Protection against ischaemia and reperfusion-induced ventricular arrhythmias resulting from brief periods of cardiac pacing

PhD thesis

Károly Kaszala, MD

Department of Pharmacology

Albert Szent-Györgyi Medical University

Szeged, Hungary

1996

Summary

The description of ischaemic preconditioning by *Murry et al* in 1986 has substantially altered the way in which we think about the pathophysiological processes of acute myocardial ischaemia. Thus, we know now that short periods of sublethal ischaemic stress, instead of cumulatively damaging the myocardium, protect the heart against the detrimental effects of a subsequent prolonged ischaemic insult. The principal protective effects of preconditioning include the reduction of myocardial infarct size and marked suppression of both the occlusion and reperfusion-induced ventricular arrhythmias.

The aims of the study summarised in this thesis were (*i*) to determine whether preconditioning can be induced by means other than short periods of coronary artery occlusion. We used cardiac pacing to induce preconditioning, and investigated whether protection against occlusion and reperfusion-induced ventricular arrhythmias occurs during a subsequent prolonged coronary artery occlusion in anaesthetised dogs. (*ii*) We assessed the time course of the protection afforded by cardiac pacing, i.e. we examined how long this form of protection lasts. (*iii*) We investigated what role the release of bradykinin and nitric oxide play in pacing-induced preconditioning and (*iv*) explored whether activation of protein kinase C is involved in pacing-induced preconditioning.

Mongrel dogs were anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively), thoracotomised, ventilated and the anterior descending branch of the left coronary artery was prepared for occlusion. Cardiac pacing was commenced via the right ventricle or the right atrium The hearts were paced for four 5 min periods with 5 min rest intervals and, at various time intervals (5 min, 15 min, 1h, 6h, 24h, 48h and 72h) following pacing, the anterior descending coronary artery was occluded for 25 min, and then rapidly reperfused.

To examine the role of bradykinin in this protection, icatibant, an antagonist of bradykinin at B_2 receptors was given in a dose of 0.3 mg kg^{-1} 10 min prior to or immediately after pacing. This was also followed by a 25 min occlusion-reperfusion period. In other dogs Nω-nitro-L-arginine methyl ester, an inhibitor of nitric oxide synthase was given, in an intravenous dose of 10 mg kg^{-1} 10 min before pacing, which was followed 5 min later by a 25 min period of occlusion and reperfusion of the anterior descending coronary artery. Some other dogs were paced, and 5 min or 24 h later myocardial samples were taken and biochemical analysis was performed to investigate whether protein kinase C_ε or G_i proteins play a role in the intracellular signalling process of preconditioning.

Our results indicate that four 5 min periods of right ventricular induce a marked protection against arrhythmias. Thus, ventricular pacing markedly reduces ventricular arrhythmias (such as ventricular premature beats, ventricular tachycardia and ventricular fibrillation) that occur during a subsequent prolonged (25 min) coronary artery occlusion. This protection ("early phase") is transient, disappears 15 min after pacing, and does not reappear 1h or 6h after pacing. The protection is reoccurs 24h following pacing (delayed or "second window of protection"); at this time similar reduction occurs in ventricular arrhythmias as seen 5 min following pacing. Protection against ventricular arrhythmias is largely lost by 48h or 72h.

Bradykinin is probably one of the most important endogenous triggering substances that is responsible for initiating preconditioning since blockade of bradykinin B_2 receptors before preconditioning completely prevented the protection.

We found some evidence that nitric oxide is also involved in mediating this form of protection.

An increased immunoreactivity in the membrane fraction of myocardial samples, which were taken either immediately after pacing or 24h later, indicate a role for both protein kinase C_ε and G_i proteins in the intracellular signalling pathways of preconditioning.

Contents

List of publications related to the subject of the thesis

Full papers

I. Kaszala K., Végh Á., Papp J. Gy., Parratt J. R. Time course of the protection against ischaemia and reperfusion induced ventricular arrhythmias resulting from brief periods of cardiac pacing. *J Mol Cell Cardiol* 1996; **28:** 2085-2095.

II. Wilson S., Song W., Kaszala K., Ravingerova T., Végh Á., Papp J. Gy., Pyne N. Delayed cardioprotection is associated with the subcellular relocalisation of ventricular protein kinase Cε, but not p42/44 MAPK. *Mol Cell Biochem* 1996; **160/161:** 225-230.

III. Parratt J. R., Végh Á., Kaszala K., Papp J. Gy. Protection by preconditioning and cardiac pacing against ventricular arrhythmias resulting from ischaemia and reperfusion. *Ann New York Acad Sci* 1996; 739: 98-108.

IV. Parratt J. R., Végh Á., Kaszala K., Papp J. Gy. Suppression of life-threatening ventricular arrhythmias by brief periods of ischaemia and cardiac pacing with particular reference to delayed myocardial protection. In: *Ischaemia: preconditioning and adaptation.* Eds.: Marber M. S. and Yellon D. M. Chap. 5; 85-113. BIOS Scientific Publishers Ltd, Oxford, 1996.

V. Kaszala K., Végh Á., Papp J. Gy., Parratt J. R. Modification by B_2 bradykinin receptor blockade with icatibant on the protection afforded by cardiac pacing against coronary artery occlusion-induced ventricular arrhythmias. *Eur J Pharmacol* (the revised form is submitted).

VI. Parratt J. R., Végh Á., Zeitlin I. J., Ahmad M., Oldroyd K., Kaszala K., Papp J. Gy. Bradykinin and endothelial-cardiac myocyte interactions in ischaemic preconditioning. *Am J Cardiol* (submitted).

Quotable abstracts

VII. Kaszala K., Végh Á., Parratt J. R., Papp J. Gy. Can preconditioning be induced by rapid cardiac pacing? *Cardiologia Hungarica* 1994; Suppl. I. 68.

VIII. Végh Á., Papp J. Gy., Szekeres L., Kaszala K., Parratt J. R. Antiarrhythmic effects of pacing-induced ischaemic preconditioning during the "second window of protection". *J Mol Cell Cardiol* 1994; **26:** 346.

IX. Végh Á., Papp J. Gy., Kaszala K., Parratt J. R. Cardiac pacing in anaesthetized dogs preconditions the heart against arrhythmias when ischaemia induced 24h later. *J Physiol Lond* 1994; 480P, 89P.

X. Kaszala K., Végh Á., Parratt, J. R., Papp J. Gy. Time dependent antiarrhythmic effect of pacing-induced preconditioning. *Cardiologia Hungarica* 1995; 24/Suppl. 1, 39.

XI. Kaszala K., Végh Á., Parratt J. R., Papp J. Gy. Time course of pacing-induced preconditioning in dogs. *J Mol Cell Cardiol* 1995; 27/6: A145.

XII. Végh Á., Kaszala K., Papp J. Gy., Parratt J. R. Time-dependent antiarrhythmic effects of preconditioning during the second window of protection. *Cardiologia Hungarica* 1995; 24 / Suppl. 1, 46.

XIII. Kaszala K., Végh Á., Papp J. Gy., György K., Parratt J. R. Local administration of potassium channel openers and ischaemia-induced ventricular arrhythmias. *Br J Pharmacol* 1995; 114: Proc. Suppl. 239P.

XIV. Végh Á., Kaszala K., Papp J. Gy., Parratt J. R. Bradykinin, a trigger for delayed pacing-induced preconditioning. *J Mol Cell Cardiol* 1995; 27/6: L54, A279.

XV. Végh Á., Papp J. Gy., Kaszala K., Parratt J. R. Delayed myocardial protection by pacing-induced preconditioning: a possible role for bradykinin. *Br J Pharmacol* 1995; Suppl. 1, 40.

XVI. Kaszala K., Végh Á., Papp J.Gy., Parratt J.R. The role of bradykinin in pacing-induced preconditioning. *J Mol Cell Cardiol* 1996; **28:** 91.

1. Introduction

Ischaemic heart disease and myocardial infarction are among the main causes of morbidity and mortality in the Western World and management of these diseases in the clinical practice has yet to be solved. In the majority of cases myocardial infarction results from an atherosclerotic narrowing, or complete occlusion, of a coronary artery, with serious consequences to the heart; necrosis of the myocardium and heart failure can occur as a result. Myocardial ischaemia is often accompanied by ventricular tachyarrhythmias, which frequently are the cause of death. This remains one of the most serious problems facing modern cardiology.

1.1. Ischaemia-reperfusion injury

Myocardial ischaemia occurs when the reduction of coronary flow is so severe that the supply of oxygen and nutrients to the myocardium is inadequate for the demand of the tissues. Ischaemia causes a substrate deficiency and this leads to both a depletion of high energy stores and a build-up of metabolic end-products, such as lactate, inorganic phosphates, H^+, potassium[1-4]. A fall in cytosolic ATP levels is associated with loss of membrane pump activity, development of rigor contracture[5] which is accompanied/followed by an increase in cytosolic calcium levels[6,7], and sodium loading of the cells. In addition, a release of numerous other biochemical factors occurs during ischaemia. These include catecholamines, acetylcholine, adenosine, arachidonic acid derivates, plasma kinins, histamine, angiotensin, nitric oxide, 5-hydroxytryptamine, opioids, atrial natriuretic peptide and oxygen free radicals[8-13]. These may cause further disturbances in ion channel and membrane function. In the absence of reperfusion, ischaemia inevitably leads to myocardial necrosis.

Although early (10-15 min) re-oxygenation/reperfusion of the myocardium is required to avoid the development of irreversible ischaemic damage, reperfusion can also injure the myocardium. Thus, arrhythmias, myocardial stunning, vascular damage, necrosis in cells that are non-lethally injured, and accelerated necrosis in cells already damaged by ischaemia can occur as a result of reperfusion[14,15]. One possible explanation is the washout of substances that accumulate during ischaemia, together with increased oxygen delivering, resulting in an

increased generation of oxygen free radicals. Further consequences include Ca^{2+} overload of the cells, leading to cell oedema, alterations in contractile performance, ultrastructural changes, arrhythmias and cell death.

1.2. Cardioprotection by brief periods of ischaemia; ischaemic preconditioning.

An interesting aspect of myocardial ischaemia was highlighted in 1986, when Murry and his colleagues[16] published their observation of an endogenous protective phenomenon of the heart. They had shown that multiple brief episodes of ischaemia, instead of cumulatively damaging the myocardium, paradoxically protected the heart from the consequences of a subsequent, sustained ischaemic insult. They occluded the left circumflex coronary artery four times for 5 min, each separated by 5 min of reperfusion and this was followed by a 40 min sustained occlusion of the same artery. The hearts were reperfused thereafter. Surprisingly, there was a 75% reduction of the infarct size compared to those dogs in which the circumflex coronary artery was occluded for 40 min without prior conditioning of the heart by short periods of ischaemia. They termed this protective phenomenon "ischaemic preconditioning".

Apart from limitation of myocardial infarct size[17,18], preconditioning can markedly reduce the incidence and severity of ventricular arrhythmias both during ischaemia[19,20] and reperfusion[19,21,22], decrease postischaemic contractile dysfunction[23] and enhance postischaemic functional recovery[24-26], blunt the loss in vasodilator reserve[27,28] and improve metabolic disturbances that are associated with ischaemia[25]. Intracellular levels of high energy phosphates are thus preservedand then lower lactate levels[23,29]. These effects of preconditioning seem not to be species dependent; this endogenous protective phenomenon exists in all species studied for example in dogs[16,19], rats[21,30], rabbits[31,32], pigs[18,33] and also in man[34,35].

This form of protection is not restricted to the heart; limitation of infact size may also occur in other organs such as the brain, lung, gastrointestinal tract, sceletal muscle and kidneys[36-38] etc.). when these organs are preconditioned by repeated occlusions of the supplying artery.

1.2.1. Time-dependency of preconditioning

The protective action of preconditioning is marked, but wanes quite rapidly. For example, in rats three cycles of 5 min preconditioning occlusions reduced infarct size during a 90 min occlusion period only if the interval after preconditioning was less then 1 hour[20]. Antiarrhythmic protection is also lost when the window between preconditioning and the prolonged occlusion is extended to 1 h[39].

Interestingly, it has been shown that several hours after preconditioning antiischaemic protection reappears. In this form of protection, the so-called delayed or "second window of protection", there is again a marked reduction in myocardial infarct size[40,41], in the incidence and severity of arrhythmias[42] and ST-segment elevation[43] 24h after the initial, triggering preconditioning ischaemic insult.

Our studies were limited to some aspects of the antiarrhythmic effects of preconditioning.

1.2.2. Antiarrhythmic effects of preconditioning

There were, in fact, several examples of this phenomenon before the preconditioning era. For example as early as in the 1950s Harris[44], who studied chronic heart failure, suggested a novel experimental model for chronic myocardial infarction because of the high incidence of ventricular arrhythmias following a sudden occlusion of a coronary artery. He found that ventricular arrhythmias and ventricular fibrillation was less likely to occur if a coronary artery was occluded in two stages, a partial occlusion with a great reduction of flow, followed 30 min later by complete coronary artery occlusion. Indeed, there is a reduction in the ventricular fibrillation threshold following short coronary artery occlusions[45]; further, the number of ventricular ectopic beats during repeated short (5 min) occlusions of the anterior descending branch of the left coronary artery are significantly reduced after the first occlusion period[46]. In rats reperfusion-induced ventricular arrhythmias are markedly reduced following a 5 min coronary artery occlusion when that occlusion is preceded by a 5 min occlusion-reperfusion insult[21]. Others have reported that preconditioning can markedly reduce the incidence of both occlusion[19,20] and reperfusion-induced arrhythmias[19,21,22,47]. In the canine, two 5 min occlusions of a coronary artery, with a 20 min reperfusion interval, reduces the

number of ventricular premature beats, (and tachycardiac episodes) and completely prevents ventricular fibrillation, during a subsequent prolonged occlusion and reperfusion period[39].

Apart from short complete coronary artery occlusions there are other ways of inducing preconditioning[48]. There has been increasing evidence that alterations in myocardial energy supply-demand balance could also induce preconditioning[49]. Previous data from our aboratory suggested that rapid cardiac pacing (two, 2 min episodes at a rate of 300 beats min^{-1}) can reduce arrhythmias in the same extent as seen with coronary artery occlusion-induced preconditioning[50]. It has also been shown that rapid pacing induces protection[51-53] against ischaemia.

1.2.3. Mechanisms that are involved in preconditioning

Although it is widely accepted that preconditioning as a phenomenon exists in several species, there are controversies about the mechanisms involved. In spite of the considerable data that have been collected, the precise mechanisms are still far from clear. Early suggestions that protection may be due to an increased collateral flow, or to the opening of preformed collateral vessels, were soon dismissed because (i) preconditioning occurs in species with relatively few preformed collaterals (e.g. rabbit, pig, rat), and (ii) there is no evidence that collateral blood flow is increased during a prolonged coronary artery occlusion when that is preceded by preconditioning[16,54,55]. Neither is "myocardial stunning" (contractile deficit occurring in the myocardium with spared energy utilisation following short coronary artery occlusions) a convincing explanation because there is a great difference in the time-course of the disappearance of stunning (which may take 1-2 days[56]) and preconditioning (where protection is lost in 1-2h[57]). Further, studies that examined the role of free radicals[58-61]; and the involvement of neutrophils[62-64] in the protection are also quite controversial.

Most investigators would probably agree that endogenous protective mediators are released from the myocytes, or endothelial cells, during ischaemia and act as a trigger for preconditioning. Some evidence suggest that adenosine[31,65], bradykinin[66-69], nitric oxide[47,70,71], prostacyclin[19,72], noradrenaline[73,74] and acetylcholine[75,76] are all possible candidates for inducing this form of cardioprotection. We know little as yet about the cellular pathways and effectors of the protection induced by these endogenous substances. Current theories include

increased guanylate cyclase activity[77] (with a resultant elevation in cyclic GMP levels[78]); and the involvement of G_i proteins[79,80], which are coupled directly to activation of acetylcholine and adenosine regulated potassium channels ($I_{KAch,\ Ado}$) [81]. Some evidence suggests that protein kinase C is activated following preconditioning[82-84]. This, in turn, phosphorylates and facilitates ATP-sensitive potassium channels[85].

1.3. Aims of the study

One of our aims was to assess whether cardiac pacing at a rate of 220 beats min^{-1} (which represents about a 40-50% increase in the resting heart rate in anaesthetized dogs), is sufficient to induce antiarrhythmic protection during a subsequent occlusion and reperfusion of the left anterior descending coronary artery. This method of inducing protection has several advantages over coronary artery occlusion. It produces less surgical stress and trauma to the animals, is safer in that ventricular fibrillation seldom if ever occurs during pacing, and has good reproducibility.

We evaluated the time course of the antiarrhythmic protection afforded by cardiac pacing in anaesthetized open-chest mongrel dogs and examined whether the late manifestation (second window) of antiarrhythmic protection could also be induced by repeated episodes of brief cardiac pacing.

Previous data suggested that bradykinin is an especially important substance in triggering coronary artery occlusion-induced preconditioning[67,86] and that the release of nitric oxide may be responsible for mediating the effects of bradykinin[87]. For this reason we determined what role bradykinin and nitric oxide play in the early phase of myocardial protection induced by cardiac pacing.

Another objective of our studies was to assess whether the G_i protein-protein kinase C pathway is activated following cardiac pacing.

2. Methods

2.1. Animals

Mongrel dogs (n=138) were used in these experiments, weighing in excess of 16 kg. Seven dogs were excluded from the experiments, either because the haemodynamic status was inappropriate at the start of the experiment, or because the area at risk, measured after the experiment, was less than 30%. The dogs were allowed access to food and water *ad libitum* until starting the experiment. All animals received humane treatment in compliance with both the *Guide for the care and use of laboratory animals* published by the US National Institutes of Health (NIH publication number No 85-23, revised 1985) and local institutional policy.

2.2. General preparation

Dogs were lightly anaesthetized by the intravenous administration of sodium-pentobarbitone, and anaesthesia was maintained by a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively). They were ventilated with room air using an Ugo Basile respirator at a rate of 10-15 srtokes min^{-1}. The stroke volume was adjusted to maintain pH and blood gases within normal limits (pH: 7.40±0.05, PaO_2: 85±1.5 mmHg, $PaCO_2$: 30±1.5 mmHg). Body temperature was monitored from the mid-oesophagus during the experiment and maintained at 37±0.5 ^oC by means of a heating pad.

Polyethylene catheters were introduced into the right femoral vein (for administration of drugs and anaesthetic), the right femoral artery (for measuring arterial blood pressure and taking blood samples), and the left ventricle, via the right carotid artery, for measuring ventricular pressure (LVP) and LV dP/dt. Blood pressure and ventricular pressure was measured by means of Isotec pressure transducers. All parameters, and the standard II limb lead electrocardiogram, were recorded on a Graphtec Thermal Arraycorder (Hugo Sachs, Germany).

2.3. Surgical preparation

Before starting surgical preparation, dogs were re-anaesthetized with a mixture of chloralose and urethane (60 and 200 mg kg^{-1}, respectively) given intravenously. Thoracotomy was performed at the fifth left intercostal space and the heart was suspended in a pericardiacl cradle. The anterior descending branch of the left coronary artery was dissected free about 2 cm distal from its origin, just above the first marginal branch, and a silk thread loosely placed around it. To evaluate the severity of myocardial ischaemia, a small rubber pad containing four unipolar electrodes was sutured to the myocardium supplied by the left anterior descending coronary artery. Changes in ST-segment elevation (mV) were continuously recorded from the same two selected epicardial sites and these were meaned for each dogs. The degree of inhomogeneity of conduction within the ischaemic zone was evaluated by means of a composite electrode[88,89]. This electrode was built into the same pad as that containing the unipolar electrodes used for monitoring local ST segment changes. A unipolar needle electrode was inserted into the subendocardial region of the left ventricle for measuring the subendocardial ECG. Dogs were than paced as described below and the effects of pacing on ventricular arrhythmias during a subsequent 25 min occlusion-reperfusion of the anterior descending branch of the left coronary artery was determined and compared to the same occlusion-reperfusion insult in control (unpaced) dogs.

2.4. Cardiac pacing

A Cordis F4 bipolar pacing electrode was introduced into the right ventricle via the right jugular vein for pacing, and also for measuring the endocardial ECG. The correct positioning of the electrode was confirmed from the endocardial ECG. One group of dogs was paced via the right atrium using the same experimental protocol. Pacing (double threshold, square impulses) was performed by means of an electrical stimulator designed in our department. Four 5 min periods of pacing at a rate of 220 beats min^{-1} were performed with 5 min "reperfusion" intervals.

2.5. Assessment of ventricular arrhythmias

Ventricular arrhythmias were assessed according to the Lambeth conventions[90]; thus the total number of ventricular premature beats, the number of episodes and the incidence of ventricular tachycardia (defined as a run of four or more ventricular premature beats) and the incidence of ventricular fibrillation occurring during occlusion were determined. The reperfusion period was followed for 15-20 min; only the incidence of reperfusion-induced ventricular fibrillation was assessed.

2.6. Measurement of area at risk

Because there is evidence that the area at risk can modify the severity of ventricular arrhythmias following coronary artery occlusion, after completing the experiments hearts were excised and patent blue V dye was injected into the occluded coronary artery just distal to the occlusion site. The area at risk was expressed as a percentage of the left ventricular wall together with the septum.

2.7. Design of the experiments

2.7.1. Design of studies to evaluate whether cardiac pacing is sufficient to induce preconditioning.

Dogs were subjected to cardiac pacing (four 5 min pacing periods at a rate of 220 beats min^{-1}, with 5 min reperfusion intervals) either via the right atrium (n=6) or the right ventricle (n=11) and 5 min later the LAD was occluded for 25 min; after this time the ischaemic region was reperfused (Figure 1).

2.7.2. Design of studies to evaluate the time course of cardiac pacing-induced preconditioning.

There were 9 groups of dogs with the time interval between cardiac pacing and coronary artery occlusion varying between 5 min and 72 hours (5 min, n=11; 1h, n=8; 6h, n=8; 24h, n=10; 48h, n=9; 72h, n=8). Controls (sham-control 1; n=14 and sham-control 2; n=8) were those dogs in which the pacing electrode was introduced for 40 min but which were not paced.

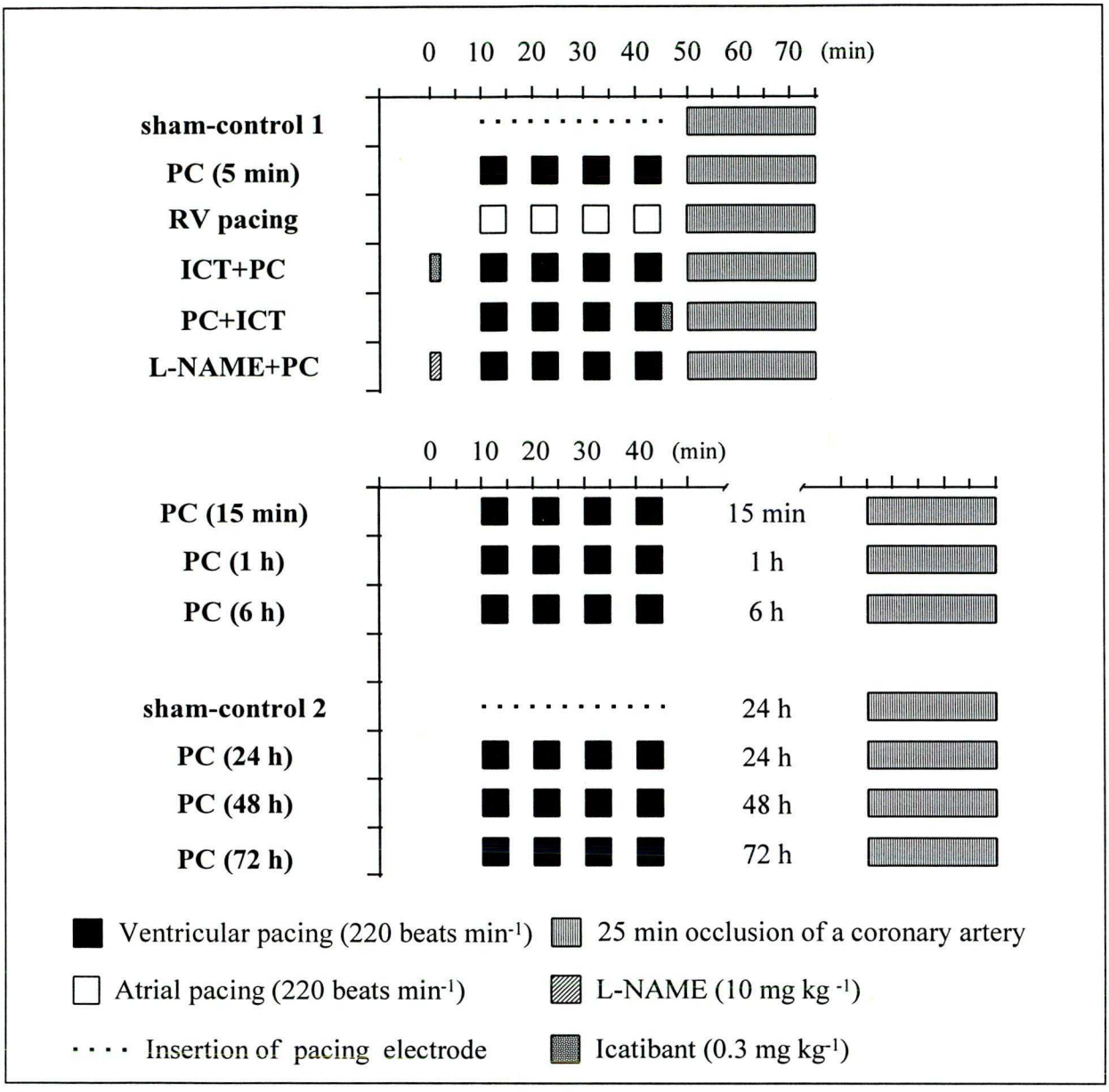

Figure 1 *Experimental protocol used to determine the time course of protection afforded by cardiac pacing and the involvement of bradykinin and nitric oxide in this form of protection. The heart was paced for four 5 min periods with 5 min rest periods between. 5 min (PC 5min, n= 11), 15 min (PC 15 min, n=8), 1 h (PC 1h, n=8), 6 h (PC 6h, n=8), 24 h (PC 24h, n=10), 48 h (PC 48h, n=9) and 72 h (PC 72h, n=8) after the last pacing period the LAD was occluded for 25 min, followed by reperfusion. The sham-control 1 (n=14) and sham-control 2 (n=8) were those dogs in which the pacing electrode was inserted for 40 min but were not paced and the LAD was occluded and reperfused 5 min or 24h later. The effects of blockade of bradykinin B_2 receptors [icatibant was given either 10 min before (ICT+PC, n=8) or immediately after pacing (PC+ICT, n=8)] or inhibition of nitric oxide synthase [Nω-nitro-L-arginine methyl ester was given 10 min prior to pacing (L-NAME, n=11)] on the protective effects of cardiac pacing was also determined.*

A 25 min occlusion of the coronary artery was performed 5 min (sham-control 1) or 24h (sham-control 2) thereafter. In all groups occlusion was followed by reperfusion (Figure 1).

2.6.3. Design of studies to evaluate the role of the release of endogenous substances (bradykinin and nitric oxide) in pacing-induced preconditioning.

In these studies preconditioning was induced by four 5 min episodes of right ventricular pacing and, 5 min after the last pacing period, the LAD was occluded for 25 min. To examine the role of the release of bradykinin in pacing-induced preconditioning, icatibant (HOE-140) an antagonist of bradykinin at B_2 receptors[91] was given as an intravenous bolus injection in a dose of 0.3 mg kg^{-1}, either 10 min before, or immediately after pacing. In another group of dogs an intravenous bolus injection of Nω-nitro-L-arginine methyl ester (L-NAME), an inhibitor of nitric oxide synthase[92] was given in a dose of 10 mg kg^{-1}, 10 min prior to pacing. The anterior descending coronary artery was then occluded for 25 min, 5 min after the last pacing period (Figure 1).

2.8. Biochemical studies to evaluate whether protein kinase C and G proteins are involved in pacing-induced preconditioning.

2.8.1. Preparation of the hearts

Dogs were lightly anaesthetized with sodium-pentobarbitone and subjected to cardiac pacing. Myocardial samples were taken immediately (n=4) or 24 h after pacing (n=4). Controls (n=4) were those dogs in which the pacing electrode was inserted for 40 min but which were not paced. Myocardial samples were then taken. Before taking these samples, the hearts were removed and, in order to remove as much blood as possible, the myocardium was quickly washed with ice-cold saline by retrograde perfusion from the aorta using a 500 ml syringe. Samples were then excised and frozen immediately in liquid nitrogen using Wollenberger clamps. The samples were stored in liquid nitrogen and transported to Glasgow in dry ice. Samples were homogenised as described below.

2.8.2. Preparation of low and high-speed particulate and supernatant fractions.

Ventricular tissue samples were weighed and homogenised in an ice-cold buffer A (0.25 M sucrose, 10mM-Tris/HCl, pH 7.4, 1mM-EDTA, 2mM-benzamidine and 0.1mM-phenylmethylsulfonyl fluoride), using a Turrex homogeniser. The subsequently obtained homogenate was filtered twice through gauze and centrifuged at 2000g for 20 min at 4°C in a Beckman Prep 65 ultracentrifuge. The particulate fraction was suspended in buffer A. This is the "low-speed nuclear pellet". The low-speed supernatant was recentrifuged at 50.000g for 20 min at 4°C. The high-speed supernatant fraction contains cytoplasm proteins. The pellet which is suspended in buffer A and contains a membrane fraction, is termed the "high-speed particulate fraction". The protein concentration of each fraction was assayed using the Bradford protein assay.

2.8.3. Detection of protein kinase C and G protein.

After equalising proteins in each sample, low-speed nuclear pellets, high-speed supernatant and high-speed particulate fractions were separately combined with sample buffer containing final concentrations of 0.125M-Tris/HCl, pH 6.7, 0.5mM-$Na_4P_2O_7$, 1.25mM-EDTA, 2.5% (v/v) glycerol, 25mM-DTT, 0.05% (w/v) bromophenol blue. Samples were boiled for 5 min and then subjected to SDS-PAGE. Resolved proteins were transferred to nitro-cellulose sheets. These were subsequently blocked in 3% (w/v) bovine serum albumin (BSA) in 20mM-Tris buffered saline, pH 6.8, containing 0.03% (v/v) Tween 20 (T-TBS). "Blocking" was performed at room temperature for 2.5 hours. After this time the nitro-cellulose sheets were probed with either anti-PKC_ε or anti-$G_{i\ \alpha\text{-}1,\ \alpha\text{-}2}$ antibodies in T-TBS containing 1% BSA (w/v). These were incubated at 37 °C for 12 hours. After this time, the nitro-cellulose sheets were washed six times in T-TBS. Detection of PKC_ε or G_i reactivity was achieved by incubating nitro-cellulose sheets for 2 hours at room temperature with either reporter horse radish peroxidase (HRP)-linked anti-mouse (PKC_ε) or anti-rabbit (G_i) antibodies; this was followed by washing the blots as described above to remove excess reporter antibody. Immunoreactive bands of PKC_ε were detected with an ECL detection kit. Nitrocelluloses that contained immunoreactive bands of G_i were incubated in 40 ml of 10mM Tris buffer (pH 7.4) and 1 ml of dianisidine solution (100mg/10ml) with additional hydrogen peroxide (3%, 10 ml) for 30

min. Horse radish peroxidase that bound to the reporter antibody breaks H_2O_2 and gives brown colorisation with dianisidine.

2.9. Chemicals

Icatibant was a gift from Hoechst AG., Germany, protein kinase C_ε antibody and $G_{i\ \alpha\text{-}1,\alpha\text{-}2}$ antibody was purchased from Affiniti Research Products Limited, Exeter, UK and Calbiochem-Novabiochem Corporation, San Diego, USA, respectively. All other chemicals were purchased from Sigma, St. Luis, USA.

2.10. Statistical methods

All results were expressed as means ± s.e.mean unless otherwise stated and differences between means were compared using a paired T-test (to compare results within the group), one-way analysis of variance with Bonferoni post hoc test (to compare results between groups). Differences in ventricular premature beats were assessed using the Kruskal-Wallis rank test, whereas incidence of arrhythmias were compared by using Chi-square test or Fisher test. Differences between groups were considered significant when $P<0.05$.

3. Results

3.1. Differences in the effects of cardiac pacing when performed via the right ventricle or the right atrium.

3.1.1. Effects of cardiac pacing on various haemodynamic parameters and on ventricular arrhythmias arising during and after the pacing periods.

During pacing at a rate of 220 beats min^{-1} (which represents an approximately 40% increase in the resting heart rate) there was a marked decrease in arterial blood pressure (about 38%), and in positive and negative LV dP/dt_{max}, whereas LV end-diastolic pressure (LVEDP) was increased significantly (Table 1).

There were pronounced elevations in both right ventricular endocardial ST-segment (measured from the pacing site, from 0.64±1.1 mV to 5.4±0.68 mV, Figure 2; this parameter

was only assessed in those dogs in which pacing was performed via the right ventricle) and subendocardial ST segment (measured from the left ventricle), either after ventricular (from 1.64±0.22 mV to 2.80±0.28 mV) or atrial pacing (from 1.58±0.45 mV to 2.75±0.44 mV).

Table 1 Haemodynamic changes induced by cardiac pacing (220 beats min^{-1})

	cardiac pacing via right ventricle			cardiac pacing via right atrium		
	baseline	**during pacing**	**after pacing**	**baseline**	**during pacing**	**after pacing**
arterial blood pressure (systolic; mmHg)	143±6	101±7†	149±7	131±9	93±9*	131±9
arterial blood pressure (diastolic; mmHg)	92±4	67±4†	92±5	91±7	63±8*	89±7
arterial blood pressure (mean; mmHg)	110±4	79±5†	116±5	107±8	75±8*	106±8
LV dP/dt_{max} (+ve; mmHg sec^{-1})	3059±185	2512±180*	3252±219	3656±354	2715±386*	3808±428
LV dP/dt_{max} (-ve; mmHg sec^{-1})	3199±185	2322±181†	3271±204	3590±301	1948±368*	3429±329
LV systolic pressure	130±5	90±6†	137±6	117±10	81±9*	114±11
LV end-diastolic pressure	4.3±0.67	12.1±2.1†	4.9±0.83	9.4±2.68	14.2±2.99*	11.3±1.09
heart rate (beats min^{-1})	159±4	220±2†	163±4	158±6	221±3†	159±7

*Values are expressed as means ± s.e.mean. *: P<0.05 vs. baseline; †: P<0.001 vs baseline.*

These modifications in haemodynamic parameters were short lasting and rapidly returned to resting values when the pacing was stopped. The haemodynamic changes occurring as a result of cardiac pacing did not differ significantly when pacing was performed via either the right atrium or the right ventricle (Table 1).

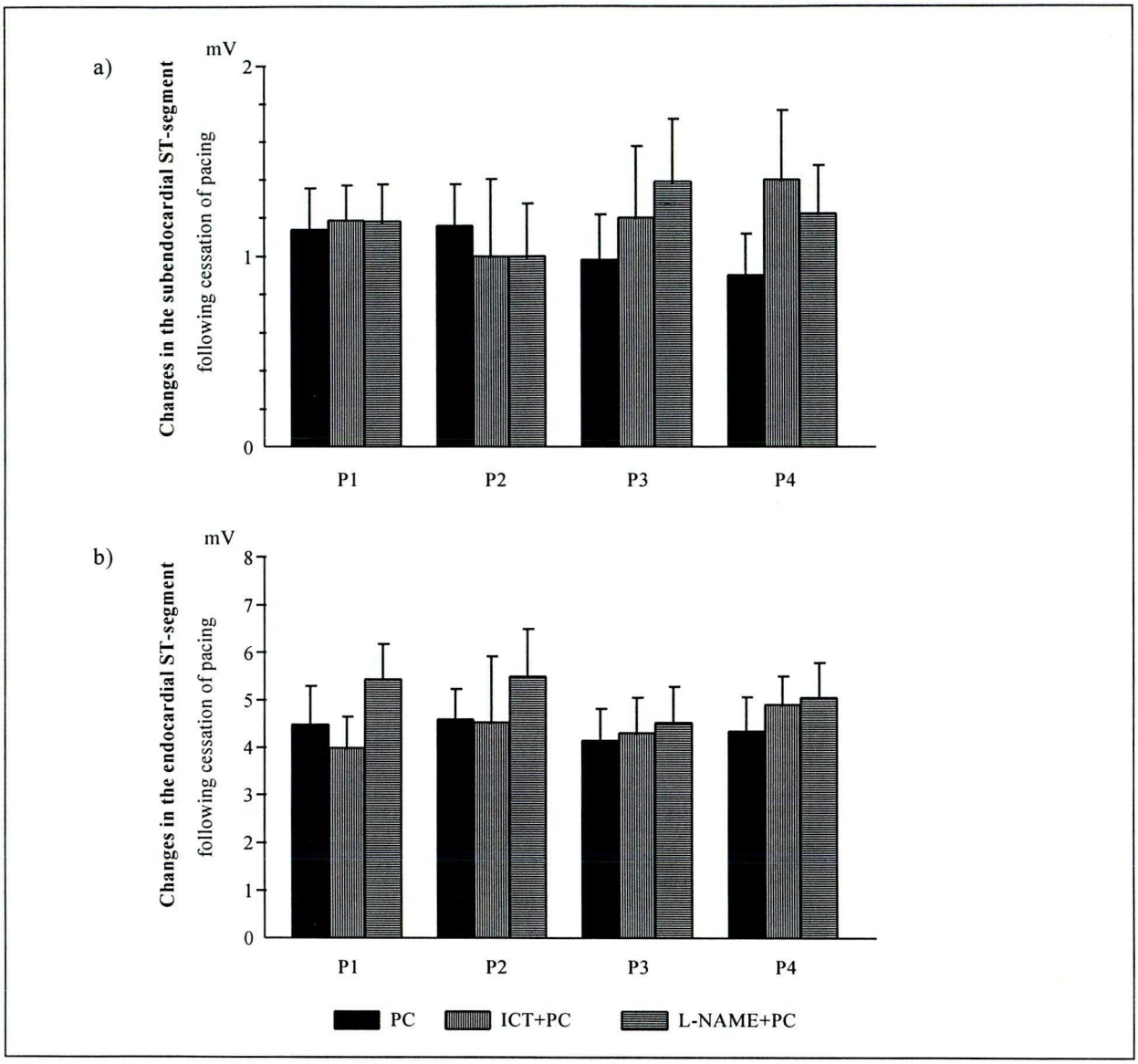

Figure 2 *Graph a and graph b shows the changes in subendocardial (recorded from the left ventricle, mV) and endocardial (recorded from the right ventricle, mV) ST-segment after stopping pacing. The filled columns represent ST-segment elevation in the untreated dogs after cessation of the four 5 min episodes (P1-P4) of cardiac pacing at a rate of 220 beats* min^{-1}*. The vertically and horizontally striped columns show the changes in the subendocardial and in the endocardial ST-segment elevation after cessation of pacing when it is performed in the presence of either icatibant (ICT+PC) or Nω-nitro-L-arginine methyl ester (L-NAME+PC), respectively.*

However, there was no change in epicardial ST-segment (measured from the surface of the left ventricle) following cardiac pacing. Endocardial, and especially left ventricular subendocardial, ST-segment elevation probably indicates that these short cycles of cardiac

pacing induce transient ischaemia in the endocardial-subendocardial region of the myocardium (Figure 2).

Only a few ventricular ectopic beats occured when the pacing was stopped (ventricular pacing: 18±0.5 vs. atrial pacing: 23±2; n.s. during the total four 5 min 'reperfusion' periods). There was no ventricular fibrillation either during or after pacing.

3.1.2. Haemodynamic and electrophysiological changes induced by a 25 min occlusion of a coronary artery; modifications by prior cardiac pacing.

Occlusion of a coronary artery resulted in haemodynamic alterations which are summarised in Table 2. There was a reduction in arterial blood pressure, left ventricular systolic pressure and both positive and negative LV dP/dt_{max}, whereas LVEDP increased significantly. Similar changes occured during occlusion when this was preceded, 5 min earlier, by atrial pacing, by ventricular pacing and when ventricular pacing was performed at different time intervals (i.e. 5 min, 15 min, 1h, 6h, 24h, 48h, 72h) prior to occlusion. However, haemodynamic changes were somewhat less marked when the time window between pacing and occlusion was either 5 min or 24 h (Table 2).

Indices of the severity of ischaemia measured from the ischaemic region of the left ventricle, such as epicardial ST-segment elevation and degree of inhomogeneity of electrical activation, were markedly increased following coronary artery occlusion. These were significantly less marked when the occlusion occured within 5 min or 24 h of pacing (Figures 3 and 4).

Table 2 Peak haemodynamic changes following coronary artery occlusion in anaesthetized dogs; modification by prior cardiac pacing at various time intervals (5 min, 15 min, 1h, 6h, 24h, 48h and 72h) previously.

	sham-control 1	PC (5min)	PC (15min)	PC (1h)	PC (6h)	sham-control 2	PC (24h)	PC (48h)	PC (72h)
	values of haemodynamic parameters before occlusion								
SABP	126±5	106±8	137±11	138±9	132±8	134±6	144±6	116±10	129±12
DABP	85±4	71±7	90±7	91±5	80±7	88±4	89±5	68±5	81±10
MABP	99±5	83±7	108±8	103±5	97±7	104±4	107±5	85±6	97±11
LVSP	138±7	110±6	116±9	117±8	129±9	151±5	151±6	123±9	148±19
LVEDP	5.6±0.4	6.0±0.5	7.2±1.2	4.0±0.6	5.5±0.6	5.2±0.2	5.1±0.3	6.7±0.8	6.3±1.3
HR	146±5	147±7	151±8	149±11	161±10	161±7	162±7	140±8	146±3
+ve LV dP/dt_{max}	3027±355	2545±102	2585±284	2079±206	2619±154	2729±211	2917±208	2887±282	2521±323
-ve LV dP/dt_{max}	2269±271	1949±120	3076±302	2056±284	2846±326	2206±105	2644±162	2653±185	2575±249
	changes in haemodynamic parameters following coronary artery occlusion								
SABP	¯17±2*	¯13±3*	¯10±3*	¯15±4*	¯16±6*	¯18±4*	¯11±2*	¯10±3*	¯11±4*
DABP	¯15±1*	¯9±3*	¯7±3	¯13±4*	¯12±6*	¯9±3	¯10±2*	¯6±2*	¯4±2
MABP	¯15±2*	¯10±2*	¯9±5*	¯12±3*	¯13±6*	¯13±3*	¯10±3*	¯8±3*	¯6±2*
LVSP	¯22±2*	¯19±3*	¯13±6	¯20±5*	¯23±7*	¯13±2*	¯9±1*	¯11±1*	¯6±5
LVEDP	⁺16.7±2.0*	⁺11.3±1.0*	⁺4.1±1.25*	⁺10.4±1.6*	⁺11.1±2.0	⁺20.1±2.0*	⁺20.6±2.6*	⁺12.8±1.5*	⁺21.3±4.3*
HR	⁺1±2	¯2±3	⁺4±1	⁺1±3	⁺6±3	⁺6±2	⁺2±1	⁺4±2	⁺9±3
+ve LV dP/dt_{max}	¯1019±159*	¯503±83*†	¯537±186	¯546±106*	¯792±222*	¯551±58*	¯350±119	¯741±101*	¯797±232*
-ve LV dP/dt_{max}	¯570±149*	¯368±72	¯758±268*	¯689±139*	¯942±327*	¯679±127*	¯306±80†	¯440±88*	¯635±221*

**: P<0.05 vs. baseline values, †: P<0.05 vs. sham-control 1 or sham-control 2. SABP: systolic arterial blood pressure (mmHg); DABP: diastolic arterial blood pressure (mmHg); MABP: mean arterial blood pressure (mmHg); LVSP: left ventricular systolic pressure (mmHg); LVEDP: left ventricular end-diastolic pressure (mmHg); HR: heart rate (beats min^{-1}); +ve LV dP/dt_{max}: peak positive left ventricular dP/dt (mmHg sec^{-1}); -ve LV dP/dt_{max}: peak negative left ventricular dP/dt (mmHg sec^{-1}).*

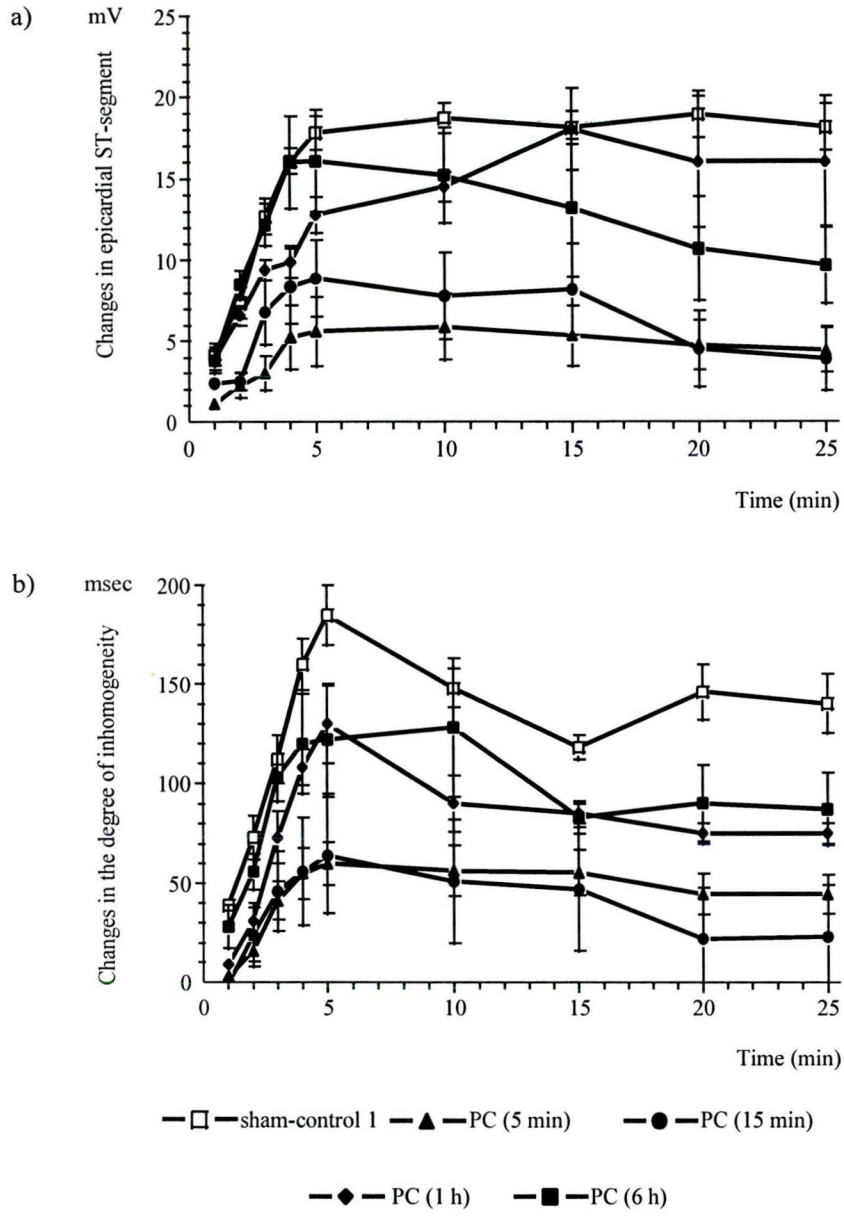

Figure 3 *Graphs showing the changes in the epicardial ST-segment elevation (mV; graph a) and in the degree of inhomogeneity of electrical activation (msec; graph b) during a 25 min coronary artery occlusion in the unpaced controls (sham-control 1), and in dogs subjected to coronary artery occlusion 5 min, 15 min, 1h and 6h following cardiac pacing.*

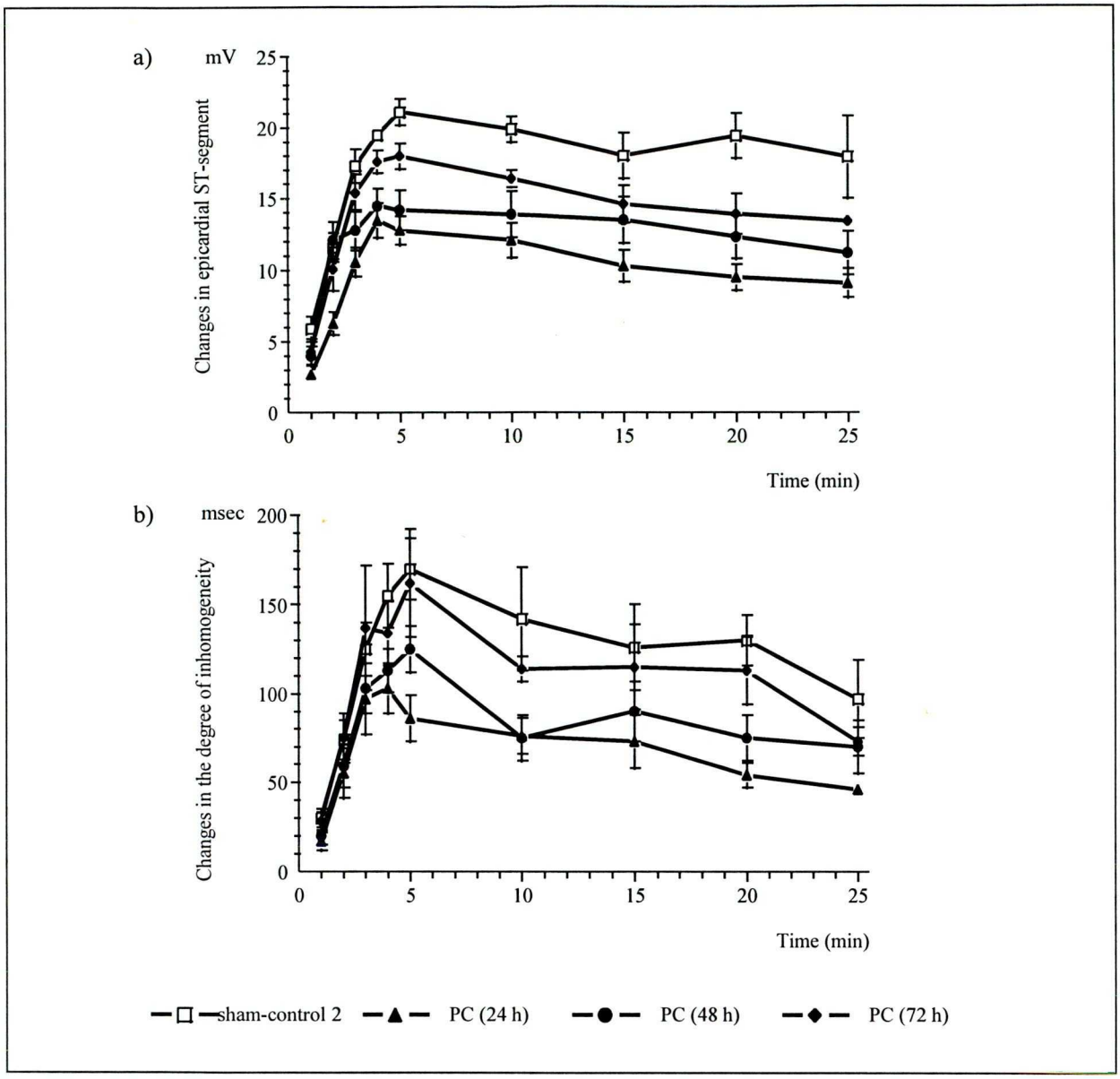

Figure 4 *Graphs show the changes in the epicardial ST-segment elevation (graph a; mV) and in the degree of inhomogeneity of electrical activation (graph b; msec) during a 25 min coronary artery occlusion in the sham-operated, unpaced controls (sham-control 2), and in those dogs in which coronary artery occlusion was performed 24h, 48h or 72h after cardiac pacing.*

3.1.3. Effects of a 25 min occlusion of the left anterior descending coronary artery on ventricular arrhythmias.

A sudden occlusion of a coronary artery, which results in an area at risk of about 30-40% of the left ventricular myocardium, precipitates severe ventricular arrhythmias and these arrhythmias often lead to ventricular fibrillation (Figure 5). There was a marked difference in

terms of arrhythmia severity between the controls (sham-control 1) and those dogs in which right ventricular pacing was performed 5 min preceding the occlusion. The most severe, life threatening arrhythmias were prevented or markedly diminished (occlusion-induced ventricular premature beats: 202±19, $P<0.05$ vs. sham-control 1; tachycardiac episodes: 4.1±2.6; incidence of ventricular tachycardia: 45%; Figure 5). Further, VF did not occur in these dogs ($P<0.05$ vs. sham-control 1) and only 45% of the paced dogs fibrillated on reperfusion, giving an overall survival from the combined occlusion-reperfusion insult of 55% (sham-control 1: 7%, $P<0.05$; Figure 5).

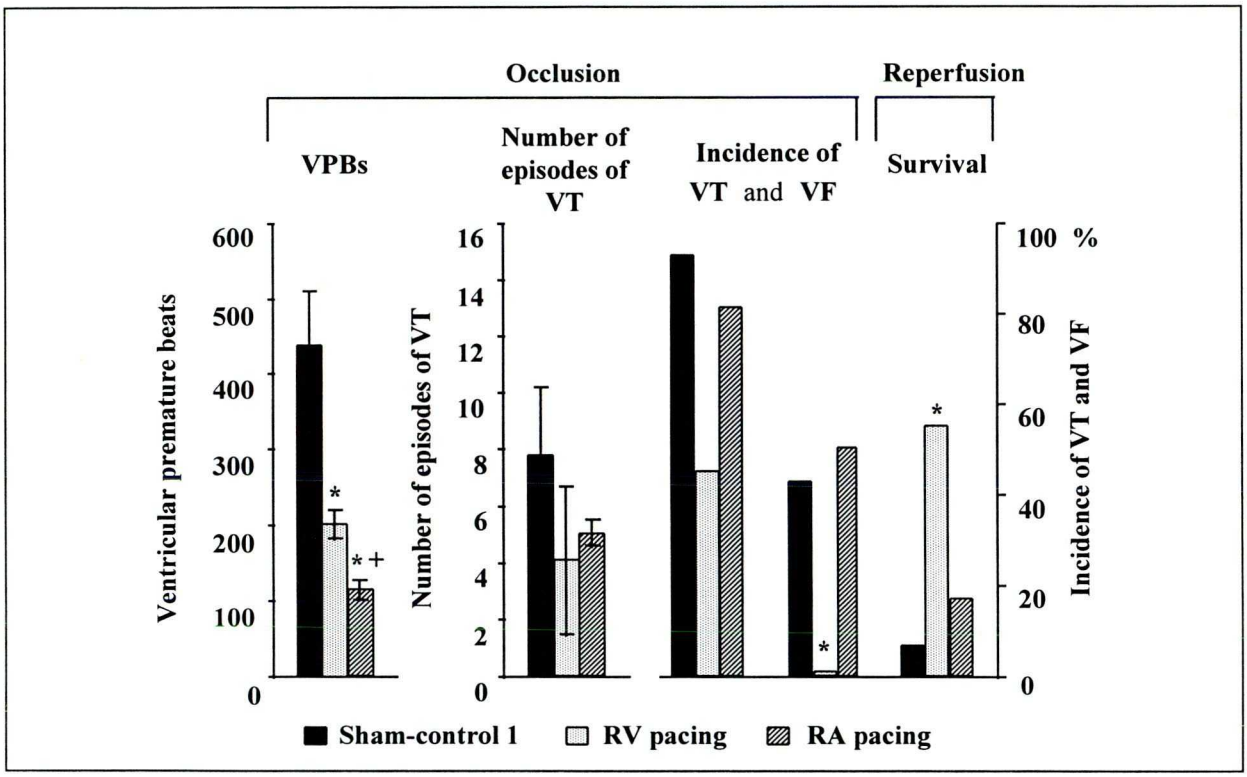

Figure 5. *The bar graph shows ventricular arrhythmias during a 25 min occlusion of the anterior descending coronary artery and the overall survival following the combined occlusion-reperfusion insult. Four 5 min episodes of right ventricular pacing 5 min prior to occlusion (RV pacing, n=11) markedly reduced ventricular arrhythmias during occlusion and significantly increased survival to the occlusion and reperfusion. When pacing was performed via the right atrium (RA pacing, n=6) ventricular premature beats and the number of episodes of ventricular tachycardia decreased, however, protection did not occur against ventricular fibrillation. *: P<0.05 vs. sham-control 1; +: P<0.05 vs. RV pacing.*

Interestingly, antiarrhythmic protection did not occur when dogs were paced using the same protocol via the right atrium, yet there was a reduction in VPBs and in VT episodes

(VPBs: 120±7; $VT_{episodes}$: 5±0.4; VT%: 83%, VF%: 50%, overall survival: 17%; Figure 5). For this reason in all further experiments dogs were paced via the right ventricle.

3.1.4. Time course of the antiarrhythmic protection afforded by right ventricular pacing. Effects of a 25 min occlusion of the left anterior descending coronary artery on ventricular arrhythmias when commenced at various time intervals following right ventricular pacing.

3.1.4.1. "Classical" or early phase of protection

As clearly seen in the controls, ventricular arrhythmias during an acute coronary artery occlusion are distributed in two distinct phases, phase Ia and phase Ib (Figure 6). In those dogs in which pacing was followed 5 min later by the occlusion period phase Ia arrhythmias decreased, whereas phase Ib arrhythmias virtually disappeared.

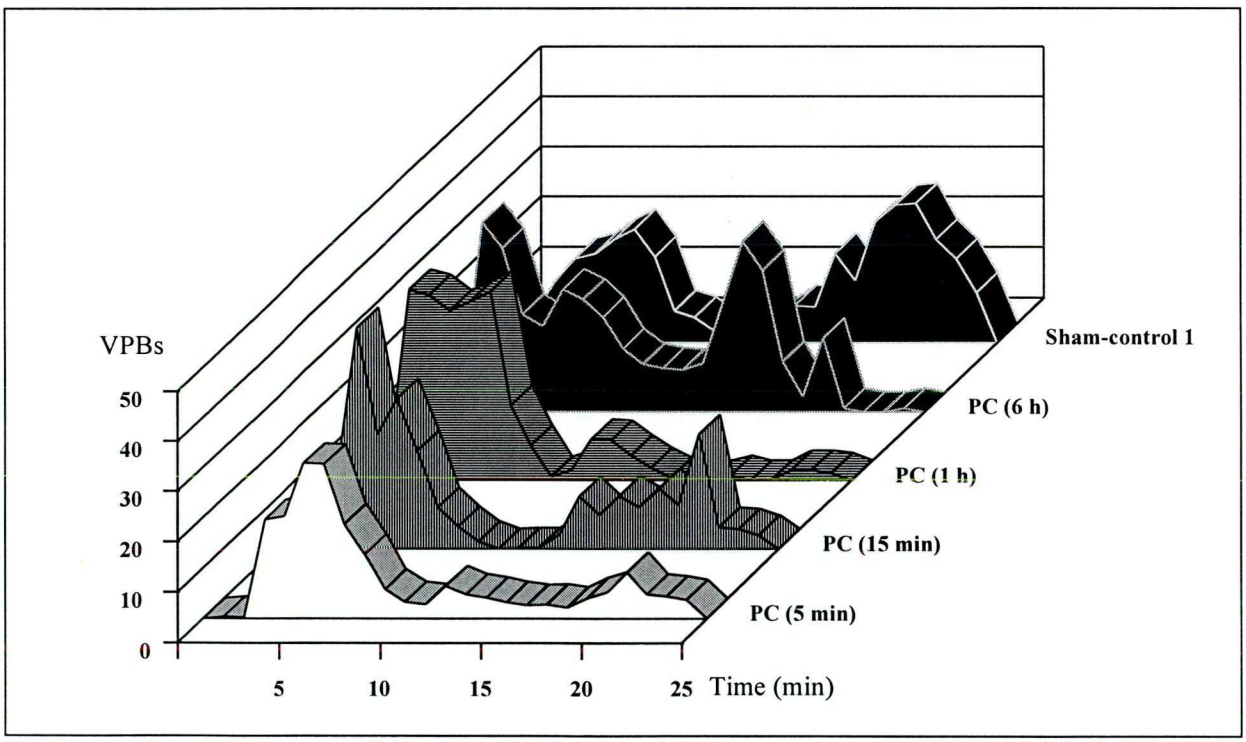

Figure 6 *Effects of cardiac pacing on the distribution of ventricular premature beats during a 25 min occlusion period. When cardiac pacing was followed 5 min later by the occlusion period, phase Ia arrhythmias were suppressed whilst phase Ib arrhythmias were completely prevented (P<0.05). When the time window between the pacing stimuli and occlusion was extented to 15 min, 1h or 6h, the ventricular premature beats were markedly increased, especially in phase Ib.*

When the time window between pacing and coronary artery occlusion was increased (to 15 min, 1h or 6h) phase Ia, but especially phase Ib arrhythmias showed a progressive increase. The mean values of ventricular premature beats in these groups were 249±24, 247±23, 346±24, respectively.

The pronounced protection by pacing against the more severe type of arrhythmias, such as ventricular tachycardia and ventricular fibrillation, was largely lost by 15 min and did not re-appear at 1h or 6h after pacing. Ventricular arrhythmias in these groups were as follows: number of episodes of VT: 11.7±1.7, 7.0±1.1, 7.3±2.7; the incidence of VF during occlusion: 63%, 75% and 63%, respectively. Only very few dogs survived the occlusion-reperfusion insult (12.5%, 13%, 0%, respectively) (Figures 7, 8 and 9).

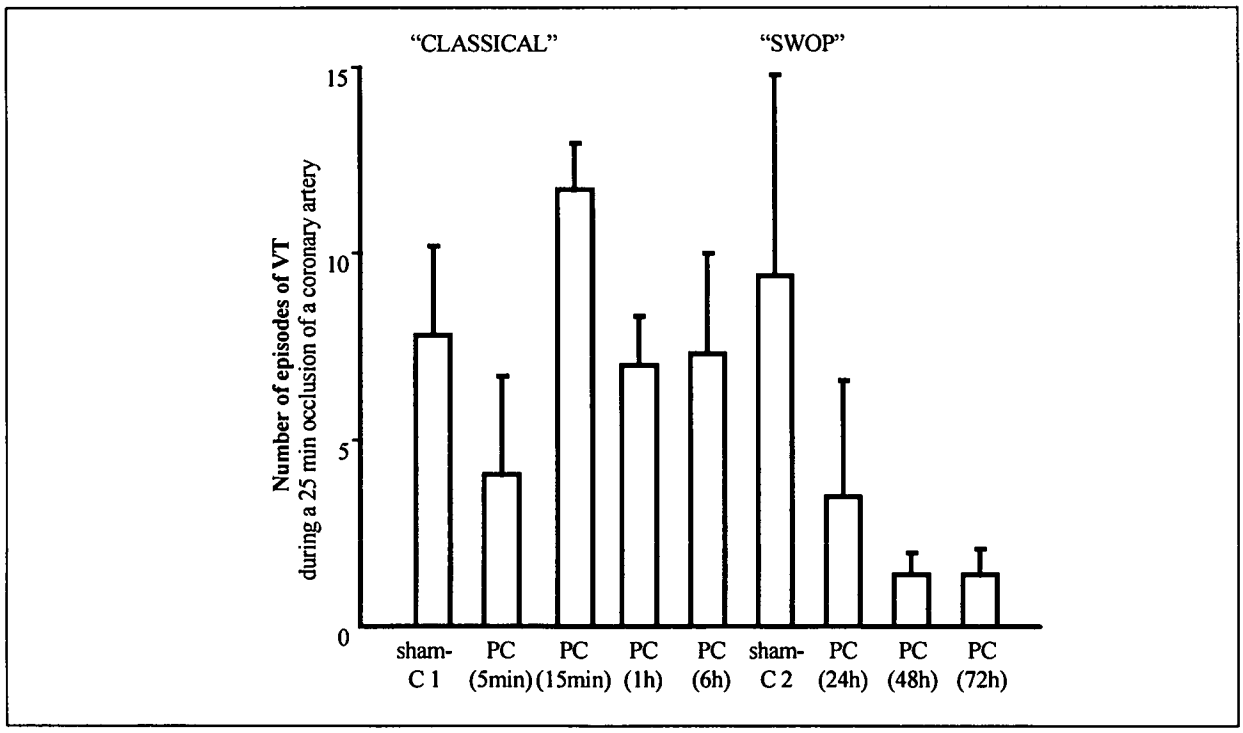

Figure 7 *The figure illustrates the number of episodes of ventricular tachycardia during a 25 min coronary artery occlusion in the "classical" or early phase and in the "second window of protection" or delayed phase following cardiac pacing. Ventricular tachycardiac episodes were markedly reduced at 5 min and at 24h, 48h ($P<0.05$) and 72h ($P<0.05$).*

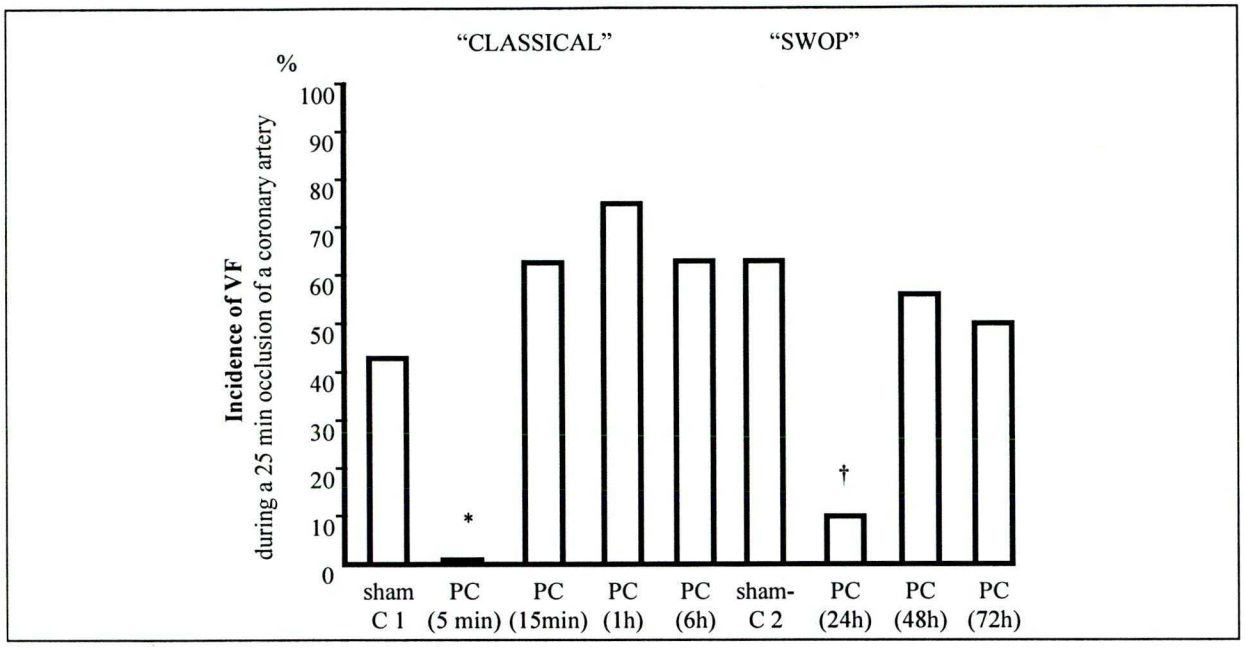

Figure 8 *The figure illustrates the incidence of ventricular fibrillation during a 25 min coronary artery occlusion in the "classical" or early phase and in the "second window of protection" or delayed phase following cardiac pacing. The incidence of ventricular fibrillation was markedly reduced at 5 min and at 24h. * and † indicate P<0.05 vs. sham-control 1 or sham-control 2, respectively.*

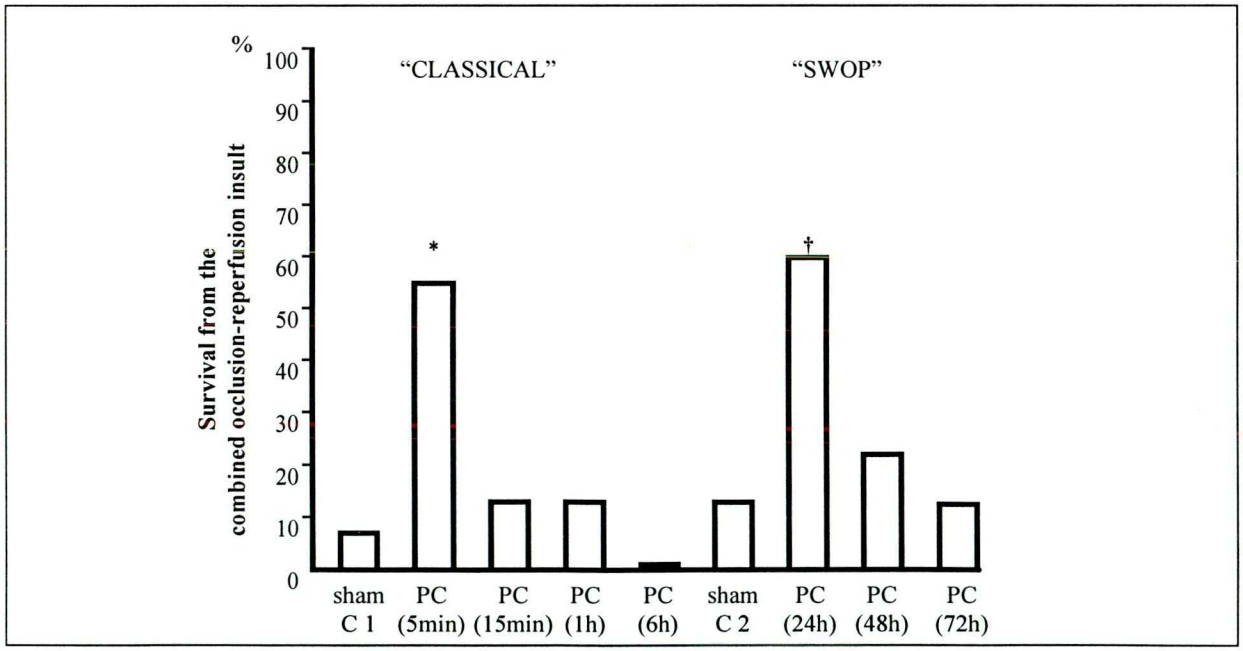

Figure 9 *The overall survival from the combined occlusion-reperfusion insult increased significantly (* and † indicate P<0.05 vs. sham-control 1 or sham- control 2, respectively) when the occlusion of the anterior descending coronary artery was performed either 5 min or 24h following cardiac pacing.*

3.1.4.2. Late phase or "second window of protection".

In those dogs in which a coronary artery was occluded 24h after pacing marked antiarrhythmic protection occured (VPBs: 145±20, P<0.05; $VT_{episodes}$: 3.5±3.1, P<0.05; incidence of VF: 10%, P<0.05 vs. sham-control 2: VPBs: 583±62; $VT_{episodes}$: 9.4±5.4; incidence of VF: 63%). Furthermore, 60% of these dogs survived the occlusion-reperfusion insult (sham-control 2: 10%). This protection was lost when the time interval between the pacing stimuli and the occlusion was extended to 48 or 72h. Thus, neither the incidence of occlusion-induced ventricular fibrillation (56% and 50%, respectively) nor the overall survival, differed significantly when compared to the sham-control 2 (22% and 12.5%, respectively; Figures 8 and 9). However, some aspects of ventricular arrhythmias were still reduced 48h or 72h after pacing (for example VPBs: 160±13, P<0.05 at 48h and 129±8, P<0.05 at 72h; Figure 7).

3.2. Endogenous mediators in pacing-induced preconditioning; is there a role for nitric oxide or bradykinin in mediating the effects of preconditioning?

3.2.1. Effects of Nω-nitro-L-arginine methyl ester.

Intravenous bolus injection of Nω-nitro-L-arginine methyl ester (L-NAME, 10 mg kg^{-1}) increased mean arterial blood pressure (+24±3 mmHg, P<0.001) and left ventricular systolic pressure (+22±4 mmHg, P<0.001), whereas heart rate was reduced significantly (Table 3). Although there were differences in the baseline haemodynamic parameters between the L-NAME treated dogs and those of the untreated dogs when pacing was commenced, similar changes occurred during pacing (Table 4).

However, both the endocardial and subendocardial ST-segment elevation was more pronounced (Figure 2). Further, 2 out of 11 dogs (18%) fibrillated during pacing, and the number of VPBs increased significantly after the pacing stimuli was terminated in the L-NAME treated dogs (63±3 vs. preconditioned (PC 5 min): 18±0.5, P<0.05).

Table 3 Haemodynamic effects of icatibant and N^G-nitro-L-arginine methyl ester (L-NAME).

	baseline values	icatibant	baseline values	L-NAME
arterial blood pressure (systolic; mmHg)	134±6	-2±9	128±9	+24±6*
arterial blood pressure (diastolic; mmHg)	90±7	-1±8	84±8	+25±3†
arterial blood pressure (mean; mmHg)	105±6	-2±8	102±9	+24±3†
LV systolic pressure	116±7	-1±9	112±9	+22±4†
LV end-diastolic pressure	5.6±1.29	+0.6±2.31	3.9±1.1	2.6±1.2
LV dP/dt_{max} (+ve; mmHg sec^{-1})	2191±273	-155±227	2130±240	-207±155
LV dP/dt_{max} (-ve; mmHg sec^{-1})	2285±133	-158±71	2102±205	+171±133
heart rate (beats min^{-1})	142±7	-4±8	149±8	-10±3*

*Values are expressed as means ± s.e.mean. *: P<0.05 vs. baseline, †: P<0.001 vs. baseline.*

3.2.2. Effects of icatibant

Icatibant, given as an intravenous bolus injection (0.3 mg kg^{-1}), produced only slight changes in haemodynamic parameters (Table 3). Preconditioning by cardiac pacing following the blockade of bradykinin B_2 receptors was, however, rather difficult; 38% (3/8) of the dogs fibrillated during pacing and the VPBs were increased significantly during the intervals between the pacing stimuli (47±2 vs. preconditioned: 18±0.5, P<0.05).

There were more pronounced elevations in both the endocardial and subendocardial ST-segment after the pacing periods (Figure 2).

Table 4 Haemodynamic changes induced by cardiac pacing when performed in the presence of icatibant or Nω-nitro-L-arginine methyl ester.

	cardiac pacing in the presence of icatibant			cardiac pacing in the presence of L-NAME		
	baseline	**during pacing**	**after pacing**	**baseline**	**during pacing**	**after pacing**
arterial blood pressure (systolic; mmHg)	132±9	76±9†	139±11	152±16§	95±16*	163±12
arterial blood pressure (diastolic; mmHg)	89±8	47±6*	94±12	107±13§	67±12†	114±11
arterial blood pressure (mean; mmHg)	103±8	57±7†	109±11	124±15§	76±13†	131±15
left ventricular systolic pressure (mmHg)	115±9	-	122±7	133±17	73±10*	129±14
left ventricular end-diastolic pressure (mmHg)	6.2±2.31	-	4.3±1.33	6.44±1.31	13.1±1.03*	7.57±1.07*
left ventricular dP/dt_{max} (+ve; mmHg sec^{-1})	2036±227	-	2406±380	1923±264§	1255±190*	2057±218
left ventricular dP/dt_{max} (-ve; mmHg sec^{-1})	2127±71	-	2252±581	2242±269§	1296±189†	2240±308
heart rate (beats min^{-1})	138±8	220±2†	153±7	149±10	220±3†	149±10

*Values are expressed as means ± s.e.mean. *: P<0.05 vs. baseline, †: P<0.001 vs. baseline, §: P<0.05 vs. untreated, paced group (see cardiac pacing via the right ventricle in Table 1). Left ventricular pressure was not recorded in the icatibant group during pacing therefore data on left ventricular pressure and LV dP/dt_{max} is not available in this group.*

3.2.3. Effects of a 25 min occlusion of the left anterior descending coronary artery. Modifications by pretreatment with Nω-nitro-L-arginine methyl ester or icatibant.

The haemodynamic effects of coronary artery occlusion are summarised in Table 5. The changes were similar, and did not differ significantly between the groups.

Table 5 Effects of the 25 min occlusion of the anterior descending coronary artery on haemodynamic parameters.

	Icatibant before pacing		Icatibant after pacing		L-NAME	
	before occlusion	Δ occlusion	before occlusion	Δ occlusion	before occlusion	Δ occlusion
SABP	132±7	-8±4	130±7	-8±4	165±13†§	-14±9*
DABP	86±9	-8±4	88±4	-10±5	121±12†§	-9±8*
MABP	101±8	-7±2	100±5	-8±15	130±14†§	-12±10*
LVSP	104±10	-8±2	117±4	-11±17	142±17	-21±9*
LVEDP	7.0±1.4	+4.0±0.4	6.0±1.9	+3.9±1	6.8±0.6	+4.1±1.9
+ve LV dP/dt_{max}	2043±168	-341±58	4103±428	-530±495	2032±268	-270±210*
-ve LV dP/dt_{max}	1982±110	-189±26	3824±275	-428±460	2496±321	-459±183
HR	152±8	+7±1	168±5	-4±5.2	153±6	-1±9

**: $P<0.05$ vs. before occlusion values (within group comparisons, paired T-test), † $P<0.05$ vs. sham-control 1 or § $P<0.05$ vs. preconditioned group (comparing the between group baseline values; one-way analysis of variance with Bonferoni post-hoc test, see Table 2). SABP: systolic arterial blood pressure (mmHg); DABP: diastolic arterial blood pressure (mmHg); MABP: mean arterial blood pressure (mmHg); LVSP: left ventricular systolic pressure (mmHg); LVEDP: left ventricular end-diastolic pressure (mmHg); HR: heart rate (beats min^{-1}); +ve LV dP/dt_{max}: peak positive left ventricular dP/dt (mmHg sec^{-1}); -ve LV dP/dt_{max}: peak negative LV dP/dt (mmHg sec^{-1}).*

There were marked increases in both epicardial ST-segment, and the degree of inhomogeneity of electrical activation, during coronary artery occlusion. Preconditioning markedly decreased these parameters of the severity of ischaemia and this was not modified by either inhibiting the formation of nitric oxide before preconditioning (Figure 10) or by blocking bradykinin B_2 receptors before, or after, preconditioning (Figure 11).

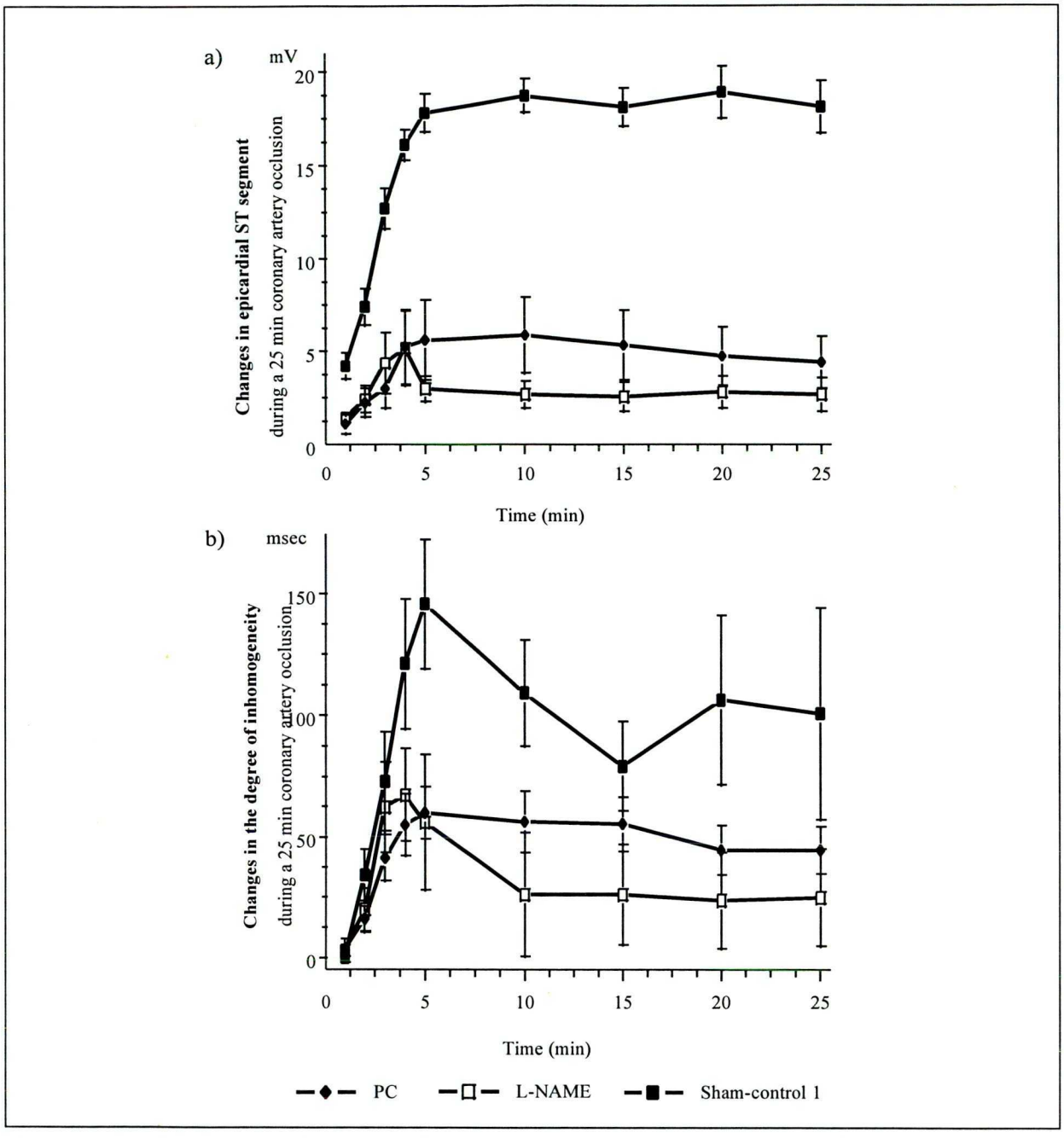

Figure 10 *Changes in the epicardial ST-segment elevation (mV) and in the degree of inhomogeneity of electrical activation during a 25 min anterior descending coronary artery occlusion in the paced group (pacing was followed 5 min later by the occlusion period, PC) in the unpaced controls (sham-control 1), and in those dogs in which the formation of nitric oxide was blocked by Nω-nitro-L-arginine methyl ester before cardiac pacing (L-NAME+PC). L-NAME did not modify the anti-ischaemic protection, afforded by cardiac pacing.*

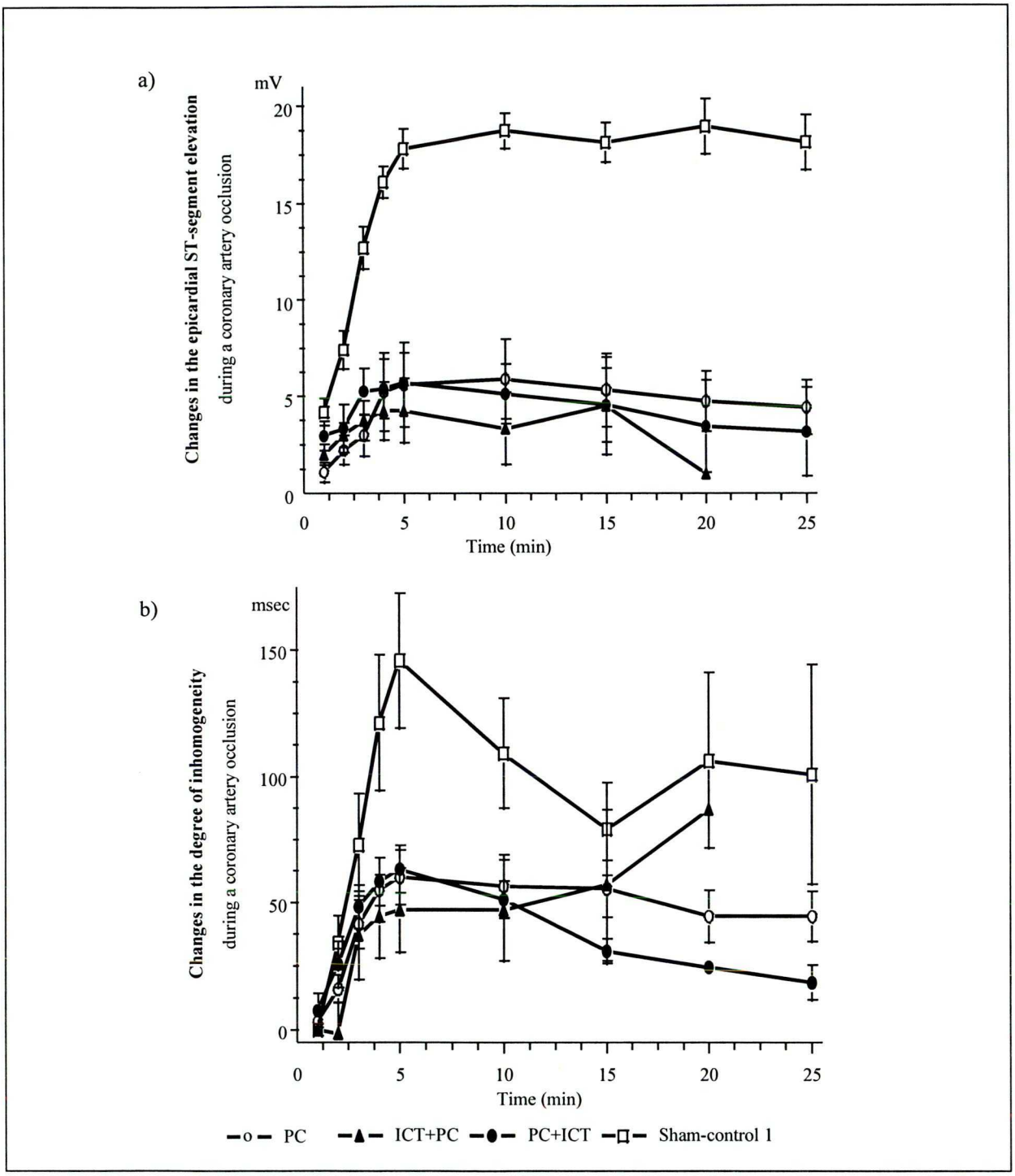

Figure 11 *Changes in the epicardial ST-segment elevation (mV) and in the degree of inhomogeneity of electrical activation during a 25 min anterior descending coronary artery occlusion in the paced group (pacing was followed 5 min later by the occlusion period, PC) in the unpaced controls (sham-control 1), and in those dogs in which icatibant was given either before (ICT+PC) or after (PC+ICT) cardiac pacing. Anti-ischaemic protection, afforded by cardiac pacing, was not modified by pretreatment with icatibant either before or after pacing.*

Occlusion-induced ventricular arrhythmias were, however, markedly increased especially when icatibant or L-NAME was given prior to pacing (total number of VPBs: 220±15 and 296±29; episodes of VT: 15±0.4 and 6.3±0.9; the incidence of VT: 100% and 78% and VF: 100% and 56%, respectively; Figure 12).

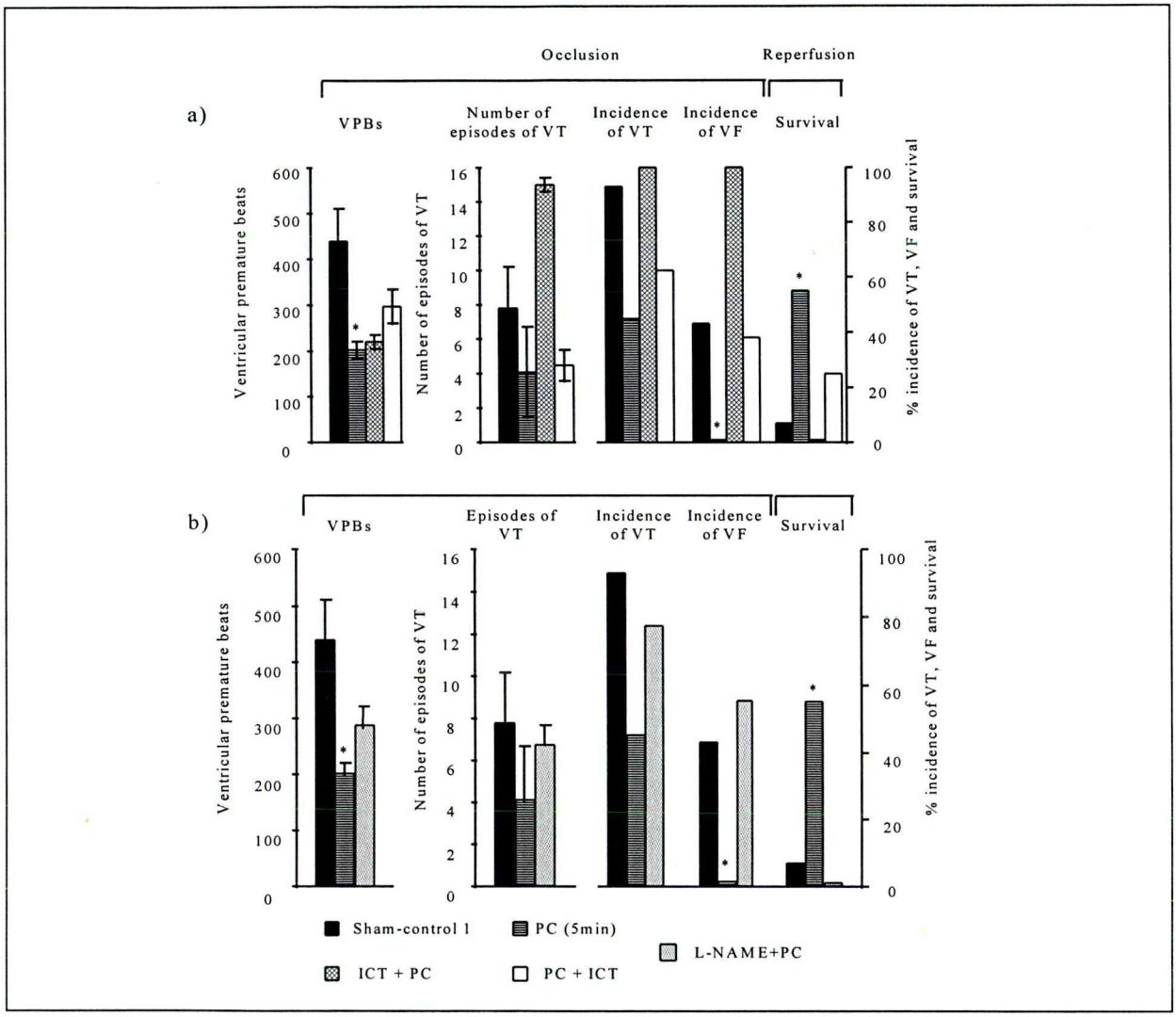

Figure 12 *Bar graph a illustrates the effects of the blockade of bradykinin B_2 receptors either before (ICT+PC) or after pacing (PC+ICT) on the total number of ventricular premature beats, the number of episodes and incidence of ventricular tachycardia, the incidence of ventricular fibrillation during a 25 min occlusion of the anterior descending branch of the left coronary artery, and the overall survival from the combined occlusion-reperfusion insult, whereas bar graph b summarises the effects of the blockade of nitric oxide synthase (L-NAME+PC) on the same arrhythmia parameters. Antiarrhythmic protection was completely lost when pacing was performed in the presence of icatibant or L-NAME, further, protection was markedly suppressed when icatibant was given after pacing but prior to the occlusion period. *: P<0.05 vs. sham-control 1.*

Further, the cardiac pacing-induced reduction of ventricular premature beats in phase Ib was reversed by both icatibant and L-NAME (Figure 13).

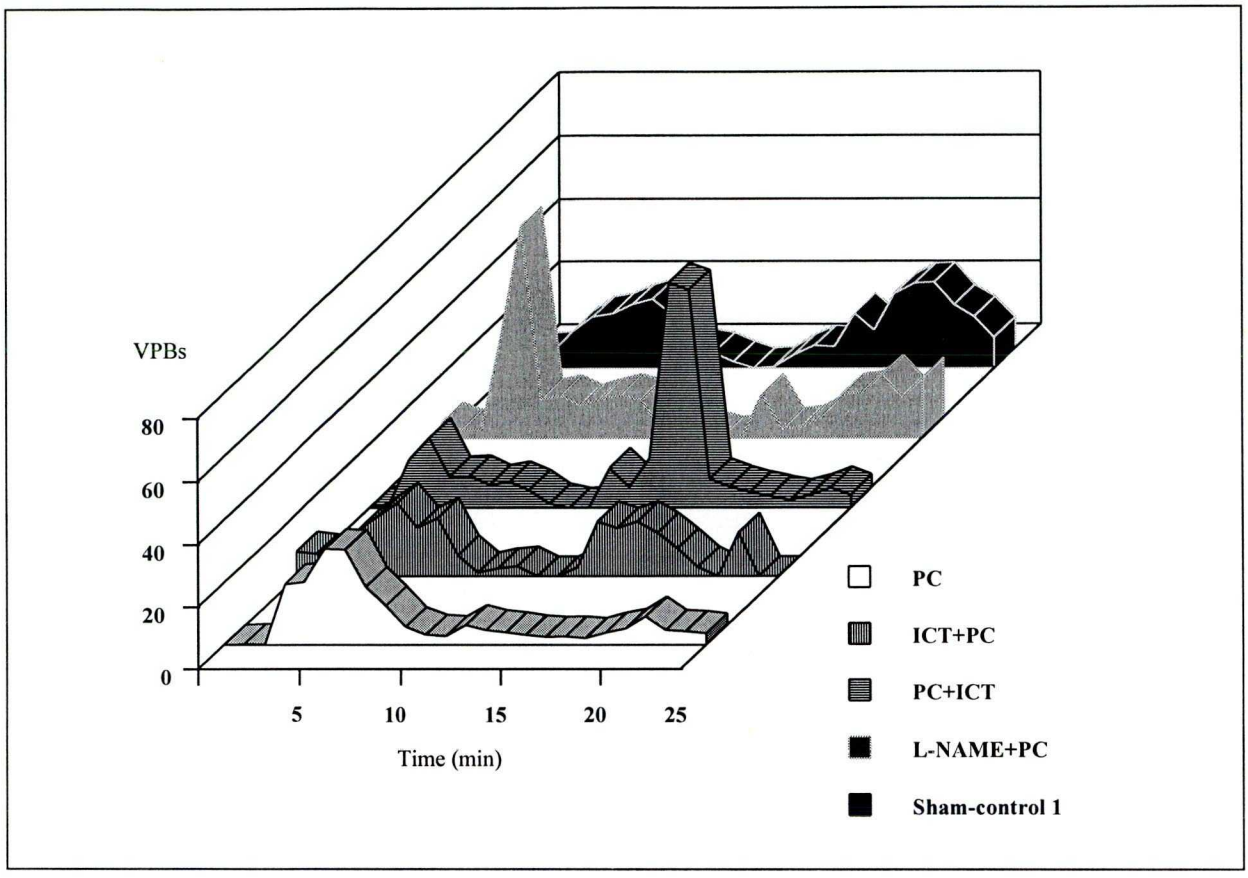

Figure 13 *Cardiac pacing-induced reduction in ventricular premature beats during a 25 min coronary artery occlusion was reversed either by blocking the nitric oxide synthase before pacing (L-NAME+PC) or the bradykinin B_2 receptors before (ICT+PC) or after pacing (PC+ICT) but prior to occlusion.*

There was no survival from the combined occlusion-reperfusion insult in all these groups. When bradykinin B_2 receptors were blocked after pacing, but prior to the occlusion period, the antiarrhythmic protection during occlusion was markedly attenuated (VPBs: 297±37; $VT_{episodes}$: 4.5±0.9; VT%: 68%). There was, however, an overall survival of 25% in this group (Figure 12). These results may suggest that the antiarrhythmic and anti-ischaemic effects of pacing-induced preconditioning are mediated by different mechanisms.

3.3. Area at risk

There was no statistically significant difference between any of the groups in the area at risk: sham-control 1 (38.5±2%); paced with 5 min (38.5±1.9%); 15 min (37.4±1.9%); 1h (40.3±1.8%) and 6h (37.6±1.8) interval before occlusion; sham-control 2 (42.7±3.3%); paced with 24h (44.1±1.4); 48h (42±1.1) and 72h (43.3±3.6) interval before occlusion; icatibant given before (32.4±1.7%) or after pacing (37.5±6.4%) and L-NAME pretreated (36±6.1%).

3.4. Involvement of the G_i protein-Protein kinase C_ε cascade in pacing-induced preconditioning.

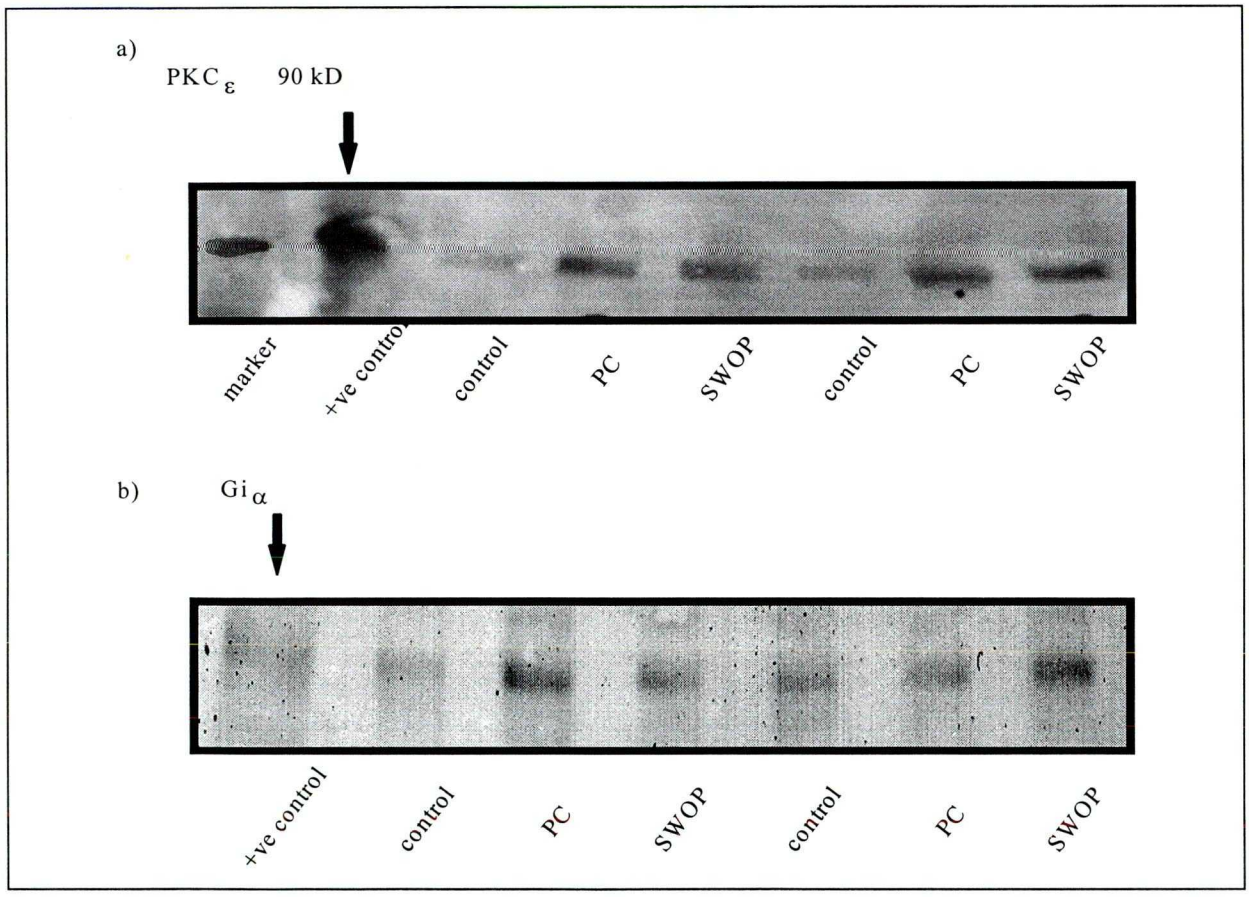

Figure 14 *Immunoreactive bands of protein kinase C_ε (top figure) and G_i α-1, α-2 (bottom figure) in the membrane fraction, isolated as described in the Methods. The hearts were paced for four 5 min periods and immediately (PC) or 24h following pacing (SWOP) myocardial samples were taken. The controls were those dogs in which samples were taken without prior cardiac pacing. PKC_ε is translocated into the cardiac membranes following cardiac pacing, and appears to be present in the membrane at 24h. An increased immunoreactivity of G_i proteins were also seen immediately and 24h after pacing.*

Most endogenous protective mediators that are believed to be involved in preconditioning act specifically on membrane receptors. If so, there should also be an increased activity in the signalling pathways linked to those receptors. To confirm this hypothesis, biochemical analysis were performed to assess whether there was an increase in G_i proteins and PKC_ε in the cell particulate (membrane) fraction. As it is illustrated in Figure 14, our preliminary data indicate that 5 min or 24h after cardiac pacing G_i protein immunoreactivity is elevated. PKC_ε seems to be translocated to the cell membrane (elevated immunoreactivity in the high speed particulate fraction) following pacing and it appears to be present in the membrane 24h after pacing.

4. Discussion

4.1. New findings

We have shown that four 5 min periods of cardiac pacing at a rate of 220 beats min^{-1} via the right ventricle, but not via the right atrium, is sufficient to protect against ventricular arrhythmias during a subsequent prolonged coronary artery occlusion (and reperfusion) when that occlusion is performed 5 min after the last pacing period. This method of inducing preconditioning proved to be safe; cardiac pacing does not induce potentially lethal ventricular arrhythmias (i.e. ventricular tachycardia or fibrillation).

We evaluated the time course of this form of preconditioning. The protection is marked (complete prevention of occlusion-induced ventricular fibrillation) but transient (15 min after pacing the protection is lost and does not re-appear 1h or 6h later). When the time window between pacing and coronary artery occlusion is extended to 24h, antiarrhythmic protection re-appears. Protection against ventricular fibrillation is lost 48h or 72h after pacing. Some arrhythmia parameters are however still reduced, even 72h following pacing (e.g. ventricular premature beats and ventricular tachycardia).

Our results indicate that the release of bradykinin may be one of the key factors responsible for initiating pacing-induced preconditioning, since blockade of bradykinin B_2 receptors before preconditioning completely prevents antiarrhythmic cardioprotection.

We have shown that the release of nitric oxide is important in mediating pacing-induced preconditioning since inhibiting the formation of nitric oxide (by L-NAME) prevented the protective effects of pacing.

We have found some evidence indicating that there is an increased concentration of G_i proteins after cardiac pacing.

We have identified for the first time a pacing-induced sustained translocation of ventricular protein kinase C_ε to the cell membrane.

4.2. Cardiac pacing-induced preconditioning

Our results indicate that, apart from short episodes of coronary artery occlusion, preconditioning may be induced by brief periods of rapid cardiac pacing. Cardiac pacing-induced preconditioning, similarly to that of "classical" (coronary artery occlusion-induced) preconditioning, is manifested by a reduction in ventricular arrhythmias and by a reduction in the severity of ischaemia (ischaemia-induced ST-segment elevation[43,50] is less). Myocardial infarct size is also reduced by pacing[93].

There is, however, some debate as to whether this form of protection falls into the category of ischaemia-induced preconditioning. ST-segment elevation, measured from the right ventricular pacing site, may indicate ischaemia but we cannot exclude the possibility that this results from a current of endocardial injury, or from other electrical disturbances following pacing. For this reason left ventricular subendocardial and epicardial electrocardiogram were also measured during the pacing procedure. Whereas significant subendocardial ST-segment elevation occured immediately after the pacing periods, no changes were observed in the epicardial leads. These findings indicate that ischaemia indeed occurs after pacing, but that is located only in the endocardial-subendocardial region. Presumably ischaemia results from an increase in myocardial oxygen supply, as a consequence of increased heart rate and elevated left ventricular filling pressure, which would reduce coronary perfusion pressure and decrease blood flow in the endocardial region of the heart.

Marber and his colleagues[94] have shown that a single 5 min atrial pacing followed 10 min later by a 45 min coronary artery occlusion failed to reduce infarct size in rabbits.

Certainly, the degree of ischaemia under these conditions, if any results at all, is less and may not be sufficient to induce preconditioning. Further, our results indicate that protection after pacing is lost between 5 min and 15 min. It is also interesting that we did not see antiarrhythmic protection when pacing was performed via the right atrium. The reason for this may be that right ventricular pacing, but not atrial pacing, compromises the outflow of blood through the aortic valve since contraction of the ventricles, initiated from the right side of the heart, proceeds from right to left. Furthermore, it has been shown that left ventricular wall stretch also increases oxygen demand and can induce protection[95]. Presumably shear stress may be greater when the heart is paced via the right ventricle.

Whatever the explanation is for the protection afforded by cardiac pacing, there is a marked reduction in occlusion and reperfusion-induced ventricular arrhythmias and this is similar in extent to that seen following short coronary artery occlusions. Protection against arrhythmias, however, lasts longer (over 1h[39]) when the heart is preconditioned by complete coronary artery occlusions, presumably because this induces a greater degree of ischaemia. Apart from the different end-points examined, this may also be one of the reasons why only some arrhythmia parameters (i.e. VPBs and episodes of VT) were reduced, whilst others (i.e. occlusion-induced VF) were not, 48h or 72h after pacing (Figures 7 vs. Figures 8 and 9), in contrast to classical preconditioning which reduced infarct size 48h later in the rabbit[96].

Another aspect of our findings should also be discussed here. Although clear antiarrhythmic protection occured 5 min or 24h following cardiac pacing, it seems ($P>0.05$) that the incidence of occlusion-induced ventricular fibrillation is increased between 15 min and 6 h following cardiac pacing, in comparison to the controls (Figure 8). Clearly, more experiments need to be done to confirm these results.

4.3. Mechanisms that may be involved in preconditioning

4.3.1. Role of endogenous mediators in preconditioning

The idea that preconditioning acts through the liberation of endogenous substances is an attractive hypothesis and may well explain this phenomenon. This evidence includes (*i*) that such substances are present in the heart, (*ii*) that they are released under conditions of

myocardial ischaemia, (*iii*) that when these substances are given exogenously in amounts similar to those that might be released, they also exert protective effects, (*iv*) that drugs that enhance their release induce cardioprotection, (*v*) that drugs that inhibit their release, or block their receptors, abolish protection.

A convincing body of work indicated a central role, in this protection, for adenosine acting on A_1 receptors. The evidence include the facts that adenosine is released under conditions of myocardial ischaemia[97,98], that intracoronary infusions of adenosine, or its selective A_1 analogues, induce protection (limiting infarct size[99,100] and reducing arrhythmias[98,101]) and that antagonists of adenosine abolish the protective effects of preconditioning[102]. Previous studies[103] from our laboratory have shown that, in the presence of non-specific adenosine receptor blockade, preconditioning by short coronary artery occlusions is difficult, 5 out of 11 dogs fibrillated during preconditioning (vs. 4/20 in the controls). However, protection during the prolonged occlusion period was not modified. This may indicate that adenosine is not the sole protective mediator that is responsible for the protection in canine hearts. Presumably, adenosine is released along with other substances. Thus, when adenosine receptors are blocked before preconditioning protection is not induced, although in some of the dogs antiarrhythmic protection is managed by other pathways.

These pathways may involve bradykinin. It has been shown that kinins are generated during coronary artery occlusion[12,104] and that kinins are released as soon as 1 min after myocardial ischaemia[105]. Exogenously given bradykinin exerts antiarrhythmic[86] and antiischaemic effects[68,69,86]. Further, protection is prevented when bradykinin B_2 receptors are blocked before preconditioning[67,68]. Bradykinin is also suggested to play a key role in the cardioprotective effects of angiotensin converting enzyme inhibitors[106,107]. In our pacing model preconditioning was very difficult in the presence of icatibant, a selective antagonist of bradykinin at B_2 receptors. In contrast to adenosine receptor blockade, in these dogs antiarrhythmic protection was completely lost during the long occlusion period; the incidences of ventricular tachycardia and ventricular fibrillation were both 100% during occlusion (Figure 12). These results indicate that the release of bradykinin may be an important triggering event that initiate preconditioning in the canine heart. When icatibant was given after pacing, but prior to a 25 min coronary artery occlusion period, protection

against occlusion-induced arrhythmias was largely lost. However, 25% of these dogs survived the combined occlusion-reperfusion insult. In this case some parallel pathways may be activated during pacing that are not prevented by icatibant. One reason may be that bradykinin B_2 receptors are coupled to the G protein-phospholipase C axis and increase the formation of inositol phosphate and diacylglycerol. These elevate intracellular Ca^{2+} concentrations and thereby release prostacyclin via stimulation of phospholipase A_2[108]. Further, intracellular elevation of Ca^{2+} also releases nitric oxide[109-111]. Both prostacyclin[19,112] and nitric oxide[47] have been shown to possess cardioprotective properties.

One possibility as to how nitric oxide is released following preconditioning is, by stimulation of bradykinin B_2 receptors. This possibility is supported by the fact that protection against occlusion-induced arrhythmias afforded by the intracoronary infusion of bradykinin is abolished when the formation of nitric oxide is prevented[113]. Others have suggested, however, that flow-mediated vasodilation activates a potassium channel (presumably the K_{Ca}) on the endothelial cell membrane and that it is this that leads to the release of nitric oxide[114]. Further, pulsatile compressions and decompressions of the coronary vessels has been shown to increase cyclic GMP levels in platelets passing through the coronary vascular beds[115], indicating nitric oxide release. Shear stress by cardiac pacing may, thus, give rise to an increased release of nitric oxide.

Indeed, antiarrhythmic protection induced by cardiac pacing or by coronary artery occlusions[47], is markedly reduced when the formation of nitric oxide is blocked before preconditioning (Figure 13). Other, indirect evidence also support this hypothesis. Nitric oxide is a substance with an ability to stimulate guanylate cyclase, thus increasing intracellular levels of cyclic GMP. Increased cyclic GMP levels can mediate cardioprotection[78,116]. Inhibition of guanylate cyclase by the intracoronary infusion of methylene blue, a non-specific inhibitor of that enzyme, has been reported to abolish preconditioning-induced protection[77].

As briefly reviewed above, there is evidence that strongly indicates a role for protective endogenous substances in preconditioning. Much less is known about how these triggering events lead finally to limitation of myocardial infarct size or reduction of ventricular arrhythmias. Our working hypothesis is summarised in Figure 15.

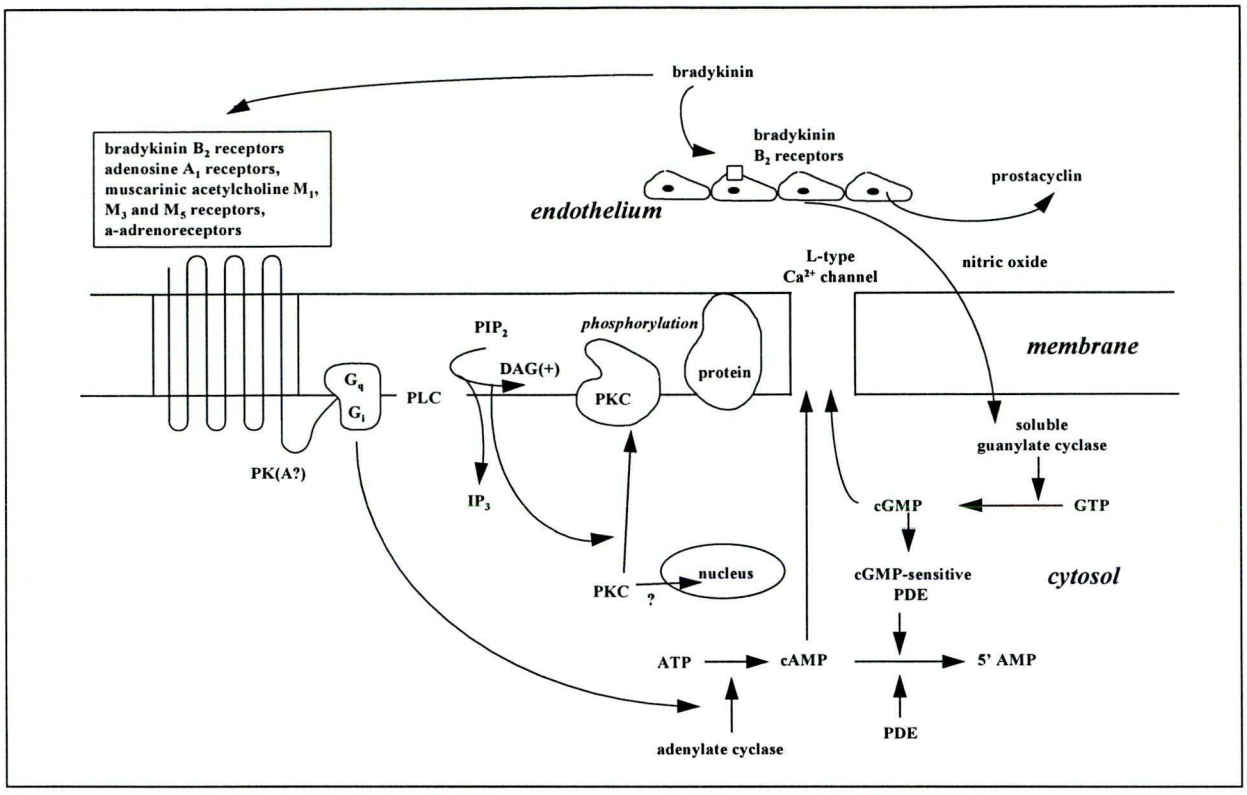

Figure 15 *A hypothesis for the antiarrhythmic effects of preconditioning. See Text for further details.*

We presume that the preconditioning stimuli release bradykinin, which then triggers the production of prostacyclin and nitric oxide. Nitric oxide activates soluble guanylate cyclase within myocytes which, in turn, increases intracellular levels of cyclic GMP. This, by activating the cyclic GMP-dependent phosphodiesterases, increases the degradation of cyclic AMP. A reduced intracellular level of cAMP and the increased cGMP levels would then inhibit the voltage-dependent L-type calcium channels[117]. This, by decreasing the trans-sarcolemmal Ca^{2+} flow, reduces intracellular Ca^{2+} concentration, prevents arrhythmias, decreases contraction, reduces energy utilisation, and reduces Ca^{2+} overload to the cells.

Noradrenaline is also released during myocardial ischaemia and may be particularly important in initiating ventricular arrhythmias[118,119], particularly in phase Ib[120]. Bradykinin can inhibit noradrenaline outflow during myocardial ischaemia[121] and thus protect against arrhythmias. This effect appears to be mediated mainly by bradykinin B_1 but also by B_2

receptors[122]. Furthermore, cardiac pacing reduced phase Ib arrhythmias, which was prevented by pretreatment with icatibant (Figure 13).

The precise mechanisms of the antiarrhythmic effects of prostacyclin or its stable analogues are still uncertain, and have been reviewed in more detail elsewhere[123]. These effects may include reduced release of noradrenaline[124], inhibition of free radical generation[125], coronary vasodilation and direct effects on the cardiac muscle action potential[126].

Other, presumably parallel, pathways are also suggested in Figure 15. Bradykinin B_2, adenosine A_1, muscarine receptors and adrenoceptors are all known to be linked to G proteins, coupling inositol triphosphate, diacylglycerol and activation of protein kinase C. Our preliminary data indicate that following cardiac pacing protein kinase C is translocated to the cell membranes[127], presumably by a G_i protein-coupled mechanism (Figure 14). Activation of protein kinase C, normally taken as being synonymous with translocation from a soluble to a particulate-bound form[128] in cell membranes[129] or nuclei[130], may be responsible for phosphorylation or activation of various proteins, for example ATP-dependent potassium channels[85], the Na^+/H^+ exchanger[131], nitric oxide synthase[132], cAMP-dependent phosphodiesterases[133] and transcription-translation factors[134].

5. References

1. Jennings R. B., Ganote C. E., Reimer K. A. Ischemic tissue injury. *Am J Pathol* 1975; **81:** 179-198.
2. Jennings R. B., Hawkins H. K., Lowe J. E., Hill M. L., Klotman S., Reimer K. A. Relation between high energy phosphate and lethal injury in myocardial ischemia in the dog. *Am J Pathol* 1978; **92:** 187-214.
3. Jennings R. B., Reimer K. A., Steenbergen C. Jr., Schaper J. Total ischemia III: Effect of inhibition of anaerobic glycolysis. *J Mol Cell Cardiol* 1989; **21** (Suppl 1): 37-54.
4. Reimer K. A., Jennings R. B., Hill M. L. Total ischemia in dog hearts, in vitro 2. High energy phosphate depletion and associated defects in energy metabolism, cell volume regulation, and sarcolemmal integrity. *Circ Res* 1981; **49:** 901-911.
5. Kingsley P. B., Sako E. Y., Yang M. Q., Zimmer S. D., Ugurbil K., Foker J. E., From A. H. Ischemic contracture begins when anaerobic glycolysis stops: a 31P-NMR study of isolated rat hearts. *Am J Physiol* 1991; **261:** H469-H478.
6. Steenbergen C., Murphy E., Levy L., London R. E. Elevation in cytosolic free calcium concentration early in myocardial ischemia in perfused rat heart. *Circ Res* 1987; **60:** 700-707.
7. Cheung J. Y., Bonventre J. V., Malis C. D., Leaf A. Calcium and ischemic injury. *N Engl J Med* 1986; **314:** 1670-1676.
8. Sole M. J., Shum A., Van Loon G. R. Serotonin metabolism in the normal and failing hamster heart. *Circ Res* 1979; **45:** 629-634.
9. Anrep G. V., Barsoum G. S., Talaat M. Liberation of histamine by the heart muscle. *J Physiol (Lond)* 1936; **86:** 431-451.
10. Olsson R. A. Changes in content of purine nucleoside in canine myocardium during coronary occlusion. *Circ Res* 1970; **26:** 301-306.
11. Berger H. J., Zaret B. L., Speroff L., Cohen L. S., Wolfson S. Regional cardiac prostaglandin release during myocardial ischemia in anesthetized dogs. *Circ Res* 1976; **38:** 566-571.
12. Kimura E., Hashimoto K., Furukawa S., Hayakawa H. Changes in bradykinin level in coronary sinus blood after the experimental occlusion of a coronary artery. *Am Heart J* 1973; **85:** 635-647.
13. Corr P. B. and Gillis R. A. Role of the vagus nerves in the cardiovascular changes induced by coronary occlusion. *Circulation* 1974; **49:** 86-97.
14. Opie L. H. Reperfusion injury and its pharmacologic modification. *Circulation* 1989; **80:** 1049-1062.
15. Hearse D. J. Reperfusion-induced injury: a possible role for oxidant stress and its manipulation. *Cardiovasc Drugs Ther* 1991; **5** (Suppl 2): 225-235.
16. Murry C. E., Jennings R. B., Reimer K. A. Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation* 1986; **74:** 1124-1136.
17. Liu Y. and Downey J. M. Ischemic preconditioning protects against infarction in rat heart. *Am J Physiol* 1992; **263:** H1107-H1112.
18. Schott R. J., Rohmann S., Braun E. R., Schaper W. Ischemic preconditioning reduces infarct size in swine myocardium. *Circ Res* 1990; **66:** 1133-1142.
19. Végh Á., Szekeres L., Parratt J. R. Protective effects of preconditioning of the ischaemic myocardium involve cyclo-oxygenase products. *Cardiovasc Res* 1990; **24:** 1020-1023.

20. Li Y. W., Whittaker P., Kloner R. A. The transient nature of the effect of ischemic preconditioning on myocardial infarct size and ventricular arrhythmia. *Am Heart J* 1992; **123:** 346-353.

21. Shiki K. and Hearse D. J. Preconditioning of ischemic myocardium: reperfusion-induced arrhythmias. *Am J Physiol* 1987; **253:** H1470-H1476.

22. Hagar J. M., Hale S. L., Kloner R. A. Effect of preconditioning ischemia on reperfusion arrhythmias after coronary artery occlusion and reperfusion in the rat. *Circ Res* 1991; **68:** 61-68.

23. Schaper W. Molecular mechanisms in "stunned" myocardium. *Cardiovasc Drugs Ther* 1991; **5:** 925-932.

24. Asimakis G. K., Innersmcbride K., Conti V. R. Attenuation of postischaemic dysfunction by ischaemic preconditioning is not mediated by adenosine in the isolated rat heart. *Cardiovasc Res* 1993; **27:** 1522-1530.

25. Kaplan L. J., Bellows C. F., Blum H., Mitchell M., Whitman G. J. R. Ischemic preconditioning preserves end-ischemic ATP, enhancing functional recovery and coronary flow during reperfusion. *J Surg Res* 1994; **57:** 179-184.

26. Cave A. C. Preconditioning induced protection against post-ischaemic contractile dysfunction: Characteristics and mechanisms. *J Mol Cell Cardiol* 1995; **27:** 969-979.

27. Hearse D. J., Maxwell L., Saldanha C., Gavin J. B. The myocardial vasculature during ischaemia and reperfusion: a target for injury and protection. *J Mol Cell Cardiol* 1993; **25:** 759-800.

28. Defily D. V. and Chilian W. M. Preconditioning Protects Coronary Arteriolar Endothelium from Ischemia-Reperfusion Injury. *Am J Physiol* 1993; **265:** H700-H706.

29. Jennings R. B., Murry C. E., Reimer K. A. Energy metabolism in preconditioned and control myocardium: effect of total ischemia. *J Mol Cell Cardiol* 1991; **23:** 1449-1458.

30. Osada M., Sato T., Komori S., Tamura K. Protective effect of preconditioning on reperfusion induced ventricular arrhythmias of isolated rat hearts. *Cardiovasc Res* 1991; **25:** 441-444.

31. Liu G. S., Thornton J., Van Winkle D. M., Stanley A. W., Olsson R. A., Downey J. M. Protection against infarction afforded by preconditioning is mediated by A_1 adenosine receptors in rabbit heart. *Circulation* 1991; **84:** 350-356.

32. Omar B. A., Hanson A. K., Bose S. K., McCord J. M. Ischemic preconditioning is not mediated by free radicals in the isolated rabbit heart. *Free Radic Biol Med* 1991; **11:** 517-520.

33. Kimura Y., Iyengar J., Subramanian R., Cordis G. A., Das D. K. Preconditioning of the heart by repeated stunning: attenuation of post-ischemic dysfunction. *Basic Res Cardiol* 1992; **87:** 128-138.

34. Yellon D. M., Alkhulaifi A. M., Pugsley W. B. Preconditioning the human myocardium. *Lancet* 1993; **342:** 276-277.

35. Alkhulaifi A. M., Yellon D. M., Pugsley W. B. Preconditioning the human heart during aorto-coronary bypass surgery. *Eur J Cardiothorac Surg* 1994; **8:** 270-275.

36. Kato H., Araki T., Itoyama Y., Kogure K., Kato K. An immunohistochemical study of heat shock protein-27 in the hippocampus in a gerbil model of cerebral ischemia and ischemic tolerance. *Neuroscience* 1995; **68:** 65-71.

37. Pang C. Y., Yang R. Z., Zhong A., Xu N., Boyd B., Forrest C. R. Acute ischaemic preconditioning protects against skeletal muscle infarction in the pig. *Cardiovasc Res* 1995; **29:** 782-788.

38. Chatson G., Perdrizet G., Anderson C., Pleau M., Berman M., Schweizer R. Heat shock protects kidneys against warm ischemic injury. *Curr Surg* 1990; **47:** 420-423.

39. Végh Á., Komori S., Szekeres L., Parratt J. R. Antiarrhythmic effects of preconditioning in anaesthetised dogs and rats. *Cardiovasc Res* 1992; **26:** 487-495.

40. Kuzuya T., Hoshida S., Yamashita N., Fuji H., Oe H., Hori M., Kamada T., Tada M. Delayed effects of sublethal ischemia on the acquisition of tolerance to ischemia. *Circ Res* 1993; **72:** 1293-1299.

41. Marber M. S., Latchman D. S., Walker J. M., Yellon D. M. Cardiac stress protein elevation 24 hours after brief ischemia or heat stress is associated with resistance to myocardial infarction. *Circulation* 1993; **88:** 1264-1272.

42. Végh Á., Papp J. Gy., Kaszala K., Parratt J. R. Cardiac pacing in anaesthetised dogs preconditions the heart against arrhythmias when ischaemia is induced 24h later. *J Physiol (Lond)* 1994; **480:** 89.

43. Szekeres L., Papp J. Gy., Szilvássy Z., Udvary É., Végh Á. Moderate stress by cardiac pacing may induce both short term and long term cardioprotection. *Cardiovasc Res* 1993; **27:** 593-596.

44. Harris A. G. Delayed development of ventricular ectopic rhythms following experimental coronary occlusion. *Circulation* 1950; **1:** 1318-1326.

45. Gulker H., Kramer B., Stephan K., Meesmann W. Changes in ventricular fibrillation threshold during repeated short-term coronary occlusion and release. *Basic Res Cardiol* 1977; **72:** 547-562.

46. Barber M. J. Effect of time interval between repeated brief coronary artery occlusions on arrhythmia, electrical activity and myocardial blood flow. *J Am Coll Cardiol* 1983; **2:** 699-705.

47. Végh Á., Szekeres L., Parratt J. R. Preconditioning of the ischaemic myocardium; Involvement of the L- arginine nitric oxide pathway. *Br J Pharmacol* 1992; **107:** 648-652.

48. Kaszala K., Végh Á., Papp J. Gy., Parratt J. R. Time course of the protection against ischaemia and reperfusion-induced ventricular arrhythmias resulting from brief periods of cardiac pacing. *J Mol Cell Cardiol* 1996; **28:** 2085-2095.

49. Iwamoto T., Bai X. J., Downey H. F. Preconditioning with supply-demand imbalance limits infarct size in dog heart. *Cardiovasc Res* 1993; **27:** 2071-2076.

50. Végh Á., Szekeres L., Parratt J. R. Transient ischaemia induced by rapid cardiac pacing results in myocardial preconditioning. *Cardiovasc Res* 1991; **25:** 1051-1053.

51. Szilvássy Z., Jakab I., Bor P., Koltai M., Tarrade T., Esanu A., Braquet P. G., Lonovics J. Cicletanine attenuates overdrive pacing-induced global myocardial ischemia in rabbits - possible role of cardiac cyclic nucleotides. *Coronary Artery Dis* 1993; **4:** 443-452.

52. Ferdinándy P., Szilvássy Z., Koltai M., Dux L. Ventricular overdrive pacing-induced preconditioning and no-flow ischemia-induced preconditioning in isolated working rat hearts. *J Cardiovasc Pharmacol* 1995; **25:** 97-104.

53. Krause E. G. and Szekeres L. On the mechanism and possible therapeutic application of delayed adaptation of the heart to stress situations. *Mol Cell Biochem* 1995; **147:** 115-122.

54. Li G. C., Vasquez J. A., Gallagher K. P., Lucchesi B. R. Myocardial protection with preconditioning. *Circulation* 1990; **82:** 609-619.

55. Hale S. L. and Kloner R. A. Effect of ischemic preconditioning on regional myocardial flow in the rabbit heart. *Coronary Artery Disease* 1992; **3:** 133-140.

56. Jennings R. B. and Reimer K. A. The cell biology of acute myocardial ischemia. *Annu Rev Med* 1991; **42:** 225-246.

57. Murry C. E., Richard V. J., Reimer K. A., Jennings R. B. Ischemic preconditioning slows energy metabolism and delays ultrastructural damage during a sustained ischemic episode. *Circ Res* 1990; **66:** 913-931.

58. Tanaka M., Fujiwara H., Yamasaki K., Sasayama S. Superoxide dismutase and N-2-mercaptopropionyl glycine attenuate infarct size limitation effect of ischaemic preconditioning in the rabbit. *Cardiovasc Res* 1994; **28:** 980-986.

59. Osada M., Takeda S., Sato T., Komori S., Tamura K. The protective effect of preconditioning on reperfusion-induced arrhythmia is lost by treatment with superoxide dismutase. *Jpn Circ J* 1994; **58:** 259-263.

60. Richard V., Tron C., Thuillez C. Ischaemic preconditioning is not mediated by oxygen derived free radicals in rats. *Cardiovasc Res* 1993; **27:** 2016-2021.

61. Iwamoto T., Miura T., Adachi T., Noto T., Ogawa T., Tsuchida A., Iimura O. Myocardial infarct size-limiting effect of ischemic preconditioning was not attenuated by oxygen free-radical scavengers in the rabbit. *Circulation* 1991; **83:** 1015-1022.

62. Litt M. R., Jeremy R. W., Weisman H. F., Winkelstein J. A., Becker L. C. Neutrophil depletion limited to reperfusion reduces myocardial infarct size after 90 minutes of ischemia. Evidence for neutrophil-mediated reperfusion injury. *Circulation* 1989; **80:** 1816-1827.

63. Engler R. and Covell J. W. Granulocytes cause reperfusion ventricular dysfunction after 15-minute ischemia in the dog. *Circ Res* 1987; **61:** 20-28.

64. Liu G. S., Stanley A. W., Downey J. M. Ischaemic preconditioning is not dependent on neutrophils or glycolytic substrate at reperfusion in rabbit heart. *Cardiovasc Res* 1992; **26:** 1195-1198.

65. Bunch F. T., Thornton J., Cohen M. V., Downey J. M. Adenosine is an endogenous protectant against stunning during repetitive ischemic episodes in the heart. *Am Heart J* 1992; **124:** 1440-1446.

66. Sun W. and Wainwright C. L. The potential antiarrhythmic effects of exogenous and endogenous bradykinin in the ischaemic rat heart in vivo. *Coronary Artery Dis* 1994; **5:** 541-550.

67. Végh Á., Papp J. Gy., Parratt J. R. Attenuation of the antiarrhythmic effects of ischaemic preconditioning by blockade of bradykinin B-2 receptors. *Br J Pharmacol* 1994; **113:** 1167-1172.

68. Wall T. M., Sheehy R., Hartman J. C. Role of bradykinin in myocardial preconditioning. *J Pharmacol Exp Ther* 1994; **270:** 681-689.

69. Goto M., Liu Y. G., Yang X. M., Ardell J. L., Cohen M. V., Downey J. M. Role of bradykinin in protection of ischemic preconditioning in rabbit hearts. *Circ Res* 1995; **77:** 611-621.

70. Hoshida S., Yamashita N., Igarashi J., Nishida M., Hori M., Kamada T., Kuzuya T., Tada M. Nitric oxide synthase protects the heart against ischemia-reperfusion injury in rabbits. *J Pharmacol Exp Ther* 1995; **274:** 413-418.

71. Cooke J. P. and Tsao P. S. Cytoprotective effects of nitric oxide. *Circulation* 1993; **88:** 2451-2454.

72. Szekeres L. and Tosaki Á. Release of 6-keto-PGF1 alpha and thromboxane B_2 in late appearing cardioprotection induced by the stable PGI analogue: 7-OXO-PGI. *Mol Cell Biochem* 1993; **119:** 129-132.

73. Thornton J. D., Daly J. F., Cohen M. V., Yang X. M., Downey J. M. Catecholamines can induce adenosine receptor mediated protection of the myocardium but do not participate in ischemic preconditioning in the rabbit. *Circ Res* 1993; **73:** 649-55

74. Banerjee A., Lockewinter C., Rogers K. B., Mitchell M. B., Brew E. C., Cairns C. B., Bensard D. D., Harken A. H. Preconditioning against myocardial dysfunction after ischemia and reperfusion by an alpha-1-adrenergic mechanism. *Circ Res* 1993; **73:** 656-670.

75. Yao Z. H. and Gross G. J. Acetylcholine mimics ischemic preconditioning via a glibenclamide-sensitive mechanism in dogs. *Am J Physiol* 1993; **264:** H2221-H2225.

76. Przyklenk K. and Kloner R. A. Low-dose i. v. acetylcholine acts as a "preconditioning-mimetic" in the canine model. *J Card Surg* 1995; **10:** 389-395.

77. Végh Á., Papp J. Gy., Szekeres L., Parratt J. R. The local intracoronary administration of methylene blue prevents the pronounced antiarrhythmic effect of ischaemic preconditioning. *Br J Pharmacol* 1992; **107:** 910-911.

78. Opie L. H. Role of cyclic nucleotides in heart metabolism. *Cardiovasc Res* 1982; **16:** 483-507.

79. Piacentini L., Wainwright C. L., Parratt J. R. The antiarrhythmic effect of ischaemic preconditioning in isolated rat heart involves a pertussis toxin sensitive mechanism. *Cardiovasc Res* 1993; **27:** 674-680.

80. Thornton J. D., Liu G. S., Downey J. M. Pretreatment with pertussis toxin blocks the protective effects of preconditioning: evidence for a G-protein mechanism. *J Mol Cell Cardiol* 1993; **25:** 311-320.

81. Ito H., Hosoya Y., Inanobe A., Tomoike H., Endoh M. Acetylcholine and adenosine activate the G protein-gated muscarinic K^+ channel in ferret ventricular myocytes. *Naunyn-Schmied Arch Pharmacol* 1995; **351:** 610-617.

82. Baxter G. F., Goma F. M., Yellon D. M. Involvement of protein kinase C in the delayed cytoprotection following sublethal ischaemia in rabbit myocardium. *Br J Pharmacol* 1995; **115:** 222-224.

83. Speechlydick M. E., Mocanu M. M., Yellon D. M. Protein kinase C - Its role in ischemic preconditioning in the rat. *Circulation Research* 1994; **75:** 586-590.

84. Li Y. W. and Kloner R. A. Does protein kinase C play a role in ischemic preconditioning in rat hearts?. *Amer J Physiol-Heart Circ Phy* 1995; **37:** H426-H431.

85. Speechlydick M. E., Grover G. J., Yellon D. M. Does ischemic preconditioning in the human involve protein kinase C and the ATP-dependent K^+ channel? Studies of contractile function after simulated ischemia in an atrial in vitro model. *Circ Res* 1995; **77:** 1030-1035.

86. Végh Á., Szekeres L., Parratt J. R. Local intracoronary infusions of bradykinin profoundly reduce the severity of ischaemia-induced arrhythmias in anaesthetized dogs. *Br J Pharmacol* 1991; **104:** 294-295.

87. Végh Á., Papp J. Gy., Szekeres L., Parratt J. R. Prevention by an inhibitor of the L-arginine-nitric oxide pathway of the antiarrhythmic effects of bradykinin in anaesthetized dogs. *Br J Pharmacol* 1993; **110:** 18-19.

88. Williams D. O., Scherlag B. J., Hope R. R., el Sherif N., Lazzara R. The pathophysiology of malignant ventricular arrhythmias during acute myocardial ischemia. *Circulation* 1974; **50:** 1163-1172.

89. Végh Á., Szekeres L., Udvary É. Effect of the blood supply to the normal noninfarcted myocardium on the incidence and severity of early post-occlusion arrhythmias in dogs. *Basic Res Cardiol* 1987; **82:** 159-171.

90. Walker M. J. A., Curtis M. J., Hearse D. J., Campbell R. W. F., Janse M. J., Yellon D. M., Cobbe S. M., Coker S. J., Harness J. B., Harron D. W. G., Higgins A. J., Julian D. G., Lab M. J., Manning A. S., Northover B. J., Parratt J. R., Riemersma R. A., Riva E., Russell D. C., Sheridan D. J., Winslow E., Woodward B. The Lambeth Conventions: guidelines for the study of arrhythmias in ischaemia, infarction and reperfusion. *Cardiovasc Res* 1988; **22:** 447-455.

91. Hock F. J., Wirth K., Albus U., Linz W., Gerhards H. J., Wiemer G., Henke S., Breipohl G., Konig W., Knolle J., et al. Hoe 140 a new potent and long acting bradykinin-antagonist: in vitro studies. *Br J Pharmacol* 1991; **102:** 769-773.

92. Rees D., Palmer R., Schulz R., Hodson H., Moncada S. Characterization of three inhibitors of endothelial nitric oxide synthase in vitro and in vivo. *Br J Pharmacol* 1990; **101:** 746-752.

93. Koning M. M. G., Gho B. C. G., Klaarwater E., Opstal R. L. J., Duncker D. J., Verdouw P. D. Rapid ventricular pacing produced myocardial preconditioning by non-ischaemic activation of K-ATP channels. *Circulation* 1996; **93:** 178-186.

94. Marber M. S., Walker D. M., Eveson D. J., Walker J. M., Yellon D. M. A single five minute period of rapid atrial pacing fails to limit infarct size in the in situ rabbit heart. *Cardiovasc Res* 1993; **27:** 597-601.

95. Ovize M., Kloner R. A., Przyklenk K. Stretch preconditions canine myocardium. *Am J Physiol* 1994; **266:** H137-H146.

96. Yellon D. M. and Baxter G. F. A "second window of protection" or delayed preconditioning phenomenon: Future horizons for myocardial protection?. *J Mol Cell Cardiol* 1995; **27:** 1023-1034.

97. Belardinelli L., Linden J., Berne R. M. The cardiac effects of adenosine. *Prog Cardiovasc Dis* 1989; **32:** 73-97.

98. Wainwright C. L. and Parratt J. R. An antiarrhythmic effect of adenosine during myocardial ischaemia and reperfusion. *Eur J Pharmacol* 1988; **145:** 183-194.

99. Thornton J. D., Liu G. S., Olsson R. A., Downey J. M. Intravenous pretreatment with A_1-selective adenosine analogues protects the heart against infarction. *Circulation* 1992; **85:** 659-665.

100. Auchampach J. A. and Gross G. J. Adenosine A_1 receptors, K-ATP channels, and ischemic preconditioning in dogs. *Am J Physiol* 1993; **264:** H1327-H1336.

101. Fagbemi O. and Parratt J. R. Antiarrhythmic actions of adenosine in the early stages of experimental myocardial ischaemia. *Eur J Pharmacol* 1984; **100:** 243-244.

102. Thornton J. D., Thornton C. S., Downey J. M. Effect of adenosine receptor blockade: preventing protective preconditioning depends on time of initiation. *Am J Physiol* 1993; **265:** H504-H508.

103. Végh Á., Papp J. Gy., Parratt J. R. Pronounced antiarrhythmic effects of preconditioning in anaesthetized dogs: Is adenosine involved? *J Mol Cell Cardiol* 1995; **27:** 349-356.

104. Hashimoto K., Hirose M., Furukawa S., Hayakawa H., Kimura E. Changes in hemodynamics and bradykinin concentration in coronary sinus blood in experimental coronary artery occlusion. *Jpn Heart J* 1977; **18:** 679-689.

105. Parratt J. R., Végh Á., Papp J. Gy. Bradykinin as an endogenous myocardial protective substance with particular reference to ischemic preconditioning - A brief review of the evidence. *Can J Physiol Pharmacol* 1995; **73:** 837-842.

106. Hartman J., Wall T., Hullinger T., Shebuski R. Reduction of myocardial infarct size in rabbits by ramiprilat: reversal by the bradykinin antagonist HOE 140. *J Cardiovasc Pharmacol* 1993; **21:** 996-1003.

107. Linz W., Scholkens B., Kaiser J., Just M., Qi B., Albus U., Petry P. Cardiac arrhythmias are ameliorated by local inhibition of angiotensin formation and bradykinin degradation with the converting-enzyme inhibitor ramipril. *Cardiovasc Drugs Ther* 1989; **3:** 873-882.

108. Regoli D. Kinins. *Br Med Bull* 1987; **43:** 270-284.

109. Kelm M. and Schrader J. Control of coronary vascular tone by nitric oxide. *Circ Res* 1990; **66:** 1561-1575.

110. Palmer R. M., Ferrige A. G., Moncada S. Nitric oxide release accounts for the biological activity of endothelium-derived relaxing factor. *Nature* 1987; **327:** 524-526.

111. Linz W., Wiemer G., Scholkens B. ACE-inhibition induces NO-formation in cultured bovine endothelial cells and protects isolated ischemic rat hearts. *J Mol Cell Cardiol* 1992; **24:** 909-919.

112. Szekeres L., Pataricza J., Szilvássy Z., Udvary É., Végh Á. Cardioprotection: endogenous protective mechanisms promoted by prostacyclin. *Basic Res Cardiol* 1991; **86** (Suppl 3): 215-221.

113. Végh Á., Papp J. Gy., Szekeres L., Parratt J. R. Prevention by an inhibitor of the L-arginine-nitric oxide pathway of the antiarrhythmic effects of bradykinin in anaesthetized dogs. *Br J Pharmacol* 1993; **110:** 18-19.

114. Cooke J. P., Rossitch E., Jr., Andon N. A., Loscalzo J., Dzau V. J. Flow activates an endothelial potassium channel to release an endogenous nitrovasodilator. *J Clin Invest* 1991; **88:** 1663-1671.

115. Lamontagne D., Pohl U., Busse R. The mechanical activity of the heart stimulates endothelium-derived relaxing factor release in the coronary vascular bed. *J Cardiovasc Pharmacol* 1991; **17** (Suppl. 3): S89-S94.

116. Pabla R., Blandward P., Moore P. K., Curtis M. J. An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart. *Br J Pharmacol* 1995; **116:** 2923-2930.

117. Blatter L. A. and Wier W. G. Nitric oxide decreases $[Ca^{2+}]_{(I)}$ in vascular smooth muscle by inhibition of the calcium current. *Cell Calcium* 1994; **15:** 122-131.

118. Martin C. and Meesmann W. Antiarrhythmic effect of regional myocardial chemical sympathectomy in the early phase of coronary artery occlusion in dogs. *J Cardiovasc Pharmacol* 1985; **7** (Suppl 5): S76-S80.

119. Sharma A. D. and Corr P. B. Adrenergic factors in arrhythmogenesis in the ischemic and reperfused myocardium. *Eur Heart J* 1983; **4** (Suppl D): 79-90.

120. Knopf H., McDonald F. M., Bischoff A., Hirche H., Addicks K. Effect of propranolol on early postischemia arrhythmias and noradrenaline and potassium release of ischemic myocardium in anesthetized pigs. *J Cardiovasc Pharmacol* 1988; **12** (Suppl 1): S41-S47.

121. Ribuot C., Yamaguchi N., Godin D., Jette L., Adam A., Nadeau R. Intracoronary infusion of bradykinin: Effects on noradrenaline overflow following reperfusion of ischemic myocardium in the anesthetized dog. *Fundam Clin Pharmacol* 1994; **8:** 532-538.

122. Chahine R., Adam A., Yamaguchi N., Gaspo R., Regoli D., Nadeau R. Protective effects of bradykinin on the ischaemic heart: implication of the B1 receptor. *Br J Pharmacol* 1993; **108:** 318-322.

123. Szekeres L. On the mechanism and possible therapeutic application of delayed cardiac adaptation to stress. *Can J Cardiol* 1996; **12:** 177-185.

124. Schror K., Addicks K., Darius H., Ohlendorf R., Rosen P. PGI2 inhibits ischemia-induced platelet activation and prevents myocardial damage by inhibition of catecholamine release from adrenergic nerve terminals. Evidence for cAMP as common denominator. *Thromb Res* 1981; **21:** 175-180.

125. Thiemermann C., Steinhagen Thiessen E., Schror K. Inhibition of oxygen-centered free radical formation by the stable prostacyclin-mimetic iloprost (ZK 36 374) in acute myocardial ischemia. *J Cardiovasc Pharmacol* 1984; **6:** 365-366.

126. Szekeres L., Németh M., Papp J. Gy., Udvary É. Short incubation with 7-oxo-prostacyclin induces long lasting prolongation of repolarisation time and effective refractory period in rabbit papillary muscle preparation. *Cardiovasc Res* 1990; **24:** 37-41.

127. Wilson S., Song W., Kaszala K., Ravingerova T., Végh Á., Papp J. Gy., Tomisawa S., Parratt J. R., Pyne N. J. Delayed cardioprotection is associated with the subcellular relocalisation of ventricular protein kinase C_ε, but not p42/44MAPK. *Mol Cell Biochem* 1996; **160/161:** 225-230.

128. Kraft A. S. and Anderson W. B. Phorbol esters increase the amount of Ca^{2+}, phospholipid-dependent protein kinase associated with plasma membrane. *Nature* 1983; **301:** 621-623.

129. Brew E. C., Mitchell M. B., Rehring T. F., Gamboni Robertson F., McIntyre R. C. Jr., Harken A. H., Banerjee A. Role of bradykinin in cardiac functional protection after global ischemia-reperfusion in rat heart. *Am J Physiol* 1995; **269:** H1370-H1378.

130. Parratt J. R. and Szekeres L. Delayed protection of the heart against ischaemia. *Trends Pharmacol Sci* 1995; **16:** 351-355.

131. Yasutake M. and Avkiran M. Exacerbation of reperfusion arrhythmias by alpha(1) adrenergic stimulation: A potential role for receptor mediated activation of sarcolemmal sodium-hydrogen exchange. *Cardiovasc Res* 1995; **29:** 222-230.

132. Sterinborda L., Echague A. V., Leiros C. P., Genaro A., Borda E. Endogenous nitric oxide signalling system and the cardiac muscarinic acetylcholine receptor-inotropic response. *Br J Pharmacol* 1995; **115:** 1525-1531.

133. Lee H. C., Cai J. J., Yu H. W. Effect of protein kinase C on cyclic 3',5'-adenosine monophosphate-dependent phosphodiesterase in hypertrophic cardiomyopathic hamster hearts. *J Pharmacol Exp Ther* 1994; **270:** 1171-1176.

134. Marshall C. J. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. *Cell* 1995; **80:** 179-185.

6. Acknowledgements

I would like to thank Professor Julius Gy. Papp, Chairman of the Department of Pharmacology, Albert Szent-Györgyi Medical University, for providing me with the facilities to carry out my studies, and also for his support in development of my thesis.

I am deeply indebted to Professor Ágnes Végh, my friend and teacher, not only for her excellent scientific advices and encouragement, but also for ensuring that the past 3 years have been enjoyable as well as productive.

I must also thank Professor James R. Parratt, Chairman of Cardiovascular Research, Department of Physiology and Pharmacology, University of Strathclyde, Glasgow, who has guided and supported me in many ways, both as a friend and an adviser.

I wish to acknowledge the collaboration with Dr. Nigel Pyne and Susan Wilson within the Glasgow Department.

It is a pleasure also to acknowledge the significant help of Erica Tóth, Ágnes Krajevszki and Gábor Girst in the technical parts of my experiments.

A special thank-you goes to my family, without whom I would not have had the opportunity to complete this thesis and finally to Gabriella Szabó, for her support, understanding and patience during the past years.