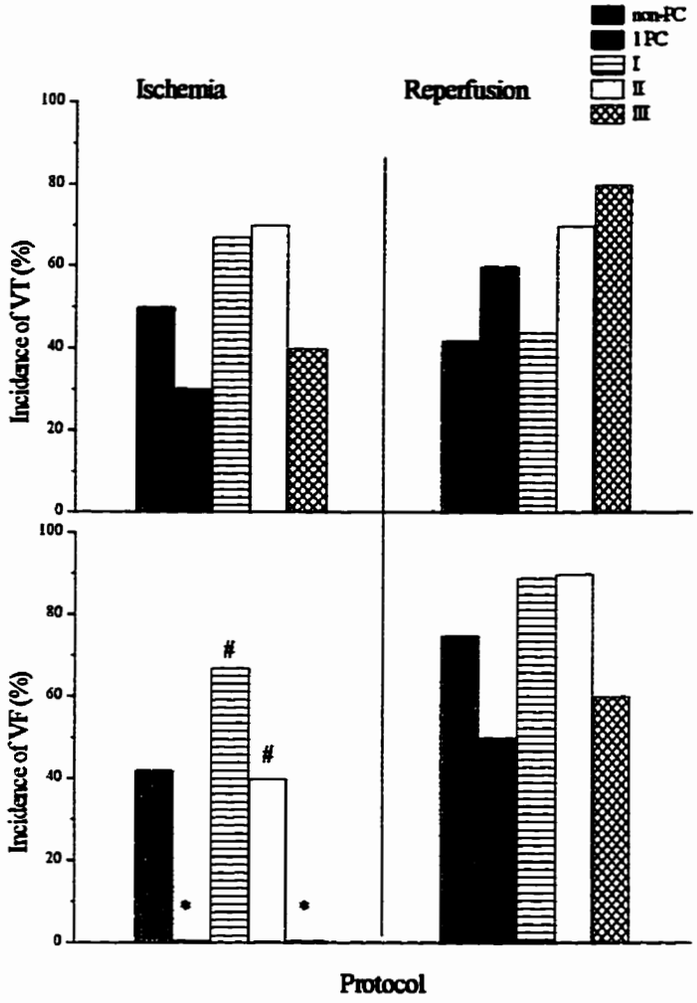

Figure 15. Effects of PKC inhibition at different stages during the preconditioning protocols on the incidences of arrhythmias during the *test* ischemia/reperfusion. Incidences of ventricular tachycardia and ventricular fibrillation are plotted for non-preconditioned hearts and hearts preconditioned with one PC and those pretreated with 2 μM chelerythrine; only during the preconditioning ischemia (I), only during the *test* ischemia/reperfusion (II), or for the entire duration of the experiment (III). Groups include the following number of hearts; non preconditioned hearts (n=14), 1 preconditioning cycle (n=10), I (n=9), II (n=10) and III (n=10). * $P < 0.05$ versus non-preconditioned hearts. # $P < 0.05$ versus 1 preconditioning cycle hearts.

(before the preconditioning cycle) or 10 minutes (before the test ischemia) exposure period are summarized in Table 4.

Table 5 summarizes the effects of chelerythrine on the duration of arrhythmias observed during ischemia in the various preconditioning protocols. The duration of VT in 1 preconditioning cycle hearts during the *test* ischemia was 2.5 ± 0.9 minutes, which was unaltered when chelerythrine was present only during the single preconditioning ischemia (2.0 ± 0.6 min), during the test ischemia/reperfusion (2.6 ± 0.8 min) or throughout the protocol (3.2 ± 1.4 min). For ischemia-induced VF, protocol I resulted in no change in the duration of this arrhythmia (5.1 ± 1.2 min) from that of non-preconditioned hearts (5.2 ± 1.3), while protocol II showed a small non-significant reduction in duration of VF (2.5 ± 0.7 min).

Chelerythrine treatment during the various stages of preconditioning had no effect on the duration of VF during reperfusion, which remained similar to that of non-preconditioned hearts (19.4 ± 5.4 minutes in 1 preconditioning cycle versus 19.4 ± 5.4 minutes in protocol I, 18.6 ± 4.8 minutes in protocol II and 16.0 ± 7.3 minutes in protocol III). Therefore, chelerythrine treatment had no effect on the duration of arrhythmias regardless of when it is administered during the preconditioning protocol.

Table 4. Effects of the PKC inhibitor chelerythrine on contractile and electrophysiologic parameters in hearts exposed for a 5 minute (prior to preconditioning) or 10 minute period (prior to the test ischemia).

Parameter	5 minute exposure		10 minute exposure	
	Control	Chelerythrine 5 min	Control	Chelerythrine 10 min
LVDP (mm Hg)	33.8 ± 2.9	33.0 ± 2.9	52.5 ± 5.7	51.0 ± 4.4
EDP (mm Hg)	5.5 ± 0.8	4.4 ± 1.3	2.8 ± 0.7	2.5 ± 0.8
$MAPD_{EPI}$ (ms)	176 ± 7	180 ± 7	174 ± 8	176 ± 6
$MAPD_{ENDO}$ (ms)	145 ± 5	148 ± 4	170 ± 8	169 ± 8

Values are expressed as means ± S.E. For hearts exposed for 5 minutes, n=7. For hearts exposed for 10 minutes, n=10.

TABLE 5. The effect of PKC inhibition at different stages during preconditioning on the onset and duration of sustained ischemia-induced or reperfusion-induced ventricular tachyarrhythmias in isolated rabbit hearts.

	Protocol				
	None	**1 PC**	**I**	**II**	**III**
ISCHEMIA	(n=12)	(n=11)	(n=9)	(n=10)	(n=10)
Time to first arrhythmia	23.8 ± 1.0	22.8 ± 1.1	21.7 ± 0.5	23.5 ± 1.4	23.7 ± 1.2
Mean VT duration (min)	3.3 ± 1.1	2.5 ± 0.9	2.0 ± 0.6	2.6 ± 0.8	3.2 ± 1.4
Mean VF duration (min)	5.2 ± 1.3	0*	5.1 ± 1.2	2.5 ± 0.7	0*
REPERFUSION					
Time to first arrhythmia	0.2 ± 0.1	0.3 ± 0.1	1.1 ± 0.8	0.8 ± 0.2	0.8 ± 0.5
Mean VT duration (min)	2.7 ± 0.9	6.2 ± 5.5	7.6 ± 3.3	2.2 ± 1.2	3.5 ± 1.9
Mean VF duration (min)	19.3 ± 4.1	22.4 ± 7.9	19.37 ± 5.4	18.6 ± 4.8	16.0 ± 7.3

Time to first arrhythmia - time to onset of either ventricular tachycardia (VT) or fibrillation (VF) during the prolonged *test* ischemia, or during reperfusion following the *test* ischemia. Values are means ± S.E. $^{*}P < 0.05$ versus non-preconditioned hearts.

I. Effects of PKC Inhibition on Contractile Function

All hearts preconditioned in the absence or presence of chelerythrine displayed a similar reduction of LVDP during the first 5 minutes of the *test* ischemia (Figure 16, top panel). This was not the case for EDP (Figure 16, lower panel). When the PKC inhibitor was present throughout the experiment (protocol III), EDP was significantly lower than that of 1 preconditioning cycle hearts. At the end of the *test* ischemia, all of the groups exhibited a significant increase in EDP, increasing by 14.9 ± 1.2 mm Hg, 9.7 ± 3.1 mm Hg, 12.1 ± 2.2 mm Hg, 16.8 ± 2.8 mm Hg, 29.0 ± 2.4 mm Hg in non-preconditioned hearts, hearts exposed to 1 preconditioning cycle, or hearts exposed to each of the three chelerythrine treatment protocols, respectively ($P< 0.05$). Interestingly, hearts with chelerythrine present throughout the protocol (protocol III) displayed the largest increase in EDP over the duration of the *test* ischemia (from 2.3 ± 0.7 mm Hg to 31.3 ± 6.2 mm Hg; ($P < 0.05$) even though this group exhibited the lowest EDP at the start of the *test* ischemia.

Upon reperfusion, chelerythrine treatment delayed the recovery of LVDP (Figure 16, top panel). Pretreatment with chelerythrine during the three different stages of the protocol (protocol I, II, III) produced a similar depression of LVDP during the *test* reperfusion compared to preconditioned and non-preconditioned hearts. However, LVDP of hearts treated 5 minutes prior to preconditioning (protocol II) and hearts treated 10 minutes prior to the *test* ischemia (protocol III) was significantly depressed for the first 30 minutes of reperfusion compared to that of hearts exposed to 1 preconditioning cycle.

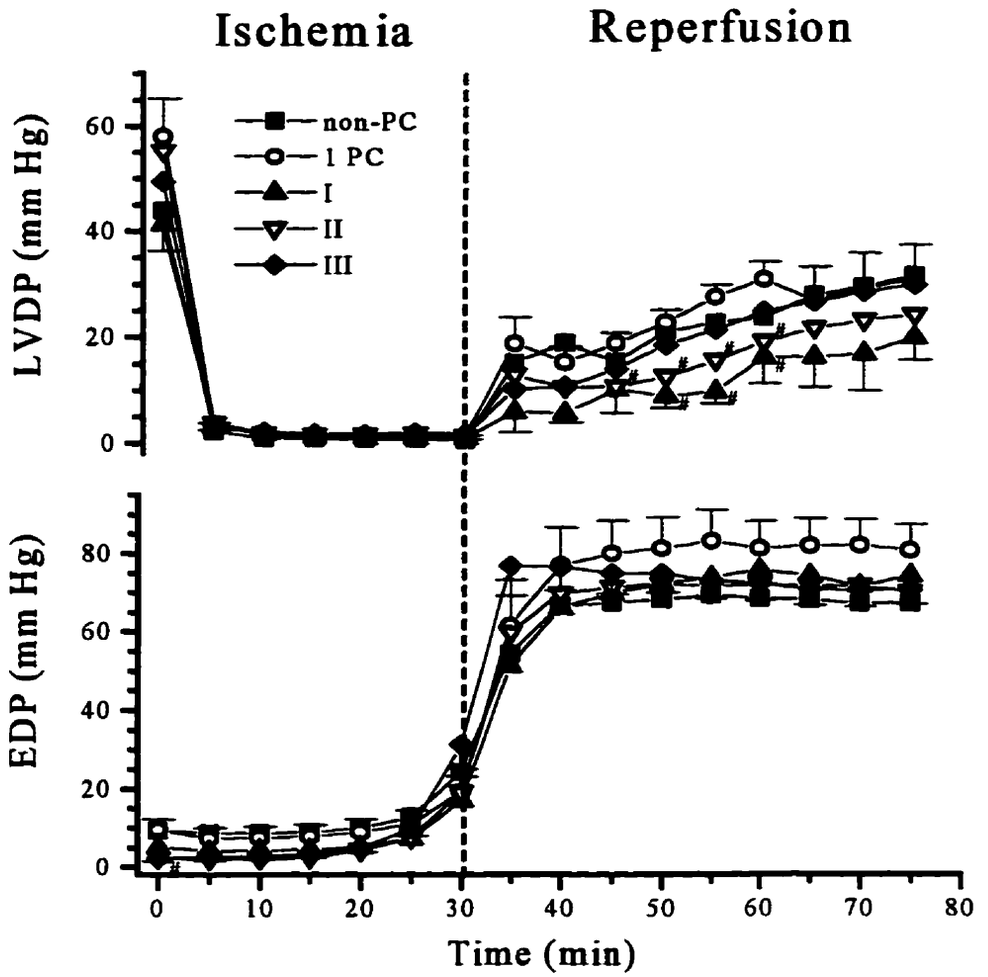

Figure 16. Effects of PKC inhibition at different stages during the preconditioning protocol on changes in left ventricular developed pressure (LVDP) and end diastolic pressure (EDP). Effects are shown for the subsequent *test* ischemia/reperfusion period. Changes in LVDP (upper panel) and EDP (lower panel) are shown for non-preconditioned hearts, 1 preconditioning cycle and pretreatment with 2 μM chelerythrine only during the preconditioning ischemia (I), only during the *test* ischemia/reperfusion (II), or the entire duration of the experiment (III). Data are means ± SE for 9-10 hearts in each group. Significance was determined using a student's *t* test (2-tailed).
$^{\#}$ $P< 0.05$ versus 1 preconditioning cycle hearts.

None of the protocols in the absence or presence of chelerythrine protected against the elevation in EDP that occurred during reperfusion (postischemic contractile recovery).

J. Effects of PKC Inhibition on $MAPD_{90}$ During Prolonged Ischemia

The effects of chelerythrine on $MAPD_{90}$ in the epicardium and endocardium are shown in Figure 17. Chelerythrine treatment throughout the protocol (protocol III) resulted in significant prolongation (184 ± 7 msec) of $MAPD_{90}$ in epicardium at the end of the long intervening reperfusion compared to both non-preconditioned (164 ± 4 ms) or hearts exposed to 1 preconditioning cycle (163 ± 3 ms). Chelerythrine treatment did not significantly prolong $MAPD_{90}$ in endocardium by the end of the intervening reperfusion (lower panel). In epicardium, treatment with chelerythrine during the single preconditioning ischemia only, the *test* ischemia only, or throughout the entire protocol reduced the degree of $MAPD_{90}$ shortening in epicardium during the first 25 minutes of ischemia.

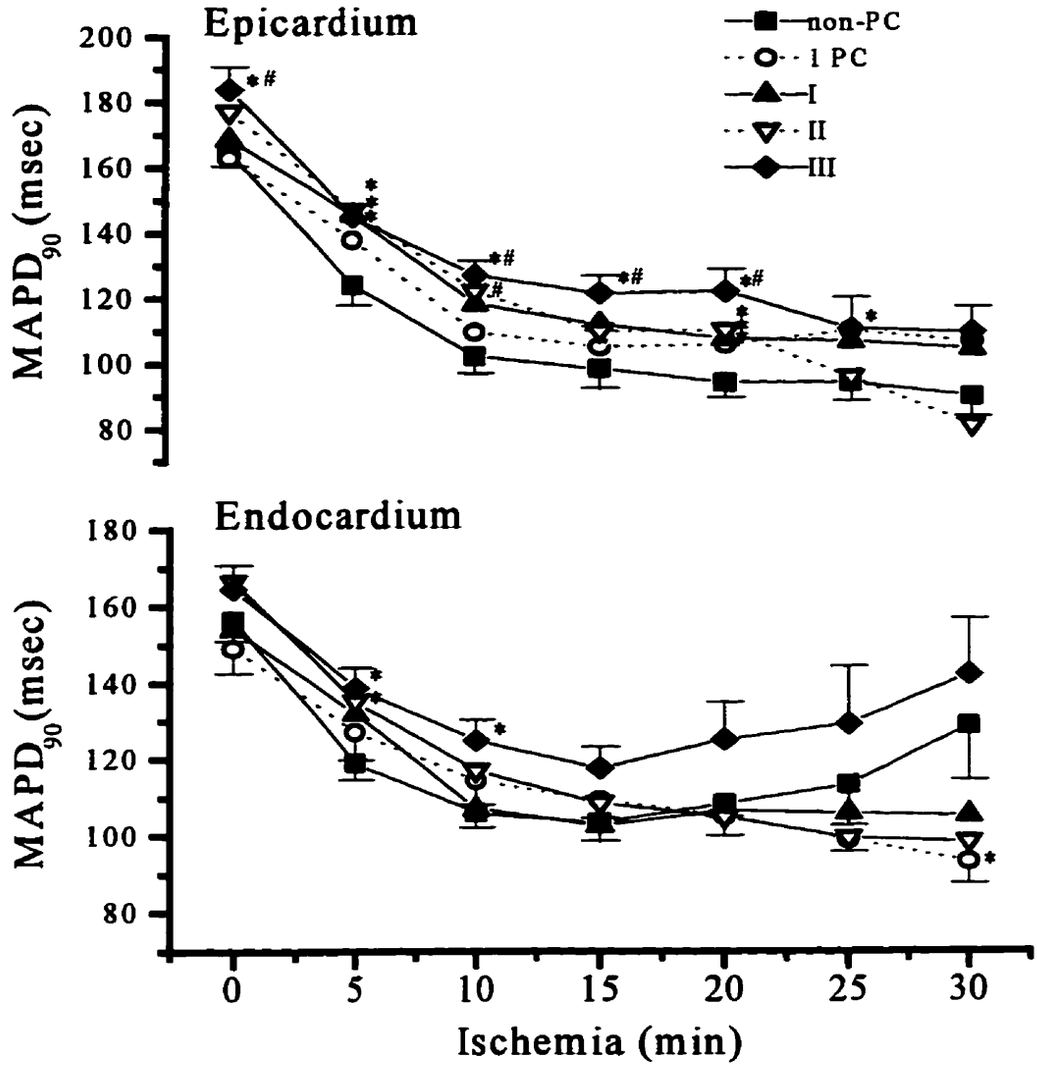

Figure 17. Electrophysiological changes occurring in epicardium and endocardium during the *test* ischemia in hearts subjected to various chelerythrine protocols. The protocols are described in the legend of Figure 15. Changes in monophasic action potential duration at 90% repolarization ($MAPD_{90}$) are shown for epicardium (upper panel) and endocardium (lower panel). Data are means ± SE for 9-10 hearts in each group. * $P < 0.05$ versus 1 preconditioning cycle hearts. # $P < 0.05$ versus non-preconditioned hearts.

VI. DISCUSSION

A. Ischemic Preconditioning and Arrhythmogenesis

Experimentally, ischemic preconditioning is the most powerful tool discovered to date to protect the heart against myocardial necrosis during ischemia. Increasing evidence indicates that this intervention may be very potent in protecting the heart against arrhythmias and contractile dysfunction caused by sustained ischemia and reperfusion. In rats, preconditioning decreases the incidences of both ischemia-induced (Li et al., 1992; Vegh et al., 1992; Lawson et al., 1993) and reperfusion-induced VT and VF (Shiki and Hearse, 1987; Osada et al., 1991; Hagar et al., 1991; Tosaki et al., 1994). The antiarrhythmic effect of preconditioning is controversial in other species. Vegh et al. (1992) found that ischemic preconditioning decreased the incidence of ischemia- and reperfusion-induced arrhythmias in dogs, whereas other studies report an increase in arrhythmias with preconditioning (Reimer et al., 1990). Studies of the effects of ischemic preconditioning on arrhythmogenesis are limited in xanthine-oxidase deficient species. In the pig, ischemic preconditioning increases ischemia-induced VF and actually decreases the time to onset of VF (Ovize et al., 1995). In conscious rabbits, one preconditioning period decreased the incidence of tachyarrhythmias during a subsequent sustained ischemic episode (Cohen et al., 1994). Thus, it remains unclear whether ischemic preconditioning actually confers protection against lethal arrhythmias in xanthine oxidase-deficient hearts and ultimately in humans.

The present study systematically evaluated the effect of multiple preconditioning periods on arrhythmogenesis in rabbit, a xanthine-oxidase deficient species (Muxfeldt and Schaper, 1987; Downey et al., 1987). In hearts subjected to 1 preconditioning cycle, the incidence of VF during ischemia dropped from 42% in non-preconditioned hearts to 0% ($P<0.05$). This dramatic reduction in ischemia-induced VF is similar to the level of protection reported by Yang et al. (1996). These authors demonstrated a 43% decrease in the incidence of VF during prolonged regional ischemia in a conscious rabbit model. In that study, however, hearts were preconditioned with 4 periods (5 min) of regional ischemia whereas hearts in our study received 1 period (5 min) of global ischemia. The apparent difference in the stimuli required to achieve preconditioning in these two models could reflect an inherent difference in the threshold of activation for preconditioning (Cohen and Downey, 1996). Perhaps 1 period of global ischemia is equivalent to 4 periods of regional ischemia with respect to protection against ischemia-induced VF.

In the present study, two cycles of preconditioning provided moderate protection against VF, whereas three and four cycles actually increased the incidence of VF. Our findings in rabbits differ from those in rats, where protection against arrhythmias is proportional to the number of preconditioning cycles (Lawson et al., 1993). In dogs, Vegh et al. (1992) reported that one or two preconditioning periods were equally effective in preventing VF, although they tested only a maximum of two cycles. However, more animals survived following 2 preconditioning cycles (40%) than 1 cycle (17%) in that study. The present study showed that 2, 3 and 4 preconditioning cycles resulted in an increased incidence of arrhythmias compared to hearts exposed to 1 preconditioning cycle.

Unfortunately, limited data are available for the effects of varying numbers of preconditioning cycles on arrhythmogenesis since most studies test only a fixed number of preconditioning cycles.

The protection afforded by preconditioning against arrhythmias also extended into reperfusion in the present study. One preconditioning cycle decreased the incidence of VF during reperfusion by 25%, whereas 2, 3 or 4 cycles increased the VF incidence by 8%, 16% and 25%, respectively. Although the decrease with one preconditioning cycle was not significant in the present study, significance may be reached with larger sample numbers. To our knowledge, this is the first report of a possible protective effect of preconditioning on reperfusion arrhythmias in rabbit hearts. However, the profile of protection induced by preconditioning in rabbits is different than that observed in other species. A single preconditioning cycle provided optimal protection against ischemia and reperfusion arrhythmias, while additional preconditioning cycles provide no protection or worsen arrhythmias in the rabbit. In contrast, three preconditioning cycles provide significantly greater protection than one cycle in the rat (Osada et al., 1991; Li et al., 1992), suggesting that additional cycles may lead to a cumulative increase in protection in this species. The basis for the differing profiles of protection seen in rabbits versus rats remains unknown. However, the notable differences in myocardial xanthine-oxidase levels in these species may play a significant role in the protective effects of preconditioning against reperfusion arrhythmias (Muxfeldt and Schaper, 1987; Downey et al., 1987).

B. Ischemic Preconditioning and Contractile Function

The *test* ischemia produced a similar degree of depression of LVDP in all groups of hearts tested. In contrast, the changes in EDP differed among the various groups of hearts. Non-preconditioned hearts exhibited a progressive and significant rise in EDP ('ischemic contracture') during the 30 minutes *test* ischemia (Figure 4). In contrast, hearts subjected to 2 or 3 preconditioning cycles exhibited significant protection against the ischemic contracture, whereas 1 or 4 preconditioning cycles had the least effect on the development of contracture. These results suggest the presence of a steep 'bell shaped' relation for the effects of preconditioning on EDP. One and 4 preconditioning cycles provide no protection against the development of ischemic contracture, whereas 2 and 3 preconditioning cycles provide maximal protection.

We also tested the effects of preconditioning on postischemic contractile recovery during the reperfusion period following the *test* ischemia. LVDP recovered gradually during the 45 minute reperfusion period in non-preconditioned hearts. Suprisingly, none of the preconditioning protocols significantly altered either the rate or extent of recovery of LVDP during reperfusion. In fact, non-preconditioned hearts exhibited the greatest recovery of LVDP among the groups, recovering to 72% of the pre-*test* LVDP. These results are in agreement with Lasley and Mentzer, Jr. (1995b) who found improved postischemic LVDP in rats but not in rabbit hearts exposed to one 5 minute preconditioning ischemia. Similarly, Sandhu et al. (1993) and Quantz et al. (1994) reported no protection against contractile dysfunction in rabbit hearts preconditioned with 1 period of global ischemia. In contrast, our results disagree with those of Omar et al.

(1991) who reported significant improvement of postischemic LVDP after one preconditioning period in isolated rabbit hearts. However, the present study used paced hearts whereas hearts were not paced in the study by Omar et al. (1991). In species such as rat (Hendrikx et al., 1993b; Cave et al., 1993; Kolocassides et al., 1996; Lasley et al., 1993) and swine (Kimura et al., 1992), improved postischemic contractile recovery is often observed following preconditioning. In dog, however, this improvement is not observed subsequent to preconditioning (Ovize et al., 1992).

None of the preconditioning protocols improved or worsened the rise in EDP that occurred during reperfusion in non-preconditioned hearts. Our results are in accord with a preliminary report that also found no effect of 3 preconditioning cycles on postischemic recovery of LVDP or EDP in isolated rabbit hearts (Asimakis et al., 1996). These results suggest that multiple periods of ischemic preconditioning cannot improve postischemic contractile function in the rabbit heart, which is not the case in species like rat (Perchenet and Kreher, 1995; Kolocassides et al., 1996).

A controversial issue in preconditioning research centers on whether myocardial stunning is a necessary prerequisite for preconditioning-induced protection to occur. In our study, we saw no evidence of myocardial stunning following the 5 minutes ischemic periods used to precondition the hearts (Figure 6). In all protocols, LVDP recovered completely during the 10 minute reperfusion periods that separated the preconditioning ischemia. Also, all preconditioned and non-preconditioned hearts attained identical values of LVDP prior to the prolonged *test* ischemia. Omar et al. (1991) also found no evidence of myocardial stunning in rabbit hearts following a single 5 minute preconditioning

ischemia. Further, Murry et al. (1991) dissociated the phenomena of stunning and preconditioning by showing that the effects of preconditioning disappear well before those of stunning in dog. As well, Miura et al. (1991) demonstrated that preconditioning protocols that produce different degrees of stunning provide a similar degree of protection against infarction. Thus, data from our study and others indicates that stunning is not a prerequisite for preconditioning-induced cardioprotection to occur in the rabbit.

It is noteworthy that the preconditioning protocols that prevented the ischemic contracture (2 or 3 preconditioning cycles) in our study provided no protection against arrhythmias. In fact, 3 preconditioning cycles actually increased the incidence of VT and VF. Similarly, the preconditioning protocol that provided the greatest protection against ischemia and reperfusion arrhythmias (1 preconditioning cycle) provided the least protection against the ischemic contracture and caused the largest rise in EDP during reperfusion. Thus, the "dose" of preconditioning needed to achieve protection is dependent on the endpoint studied (i.e., arrhythmias or contractile recovery). Fortunately in rabbits, the optimal "dose" of preconditioning that prevents arrhythmias is also a "dose" that reduces myocardial infarct size (Miura et al., 1991; Cohen et al., 1991).

C. Discordant Effects of Preconditioning on Contractile versus Electrical Parameters

Hearts subjected to multiple preconditioning cycles exhibited an initial prolongation followed by a shortening of $MAPD_{90}$ during the preconditioning ischemia. With each successive preconditioning cycle, the initial increase in $MAPD_{90}$ was greater and the extent of $MAPD_{90}$ shortening was less during the final phase. In contrast, the degree of LVDP depression during the ischemic phases of the preconditioning cycles remained the same for hearts exposed to 1-4 preconditioning cycles. Moreover, repeated preconditioning cycles produced a progressively greater recovery of LVDP during each reperfusion to above pre-ischemic values, whereas the $MAPD_{90}$ changes of both the epicardium and endocardium returned only to pre-ischemic values. The altered electrical response observed during the ischemic phase of the preconditioning cycles could be detrimental to the heart. Shortening of action potential duration (APD) during ischemia may protect the heart by reducing Ca^{2+} entry during the action potential (Cole et al., 1991), thereby decreasing ATP utilization (Cole, 1993). Any loss of the APD shortening response during repeated preconditioning cycles would therefore tend to increase $[Ca^{2+}]_i$. This increase in $[Ca^{2+}]_i$ may differentially affect the activation of *classical* (Ca^{2+}-dependent) isoforms of PKC (see below). Activation of PKC by phorbol esters has been implicated in arrhythmogenesis (Black et al., 1993), however, whether Ca^{2+}-dependent isoforms are involved in arrhythmogenesis is unknown. This finding could explain the higher prevalence of arrhythmias during the subsequent prolonged ischemia/reperfusion in hearts preconditioned with 3 or 4 preconditioning cycles in the present study.

Recently, Perchenet and Kreher (1995) also reported biphasic changes in APD during the preconditioning ischemia in isolated rat hearts. The authors measured action potentials using microelectrodes and observed an initial prolongation followed by a shortening of APD during repeated preconditioning cycles. These authors did not see a reduction in the degree of ischemia-induced APD shortening with successive cycles, although they tested only two preconditioning periods.

D. Electrophysiologic Responses of Epicardium and Endocardium to Preconditioning

Significant heterogeneity exists across the ventricular wall with respect to sensitivity to ischemia (Lukas and Antzelevitch, 1993; Kimura et al., 1986; Furukawa et al., 1991). Electrical activity in epicardium is much more sensitive than endocardium to depression during ischemia in most species (Lukas and Antzelevitch, 1993; Gilmour, Jr. and Zipes, 1980; Kimura et al., 1990; Kimura et al., 1986). Whether preconditioning occurs across the entire ventricular wall or only in specific layers is not known at present. Koning et al. (1995) showed that preconditioning the swine heart using partial coronary artery occlusion preferentially limited infarct area in the *epicardium*. This study was carried out *in situ* and did not account for the preferential shunting of blood flow to epicardium during ischemia. Therefore, these results may reflect the shunting of blood flow and not intrinsic differences in the sensitivity of the cells to ischemia. No study to date has tested the effects of preconditioning on electrophysiologic activity in specific ventricular layers during the preconditioning or prolonged ischemia. To address this concern, the present

study examined the effects of repeated preconditioning cycles on monophasic action potentials in the epicardial and endocardial layers of the isolated rabbit ventricle. In our study, the major difference in the responses of epicardium and endocardium in non-preconditioned hearts were limited to the last 15 minutes of the *test* ischemia. Significant shortening of $MAPD_{90}$ occurred in both epicardium (74.3 ± 5.0 ms, $P< 0.05$) and endocardium (52.8 ± 9.7 ms, $P< 0.05$) during the first 15 minutes of ischemia. There were no significant differences between the epicardial and endocardial responses, although a trend towards greater shortening was seen in epicardium. Unlike the monotonic shortening of $MAPD_{90}$ in epicardium, endocardium demonstrated biphasic changes in $MAPD_{90}$ during the *test* ischemia. This consisted of an initial shortening in $MAPD_{90}$ followed by a lengthening to 128.8 ± 14.2 ms. This corresponded to an overall shortening of only 27.43 ± 9.7 ms from pre-ischemic $MAPD_{90}$ for endocardium as compared to 74.3 ± 5.4 ms for epicardium ($P< 0.05$). These findings are similar to those observed in feline ventricular cells by (Kimura et al., 1986). These investigators showed that the magnitude of the reduction of action potential duration was greater in epicardial cells than in endocardial cells during ischemia. As well, APD of endocardial cells decreased progressively during 30 minutes of ischemia, whereas APD of epicardial cells was reduced maximally at 10 minutes and then partially recovered.

Preconditioned hearts exhibit less shortening of $MAPD_{90}$ in both epicardium and endocardium during early ischemia. In epicardium, this difference was statistically significant in hearts preconditioned with 2 or 3 cycles versus non-preconditioned hearts

during the first 10 minutes of the *test* ischemia. Similarly, the endocardial responses exhibited less shortening in hearts preconditioned with 2, 3 or 4 cycles at 5 minutes of ischemia and this trend was still present at 10 minutes of ischemia. By 15 minutes of ischemia, however, there were no differences in the epicardial and endocardial responses between preconditioned and non-preconditioned hearts. During the final 15 minutes of ischemia, the degree of $MAPD_{90}$ shortening in epicardium was similar in non-preconditioned hearts and those preconditioned with 1-4 cycles. In contrast, the endocardial response was much more variable in preconditioned hearts during the later stage of ischemia. $MAPD_{90}$ in endocardium of hearts exposed to 1 preconditioning cycle did not exhibit the biphasic changes that occurred in non-preconditioned hearts, but continued to shorten throughout the *test* ischemia to 93.3 ± 5.6 ms (versus 128.8 ± 14.2 ms for non-preconditioned hearts, $P< 0.05$). Thus, hearts preconditioned with 1 cycle exhibited the least dispersion in $MAPD_{90}$ between epicardium and endocardium. This finding is noteworthy since 1 preconditioning cycle also conferred complete protection against ischemia-induced arrhythmogenesis. However, further studies are required to establish a causal relationship between these two parameters.

The limited number of mapping sites used in our model make it difficult to precisely identify the mechanism(s) responsible for VF and VT during ischemia. Di Diego and Antzelevitch (1993) have suggested that ischemia results in marked dispersion of repolarization and refractoriness between epicardium and endocardium leading to the development of extrasystolic activity via a reentrant mechanism in dog. Kimura et al.

(1986) suggested that dispersion of repolarization and development of postrepolarization refractoriness between endocardial and epicardial cells may be related to the development of arrhythmias during early ischemia in the cat ventricle. Whether these mechanisms apply to rabbit hearts cannot be determined from our results. However, preconditioning does appear to affect the dispersion of repolarization seen between epicardium and endocardium. Arrhythmias in our isolated rabbit heart model typically occurred at ~19-24 minutes of ischemia (Table 3). At this time, hearts preconditioned with 1 cycle exhibited the least dispersion of $MAPD_{90}$ between epicardium and endocardium and the greatest protection against arrhythmias. We cannot state with certainty that the MAPD changes observed during late ischemia with 2-4 preconditioning cycles are typical, however, due to the small number of hearts that did not exhibit VT or VF in these groups.

E. Role of PKC in Preconditioning Against Arrhythmogenesis

Since the initial report of ischemic preconditioning in the heart in 1986 (Murry et al., 1986), intense investigation has been carried out to determine the mechanism underlying preconditioning. In 1994, Ytrehus et al. (1994) hypothesized that endogenous ligands such as adenosine initiate an intracellular pathway in which PKC plays a central role. Once activated, PKC phosphorylates a secondary effector protein which in some way induces protection. Shortly after this, Liu et al. (1994) proposed the translocation theory of PKC activation in preconditioning against infarction. In the present study, we tested this translocation theory using the PKC inhibitor chelerythrine and incidence of

arrhythmias as an endpoint. As well, our model allowed us to monitor contractile function during the test ischemia reperfusion to determine the role of PKC in contractile function during preconditioning.

Increasing concentrations of chelerythrine administered only during the preconditioning ischemia abolished the protective effects on preconditioning on both ischemia-induced VT and VF. This finding is similar to that of Tosaki et al. (1996) who showed that the selective PKC inhibitor calphostin C equally blocked the effects of preconditioning on VT and VF in rats, causing a 50% increase in the incidence of both arrhythmias. The protection against ischemia induced VF was not only blocked, but hearts treated with chelerythrine during the preconditioning ischemia exhibited a higher incidence of VF (67%) than non-preconditioned hearts (42%). These results agree with those of Baxter et al. (1995) who found that chelerythrine administered just before the preconditioning protocol abolished the delayed protection against infarction 24 hours later in rabbits. As well, Armstrong et al. (1996) showed that the protection of preconditioning induced by a protein phophatase inhibitor (calyculin A) in rabbit cardiomyocytes was abolished in the presence of calphostin C. In contrast, Liu et al. (1994) found that inhibition of protein kinase C activity with staurosporine during the preconditioning episode did not block protection against infarction in five of eight rabbit hearts. Similar findings by Przyklenk et al. (1995) in dog heart showed no effect of PKC inhibition on preconditioning against infarct size reduction. Our findings suggest that PKC activity is necessary during the preconditioning ischemia for protection against ischemia-induced VF

to occur in the rabbit heart. In this respect, our results do not support the translocation hypothesis for PKC in ischemic preconditioning.

Inhibition of PKC activity with chelerythrine during the *test* ischemia also abolished the protective effect against ischemia-induced VF seen with 1 preconditioning cycle (40% versus 0% for 1 preconditioning cycle hearts, $P<0.05$). Moreover, chelerythrine not only blocked the effects of preconditioning against ischemia VT and reperfusion VF, but actually exacerbated the incidence of these arrhythmias. These findings are consistent with the translocation theory. Ytrehus et al. (1994) also found that pretreatment with staurosporine 5 minutes prior to the 30 minute ischemic insult abolished the protective effect of preconditioning on infarct size in the rabbit heart. As well, studies in the rat (Speechly-Dick et al., 1994) and human atrium (Speechly-Dick et al., 1995) found similar blocking of protection seen with preconditioning by coronary occlusion and simulated ischemia against infarct size and contractile dysfunction, respectively. In contrast, studies in dog (Przyklenk et al., 1995) and rabbit cardiomyocytes (Armstrong et al., 1996) have shown that pretreatment with a PKC inhibitor just prior to the prolonged ischemia has no effect on preconditioning afforded by ischemic preconditioning or protein phosphatase inhibition, respectively.

Interestingly, PKC inhibition during the preconditioning phase, the intervening reperfusion, as well as during the *test* ischemia/reperfusion (protocol III), did not abolish the protective effect of 1 preconditioning cycle against VT or VF during the *test* ischemia/reperfusion. Similarly, Przyklenk et al. (1995) showed that PKC inhibition during the preconditioning phase and *test* ischemia/reperfusion also had no effect on

protection against infarct size achieved with ischemic preconditioning in dog. These results suggest that PKC activity during the intervening reperfusion may be important in the preconditioning process. Possibly, certain isoforms of PKC exert detrimental or beneficial effects during preconditioning. Thus, the predominant effect of chelerythrine would depend on which isoforms are blocked by the concentration of chelerythrine.

F. Role of PKC in Preconditioning and Contractile Function

Chelerythrine treatment during the preconditioning ischemia (protocol I) or *test* ischemia (protocol II), resulted in a delayed rate of recovery of LVDP during reperfusion. However, there was no difference in the final recovery of LVDP between groups in our study. In contrast, Speechly-Dick et al. (1995) found that PKC inhibition during the *test* ischemia blocked the increased recovery of contractile function seen with preconditioning in human right atrial trabeculae. As well, Mitchell et al. (1995) showed that PKC inhibition abolished the improved recovery obtained with preconditioning on postischemic contractile dysfunction in the rat heart. However, these studies demonstrated a significant difference in recovery of contractile function with preconditioning (in the absence of PKC inhibition) which we did not see in the rabbit heart.

Sustained PKC inhibition throughout the protocol (protocol III) resulted in a significant decrease in EDP at the end of the intervening reperfusion. However, these hearts exhibited the greatest rise in EDP (ischemic contracture) during the subsequent *test* ischemia. Whether this is due to differences in the handling of intracellular Ca^{2+} during reperfusion and ischemia associated with PKC is not known.

G. Role of PKC in Preconditioning and MAPD Changes During Prolonged Ischemia

The electrophysiologic responses of epicardium and endocardium to ischemic preconditioning had not been assessed prior to the present study. In our study, we measured the effect of PKC inhibition on MAPD in two layers of the myocardium (Figure 17). Hearts treated with chelerythrine exhibited significantly longer $MAPD_{90}$ than control hearts regardless of when it was present during the protocol. When chelerythrine was present throughout the experiment (protocol III), epicardial $MAPD_{90}$ remained significantly longer than that of non-preconditioned and hearts exposed to 1 preconditioning cycle during the first 20 minutes of ischemia. Interestingly, this drug regime showed the least effect on preconditioning against arrhythmias during this time period, which is the critical time for the appearance of arrhythmias (Table 2). After this time, the $MAPD_{90}$ for hearts treated with chelerythrine throughout the experiment (protocol III) was not different from hearts exposed to 1 preconditioning cycle.

Treatment with chelerythrine throughout the experiment (protocol III) resulted in less abbreviation of $MAPD_{90}$ in endocardium compared to non-preconditioned hearts. The other chelerythrine inhibition protocols, however, showed no difference from non-preconditioned or 1 preconditioning cycle hearts during the first 15 minutes of ischemia. As well, only hearts that were exposed to chelerythrine throughout the experiment (protocol III) showed the biphasic changes in $MAPD_{90}$ that were characteristic of non-preconditioned hearts. The only significant differences between the endocardial $MAPD_{90}$ of hearts exposed to 1 preconditioning cycle and non-preconditioned or chelerythrine

treated hearts appears at 30 minutes of ischemia. At this time, hearts treated with chelerythrine throughout the experiment (protocol III) exhibited significantly longer MAPD in endocardium than preconditioned hearts. This is noteworthy since protection against ischemia-induced arrhythmias is identical between 1 preconditioning cycle and protocol III for VF (0%) and similar for VT (30%;1 preconditioning cycle and 40%; protocol III). Unlike the epicardium, however, the time at which this difference in $MAPD_{90}$ between 1 preconditioning cycle and protocol III hearts occurred was only prior to the *test* reperfusion.

These findings suggest that there is no causal relationship between PKC induced $MAPD_{90}$ changes and arrhythmogenesis.

VII. SUMMARY AND CONCLUSIONS

The phenomenon of preconditioning has been intensively studied in recent years in order to exploit this endogenous form of cardioprotection. This protection has many components that work in orchestration to attain a common goal. Subtle changes in any of these components are undoubtedly responsible for the many variations that are seen with preconditioning in the different species and against the different endpoints. The present study demonstrates that ischemic preconditioning is more effective at protecting against ischemia-induced versus reperfusion induced arhythmias. Ischemic preconditioning with 1 or 2 preconditioning cycles protect the heart against ischemia-induced VF, while 3 or 4 cycles potentiate this arrhythmia. Protection against reperfusion-induced VF is seen with 1 preconditioning cycle, however, to a lesser degree. This study also suggests that

preconditioning does not protect against contractile dysfunction during ischemia or reperfusion. The findings from the current study also suggest that preconditioning may affect the dispersion of repolarization seen between epicardium and endocardium. However, a causal relationship between the decreased dispersion seen with 1 preconditioning cycle and arrhythmogenesis remains to be established.

From the chelerythrine experiments, this study shows that PKC inhibition during either the preconditioning ischemia or *test* ischemia abolishes the protection against arrhythmias conferred by 1 preconditioning cycle. In contrast, PKC inhibition throughout the protocol did not alter the preconditioning response against arrhythmias. However, hearts exposed to chelerythrine throughout the protocol exhibited less shortening of MAPD than non-preconditioned hearts or hearts exposed to 1 cycle during the *test* ischemia. These results suggest that the effects of PKC inhibition on MAPD changes during ischemia do not correlate with effects on arrhythmogenesis.

The substantial suppression of arrhythmias seen with ischemic preconditioning suggests that exploitation of the mechanism of ischemic preconditioning may represent an important new direction in antiarrhythmic drug development.

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