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Gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischaemic preconditioning

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Abstract

Objective: The aim of this study was to determine whether uncoupling of gap junctions (GJ) prior to ischaemia would modify the antiarrhythmic effect of ischaemic preconditioning (PC) in a canine model of ischaemia/reperfusion.
Methods: Twenty control dogs, anaesthetised with chloralose and urethane, were thoracotomised and subjected either to a 25 or a 60 min occlusion of the left anterior descending (LAD) coronary artery. This prolonged ischaemia was preceded 20 min earlier by a single 5 min LAD occlusion in preconditioned dogs (PC group; $n=14$) or by a 20 min intracoronary infusion of 50 μM carbenoxolone (CBX group; $n=15$), a relatively selective uncoupler of gap junctions. CBX was also infused in PC dogs (CBX+PC group; $n=11$). The severity of ischaemia (epicardial ST-segment changes, inhomogeneity of electrical activation) and of ventricular arrhythmias, such as ventricular premature beats (VPBs), ventricular tachycardiac (VT) episodes and ventricular fibrillation (VF), as well as changes in electrical impedance was assessed throughout the experiments. Connexin 43 (Cx43) phosphorylation and GJ permeability were determined at the end of the occlusion periods.
Results: Compared to the controls PC and, interestingly, CBX markedly reduced, e.g. the total number of VPBs (440±104 vs 47±11 and 60±15; $P<0.05$) during the prolonged occlusion. This protection was, however, attenuated when CBX was infused in PC dogs (VPBs: 203±32). Changes in electrical impedance, GJ permeability and Cx43 dephosphorylation were significantly less in the PC and CBX groups than in the controls but these were again increased in the CBX+PC group.
Conclusions: Uncoupling of GJs prior to ischaemia either by PC or CBX preserves the electrical coupling of cells and results in an antiarrhythmic effect during a subsequent ischaemic insult, indicating that a partial closure of gap junctions may play a trigger role in the protection. In contrast, when CBX is administered in PC dogs the protection both against GJ uncoupling and arrhythmias is markedly attenuated, suggesting that the antiarrhythmic protection, at least in part, is mediated through GJs.

Keywords: Preconditioning; Ventricular arrhythmias; Gap junctions; Electrical impedance; Connexin 43

This article is referred to in the Editorial by Lascano and Negroni (pages 341–342) in this issue.

1. Introduction

One of the most important consequences of the abrupt reduction in coronary blood flow that results from coronary artery occlusion in both humans and experimental animals is the occurrence of those severe ventricular arrhythmias which are responsible for sudden cardiac death. It is well established that in dogs (and indeed in other large animals) these early ischaemia-induced ventricular arrhythmias occur in two distinct phases, termed as phase Ia and phase Ib [1]. There is also good evidence that the mechanisms of these two arrhythmia phases are different [reviewed in [2,3]]. Phase Ia arrhythmias, that occur between 3 to 8 min of the occlusion, are associated with rapid changes in the membrane electrical properties, derived from early metabolic and the consequent ionic alterations, which modify excitation and impulse conduction within the ischaemic tissue [4–6]. In contrast, phase Ib arrhythmias that appear at around 15 min of ischaemia and frequently terminate in ventricular

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fibrillation [1,7,8], are thought to relate to electrical uncoupling of cardiac myocytes [9], since their appearance coincides with a marked increase in tissue resistivity [10]. This arrhythmia phase, at least in dogs, lasts until 30 min of ischaemia; after this the arrhythmias fade spontaneously [8,11].

We have previously demonstrated that brief periods of coronary artery occlusion in anaesthetised dogs markedly reduce the severity of ventricular arrhythmias that occur during a subsequent, more prolonged ischaemia [8]. This protection is "real", i.e. there is an absolute reduction in the number and incidence of ventricular arrhythmias over the entire occlusion period, and there is no shift in their distribution to a later time of the occlusion [8].

In a more recent study Cinca et al. [12] have shown that in anaesthetised pigs ischaemic preconditioning delays both gap junctional uncoupling and the occurrence of phase Ib arrhythmias. This particular study, however, did not examine how the events, occurring during the preconditioning stimulus, contribute to this protection. Is the uncoupling of gap junctions, that may happen during the preconditioning ischaemia, the stimulus which would modify gap junctional coupling during the subsequent ischaemia, and result in a delay in arrhythmogenesis? Furthermore, as DeGroot and Coronel have recently pointed out "it is unknown whether either accelerated uncoupling or maintenance of gap junctional communication is antiarrhythmic" [13]. Although there is some evidence that uncoupling of gap junctions either prior to or during ischaemia limits infarct size [14,15], there have been no such studies, as yet, in relation to arrhythmias. Furthermore, Li et al. [16] have found that the infarct size limiting effect of preconditioning is abrogated by closing of gap junctions prior to preconditioning. These studies, however, used heptanol which is not a selective uncoupler of gap junctions; indeed this drug has a cardioprotective (antiischaemic) effect which is unrelated to gap junctional uncoupling [15], and particularly in higher doses it may affect other transmembrane ion currents as well [17]. More recent evidence, however, suggests that glycyrrhizic acid derivatives, such as carbenoxolone, are more selective for gap junctions than heptanol. These drugs do not have an antiischaemic effect [15] and carbenoxolone, at least in the myocardium, does not affect sodium, potassium or calcium currents [18]. Nevertheless, in non-cardiac tissues these drugs have been found to increase the expression of Hsp70 protein [19], may affect other ionic channels than gap junctions [20], and downregulate Cx43 protein and mRNA expression [21].

The present study was concerned with the question as to whether uncoupling of gap junctions prior to the prolonged ischaemia would modify arrhythmogenesis or the antiarrhythmic effect of ischaemic preconditioning in the anaesthetised dog. For this purpose, we used carbenoxolone, and the effects on arrhythmias, in comparison to changes in electrical impedance, were assessed. Gap junctional permeability and the phosphorylation status of connexin 43 (Cx43) were also determined. A preliminary account of these results was presented at the European Section Meeting of the ISHR [22] and at the World Congress of Cardiology [23].

2. Materials and methods

2.1. Surgical preparation

Adult mongrel dogs of either sex with a mean body weight of 21.2±3.2 kg were used in this study. The origin and upkeep of these dogs were in accord with Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals which conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). Under light anaesthesia (30 mg kg^{-1} intravenous pentobarbitone), the right femoral vein was catheterised through which a mixture of α-chloralose and urethane (80 and 200 mg kg^{-1}, respectively) was given to maintain anaesthesia. The dogs were then intubated and ventilated with room air using a Harvard respirator (Harvard Apparatus, Natick, MA, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits [8]. Body temperature was measured from the oesophagus and maintained by means of a heating pad between 36.5 and 37.5 °C throughout the experiments.

The surgical interventions have been described in details elsewhere [8]. In brief, catheters were inserted into the right femoral artery and, via the left carotid artery, into the left ventricle to measure arterial blood pressure (systolic and diastolic) and left ventricular pressure changes (systolic and end-diastolic) as well as positive and negative dP/dt. A thoracotomy was performed at the fifth intercostal space and the anterior descending branch of the left coronary artery (LAD) was prepared for occlusion just proximal to the first main diagonal branch. Distal to the occlusion site a small side branch of this artery was cannulated for the intracoronary administration of saline or carbenoxolone. To evaluate the severity of myocardial ischaemia, epicardial ST-segment changes and the degree of inhomogeneity of electrical activation were measured with a composite electrode positioned within the potentially ischaemic area as previously described [8,24]. This electrode gives a summarised recording of R-waves from 24 epicardial measuring points. In the normal, adequately perfused myocardium all sites are activated almost simultaneously, resulting in a single large spike. However, following occlusion widening and fractionation of this summarised R-wave occur, indicating that the adjacent fibers are not simultaneously activated because of the inhomogenous conduction [11]. We expressed this as the greatest delay in activation (in ms) within the ischaemic area. All these parameters, together with a chest lead electrocardiogram were monitored with a Plugsys Haemodynamic Apparatus (Hugo Sachs Electronics, Germany), and recorded on a Graphtec Thermal Array Recorder.

Ventricular arrhythmias were evaluated as previously outlined [8]. In brief, the total number of ventricular premature beats (VPBs), the number of episodes of ventricular

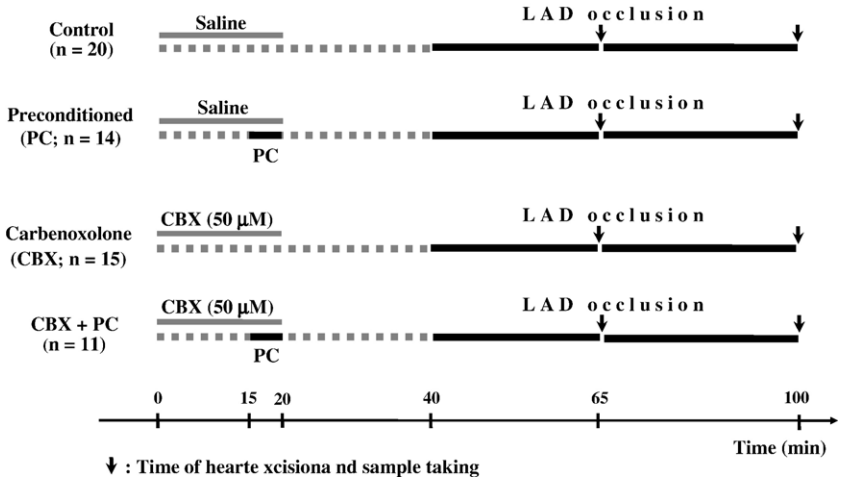

Fig. 1. Experimental protocol. Control dogs were subjected either to a 25 or a 60 min occlusion of the left anterior descending (LAD) coronary artery. This was preceded 20 min earlier by a single 5 min preconditioning occlusion (PC group) or by a 20 min intracoronary infusion of 50 μM carbenoxolone (CBX group). In another group of dogs the PC occlusion was performed during the last 5 min of CBX infusion (CBX+PC).

tachycardia (VT; defined as a run of four or more consecutive VPBs at a rate faster than the resting heart rate), and the incidence of ventricular fibrillation (VF) were assessed during the occlusion period.

2.2. Measurement of myocardial electrical impedance

Myocardial electrical impedance was measured using a method described by Padilla et al. [25]. The electrode probe consisted of four stainless steel pins (5 mm long, 0.4 mm in diameter and electrically insulated, except 1 mm at the top) mounted on a non-conductive panel with an interelectrode distance of 2.5 mm. Following calibration in saline (0.9%; resistivity: 71 Ω cm) the electrode was inserted into the ischaemic area, diagonal to the main branch of the LAD. An alternating current (10 μA, 8 kHz) was applied through the outer pair of electrodes, and voltage was measured between the inner electrode pair using a lock-in amplifier (SR830 DSP; Stanford Research Systems, California, USA). A current frequency of 8 kHz was selected in order to detect maximal changes in phase angle without impairing the assessment of resistivity [25]. Changes in resistivity (in Ω cm) and in phase angle (in °) were recorded by a computer with an acquisition time of 4 s and plotted at 1 min intervals. To eliminate small oscillations, resulting from ventilation, 5 consecutive 4 s measures were meaned at each minute.

2.3. Measurement of gap junctional metabolic coupling

This was assessed by the measurement of permeability using double dye-loading, based on a method of Ruiz-Meana et al. [26]. Freshly excised transmural tissue blocks, from both the ischaemic and non-ischaemic areas, were submerged in a mixture of Lucifer Yellow (LY, 1.5 mg ml^{-1}) and TRITC-Dextrane (TD, 3.5 mg ml^{-1}) for 15 min, followed by fixation in 4% paraformaldehyde at pH 7.4. Cryosections (25 μm) were prepared from the midmyocardial layer of the blocks and 10 fluorescent image pairs were taken from each sample with a CCD-camera connected to an Olympus IX70 fluorescent microscope. The ratio of LY and TD stained areas was calculated using the ImageJ software. Gap junction permeability within the ischaemic area was expressed as a percentage of permeability measured within the non-ischaemic wall region.

Table 1
Haemodynamic changes during coronary artery occlusion

	Control		PC		CBX		CBX+PC	
	Baseline	Change	Baseline	Change	Baseline	Change	Baseline	Change
SABP (mm Hg)	144±6	−10±1*	139±7	−7±1*	141±5	−10±1*	142±6	−10±1*
DABP (mm Hg)	91±4	−10±1*	86±4	−7±1*	89±4	−9±1*	89±6	−8±1*
MABP (mm Hg)	109±4	−10±1*	104±5	−7±1*	106±4	−9±1*	107±6	−8±1*
LVSP (mm Hg)	143±6	−11±1*	140±8	−8±1*	137±5	−11±2*	141±7	−9±1*
LVEDP (mm Hg)	7.1±0.3	8.5±0.9*	7.9±0.5	5.9±0.8*	7.3±0.7	8.1±1.0*	8.1±0.5	7.9±0.9*
+d*P*/d*t* (mm Hg/s)	3088±163	−608±66*	2860±174	−581±55*	2867±169	−621±68*	2923±149	−629±62*
−d*P*/d*t* (mm Hg/s)	2625±174	−536±60*	2426±185	−494±81*	2562±170	−455±61*	2598±123	−457±34*
HR (bpm)	163±6	2±2	162±6	4±3	160±4	2±3	162±4	−1±2

Abbreviations: SABP = systolic arterial blood pressure; DABP = diastolic arterial blood pressure; MABP = mean arterial blood pressure; LVSP = left ventricular systolic pressure; LVEDP = left ventricular end-diastolic pressure; HR = heart rate. Values are means±S.E.M.
* $P<.05$ cp baseline value.

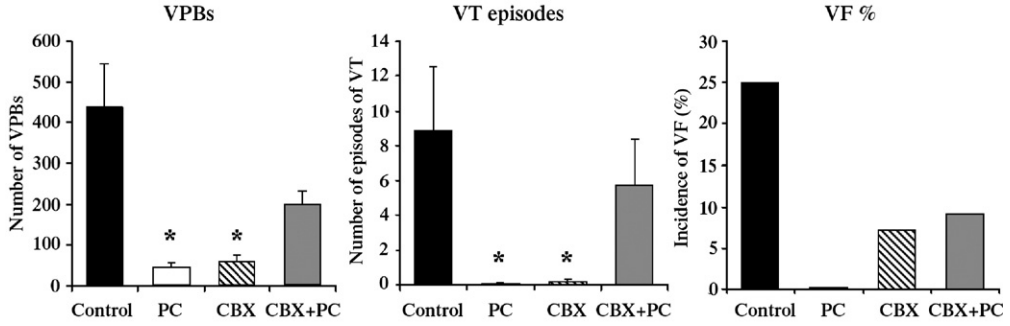

Fig. 2. Arrhythmia events (VPBs, VT episodes and VF) occurring during a 25 min occlusion of the LAD in all dogs, irrespective of the duration of the occlusion. Both PC and CBX significantly reduced the numbers of VPBs and VT episodes and suppressed the incidence of VF. This protection was attenuated in the CBX+PC group. Values are means±S.E.M. *$P<0.05$ compared with controls.

2.4. Western blot analysis of Cx43 phosphorylation

Ischaemic and non-ischaemic tissue samples were immediately frozen in liquid nitrogen and stored at −80 °C. For the isolation of membrane proteins the samples were homogenized in ice-cold lysis buffer (20 mM Tris–HCl pH 7.4, 250 mM sucrose, 0.1% protease inhibitor cocktail (Sigma), 10 mM β-mercaptoethanol, 10 mM sodium orthovanadate), and centrifuged at 2000 *g* for 15 min. The supernatant was then ultracentrifuged at 50 000 *g* for 45 min and the pellet (membrane fraction) was resuspended in lysis buffer. After the determination of protein concentration by the method of Lowry, 15 μg of each sample was separated on 12% polyacrylamide gels and transferred to PVDF membranes (Millipore). The blots were blocked with 5% non-fat milk for 1 h and immunolabeled overnight with a polyclonal rabbit anti-Cx43 primary antibody (Zymed) diluted to 1:2500. This was followed by 1 h incubation with an HRP-conjugated anti-rabbit goat secondary antibody (Santa Cruz) in a dilution of 1:8000. Blots were developed with the ECL Plus kit (Amersham) and scanned with a Typhoon laser scanner. Band intensities were determined by the Image Quant

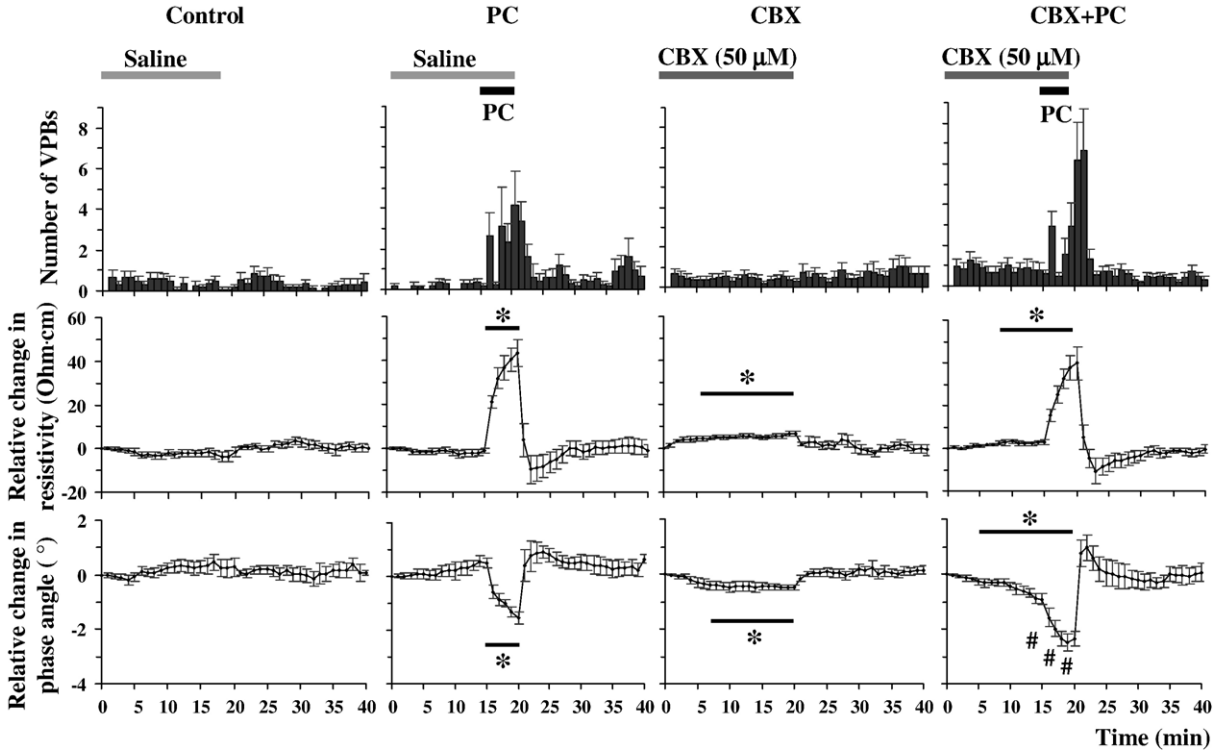

Fig. 3. Distribution of VPBs and relative changes in tissue impedance during preconditioning and carbenoxolone infusion. Compared to the infusion of saline, the local intracoronary administration of carbenoxolone slightly but significantly increased tissue resistivity and decreased phase angle. These changes were reversible, after cessation of the infusion they returned to the initial levels. The 5 min PC occlusion resulted in ectopic activity, a rise in tissue resistivity and a decline in phase angle. These impedance changes were somewhat more marked and there was a higher number of VPBs when the PC occlusion was performed in the presence of CBX infusion. Values are means±S.E.M., obtained from 8 to 12 dogs. *$P<0.05$ cp control group; $^{\#}P<0.05$ cp preconditioned group.

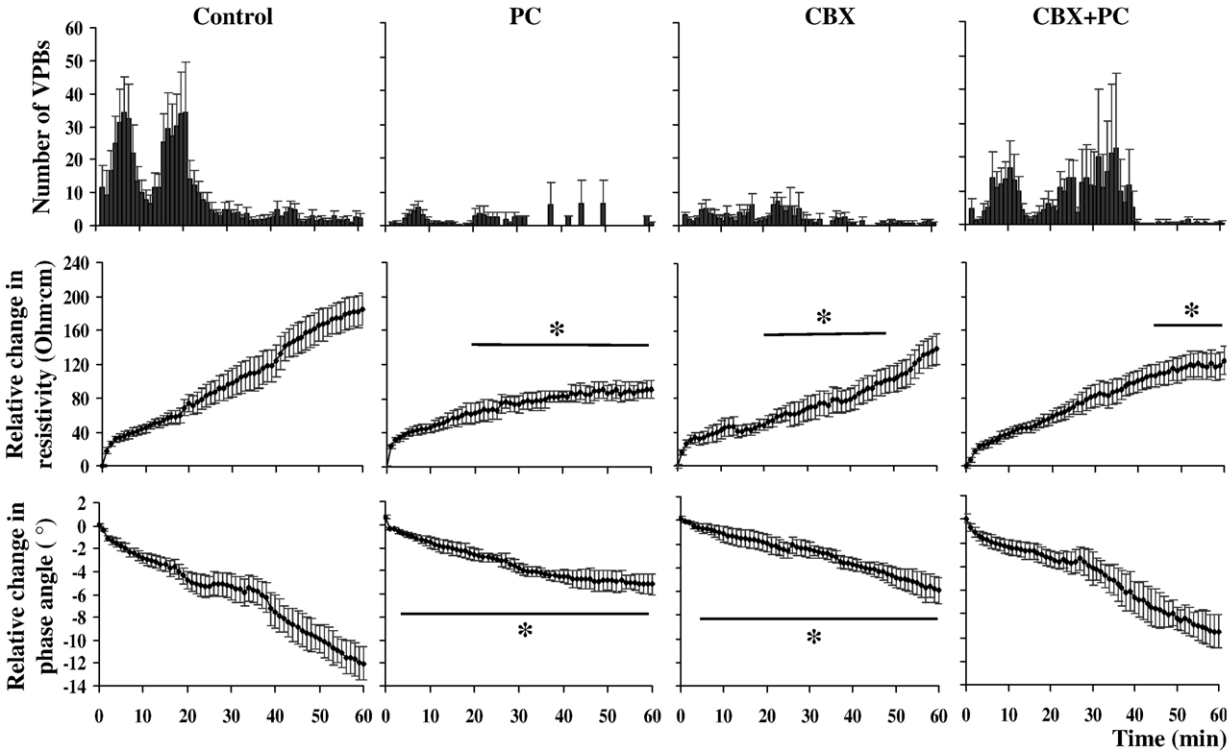

Fig. 4. Distribution of VPBs and relative changes in tissue impedance at 1 min intervals over a 60 min occlusion of the LAD. Data obtained from dogs subjected to a 25 min occlusion are also included. In control dogs a marked ectopic activity, separated in two phases, occurred during the first 30 min of the occlusion. During this period tissue resistivity and phase angle, after a marked initial increase (0–5 min), were gradually changed; an acceleration in impedance changes occurred around 17 min of ischaemia just prior to the appearance of phase Ib arrhythmias. After 30 min of the occlusion, a steep increase in resistivity and a decrease in phase angle started but by this time the ectopic activity had already disappeared. Compared to the controls, both PC and CBX significantly suppressed the number of VPBs over the entire occlusion period and reduced changes in tissue impedance. These protective effects were, however, markedly attenuated when PC was performed in the presence of CBX infusion. Values are means±S.E.M. obtained from 8 to 12 dogs. *$P<0.05$ cp control group.

software, and the relative amount of phosphorylated and non-phosphorylated Cx43 isoforms was expressed as a percentage of the total sarcolemmal connexin content.

2.5. Experimental protocols

Dogs were randomly selected into four experimental groups (Fig. 1). Control animals ($n=20$) were infused with saline locally into a side branch of the LAD for 20 min (rate: 0.5 ml min^{-1}), and 20 min later were subjected to a 25 min occlusion of the LAD. In some dogs (4 to 5 in each group) the occlusion was maintained up to 60 min in order to follow the later changes in tissue impedance. In the preconditioned group (PC; $n=14$) these prolonged ischaemic periods were preceded, 20 min earlier, by a single 5 min occlusion of the same coronary artery. Carbenoxolone (Sigma), dissolved in saline to a final concentration of 50 μM, was administered in intracoronary infusion under similar conditions as saline in another 15 control dogs (CBX group), and also in a group of preconditioned dogs (CBX+PC group; $n=11$). At the end of the ischaemic periods, without reperfusion, the hearts were arrested by intravenous administration of potassium chloride, and tissue samples were collected from both the ischaemic and the non-ischaemic ventricular regions.

2.6. Statistical analysis

All data were expressed as mean±S.E.M. and differences between means were compared by ANOVA for repeated measures or by one-way ANOVA as appropriate, using Fisher's post hoc test. The number of VPBs and VT episodes were compared with the Kruskal–Wallis rank sum test, and the incidence of VF was analysed by the Fisher's exact test. Differences were considered significant at $P<0.05$.

3. Results

3.1. Haemodynamic effects of carbenoxolone and coronary artery occlusion

Local intracoronary infusion of carbenoxolone did not substantially modify the haemodynamic parameters (data not shown). Occlusion of the LAD resulted in significant decreases in arterial blood pressure, left ventricular systolic pressure, positive and negative dP/dt, and an increase in left ventricular end-diastolic pressure whereas the heart rate was not significantly affected. These changes were similar in all experimental groups (Table 1).

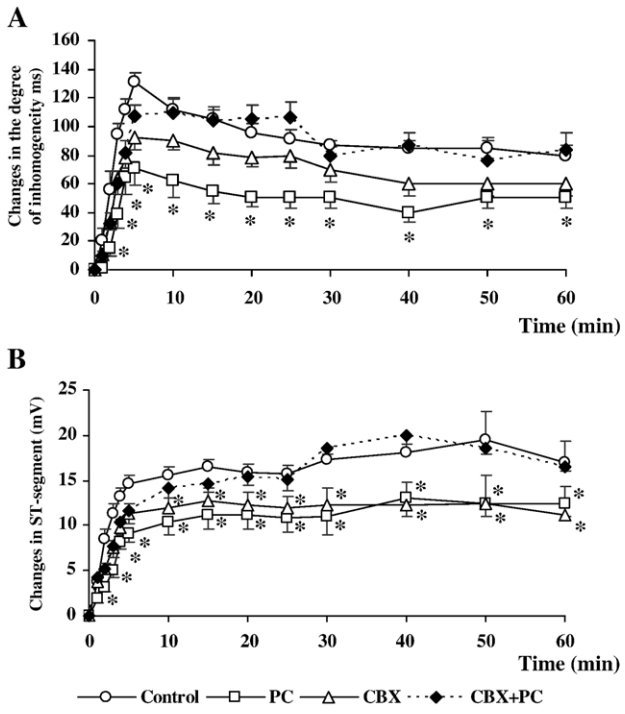

Fig. 5. Changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment during a 60 min occlusion of the LAD. In control dogs both indices of ischaemia severity were markedly increased especially during the initial 5 min of the occlusion. These ischaemic changes were markedly attenuated in the PC, and somewhat less in the CBX treated dogs, but they were again increased in the CBX+PC group. Values are means±S.E.M. *$P<0.05$ cp control group.

3.2. Severity of ventricular arrhythmias during a 25 min occlusion of the LAD

This is shown in Fig. 2. Compared to the controls preconditioning significantly reduced the total number of VPBs and the number of VT episodes during the prolonged occlusion. Interestingly, carbenoxolone, infused 20 min prior to ischaemia, also significantly suppressed these ischaemia-induced arrhythmias. In contrast, when carbenoxolone was administered in preconditioned dogs the numbers of VPBs and VT episodes were again increased. The incidence of VF was reduced in all groups but these changes were not significantly different from the controls.

3.3. Distribution of VPBs and changes in myocardial electrical impedance during pretreatment and the subsequent prolonged ischaemia

These are illustrated in Figs. 3 and 4. The mean values of resistivity and phase angle measured at baseline were 342±14 Ω cm and 9.3±2.1°, respectively. Compared to saline infusion which did not modify tissue impedance, intracoronary administration of carbenoxolone resulted in a slight but significant increase in tissue resistivity (by 6.7±1.0 Ω cm) and a decrease in phase angle (by −0.9±0.2°). These changes were reversible, after cessation of carbenoxolone infusion they returned to the initial values. Comparing the effects of preconditioning in the absence and in the presence of carbenoxolone, a somewhat more marked reduction in phase angle (−1.6±0.2 vs −2.5±0.3°) and a higher total number of VPBs (9.6±2.8 vs 19.0±5.1; $P<0.05$) occurred in dogs that were preconditioned in the presence of carbenoxolone (Fig. 3). Such a difference was not observed in tissue resistivity (43.2±6.0 vs 39.8±8.0 Ω cm).

When dogs were subjected to a prolonged coronary artery occlusion (Fig. 4), there was an immediate increase in tissue resistivity and a decrease in phase angle in the control animals, followed, a few minutes later, by severe ectopic activity (phase Ia). This phase lasted for about 7 min of ischaemia after which the number of VPBs was reduced, whereas tissue resistivity was gradually increased and phase angle decreased. A second steeper rise in resistivity and a decline in phase angle appeared at around 16 min of the occlusion (15.8±1.3 min and 16.1±1.7 min, respectively) followed by a marked increase in ectopic activity at 16.8±0.9 min (peaked at 19.1±1.0 min). These phase Ib arrhythmias faded at around 30 min of ischaemia whereas a third steep increase in resistivity and decline in phase angle started and maintained over the rest of the occlusion. Both preconditioning and carbenoxolone profoundly suppressed the number of VPBs and significantly attenuated the ischaemia-induced changes in resistivity and phase angle (Fig. 4). In these groups neither a second nor a third steep change in impedance could be observed. In contrast, when carbenoxolone was infused in preconditioned dogs, the number of VPBs was again increased, particularly during phase Ib, and the change in phase angle was almost the same as in the controls.

3.4. Changes in the degree of inhomogeneity of electrical activation and in epicardial ST-segment during coronary artery occlusion

These are shown in Fig. 5. In control dogs these indices of ischaemia severity were especially marked during the initial 5 min of the occlusion. For example, the degree of inhomogeneity, which is approximately 50 ms in the normal non-ischaemic myocardium, rapidly increased by around 130 ms within 5 min of the commencement of the occlusion. After this time the inhomogeneity was slightly decreased and maintained over the rest of the occlusion. Both preconditioning and carbenoxolone alone significantly attenuated

Table 2
Changes in gap junction permeability within the ischaemic area following a 25 and 60 min coronary artery occlusion

Gap junction permeability (% of the non-ischaemic value)				
	Control	PC	CBX	CBX+PC
25 min	60±3*	108±4	99±8	84±2*
60 min	66±6*	96±11	106±13	79±9*

Values are means±S.E.M. obtained from 5 to 6 samples in each group.
* $P<0.05$ cp non-ischaemic value.

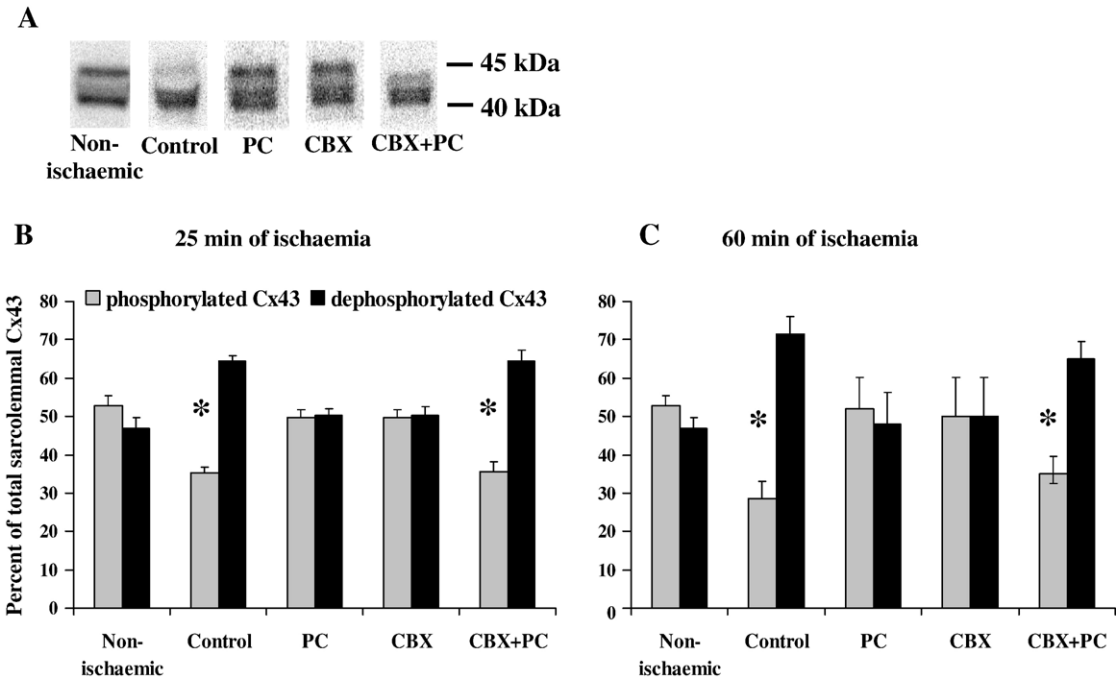

Fig. 6. A representative Western blot (A), and changes in the phosphorylated and dephosphorylated Cx43 isoforms as a percentage of the total sarcolemmal Cx43 content, assessed at 25 (B) and 60 min (C) of coronary artery occlusion. In control hearts a marked dephosphorylation of Cx43 was observed at both 25 and 60 min of ischaemia. PC and also CBX preserved the phosphorylated form of Cx43, whereas in the CBX+PC group the phosphorylation pattern of Cx43 was similar to the controls. Values are means±S.E.M., obtained from $n=3$ to 6 samples in each group. *$P<0.05$ cp control group.

epicardial ST-segment elevation and the degree of inhomogeneity but these were again increased when the two stimuli (CBX+PC) were applied together.

3.5. Changes in gap junctional permeability following coronary artery occlusion

These data are summarised in Table 2. Gap junctional permeability, assessed 25 or 60 min after coronary artery occlusion in the ischaemic area of the control hearts, was significantly decreased to around 60% of the normal non-ischaemic (100%) value. This reduction was abolished by both preconditioning and carbenoxolone, however, when preconditioning was performed during carbenoxolone infusion, permeability within the ischaemic wall region was again markedly reduced.

3.6. Changes in the phosphorylation status of Cx43 following coronary artery occlusion

These are illustrated on a representative Western blot (Fig. 6A) and summarised in Fig. 6A and B. In tissue samples taken from the non-ischaemic area the phosphorylated and the dephosphorylated forms of Cx43 were almost equally distributed. This ratio was shifted towards dephosphorylation in the ischaemic control hearts, where almost 70% of the sarcolemmal Cx43 content was dephosphorylated. This marked dephosphorylation was prevented by both preconditioning and carbenoxolone. In contrast, when preconditioning and carbenoxolone were simultaneously applied, the phosphorylation pattern of Cx43 was similar to the controls.

4. Discussion

These studies confirm our previous observations that in anaesthetised dogs a single, brief (5 min) period of coronary artery occlusion provides protection against arrhythmias occurring during a subsequent, more prolonged ischaemia [27]. We have now demonstrated that this marked antiarrhytmic effect is, at least in part, mediated through gap junctions, and associated with a reduced electrical uncoupling of cells during the prolonged ischaemia. The evidence for this comes from the result that carbenoxolone, a relatively selective gap junction uncoupler, administered prior to preconditioning increases uncoupling and attenuates the antiarrhythmic protection during a subsequent ischaemic insult. Furthermore, the fact that carbenoxolone results in similar protective effects as ischaemic preconditioning suggests that a partial and reversible closure of gap junctions prior to ischaemia may play a trigger role in the protection.

There has been recent interest concerning the role of gap junctions in the protective effects of ischaemic preconditioning [28,29]. Most of these studies, however, primarily focused on other endpoints of preconditioning than arrhythmias. There is, indeed, only one study by Cinca et al. [12] which is relevant to our experiments. They showed in anaesthetised pigs that preconditioning delays gap junctional uncoupling and

postpones the occurrence of phase Ib arrhythmias [12]. There are, however, significant differences between this and our studies. Firstly, we have consistently found in our canine model that preconditioning, induced either by brief coronary artery occlusions [8], cardiac pacing [30] or physical exercise [31], provides an absolute reduction in the number and incidence of arrhythmias, and that their appearance is not shifted to a later time of the occlusion [8]. Secondly, in dogs subjected to a longer period of coronary artery occlusion (i.e. 60 or 120 min), ventricular arrhythmias fade by the 30 min of ischaemia, and no further arrhythmia phase, as described in anaesthetised pigs [10], occurs during the rest of the occlusion. One possible explanation for this dissimilarity in arrhythmogenesis might be that in these two species the extent of the pre-existing collateral circulation is different. This assumption is also confirmed by the measurement of tissue impedance. Whereas in pigs tissue resistivity steeply increases and the phase angle markedly declines prior to the appearance of phase Ib arrhythmias [10,12], in dogs these impedance changes develop more gradually, and their acceleration, which just precedes the onset of phase Ib arrhythmias, is not as marked as in pigs. This gradually developing uncoupling of gap junctions in dogs may also explain that in this species many VPBs, couplets and salvos occur during phase Ib, in contrast to pigs where ventricular fibrillation may suddenly appear without any previous ectopic activity. There were, however, two further time intervals during the long occlusion where tissue impedance was significantly changed. An immediate increase in tissue resistivity and a decrease in phase angle could be observed at the beginning of the occlusion, when phase Ia arrhythmias were apparent. Although there is still a debate whether uncoupling of gap junctions takes place already during the early course of ischaemia [6], a number of evidence suggests that the occurrence of phase Ia arrhythmias is unlikely due to gap junctional uncoupling [2,3,9]. Indeed, in the early stage of ischaemia conduction disturbances, resulting mainly from ionic alterations [2,3,32], are the major causes of arrhythmogenesis [11,33]. These are shown, in our studies, by the rapidly developing increases in the degree of inhomogeneity and in epicardial ST-segment. Although we have no direct evidence for an early closure of gap junctions, we presume that there might be cells within the ischaemic area which are severely injured and uncoupled soon after the commencement of the coronary artery occlusion. This assumption is difficult to prove since, as the findings of Jongsma and Wilders [34] showed, a substantial portion of gap junctions needs to be closed to detect changes in tissue resistivity. However, there is some evidence, albeit in a different model, which might favour our supposition. Two recent studies, assessing the intracellular distribution of Cx43, have clearly shown that gap junctions can significantly be altered even during brief periods of ischaemia [35,36]. Thus, our proposal is that gap junctional uncoupling needs to reach a certain critical level and/or rate to contribute to conduction disturbances and to the generation of arrhythmias. At the beginning of the ischaemia this is not, as yet, sufficient to substantially affect conduction [3], however, as the ischaemia progresses the uncoupling of adjacent cells is accelerated and, perhaps, becomes inhomogeneous, resulting in meandering impulse propagation [33] and ultimately arrhythmias [37,38]. In contrast, when gap junctional uncoupling becomes even more advanced and perhaps more homogeneous, the conduction normalises [38,39] and the arrhythmias fade since the substrate for arrhythmogenesis has already waned. Such a dissociation between arrhythmias and gap junctions can be seen in dogs subjected to a 60 min occlusion, where around 30 min of ischaemia a third steep change in electrical impedance occurs whereas the arrhythmias disappear.

In the present study both preconditioning and, interestingly, carbenoxolone almost equally protected against arrhythmias and modified tissue impedance during the prolonged ischaemia. In both cases, changes in tissue resistivity and phase angle were much less than in the controls, indicating that a reduced (or perhaps a slower) gap junctional uncoupling is responsible for the antiarrhythmic effect. There were, however, differences between preconditioning and carbenoxolone in modifying these ischaemia-induced changes in tissue impedance. Whereas preconditioning completely abolished the marked changes in resistivity and phase angle that occurred in control dogs, carbenoxolone only shifted these to a later time of the occlusion (Fig. 4). Furthermore, carbenoxolone was less effective in reducing inhomogeneity and ST-segment elevation than preconditioning. We do not know the explanation for these differences, but it might well be that the preconditioning ischaemia is a stronger stimulus for inducing protection than the infusion of carbenoxolone. This assumption is supported by the impedance changes which were more marked during preconditioning than during carbenoxolone treatment. Nevertheless, the potential antiarrhythmic effect of carbenoxolone might be of some interest. The fact that carbenoxolone results in a slight but detectable change in tissue impedance suggests that gap junctions are closing during the infusion period. We propose that this partial and reversible closure of gap junctions, in the absence of ischaemia, might act as a trigger, and induce such processes which then prevent gap junctions from further uncoupling and result in antiarrhythmic protection during the prolonged occlusion. We are aware that the precise mechanisms of ischaemia-induced re-entry type arrhythmias or their modification by an antiarrhythmic procedure cannot be explained without measuring the actual values of impulse conduction and refractoriness. Therefore, we can only speculate that carbenoxolone, by inhibiting the further uncoupling of cells, modifies the gap junction-mediated slowing of impulse conduction in such a way that the formation of re-entry circuits within the ischaemic and/or border areas is prevented.

Although we do not know how this partial and reversible closure of gap junctions occurring during carbenoxolone infusion and, perhaps also during preconditioning would result in a reduced uncoupling during the subsequent ischaemia, it seems likely that this effect can somehow be connected to changes in the Cx43 protein itself. There is some recent evidence that this protein, besides the pore forming and

gating properties, may also play a part in signal transduction [28,40]. In our own studies, the marked dephosphorylation of Cx43 that occurred within the ischaemic area of the control hearts was shifted towards phosphorylation again by both carbenoxolone and preconditioning. Although it is still not known precisely how the phosphorylation of Cx43 modifies gap junction function, a number of evidence suggest that ischaemia results in dephosphorylation of Cx43 [41,42], and preconditioning, by preserving the phosphorylated form of Cx43 [28], attenuates gap junctional uncoupling and thus results in protection. This attenuated gap junctional uncoupling following preconditioning and carbenoxolone is also confirmed by permeability measurements showing a preserved metabolic coupling of cells within the ischaemic area.

Our starting hypothesis was that if the preservation of gap junctional coupling plays a role in the antiarrhythmic effect of preconditioning [12], then a stimulus, which is known to uncouple gap junctions, would attenuate or abolish this protection. Indeed, when carbenoxolone was infused in preconditioned dogs the antiarrhythmic effect was attenuated, i.e. the number of VBPs was again increased particularly during phase Ib, and the decrease in phase angle was almost the same as in the controls. However, it should be noted that in some dogs there were still signs of the preconditioning-induced protection. For example, the development of inhomogeneity and ST-segment elevation during the initial 5 min of ischaemia, as well as changes in tissue resistivity were rather similar to the preconditioned dogs. In contrast, the phosphorylation pattern of Cx43 was almost the same as in the controls, and gap junction permeability was again reduced. There are a number of possible explanations for the attenuation of the protection. One of these might be that closing of gap junctions prior to preconditioning inhibits the transport of mediators which are generated during the preconditioning procedure [43,44], and use gap junctions for their propagation. Thus, inhibition of their transfer would influence signaling pathways leading to protection [16,45]. It may also be likely that in the presence of carbenoxolone infusion preconditioning results in a stronger uncoupling of gap junctions than either preconditioning or carbenoxolone alone. This stronger uncoupling then initiates such processes which lead to the attenuation or loss of the protection. This assumption is supported by the results which clearly show that in the presence of carbenoxolone infusion preconditioning results in a more marked decline in phase angle and a significantly higher number of VPBs than in the absence of carbenoxolone. Such an adverse effect is not surprising since there is good evidence that a stronger preconditioning stimulus abrogates rather than induces protection [8,46,47].

We conclude that the maintenance of the electrical and metabolic coupling of gap junctions during ischaemia may contribute to the antiarrhythmic effects of ischaemic preconditioning. This protection is, however, attenuated if carbenoxolone is administered prior to the preconditioning occlusion. Furthermore, we also suggest that uncoupling of gap junctions prior to ischaemia may play a trigger role in the protection, since carbenoxolone itself, simply by closing a part of gap junctions, prevents these channels from further uncoupling during the prolonged ischaemia and results in antiarrhythmic protection similar to preconditioning.

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