Role of gap junctions in acute ischaemia-induced arrhythmias and in the antiarrhythmic effect of ischaemic preconditioning

PhD Thesis

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LIST OF PUBLICATIONS

Full papers

I. **Papp R,** Gönczi M, Kovács M, Seprényi G, Végh Á. Gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischaemic preconditioning. Cardiovasc Res 2007; 74: 396-405.

II. **Papp R**, Gönczi M, Végh Á. A réskapcsolatok szerepe akut miokardiális iszkémia okozta aritmiákban. Cardiol Hung 2008; 38: 109-15.
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Abstracts

1. Abstracts of oral presentations

Carbenoxolone, a gap junction uncoupler, reduces the antiarrhythmic effect of preconditioning.
R Papp, M Gönczi, JR Parratt, Á Végh.
International Society for Heart Research (ISHR) European Section Meeting, 2005.

Carbenoxolone, a gap junction uncoupler, mimics the antiarrhythmic effects of ischaemic preconditioning.
R Papp, M Gönczi, Á Végh.
World Congress of Cardiology, Barcelona, 2006.

A réskapcsolatok szerepe a prekondicionálás antiaritmiás hatásának kiváltásában.
Papp R, Gönczi M, Kovács M, Seprényi Gy, Végh Á.
Magyar Kardiológus Társaság Kongresszusa, 2007.

A rotigaptide védelmet biztosít az akut iszkémia okozta kamrai aritmiákkal szemben altatott kutyában.
Papp R, Gönczi M, Végh Á.
Magyar Kardiológus Társaság Kongresszusa, 2008.

2. Abstracts of poster presentations

A réskapcsolatokat záró carbenoxolone gyengíti a prekondicionálás antiaritmiás hatását
Papp R, Kovács M, Gönczi M, Seprényi Gy, Végh Á.
Magyar Élettani Társaság Vándorgyűlése, 2006.

A gap junction blokkoló carbenoxolone csökkenti az iszkémiás prekondicionálás antiaritmiás hatását.
Papp R, Gönczi M, Végh Á.
Magyar Farmakológus Társaság Tavaszi Szimpóziuma, 2005.

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R Papp, M Gönczi, Á Végh.
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Quantification of the surface expression of ion channel and gap junction proteins on cardiomyocytes with confocal microscopy.
G Seprényi, **R Papp,** M Kovács, K Acsai, Á Végh, A Varró.
International Society for Heart Research (ISHR) European Section Meeting, 2006.

Involvement of gap junctions in the antiarrhythmic effect of preconditioning: a molecular study.
R Papp, M Kovács, M Gönczi, G Seprényi, Á Végh.
International Symposium on Myocardial Cytoprotection, 2006.

A réskapcsolatok szerepe az akut iszkémia okozta aritmiákban és a prekondicionálás antiaritmiás hatásában.
Papp R, Gönczi M, Kovács M, Seprényi Gy, Végh Á.
Magyar Élettani Társaság Vándorgyűlése, 2007.

Protective effect of rotigaptide against acute ischaemia-induced arrhythmias in dogs.
R Papp, M Gönczi, Á Végh.
International Society for Heart Research (ISHR) European Section Meeting, 2008.

SUMMARY

Gap junctions, as low-resistance pathways for intercellular communication, have been suggested to play a role in acute ischaemia-induced arrhythmias and also in the antiarrhythmic effect of ischaemic preconditioning. However, the mechanism of their contribution has not been clarified.

Therefore, we aimed (1) to examine gap junction function, changes in impulse conduction and the severity of arrhythmias during a 60 min occlusion of the LAD; (2) to evaluate the effects of gap junction coupler/uncoupler drugs (rotigaptide and carbenoxolone) applied before and continuously during ischaemia; (3) to assess the effect of preconditioning on ischaemia-induced uncoupling, conduction impairments and arrhythmias, and (4) to study the possible trigger role of gap junctions in ischaemic preconditioning with the use of gap junction modifier drugs. The severity of arrhythmias and changes in impulse conduction were investigated in anaesthetised, open-chest dogs whereas gap junction function was studied both *in vivo* and *in vitro*.

We have confirmed the role of gap junctional uncoupling in the 1b phase of acute ischaemia-induced arrhythmias but also pointed out that closure of gap junctions can lead to arrhythmias only during a certain period of ischaemia. The use of gap junction modifier drugs corroborated that the timing of gap junctional uncoupling is crucial in arrhythmogenesis.

We have shown that improvement in impulse conduction due to reduced gap junctional uncoupling contributes to the antiarrhythmic effect of preconditioning.

We have also found that whereas the gap junction opener rotigaptide did not influence, the gap junction uncoupler carbenoxolone attenuated the protective effect of preconditioning when it was applied during the preconditioning ischaemia. This emphasizes the role of gap junctions in the transfer of those endogenous mediators, which are released by the preconditioning stimulus and induce the protective effect. The fact that carbenoxolone, applied by itself before the sustained ischaemia, resulted in a protective effect similar to preconditioning, raised the supposition that gap junctional uncoupling or some other function of connexin proteins may also serve as a trigger for preconditioning.

These experiments show that modification of gap junctions either prior to or during ischaemia significantly influence the severity of ischaemia-induced arrhythmias.

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LIST OF ABBREVIATIONS

ARI	Activation-recovery interval
ARI_{n400}	Activation-recovery interval normalized to a cycle length of 400 ms
CBX	Carbenoxolone
CL	Cycle length
Cx43	Connexin 43
DABP	Diastolic arterial blood pressure
HR	Heart rate
HRP	Horseradish peroxidase
LAD	Left anterior descending coronary artery
LVEDP	Left ventricular end-diastolic pressure
LVSP	Left ventricular systolic pressure
LY	Lucifer Yellow
MABP	Mean arterial blood pressure
PC	Preconditioning
PVDF	Polyvinylidene Fluoride
RG	Rotigaptide
SABP	Systolic arterial blood pressure
TAT	Total activation time
TAT_{n400}	Total activation time normalized to a cycle length of 400 ms
TRITC	Tetramethyl-rhodamine isothyocyanate
VF	Ventricular fibrillation
VPB	Ventricular premature beat
VT	Ventricular tachycardia

1. INTRODUCTION

Sudden cardiac death, resulting from malignant ventricular arrhythmias of acute myocardial ischaemia, is one of the major causes of death in the developed countries. Unfortunately, currently available antiarrhythmic drugs, which usually target cardiac ion channels and pumps, are not adequately safe since they may also induce severe arrhythmias, especially under ischaemic conditions. Therefore, interventions either targeting structures other than sarcolemmal ion channels, such as gap junctions, or utilizing endogenous adaptive mechanisms of the heart such as ischaemic preconditioning, could be the bases of new therapeutic strategies to prevent and/or attenuate acute ischaemia-induced ventricular arrhythmias.

1.1 Time course and mechanisms of acute ischaemia-induced arrhythmias

Acute myocardial ischaemia, resulting from severe stenosis or complete occlusion of a coronary artery, is usually accompanied by such metabolic and electrophysiological alterations in the affected tissue that lead to severe, often lethal ventricular arrhythmias. In large animals, and probably also in humans, these arrhythmias are most severe during the first 30 min of ischaemia and occur in two distinct phases [1]. The 1a phase of these arrhythmias appears between 3 and 8 min of ischaemia; this is followed by a short arrhythmia-free period and, around 15 min of ischaemia, by a new arrhythmia phase. This 1b phase of arrhythmias, during which the risk of ventricular fibrillation is the highest, lasts up to 30 min of ischaemia. Thereafter, ectopic activity diminishes and remains low; there are no or only a few and less malignant arrhythmias in the subacute phase (24-72 hours of ischaemia).

There are different mechanisms underlying the two phases of early arrhythmias. Already within the first minutes of ischaemia, the lack of glucose and oxygen supply results in intracellular acidosis and a reduction of high-energy phosphate deposits [2, 3]. Since the energy produced by intact cellular metabolism is essential for the proper function of membrane ion transporters, this ischaemia-induced energy deficit rapidly leads to severe ionic imbalance, including the loss of K^+ and an increase in intracellular Na^+ and Ca^{2+} [4]. The subsequent depolarization, further enhanced by the accumulation of lysophosphoglycerides [5], partially inactivates the Na^+ channels and produces those conduction abnormalities, which are responsible for the phase 1a of ischaemia-induced arrhythmias [6].

The causes of the short arrhythmia-free period between phase 1a and 1b are less known but it is usually explained by the enhancement of membrane pump function as catecholamines are released from sympathetic nerve endings [7, 8]. However, this mechanism can counteract the ischaemic damage only for several minutes and totally depletes the intracellular energy sources.

During the 1b phase, both the loss of ATP and further accumulation of catecholamines end in intracellular Ca^{2+} overload [7], which may enhance triggered activity [9, 10]. Catecholamines are arrhythmogenic also by increasing the spatial heterogeneity of the effective refractory period throughout the border of the ischaemic and the normally perfused myocardium [11]. The closure of gap junctions, channels specialized for direct intercellular communication, is also suggested to be an important determinant of phase 1b arrhythmias.

1.2 Structure, function and pharmacology of myocardial gap junctions

Gap junctions are low-resistance pathways for direct intercellular communication, allowing the exchange of molecules smaller than 1 kDa with minor selectivity for charge or molecule weight. These structures, together with other cell adhesion proteins, are packed into gap junction plaques, which are located predominantly at the end-to-end connections of neighbouring cells. To form a direct cytoplasmic connection, each cell provides a hemichannel, a hexamer of connexin protein subunits.

Connexins are membrane proteins with four transmembrane domains and intracellular N- and C-terminals. Based on their molecule weight, more than 20 connexin isoforms have been identified [12], three of which are abundant in the heart muscle. Connexin 40 and 45 occur predominantly in the atria and in the conduction system, whereas connexin 43 (Cx43) is the main gap junction forming protein in the working myocardium of the ventricles [13].

These isoforms differ mainly in the length of their C terminal, a domain that contains several Ser and Tyr amino acid residues as targets of phosphorylation/dephosphorylation by several kinases and phosphatases of the cellular signal transduction cascades [14, 15, 16]. Phosphorylation/dephosphorylation regulates both membrane delivery, internalization, degradation, opening/closure and conductance of gap junctions [16]. On the intracellular loop, there is a pH sensor sequence as well; this domain is responsible for the closure of gap junctions at low pH [17]. Furthermore, many other factors, which indicate the metabolic state of the cell and are altered by ischaemia (intracellular Ca^{2+}, ATP and lipid metabolites), are able to regulate the function of these intercellular connective structures [18, 19, 20].

Besides cellular signalling and metabolism, there are many agents that can be used as pharmacological modifiers of gap junction function. Volatile anaesthetics, some long-chain fatty acids and polyalcohols such as palmitoleic acid and heptanol, dicoumarol, glycyrrhetinic acid and its derivatives, as well as peptides mimicking the extracellular domains of connexins are all known to uncouple gap junctions [21]. The gap junction specificity of these substances, however, largely differs. For example, heptanol may affect other ion channels especially in doses higher than 0.5 mM [22], whereas the glycyrrhetinic acid derivative carbenoxolone is reported to be rather selective for gap junctions [23, 24].

There are pharmacological means to keep gap junctions open as well, even in the presence of triggers for gap junction closure. In 1980, an antiarrhythmic peptide with gap junction coupler properties was found in bovine atria [25]. Synthetic analogues of this peptide are also shown to maintain gap junctional communication under such circumstances which otherwise favour uncoupling [26]. Rotigaptide (formerly known as ZP123), due to its increased protease resistivity afforded by the D-amino acids contained in the molecule, is known to be the most stable synthetic antiarrhythmic peptide [27].

The importance of the cellular regulation and/or pharmacological modulation of gap junction function lies not only in the direct metabolic connection they provide, but also in their role in cardiac impulse propagation, therefore impairments of gap junction function may lead to severe arrhythmias.

1.3 Role of gap junctions in acute ischaemia-induced arrhythmias

During the course of acute myocardial ischaemia, the loss of ATP, intracellular acidosis, as well as accumulation of lipid metabolites and intracellular Ca^{2+} trigger gap junctional uncoupling. This is clearly exemplified by that the increase in intracellular Ca^{2+} coincide with a steep increase in tissue resistivity, a parameter indicating gap junctional uncoupling in the ischaemic rabbit papillary muscle [28]. In the same model, Kléber *et al.* [29] used the cable theory to separate extra- and intracellular components of tissue resistivity and provided further evidence that increase in intracellular resistivity, a more proper indicator of gap junctional uncoupling, starts at around 15 min of ischaemia. Furthermore, it has also been demonstrated in anaesthetised pigs that gap junctional uncoupling, determined by a steep rise in tissue impedance, coincided with the appearance of 1b arrhythmias [30].

The role of gap junctions in arrhythmogenesis can also be studied by pharmacological modulation of gap junctional coupling. In myocyte strands *in vitro* [31], as well as in isolated

rabbit hearts [32], uncoupling of gap junctions by palmitoleic acid results in a markedly slower and meandering impulse conduction. Slowing of impulse conduction in the presence of the relatively selective gap junction uncoupler, carbenoxolone, has also been demonstrated in both isolated rabbit hearts [23] and human hearts *in vivo* [33]. Conduction slowing mediated by gap junctional uncoupling may favour the formation of reentry by decreasing its wavelength (the product of conduction velocity and the effective refractory period). Indeed, in genetically engineered mice in which the expression of connexin 43 was substantially reduced, an increased propensity for arrhythmias could be observed [34], especially if the loss of Cx43 expression was heterogenous throughout the heart [35, 36] or if those mice were subjected to myocardial ischaemia [37]. There is also evidence that reentrant arrhythmias triggered after 3 hours of ischaemia [38], or formed spontaneously during reperfusion [39], as well as ouabain-, hypokalaemia-, acidosis- or ischaemia-induced conduction slowing were effectively attenuated by the gap junction coupler rotigaptide [27, 40, 41].

It must be noted that on one hand, closure of gap junctions by isolating dying, unexcitable myocytes from their surviving, excitable neighbours may limit both infarct size [42, 43] and the spread of triggered activity [10, 44]. On the other hand, however, it may end in severe, even life-threatening ventricular arrhythmias, which provide a reason for the research of drugs or other treatments to reduce these arrhythmias.

1.4 The antiarrhythmic effect of ischaemic preconditioning and possible role of gap junctions in the protection

In 1986, Murry and his co-workers reported that short, sublethal cycles of myocardial ischaemia/reperfusion, prevent both the loss of ATP and cell death during a subsequent, more prolonged ischaemic insult [45]. This phenomenon has been termed as ischaemic preconditioning. It has also been shown that preconditioning not only reduces ischaemia/reperfusion induced cell death (infarct size) [45, 46], but improves cardiac function [47], and markedly reduces ventricular arrhythmias [48] both during ischaemia and reperfusion. This protection lasts for only 1 or 2 hours after the preconditioning stimulus (early phase or first window of protection), but reappears 16-24 hours later and lasts for 48-72 hours (late phase or second window of protection) [49, 50, 51]. The present study, however, focused only on the early phase of preconditioning-induced protection.

During the preconditioning stimulus, many endogenous substances (such as adenosine, bradykinin, reactive oxygen species, nitric oxide [51, 52, 53]) have been proved to be released

and, by the activation of cellular signalling cascades, induce protection (triggers). Although the precise mechanisms by which preconditioning exerts its protective effect are not fully understood, the proposed end-effectors include sarcolemmal ion channels/transporters, antiapoptotic- and mitochondrial proteins [51, 53, 54].

Gap junctions, as key factors in cardiac impulse propagation and arrhythmogenesis, are also suggested to participate in preconditioning. However, there is only one study, which examined directly the role of gap junctions in the antiarrhythmic effect of ischaemic preconditioning: Cinca *et al.* [55] have shown that in the *in vivo* pig heart, both the occurrence of phase 1b arrhythmias and gap junctional uncoupling (determined as a steep rise in tissue resistivity) were delayed by preconditioning. Although Shome *et al.* [56] have also suggested that gap junctional uncoupling is reduced by preconditioning in anaesthetised dogs, they concluded to the extent of gap junctional uncoupling only from the decrease in ST-segment elevation due to increased intercellular resistance and from changes in conduction velocity, instead of any, more direct measurement.

The concept that several endogenous substances are released during the trigger phase of preconditioning leads to the supposition that gap junctions, by facilitating the propagation of at least some of these substances, may play a role already in the induction of the protective effect. Indeed, Li *et al.* [57] have found that heptanol, administered in a dose of 0.5 mM before the preconditioning stimulus, abrogated the infarct size-reducing effect of preconditioning. Similarly, in isolated rabbit hearts, IP_3-induced cytoprotection was completely abolished if gap junctions were simultaneously uncoupled by heptanol or by the peptide Gap27 [58]. Unfortunately, in these studies only infarct size was determined as an endpoint of the protective effect and, as to our present knowledge, there is no study on the trigger role of gap junctions in the antiarrhythmic effect of preconditioning.

1.5 Aims of the study

On the basis of our presently incomplete knowledge about the role of gap junctions in acute ischaemia-induced arrhythmias, and of the potential benefits of gap junction modification, either by drugs or by ischaemic preconditioning as tools to reduce these arrhythmias, this study aimed:

(1) To assess the role of gap junctions in ischaemia-induced arrhythmias in anaesthetised, open-chest dogs by comparing arrhythmia severity and changes in impulse conduction with gap junction function;

(2) To examine the effect of gap junction coupler/uncoupler drugs, given prior to and during ischaemia on these parameters, thus to further investigate the importance of gap junction function in arrhythmogenesis;

(3) To study the effects of ischaemic preconditioning on ischaemia-induced gap junctional uncoupling, conduction abnormalities and ventricular arrhythmias in this model;

(4) To reveal how gap junctions, if they do, contribute to the trigger phase of ischaemic preconditioning.

2. MATERIALS AND METHODS

2.1 Experimental animals

Adult mongrel dogs of either sex weighing between 8.5 and 46 kg (mean 21 ± 7 kg) were used in these studies. The origin and upkeep of the dogs were in accordance with Hungarian law (XXVIII, chapter IV, paragraph 31) regarding large experimental animals which conforms to EU laws.

2.2 Surgical preparation

Anaesthesia was induced by pentobarbital (30 mg/kg, i.v.) and maintained with a mixture of chloralose and urethane (60 and 200 mg/kg, respectively) administered into the femoral vein. Catheters were inserted into the right femoral artery and, via the left carotid artery, into the cavity of the left ventricle to measure arterial blood pressure and left ventricular pressure changes. From left ventricular pressure changes, positive and negative dP/dt_{max} values were calculated to evaluate ventricular contraction and relaxation. All these parameters were measured with a Plugsys Haemodynamic Apparatus (Hugo Sachs Electronics, Germany) and registered on a Graphtec Thermal Array Recorder.

Dogs were intubated and ventilated with room air with a Harvard respirator (Harvard Apparatus, Natick, MA, USA) at a rate and volume sufficient to maintain arterial blood gases and pH within physiological limits. The chest and the pericardium were opened and the left anterior descending coronary artery (LAD), proximal to its first diagonal branch, was prepared for occlusion. Distal to the occlusion site, a cannula was introduced into a small side branch of the LAD for drug administration. After surgery, the chest was covered to avoid cooling and drying. Body temperature was regularly monitored and kept at 37 ± 0.5 °C by means of a heating pad.

2.3 Evaluation of ventricular arrhythmias

Ventricular arrhythmias were evaluated from a lead II ECG according to the Lambeth conventions [59], modified as follows: the numbers of ventricular premature beats (VPBs) and tachycardiac episodes (VT, defined as four of more consecutive VPBs) as well as the incidence of ventricular fibrillation (VF%) were assessed.

2.4 Electrophysiological techniques to assess conduction and action potential properties

In some of the experiments, a composite electrode was sutured onto the ventricular surface expected to be ischaemic upon LAD occlusion. The composite electrode gives a summerized recording of bipolar electrograms measured from 24 epicardial points, resulting in a single large spike in case of normal activation, whereas it is prolonged and fractionated if the activation is slow and/or inhomogeneous. This signal was measured by a custom-made amplifier unit and recorded on a Graphtec Thermal Array Recorder; the inhomogeneity of electrical activation was expressed as the duration of the signal in ms.

In the rest of the experiments, instead of the composite electrode, an activation mapping electrode containing 31 unipolar leads was sutured above the ischaemic area. The 31 unipolar ECGs, together with the lead II ECG, were recorded on a computer and analysed off-line. Activation maps, showing local activation times in ms for each electrode point, were created from representative normal beats in every 2 min of the experiments. Local activation times were defined as the dV/dt_{min} of the QRS complex; the beginning of the QRS on the lead II ECG was considered as time zero. From activation maps, the total activation time (TAT) was determined as the time difference between the first and the last activation and normalized for the average cycle length of 400 ms (TAT_{n400}). In order to exclude the potential effect of rotigaptide on action potential upstroke velocity, dV/dt_{min} values of the unipolar recordings (reflecting the dV/dt_{max} of the local action potential) were measured from five randomly selected electrode points at baseline and after 10 min of rotigaptide (1 μg/kg/min) infusion. On the same manner, activation-recovery intervals (ARI), which are proportional to action potential duration, were also determined as the difference between the local activation and local repolarization times (the latter assessed as the dV/dt_{max} of the T wave) and normalized to the average cycle length of 400 ms.

2.5 Measurement of myocardial electrical impedance

Tissue impedance was measured with a four-pinned electrode (for detailed description of the electrode, see [60]) inserted into the center of the potentially ischaemic myocardium and fixed to the epicardium with sutures. The whole electrode was electrically insulated except for the tip of the electrode pins, which were in contact with the midmyocardium. Through the outer pair of electrode pins, a 10 μA alternating current with a frequency of 8 kHz was applied.

Voltage was measured between the inner pair of electrodes with a custom-made signal processing unit connected to a lock-in amplifier (SR830 DSP; Stanford Research Systems, California, USA). According to the concept of impedance measurement, gap junctional uncoupling increases the capacity of the cell membranes, thus increases resistivity and shifts the voltage curve to the right compared to the current. This phase delay can be expressed in degrees, the more negative the phase angle value, the more pronounced is the phase delay induced by gap junctional uncoupling. Raw data, together with tissue resistivity (in Ohm·cm) and phase angle values (in °) gained in every 4 sec, were stored on a computer. During off-line analysis, relative changes in resistivity and phase angle were plotted at 1 min intervals. To attenuate small oscillations due to ventilation movements, the mean of five consecutive 4-sec measurements was used for each minute.

2.6 Myocardial tissue sampling at the end of the occlusion

At the end of the occlusion period, hearts were arrested by an intravenous bolus of saturated KCl, excised rapidly and washed in ice-cold saline.

For the measurement of gap junctional metabolic coupling, solutions containing both Lucifer Yellow (Sigma, 1.5 mg/ml) and TRITC-dextrane (Sigma, 3.5 mg/ml) were prepared previously with Tyrode solution and stored at -20 °C until use. Freshly excised transmural tissue blocks originating from both the ischaemic region and a further, non-ischaemic region of the left ventricle were submerged into the dye mix for 15 min at 20 °C; the solution containing the normoxic sample was oxygenated during the staining period. After staining, samples were fixed in 4% paraformaldehyde and stored at 4 °C.

For the purpose of western blot analysis, transmural tissue samples from both the ischaemic and a further, non-ischaemic area of the left ventricle, were excised and snap-frozen in liquid nitrogen, then stored at -80 °C until use.

2.7 Assessment of gap junctional metabolic coupling

Samples stained and fixed previously, were dehydrated (30% sucrose, for at least 1 day) and 25 μm sections from the midmyocardial layer were made at -20 °C in a cryostat (Leica). 10 image pairs per sample were taken with a fluorescent microscope (Olympus IX 70) coupled to a CCD-camera and the penetration of the two dyes was quantified with the ImageJ software by expressing the areas stained by Lucifer Yellow (LY) and TRITC-dextrane (TD) in square

pixels. Since the penetration of the small-molecule weight Lucifer Yellow depends on the open/closed state of gap junctions, whereas large molecules of TRITC-dextrane label only cells injured during sample excision, gap junctional metabolic coupling can be determined as the ratio of LY/TD areas. Gap junction permeability measured in the ischaemic samples was expressed as a percentage of the permeability measured in the non-ischaemic self-controls.

2.8 Western blot analysis of the phosphorylational status of connexin 43

Frozen tissue samples were grinded in liquid nitrogen with a mortar and pestle, then 180-200 mgs of the powdered tissue were further homogenized in ice-cold lysis buffer (250 mM sucrose, 20 mM Tris-HCl, 10 mM β-mercaptoethanol, 10 mM sodium orthovanadate, 0.5 % protease inhibitor cocktail (Sigma), at pH 7.4). Tissue homogenates were centrifuged at 2000 g for 15 min at 4 °C, supernatants were collected and the homogenization step, together with the centrifugation was repeated with the pellets. The collected supernatants (total protein extracts lacking nuclear and contractile elements as well as un-homogenized connective tissue) were ultracentrifuged at 100 000 g for 45 min at 4 °C, and the pellets (the membrane fraction) were dissolved in lysis buffer and stored at -80 °C. The protein content of these membrane isolates were determined according to the method of Lowry and 20 μg protein of each sample was separated on 12% polyacrylamide gels. Proteins were then transferred to PVDF membranes (Millipore). Unspecific binding capacity of the membrane was blocked by incubation in 5% milk for 1 hour, then blots were labeled overnight with the primary antibody (Rabbit polyclonal anti-Cx43 (Zymed), dilution 1:2000). After subsequent incubation with the secondary antibody (HRP-conjugated goat anti-rabbit IgG (Santa Cruz), dilution 1:8000) for 1 hour, blots were developed with the ECL Plus kit (Amersham) and scanned with a Typhoon laser scanner. The densities of the phosphorylated (43-46 kDa) and dephosphorylated Cx43 bands (40-43 kDa) were quantified with the ImageQuant software and their amounts were expressed as a percentage of the total membrane Cx43 content.

Although the effect of Cx43 phosphorylation, as mentioned under point 1.2, depends largely on the kinase and the site of phosphorylation [16], this method still can be used for the determination of gap junctional coupling since it is commonly accepted that highly phosphorylated Cx43 forms are related to the open state of gap junctions, while dephosphorylation is associated with gap junction closure.

2.9 Statistical analysis

All data are expressed as mean ± SEM. The total number of VPBs and VT episodes were analysed with the Kruskal-Wallis test and the incidence of VF was analysed with the Fisher exact test. Impedance changes were compared to the control group in each 5 min of the ischaemia with Student's t-test. Changes in TAT_{n400} were also compared to changes in the control group with Student's t-test, in every 2 min between 2-8 and 14-30 min of ischaemia and in every 6 min between 30 and 60 min of ischaemia. Gap junction permeability and connexin 43 phosphorylation results were analysed with one-way ANOVA using Fisher's LSD post hoc test. Changes were considered significant at $p < 0.05$.

2.10 Drugs

Stock solutions of carbenoxolone (Sigma) and rotigaptide (formerly known as ZP123, synthetized at the Department of Medical Chemistry, University of Szeged, Hungary) were made with saline (carbenoxolone: 500 μM, stored at 4 °C no longer than 1 week; rotigaptide: 2 mg/ml, stored at -20 °C until use). Stocks were diluted with saline to the final concentration of the infusion just before use.

2.11 Experimental protocols

2.11.1 Protocols to study the effects of gap junction coupler/uncoupler agents administered before and during myocardial ischaemia

These are illustrated on *Figure 1*. Dogs were randomly selected into five groups. Control dogs (n=6) were subjected to a 60 min occlusion of the LAD; intracoronary infusion of saline was started 10 or 15 min before and maintained throughout the occlusion period. In the CBX group (n=8), 50 μM carbenoxolone was administered in intracoronary infusion 15 min prior to and during the LAD occlusion. The timing of the infusion was selected using literature data; it has been reported earlier that in the isolated rabbit heart, carbenoxolone exerted its maximal effect on conduction velocity after 15 min of continuous administration [23]. In other three groups of dogs, rotigaptide (RG) infusion in a dose of 0.04 μg/kg/min (n=7), 0.2 μg/kg/min (n=8) or 1 μg/kg/min (n=7), was started 10 min prior to and maintained over the whole occlusion period.

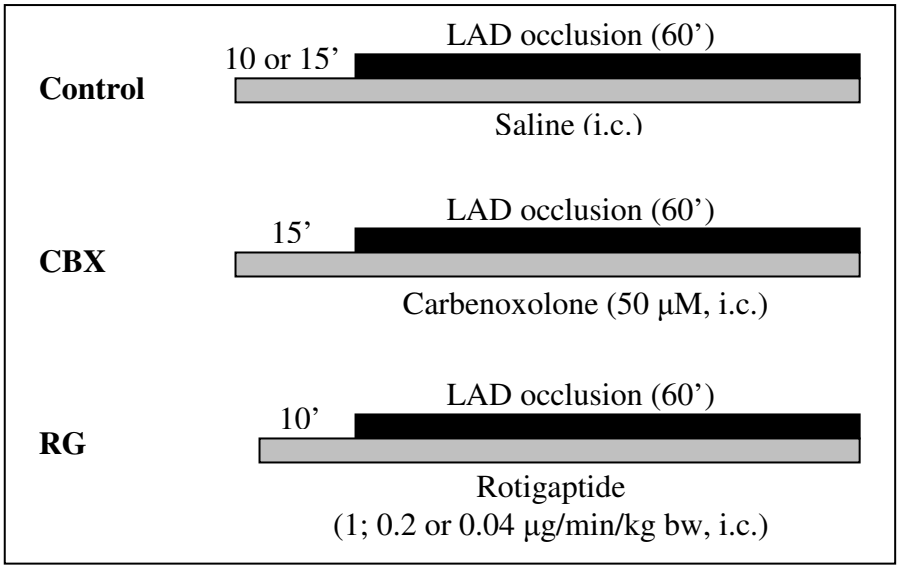

Figure 1. Protocols to study the effects of gap junction coupler/uncoupler agents administered before and during myocardial ischaemia.

2.11.2 Protocols to study the role of gap junctions in the antiarrhythmic effect of ischaemic preconditioning

As illustrated on *Figure 2*, dogs were randomly selected into six groups. Control dogs (n=6) were infused with saline on the intracoronary route for 20 min, and subjected to a 60 min LAD occlusion 20 min later. In the preconditioned group (PC; n=8), in the presence of the same saline infusion, a 5 min preconditioning ischaemia/reperfusion was performed 20 min

prior to the prolonged ischaemia. To study the possible trigger role of gap junctions in PC, carbenoxolone was administered in an intracoronary infusion 20 min prior to the LAD occlusion, in the presence (CBX+PC, n=11) or absence (CBX bef.; n=15) of preconditioning. In two other groups, rotigaptide was infused via the coronary side branch 20 min prior to the prolonged occlusion, either by itself (RG bef.; n=8) or in combination with PC (RG+PC; n=8).

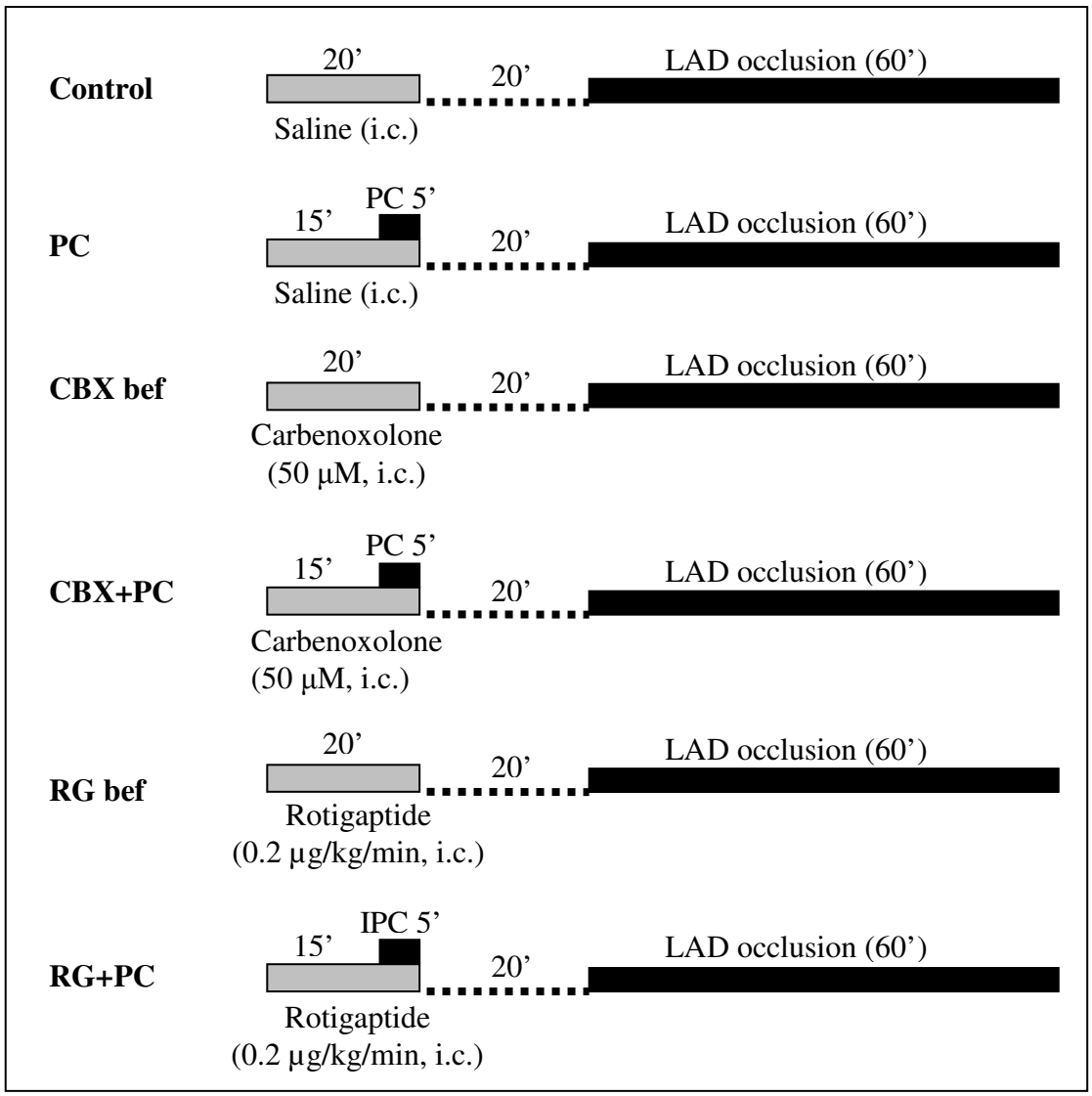

Figure 2. Protocols to study the role of gap junctions in the antiarrhythmic effect of ischaemic preconditioning.

2.11.3. Data handling in the control groups

Since the time of saline administration (prior to and during LAD occlusion or prior to only) did not cause any significant difference in any of the measured parameters between the two control groups, their data were pooled and handled as one group with a case number of 12.

3. RESULTS

3.1. Effects of gap junction coupler/uncoupler agents on acute ischaemia-induced haemodynamic and electrophysiological alterations

3.1.1 Haemodynamic changes following saline, carbenoxolone, and rotigaptide infusion

These are shown in *Table 1*. The 20 min administration of carbenoxolone/rotigaptide, similar to the 20 min infusion of saline, resulted in no significant change in any of the measured haemodynamic parameters.

3.1.2 Haemodynamic changes during LAD occlusion

Table 2 shows maximal changes in the measured haemodynamic parameters during the LAD occlusion in control as well as in carbenoxolone- and rotigaptide-treated dogs. Coronary occlusion resulted in a significant decrease in arterial blood pressure and left ventricular pressure. The dP/dt_{max} values were also significantly reduced, whereas left ventricular end-diastolic pressure was elevated following occlusion. All these changes were similar to the controls in the CBX- and rotigaptide-treated dogs. Heart rate did not show any significant change in any of the groups.

3.1.3 Verification of the gap junction specific effect of rotigaptide

dV/dt_{min} values of local electrograms and activation-recovery intervals (ARI) corrected to cycle length, measured during the 10 min administration of rotigaptide (1 μg/kg/min) in five dogs, are listed in *Table 3*. The lack of any significant change verifies that neither the upstroke velocity, nor the duration of local action potentials was affected by this concentration of the peptide. In whole-cell patch clamp studies performed on isolated canine myocytes, the peptide proved to be ineffective on sarcolemmal $I_{Ca,L}$, I_{to}, I_{Kr} and I_{K1} currents as well (data not shown).

Table 1. Haemodynamic changes following intracoronary saline/carbenoxolone/rotigaptide administration (mean ± SEM).

	Saline		CBX		Rotigaptide (µg/kg/min) 0.04		0.2		1	
	pre	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**	**pre**	**post**
SABP (Hgmm)	147±3	145±3	145±8	147±7	149±5	145±6	145±8	146±8	146±12	142±11
DABP (Hgmm)	102±2	102±2	98±6	101±5	101±3	99±3	96±6	98±6	107±8	102±8
MABP (Hgmm)	117±2	117±2	115±6	118±6	117±4	114±4	112±7	114±7	120±9	115±9
LVSP (Hgmm)	148±3	146±3	141±7	142±7	147±67	143±67	144±8	144±8	145±11	139±9
LVEDP (Hgmm)	6.4±0.3	6.5±0.2	6.2±0.7	7±0.6	6.7±0.4	6.9±0.3	6.1±0.3	6.4±0.4	6.6±0.7	6.4±0.4
+dP/dt (Hgmm/s)	3337±67	3295±78	3098±139	3211±187	3395±109	3281±135	3196±162	3117±155	3336±329	3267±403
-dP/dt (Hgmm/s)	2901±19	2890±70	2812±173	2700±164	2769±119	2712±108	2948±199	2962±186	2926±138	2977±184
HR (bpm)	165±3	164±3	161±10	156±9	156±8	156±7	159±6	159±6	165±9	165±9

*Table 2. Haemodynamic changes following LAD occlusion I (mean ± SEM, * p< 0.05 vs. baseline).*

	Control		CBX		Rotigaptide (µg/kg/min) 0.04		0.2		1	
	baseline	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**
SABP (Hgmm)	142±6	130±6*	147±7	133±9*	146±6	134±6*	145±7	136±8*	141±11	129±12*
DABP (Hgmm)	89±5	80±5*	101±5	91±7*	99±3	88±3*	97±6	89±6*	102±8	89±8*
MABP (Hgmm)	107±5	97±5*	118±6	106±7*	115±4	103±7*	113±7	104±7*	115±9	103±9*
LVSP (Hgmm)	141±6	128±6*	142±7	133±8*	143±7	134±7*	143±7	135±7*	139±9	131±12*
LVEDP (Hgmm)	7.1±0.4	13.8±0.6*	7±0.6	13.2±0.7*	6.8±0.3	11.8±0.4*	6.3±0.4	11±0.4*	6.6±0.4	12.8±1*
+dP/dt (Hgmm/s)	3107±164	2518±142*	3211±187	2715±192*	3264±130	2807±86*	3063±152	2638±179*	3267±403	2860±431*
-dP/dt (Hgmm/s)	2692±146	2133±121*	2700±164	2142±215*	2725±107	2455±133*	2924±178	2420±242*	2905±167	2404±172*
HR (bpm)	160±6	161±6	156±9	158±12	156±7	162±7	159±6	162±6	165±9	171±10

Table 3. Changes in dV/dt_{min} of local electrograms (in V/s) and in ARI normalized to a cycle length of 400 ms (ARI_{n400}, in ms) as well as cycle length (ms) at baseline (0 min) and after 10 min administration of rotigaptide (1 µg/kg/min, i.c.). Values are mean ± SEM.

	dV/dt_{min}		ARI_{n400}		Cycle length	
	0 min	**10 min**	**0 min**	**10 min**	**0 min**	**10 min**
Dog 1	-7.6±0.4	-7.5±0.3	169.3±1.7	168.4±2.6	387	389
Dog 2	-7.7±0.7	-7.8±0.7	147.9±19	158.4±20.5	431	412
Dog 3	-9.4±1	-9.9±0.9	164.7±0.6	167±1.1	453	458
Dog 4	-5.6±0.7	-5.4±0.7	151.4±2	150.5±1	512	523
Dog 5	-7.7±0.9	-7.9±0.8	157±1.3	158.6±1	557	561

3.1.4 Severity and distribution of ventricular arrhythmias induced by acute ischaemia in the presence of saline, carbenoxolone or rotigaptide

The distributions of VPBs over the 60 min LAD occlusion are illustrated in *Figure 3*. In the control dogs, arrhythmias occurred in two distinct phases termed as 1a and 1b, thereafter, ectopic activity faded. Both arrhythmia phases were reduced by carbenoxolone as well as by rotigaptide. Interestingly, while CBX and the lowest dose of rotigaptide attenuated both phases of arrhythmias equally, higher doses of rotigaptide were particularly effective against phase 1a arrhythmias.

Figure 4 depicts the total numbers of ventricular premature beats (VPBs) and tachycardiac episodes (VT) as well as the incidence of ventricular fibrillation (VF %) during the 60 min LAD occlusion. In the control group, there were a large number of VPBs and VT episodes, occurring predominantly in the first 25 min of the LAD occlusion, and 2 dogs out of 12 fibrillated during ischaemia. These were markedly reduced by both CBX and rotigaptide. Although there was no significant difference between the three rotigaptide dose groups, the number of VPBs slightly increased when the two higher dose were applied, and the incidence of VF in the RG 1 group was not decreased but even higher than in the controls. VF incidence, however, showed no significant difference between any of the groups, probably because its low incidence even in the controls.

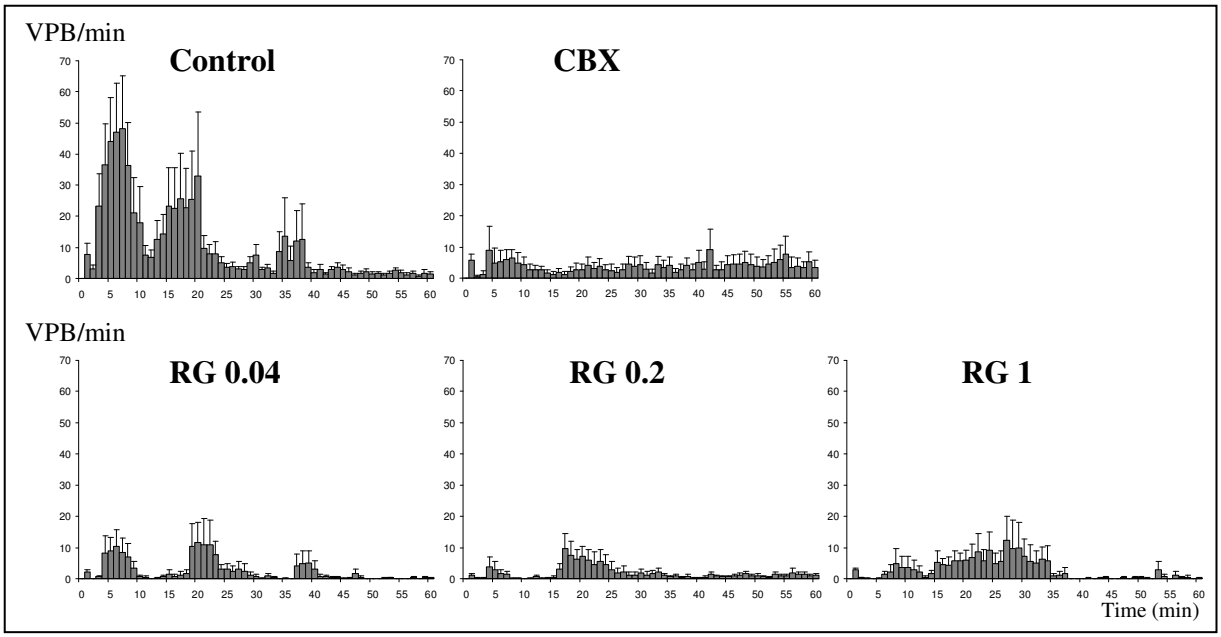

Figure 3. Distribution of VPBs during the 60 min LAD occlusion.

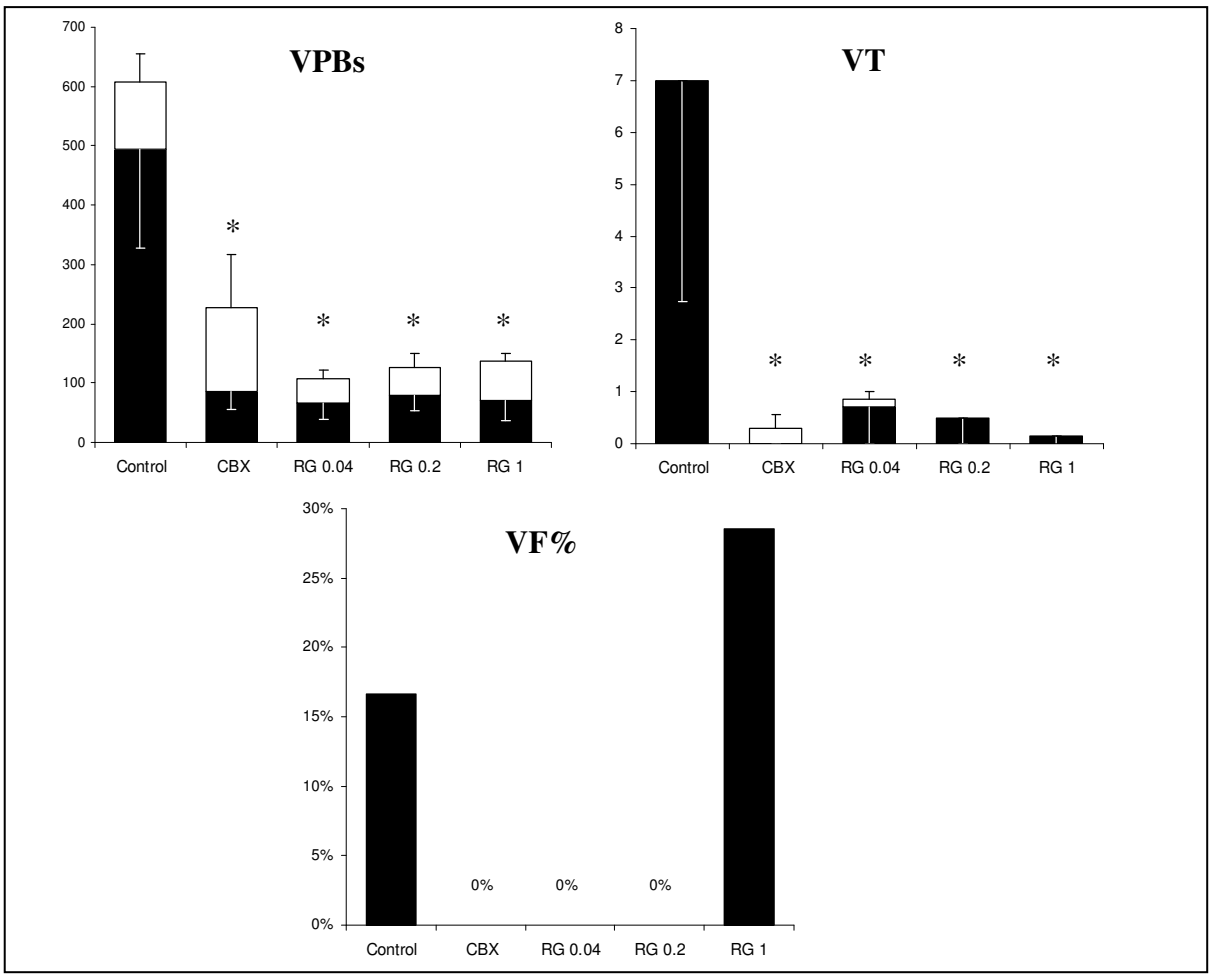

*Figure 4. Total numbers of VPBs, VT episodes and the incidence of VF during the first 25 min (filled bars) and during 25-60 min of ischaemia (open bars). Values are means ± SEM; *p <0.05 vs. control.*

3.1.5 Effects of carbenoxolone and rotigaptide on conduction abnormalities caused by ischaemia

The inhomogeneity of impulse conduction, shown on *Figure 5A*, was measured with the composite electrode in some control dogs and in the carbenoxolone group. The inhomogeneity of electrical activation increased steeply already in the first few minutes of ischaemia, which was followed by some decrease and a plateau phase during the rest of the occlusion period. Inhomogeneity was significantly reduced by CBX at any time point of the ischaemia.

In some control dogs and in the rotigaptide groups, impulse conduction was characterized using the total activation time (TAT_{n400}) assessed by activation mapping; this is illustrated by *Figure 5B*. Due to the better resolution of this technique, changes in TAT_{n400} in the control group follow the arrhythmia phases more sensitively: following a steep rise during phase 1a, there is a transient decrease but TAT_{n400} is elevated again during the 1b phase and remains high during the second half of the occlusion. In the rotigaptide-treated dogs, although TAT_{n400} values also increased during ischaemia, they were significantly lower than the control during both phase 1a and 1b. The three doses of rotigaptide did not show any significant difference in their effects at any time point of the coronary occlusion.

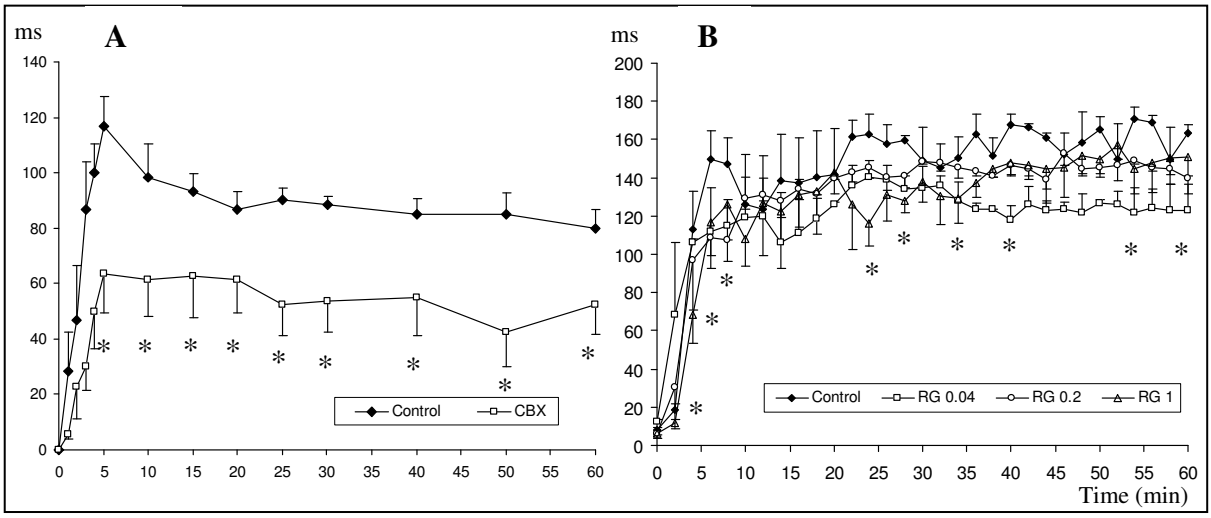

*Figure 5. Changes in the inhomogeneity of electrical activation (A) and in TAT_{n400} (B) during the 60 min LAD occlusion (mean ± SEM; *p< 0.05 vs. control).*

3.1.6 Changes of myocardial electrical impedance during the 60 min LAD occlusion; effects of gap junction modifier agents

Figure 6 depicts relative changes in tissue resistivity and phase angle during the 60 min of ischaemia. In control animals, after an initial increase in resistivity and a decrease in phase angle, both parameters reached a plateau. A second, steep change in resistivity and phase angle occurred just prior to the 1b phase of arrhythmias. Paradoxically, whereas ectopic activity faded after 30 min of ishaemia, both resistivity and phase angle showed further changes.

Intracoronary infusion of CBX attenuated impedance changes and abolished the second steep rise in tissue resistivity as well as the drop in phase angle, indicating a reduced and more gradual uncoupling.

Although impedance changes in the rotigaptide-treated dogs did not differ significantly from that observed in the controls, the biphasic manner of these changes was less apparent. In the RG 1 group, in parallel to the marked antiarrhythmic effect during phase 1a, early changes in resistivity and phase angle were significantly less than in the controls.

3.1.7 Effects of carbenoxolone and rotigaptide on ischaemia-induced gap junctional uncoupling determined by molecular analyses of the tissue samples

Gap junction permeability, assessed by double dye-loading of freshly excised tissue samples at the end of the LAD occlusion, was decreased by around 40 % in the ischaemic region of control hearts compared to the permeability measured in the non-ischaemic self-controls (*Figure 7*). This decrease was prevented by CBX as well as by all the three doses of rotigaptide: in these groups, gap junction permeability was almost identical in the ischaemic and in the non-ischaemic tissue samples.

Western blot analysis have shown a marked dephosphorylation of Cx43 in tissue samples originating from the ischaemic zone of control hearts (*Figure 8*), whereas the normal phosphorylation pattern was maintained by any dose of rotigaptide. Interestingly, despite to its protective effect against all the other parameters, CBX failed to prevent the dephosphorylation of Cx43.

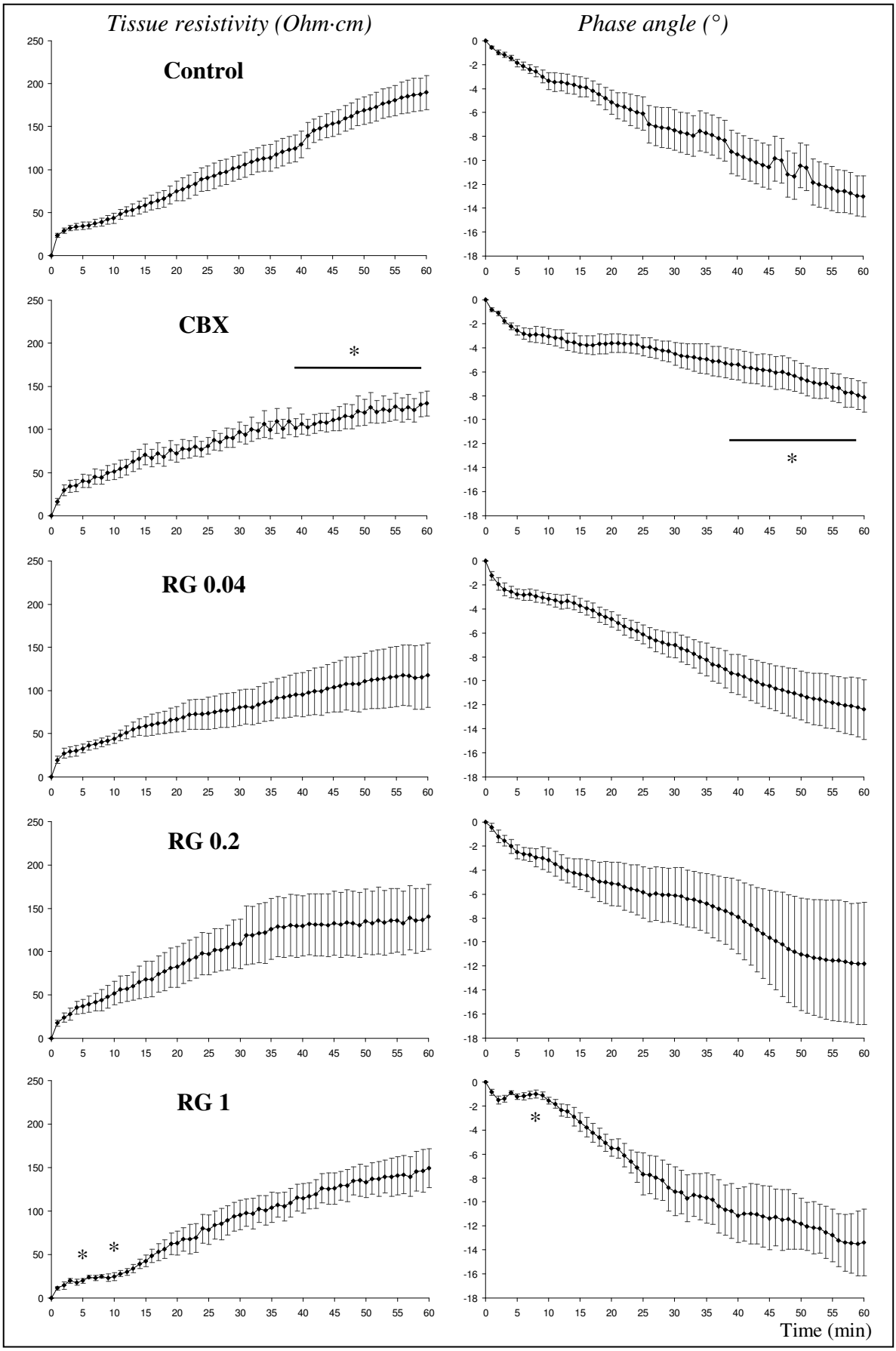

*Figure 6. Relative changes in tissue resistivity (in Ohm·cm) and in phase angle (in °) during 60 min occlusion of the LAD (mean ± SEM; *p< 0.05 vs. control).*

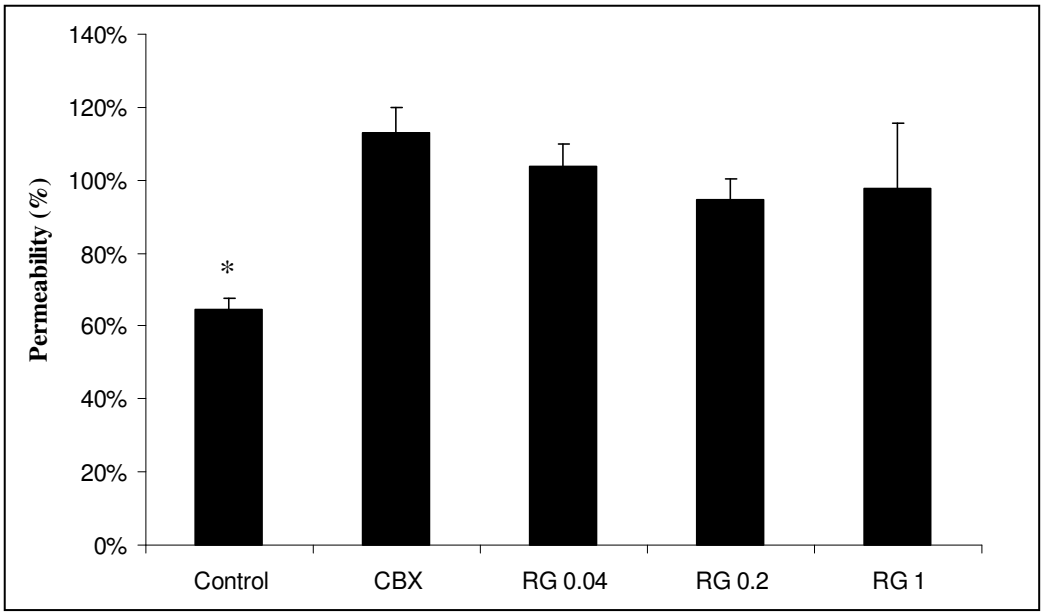

*Figure 7. Gap junction permeability after 60 min of ischaemia. Permeability of the ischaemic tissue samples are expressed as percentage of the non-ischaemic self-controls (mean ± SEM; *p< 0.05 vs. non-ischaemic self-control).*

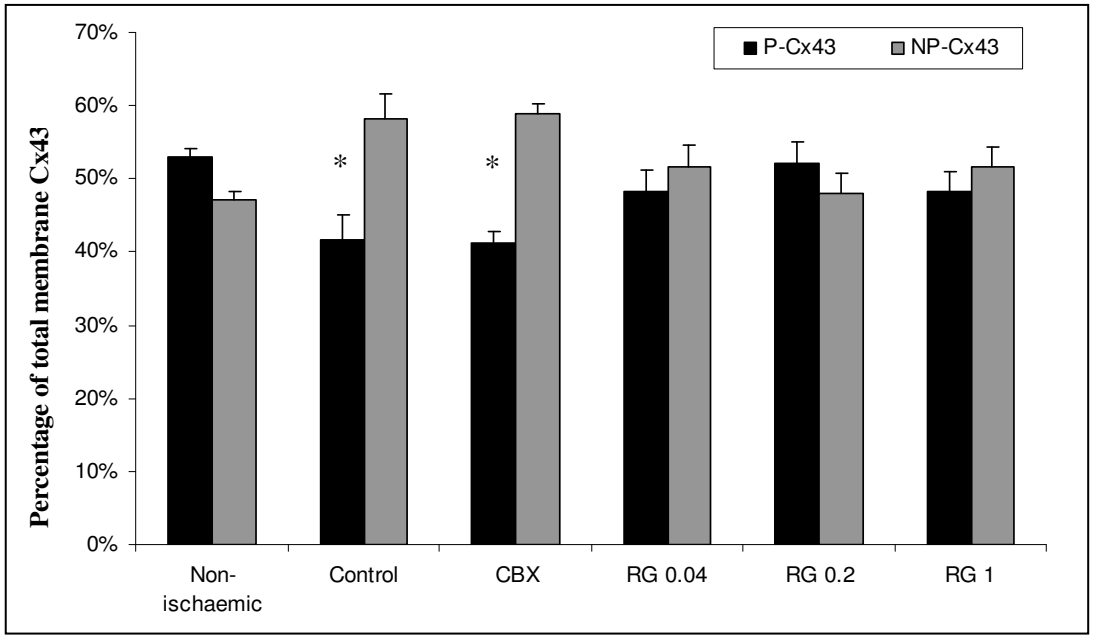

*Figure 8. The distribution of phosphorylated (black bars) and dephosphorylated (grey bars) Cx43 expressed as a percentage of total membrane Cx43 after 60 min of ischaemia (mean ± SEM; *p< 0.05 vs. non-ischaemic).*

3.2. Effects of ischaemic preconditioning on haemodynamic and electrophysiological alterations induced by acute ischaemia

3.2.1 Haemodynamic effects of LAD occlusion; effects of preconditioning and gap junction modification before ischaemia

These are shown in *Table 4*. Subsequent to LAD occlusion, arterial blood pressure and left ventricular systolic pressure, as well as positive and negative dP/dt_{max} significantly decreased in all groups. LVEDP was significantly elevated, whereas the heart rate did not show significant difference neither between the groups nor after coronary artery occlusion.

3.2.2 The antiarrhythmic effect of ischaemic preconditioning

As shown on *Figure 9*, ischaemic preconditioning has markedly reduced both phases of arrhythmias during the 60 min LAD occlusion. The total number of VPBs, as well as the number of VT episodes and the incidence of VF (*Figure 10*) were also significantly reduced in the preconditioned dogs. It is also prominent on both figures that PC afforded an absolute protection against these arrhythmias instead of shifting them to a later period of the occlusion.

3.2.3 Amelioration of conduction slowing by preconditioning

Conduction properties in this group, similar to the controls, were studied by both the composite electrode (*Figure 11A*) and activation mapping (*Figure 11B*). Independently of the technique used, preconditioning significantly improved impulse conduction during the entire occlusion period.

*Table 4. Haemodynamic changes following LAD occlusion II (mean ± SEM, * p< 0.05 vs. baseline).*

	Control		PC		CBX bef		CBX+PC		RG bef		RG+PC	
	baseline	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**	**baseline**	**occlusion**
SABP (Hgmm)	142±6	130±6*	129±9	121±11*	141±5	131±4*	132±6	122±7*	144±10	132±10*	126±5	116±6*
DABP (Hgmm)	89±5	80±5*	91±9	84±8*	89±4	80±5*	89±6	81±4*	92±7	82±9*	90±3	80±4*
MABP (Hgmm)	107±5	97±5*	103±9	96±9*	106±4	97±5*	102±6	94±7*	106±8	95±9*	101±4	92±5*
LVSP (Hgmm)	141±6	128±6*	129±9	122±10*	137±5	126±4*	131±7	122±8*	145±9	137±10*	126±6	118±5*
LVEDP (Hgmm)	7.1±0.4	13.8±0.6*	7.2±0.3	12.5±1.1*	7±1	15±1*	8±1	16±1*	6.5±0.3	12.8±1.1*	6±0.4	11.9±0.7*
+dP/dt (Hgmm/s)	3107±164	2518±142*	2843±198	2250±86*	2867±169	2446±148*	2923±149	2294±162*	3248±95	2602±31*	2732±131	2395±127*
-dP/dt (Hgmm/s)	2692±146	2133±121*	2597±41	2070±76*	2562±170	2107±68*	2598±123	2141±134*	2728±237	2363±319*	2684±187	2204±162*
HR (bpm)	160±6	161±6	161±8	165±9	160±4	162±3	162±4	161±2	166±3	169±3	158±5	165±6

3.2.4. Reduction of gap junctional uncoupling by ischaemic preconditioning

As shown by *Figure 12*, changes in tissue impedance were substantially lower in the preconditined dogs throughout the ischaemic period and there was a lack of second steep changes both in resistivity and phase angle, indicating that preconditioning resulted in a slower and more gradual uncoupling. It is also important to note that changes reflecting gap junctional uncoupling in the control group were not only delayed by preconditioning, but indeed reduced.

Molecular analysis of tissue samples originating from preconditioned hearts have found the preservation of both gap junctional metabolic coupling and the phosphorylated state of Cx43 (*Figures 13 and 14*), thus confirmed that gap junctional communication was almost intact in these hearts.

3.3 Effects of gap junction coupler/uncoupler drugs, applied instead of or together with preconditioning, on arrhythmias, conduction impairments and gap junctional uncoupling during LAD occlusion

3.3.1 Effects of gap junction modifier drugs applied either alone or together with preconditioning on arrhythmia severity during LAD occlusion

These are shown on *Figures 9 and 10*. The gap junction uncoupler carbenoxolone attenuated the antiarrhythmic effect of preconditioning as shown by the increased number of VPBs and VT episodes as well as a slight increase in VF incidence compared to the PC group. In contrast, CBX administration by itself resulted in an antiarrhythmic protection similar to preconditioning.

Arrhythmia severity following the administration of rotigaptide by itself was similar to the controls, some values, such as the number of VT episodes and the incidence of VF, although not significantly, were even higher than in the control group. When RG was administered together with PC, it did not have any significant influence on the protective effect of PC.

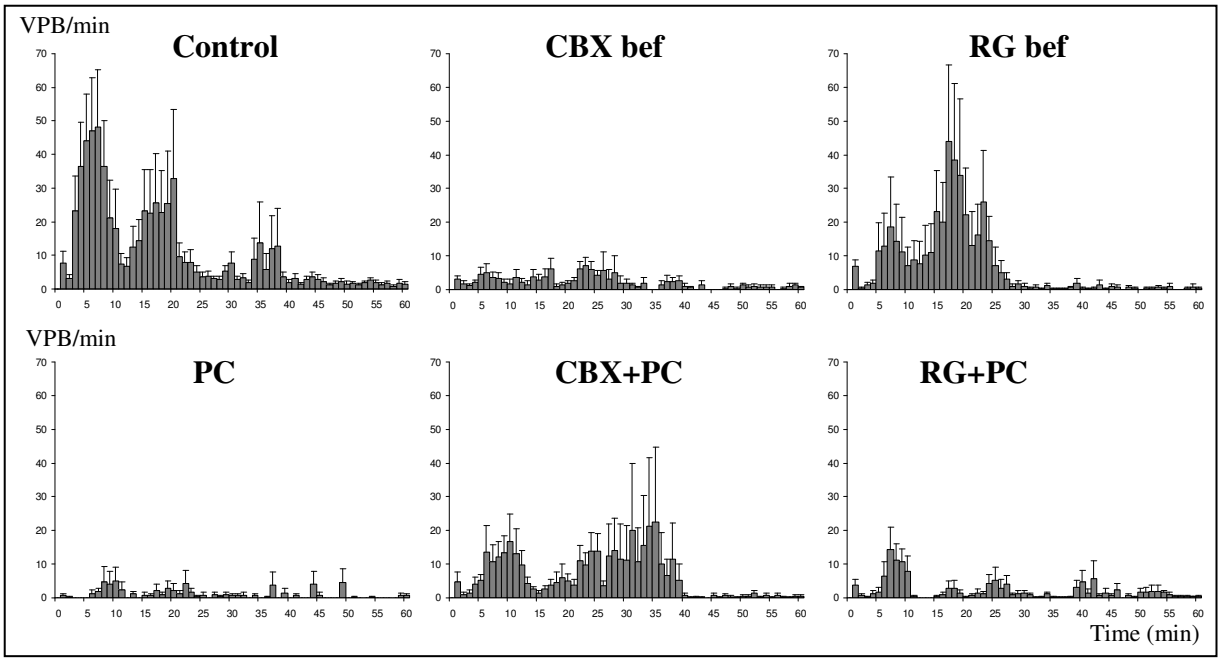

Figure 9. Distribution of VPBs during the 60 min LAD occlusion.

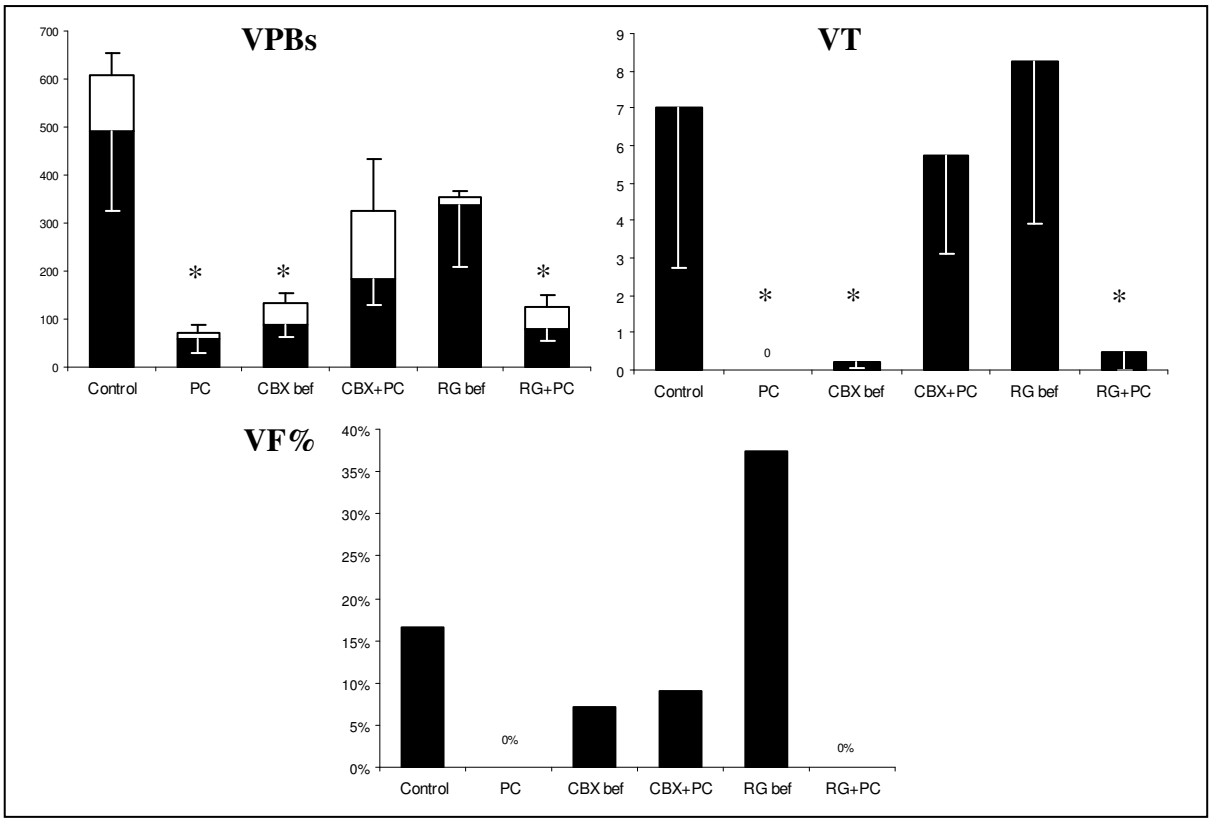

*Figure 10. Total numbers of VPBs, VT episodes and the incidence of VF during the first 25 min (filled bars) and during 25-60 min of ischaemia (open bars). Values are means ± SEM; *p< 0.05 vs. control.*

3.3.2 Changes in ischaemia-induced conduction impairments following carbenoxolone/rotigaptide infusion by itself or in combination with preconditioning

These were measured using the composite electrode in the carbenoxolone groups (*Figure 11A*) and by activation mapping in experiments with rotigaptide (*Figure 11B*). CBX, when applied alone, resulted in a similar, although somewhat less pronounced improvement in conduction slowing than PC, whereas, when applied together with preconditioning, the protective effect was abrogated by CBX.

When administered in preconditioned dogs, rotigaptide did not modify substantially the TAT_{n400} reduction afforded by PC. Interestingly, infusion of rotigaptide by itself exerted a different effect on TAT_{n400} than on arrhythmia severity since TAT_{n400} was much smaller than the control values during both arrhythmia phases. It must be noted, however, that these lower TAT_{n400} values may originate from the loss of animals with more severe TAT_{n400} changes due to VF.

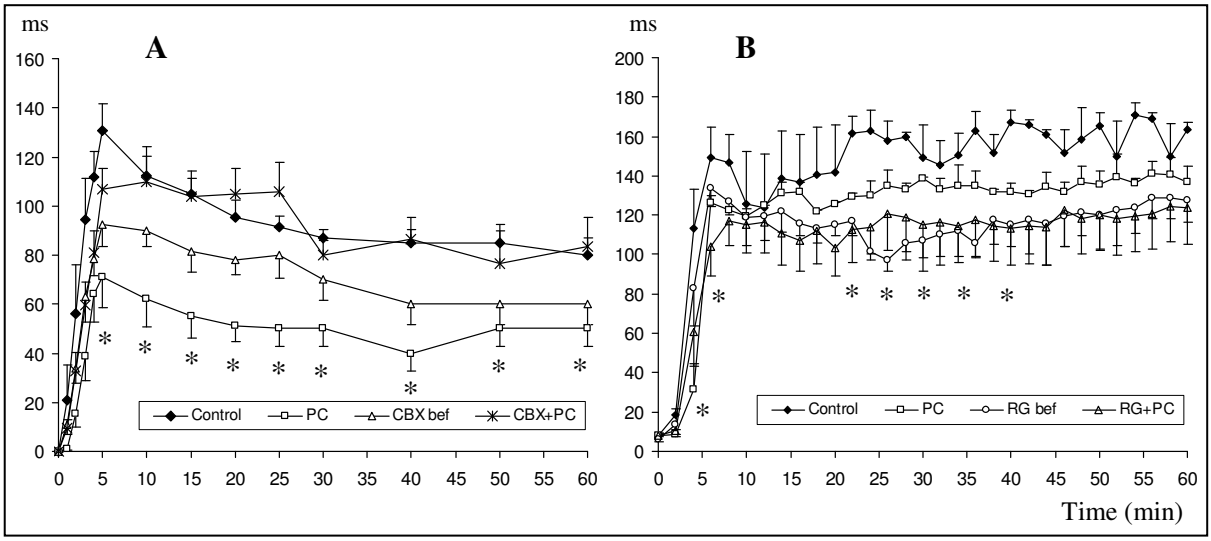

*Figure 11. Changes in the inhomogeneity of electrical activation (A) and in TAT_{n400} (B) during the 60 min LAD occlusion (mean ± SEM; *p< 0.05 vs. control).*

3.3.3 Effects of gap junction modifier drugs applied with and without preconditioning on gap junction function during the course of prolonged ischaemia

Compared to the controls, infusion of carbenoxolone by itself prior to the prolonged coronary occlusion has led to a reduced gap junctional uncoupling during the occlusion period, as it is shown by changes in tissue impedance (*Figure 12*) as well as by the preservation of gap junction permeability and Cx43 phosphorylation (*Figures 13 and 14*). In the CBX+PC group, attenuation of the antiarrhythmic effect of preconditioning was accompanied by a marked gap junctional uncoupling, as indicated by the rapid decrease in phase angle, the reduction in gap junction permeability and the dephosphorylation of Cx43.

The infusion of rotigaptide prior to ischaemia did not modify ischaemia-induced gap junctional uncoupling (although metabolic coupling was preserved in this group), nor the protective effect of preconditioning as it was clearly represented by both *in vivo* and *in vitro* analyses of gap junction function.

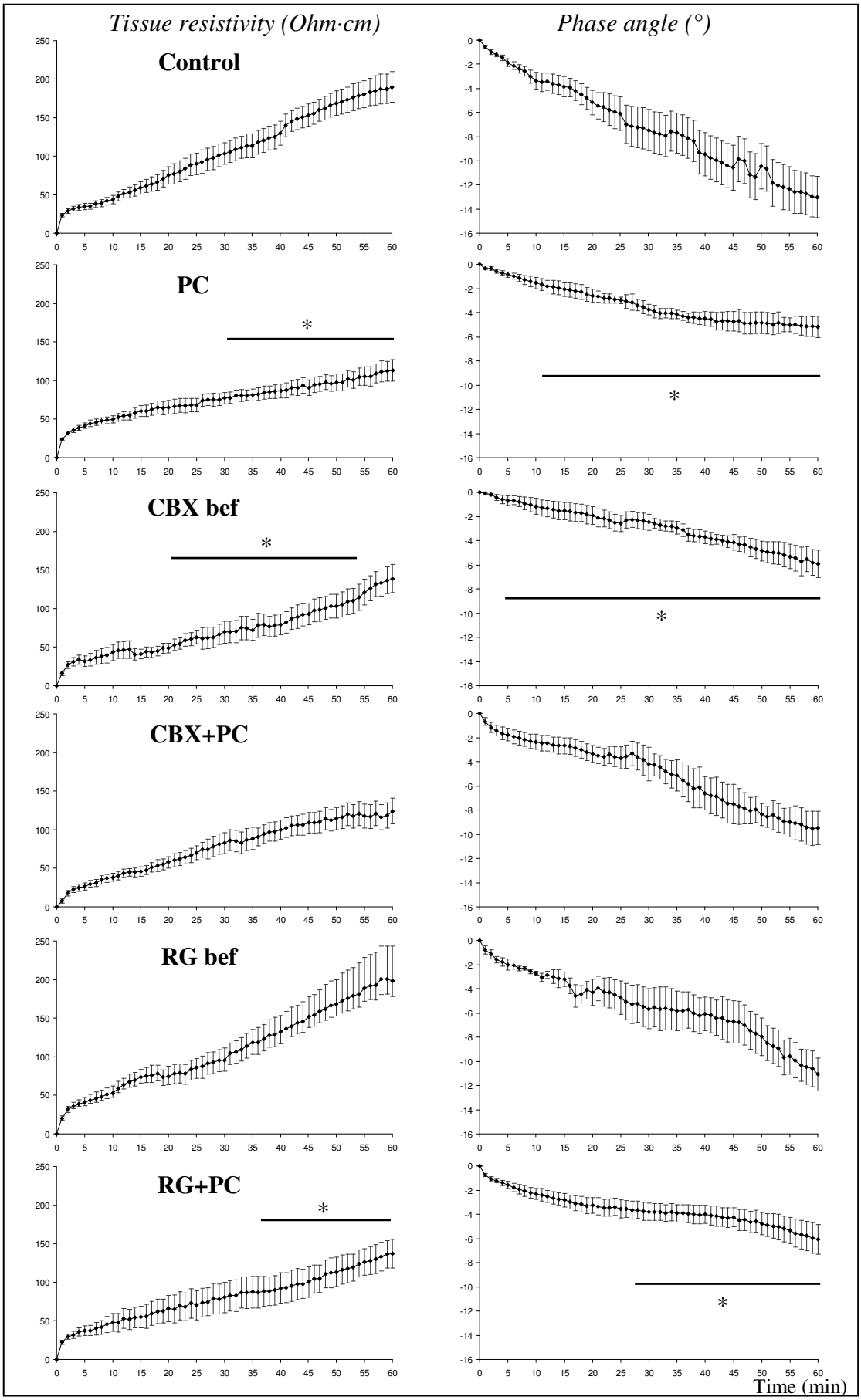

*Figure 12. Relative changes in tissue resistivity (in Ohm·cm) and in phase angle (in °) during the 60 min occlusion of the LAD (mean ± SEM; *p< 0.05 vs. control).*

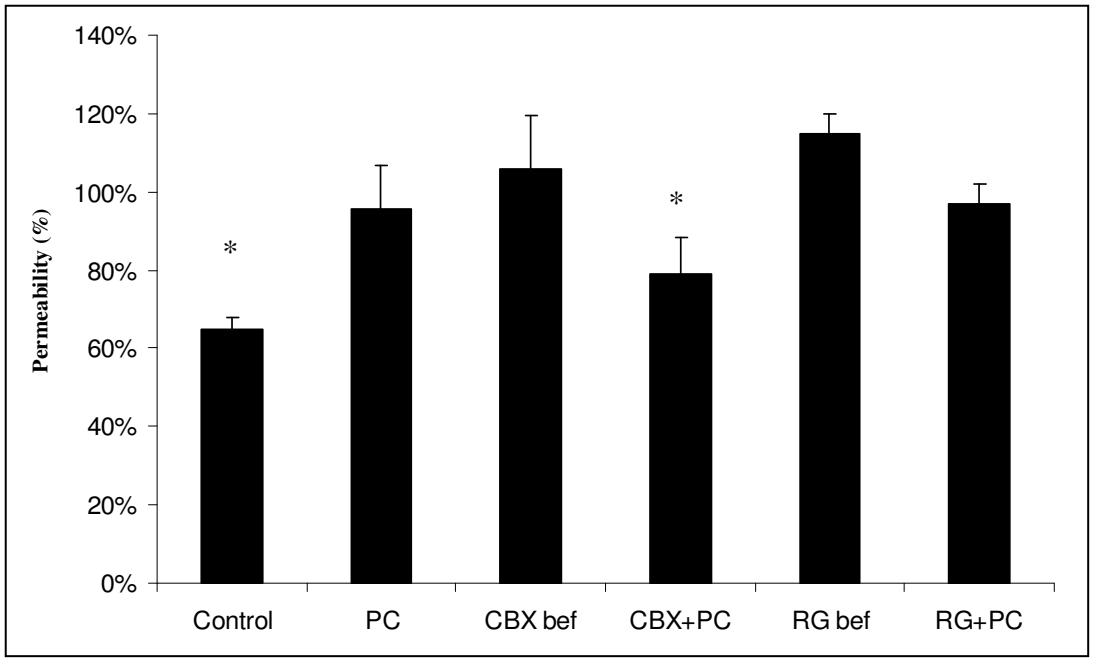

*Figure 13. Gap junction permeability after 60 min of ischaemia. Permeability of the ischaemic tissue samples are expressed as percentage of the non-ischaemic self-controls (mean ± SEM; *p< 0.05 vs. non-ischaemic self-control).*

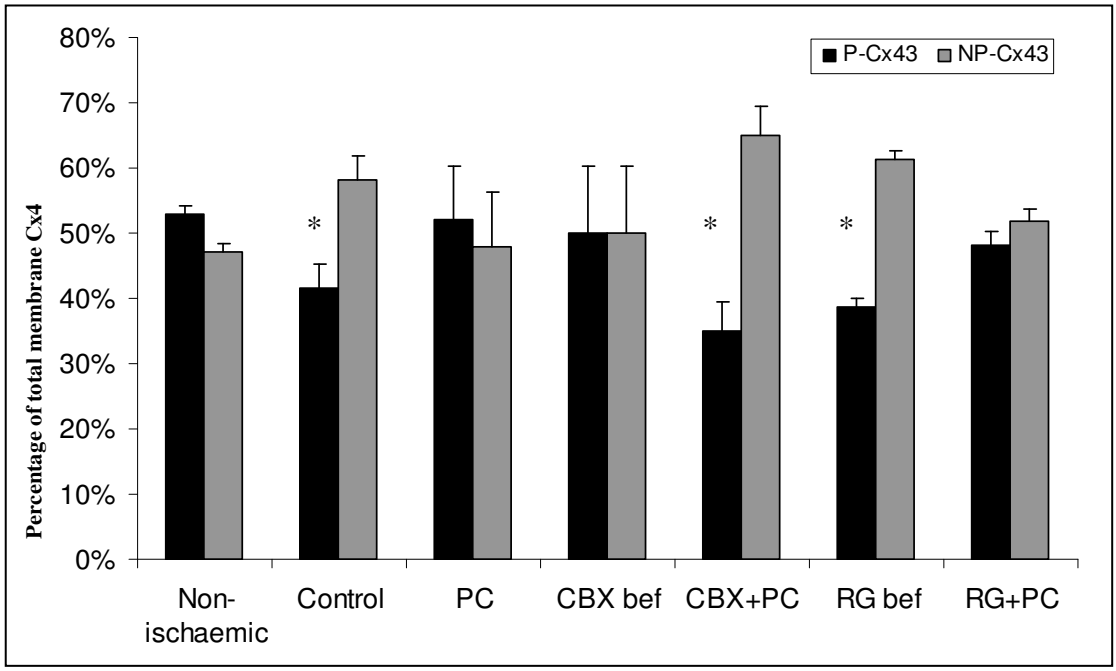

*Figure 14. The distribution of phosphorylated (black bars) and dephosphorylated (grey bars) Cx43 expressed as a percentage of total membrane Cx43 after 60 min of ischaemia (mean ± SEM; *p< 0.05 vs. non-ischaemic).*

4. DISCUSSION

4.1 New findings

(1) We have confirmed that gap junctional uncoupling and subsequent conduction slowing plays a role in the 1b phase of acute ischaemia-induced arrhythmias in anaesthetised, open-chest dogs. We have also pointed out that during the later phases (after 30 min) of ischaemia, despite further gap junctional uncoupling, ectopic activity is diminished.

(2) We have found that both gap junction coupler and uncoupler drugs, applied prior to and continuously during ischaemia, are able to improve impulse conduction and attenuate arrhythmias.

(3) Our study have demonstrated that ischaemic preconditioning markedly reduces ischaemia-induced gap junctional uncoupling, which, by the amelioration of impulse conduction, may contribute to the antiarrhythmic effect of preconditioning. It is of importance that in this model, preconditioning provides an absolute protection against gap junctional uncoupling, conduction slowing and arrhythmias, instead of shifting them to a later period of ischaemia.

(4) We have shown that the protective effect of ischaemic preconditioning can be attenuated if gap junctions are closed with carbenoxolone during the preconditioning stimulus. The protection induced by the administration of carbenoxolone by itself has suggested a possible role of gap junctional uncoupling also as a trigger for preconditioning. Although administration of the gap junction coupler rotigaptide during the preconditioning ischaemia does not influence the protective effects of preconditioning, some role of gap junctions or connexin proteins as triggers of preconditioning cannot be excluded.

4.2 Role of gap junctional uncoupling in acute ischaemia-induced arrhythmogenesis

Continuous *in vivo* assessment of gap junctional coupling by the measurement of myocardial electrical impedance allowed us to compare gap junction function with changes in impulse conduction and arrhythmias over the entire experimental period. In control dogs, occlusion of the LAD was abruptly followed by an increase in tissue resistivity and a decrease in phase angle. It is tempting to speculate whether these changes reflect gap junctional uncoupling already at this early time point of ischaemia. However, determination of gap junction permeability and Cx43 phosphorylation in samples excised after 5 min of ischaemia (data not

shown) as well as earlier studies in a model that allowed the distinction between the extra- and intracellular components of tissue resistivity failed to demonstrate any significant uncoupling in this phase of ischaemia [29]. These early changes in tissue impedance are rather signs of those rapid alterations in the ischaemic tissue such as the lack of blood due to the arrest of perfusion, changes in the extracellular space and cellular oedema [61, 62].

In contrast, gap junctional uncoupling is proved to be a contributing factor in the genesis of phase 1b arrhythmias. Similar to earlier results obtained from *in vivo* or isolated porcine hearts [30, 44, 55], we have found a second steep rise in tissue resistivity and a decrease in phase angle just preceding the occurrence of phase 1b arrhythmias. Molecular analyses of tissue samples taken after 25 min of ischaemia in some additional control dogs, by showing a significant decrease in gap junction permeability and dephosphorylation of Cx43 [60], provided further evidence for the presence of uncoupling in this phase. Closure of gap junctions during phase 1b was also reflected by a second increase in TAT_{n400}, a more precise measure of conduction abnormalities than the inhomogeneity of activation determined by the composite electrode.

Paradoxically, after 30 min of the LAD occlusion further changes in tissue impedance and still elevated TAT_{n400} values are no more accompanied by arrhythmias. The lack of arrhythmias despite advanced gap junctional uncoupling suggests that closure of gap junctions may provoke arrhythmias either only during a particular phase of ischaemia when uncoupling is too rapid or heterogeneous, or by acting synergistically with other arrhythmogenic factors. There is some evidence for this assumption established by Smith *et al.* [30]: in anaesthetised pigs, if gap junctional uncoupling has occurred much earlier than the 1b phase, this early uncoupling was able to salvage these pigs from VF.

It may well be that rapid closure of gap junctions create heterogeneities in coupling and therefore also in impulse conduction throughout the affected tissue. This applies particularly to the border zone of the ischaemic area, which is the most heterogeneous region from both the metabolic and the electrophysiological aspect. Thus, it is conceivable that some groups of cells, already severely damaged by ischaemia, uncouple more rapidly than neighborous areas with more adequate perfusion, resulting in marked heterogeneities in impulse propagation and in an increased propensity for arrhythmias. Albeit focusing on questions other than the consequences of acute ischaemia, there is some evidence for the importance of heterogeneous coupling in arrhythmogenesis, as both patchy Cx43 expression in chimeric mice [35, 36] and redistribution of Cx43 in healing canine infarcts increase the propensity for ventricular arrhythmias [63, 65]. Computer simulation studies have also reported that at a given Na^+

channel conductance, spatially heterogeneous uncoupling results in more reentry circuits than a more severe but homogenous uncoupling [65].

Others suppose that intermediate gap junctional uncoupling is responsible for phase 1b arrhythmias since it has been shown both *in vitro* and *in vivo* that partial uncoupling may allow not only the spread of cell death but, once formed, the propagation of triggered activity as well [10, 44]. It can be explained by that intermediate uncoupling, by reducing electrotonic interaction but not affecting conduction, favours both the formation and spread of focal arrhythmias, however, substantial conduction slowing by advanced gap junctional uncoupling inhibits their propagation.

On the other hand, gap junctional uncoupling is likely to influence arrhythmogenesis together with other factors. Accumulation of catecholamines in the ischaemic tissue, first, increases Ca^{2+} load of the myocytes and enhances triggered activity [7, 9, 10]; second, it may exaggerate the spatial heterogeneity of the effective refractory periods [11]; and third, it also contributes to the closure of gap junctions [66, 67]. Ventricular wall stretch, perhaps by further increasing Na^{+} and Ca^{2+} load, is also suggested to participate in arrhythmogenesis during the 1b phase [68, 69]. Concerning the possible mechanism of phase 1b arrhythmias, whereas catecholamines and wall stretch readily induce triggered activity, the slowing of impulse conduction secondary to gap junctional uncoupling form a substrate for reentry as well [23, 31, 32, 33]. This can be further enhanced by the exaggeration of spatial heterogeneity of refractoriness by both catecholamines [11] and gap junctional uncoupling [32, 70].

To conclude, it is well established that gap junctional uncoupling plays a role in acute ischaemia-induced arrhythmias, however, it is still unclear whether the extent or the timing of uncoupling is more important to determine arrhythmogenesis. Further investigations using more specific techniques and/or gap junction modifier agents may lead to a better understanding of this mechanism.

4.3 Attenuation of ischaemia-induced arrhythmias by gap junction modifier agents applied before and during ischaemia

The role of gap junctional uncoupling in acute ischaemia-induced ventricular arrhythmias was also confirmed by that maintenance of gap junctional coupling by the gap junction opener rotigaptide afforded protection against both ischaemia-induced conduction slowing and arrhythmias. Interestingly, higher doses of the peptide slightly increased the number of VPBs

and the highest dose did not protect against VF. A possible explanation for this phenomenon is, as discussed in details under point 4.2, that on one hand, maintenance of gap junctional coupling may reduce the risk of reentrant arrhythmias, however, on the other hand it may favour the formation and spread of triggered activity. Unfortunately, our activation mapping system does not allow clear distinction between these two arrhythmia mechanisms. In an attempt to distinguish clearly recognizable reentry and other types of arrhythmias, although we have found a reduction of the portion of reentry in the rotigaptide treated dogs (3 ± 1.6 % vs. 7.3 ± 2% in the controls), the low occurrence of clearly recognizable reentrant circuits even in the control group highlighted the limitations of this technique. Others, however, with the use of intramural 3D activation mapping provided evidence that after 3 hours of ischaemia, rotigaptide inhibited the induction of reentrant arrhythmias whereas focal arrhythmias could be readily provoked in the presence of the peptide [38, 71].

It was also prominent that the two higher doses of rotigaptide attenuated phase 1a arrhythmias more efficiently than the lowest dose. This is rather surprising as gap junctional uncoupling is reported to participate only in the formation of phase 1b arrhythmias. Since non-gap-junction-specific cellular electrophysiological effects of rotigaptide have been excluded, this may be explained by that gap junctional uncoupling, which is insufficient to play a role during phase 1a but may start already within the first minutes of ischaemia, is inhibited by the peptide and this improved coupling counteracts with conduction slowing caused by Na^+ channel inactivation. TAT_{n400} changes were also significantly smaller than the controls already during phase 1a.

Maintenance of gap junctional coupling in all the three rotigaptide dose groups was verified by determination of gap junction permeability and phosphorylation of Cx43, but not by tissue impedance changes. This can be originated from the lower sensitivity of this technique i.e. impedance is influenced by other ischaemia-induced electrophysiological alterations as well, and these may mask changes in gap junctional coupling [61, 62].

In contrast to the antiarrhythmic effect of rotigaptide, the attenuation of arrhythmias by the gap junction uncoupler carbenoxolone was rather surprising. In this group, the marked reduction of arrhythmias was accompanied by more homogenous electrical activation probably resulting from a more gradual gap junctional uncoupling. These results further support the concept that gap junctional uncoupling is arrhythmogenic only during a particular period of ischaemia. Thus, it may well be that by the modification of the timing of gap junctional uncoupling with a gap junction blocker, an antiarrhythmic effect can be achieved. However, since this group shows great similarities to the group when carbenoxolone was

applied 20 min prior to ischaemia, it is also conceivable that carbenoxolone exerted some indirect, even non gap junction-specific effect already before the commencement of ischaemia and this led to the protective effect (for detailed discussion, see point 4.5). In order to clarify whether carbenoxolone was protective by direct modulation of gap junctional coupling, further experiments are needed with the use of other, relatively specific gap junction uncouplers such as palmitoleic acid or the peptide Gap27.

It is also worth to note that there are already some precedents in the literature for the protective effect of both gap junction opener and blocker agents in the same setting. It has been established that defibrillation energy required upon resuscitation can be decreased by both the gap junction coupler rotigaptide and the gap junction uncouplers stearic acid and heptanol [72, 73]. Infarct size due to ischaemia/reperfusion was also significantly decreased both by rotigaptide and heptanol, given either during the course of ischaemia or only during reperfusion [74, 39, 43, 75, 76]. Unfortunately, our model does not allow the study of infarct size, which in this case could not only serve as an additional endpoint, but could have the benefit to compare the effects of both a coupler and an uncoupler on infarct size in the same animal model and revise those earlier studies performed on different species under different experimental conditions.

In summary, the timing of gap junctional uncoupling appears to be more important in arrhythmogenesis than its extent. Furthermore, the antiarrhythmic protection found with the application of both a gap junction coupler and uncoupler agent, confirms that both are possible candidates in the development of new drugs against ischaemia/reperfusion induced arrhythmias and infarction.

4.4 The effect of ischaemic preconditioning on ischaemia-induced gap junctional uncoupling and its relationship with the antiarrhythmic protection

In the preconditioned dogs, ischaemia-induced changes in tissue impedance were significantly lower than changes in the control dogs, this, together with the lack of the second steep rise of resistivity and decline in phase angle suggests a reduced and more gradual gap junctional uncoupling. Molecular analyses of tissue samples have also shown protection against ischaemia-induced gap junctional uncoupling. This protective effect probably contributed to the improved impulse conduction during the ischaemic period and to the marked reduction in arrhythmia severity.

It is important to note that in this model, i.e. in chloralose-urethane-anaesthetised, open-chest dogs, preconditioning offered an absolute protection against all the measured parameters instead of shifting them to a later phase of ischaemia. This differs from what others have observed and clearly shows the differences among the models used. In chloralose-anaesthetised pigs, a species with poor collateral circulation thus increased propensity for ischaemia-induced VF, both gap junctional uncoupling and ventricular arrhythmias were delayed by preconditioning with only a slight reduction in their severity [55]. However, a similar delay of gap junctional uncoupling and arrhythmias by preconditioning in pentobarbital-anaesthetised dogs emphasizes the importance of the anaesthetic used [56].

4.5 Gap junctions as potential triggers of ischaemic preconditioning

Gap junctions are also suggested to play a role in the trigger phase of preconditioning as well. As one may speculate, these direct cytoplasmic connections may promote the propagation of any endogenous mediators or second messengers of preconditioning, which are membrane-impermeable but smaller than 1 kDa, such as bradykinin, adenosine, some free radicals, cyclic nucleotides, IP_3 and Ca^{2+}. During the short preconditioning ischaemia, gap junctional uncoupling, if starts, does not hinder this interchange since metabolic coupling is still preserved even after 2 hours of ischaemia [77]. Indeed, intracoronary administration of the gap junction uncoupler carbenoxolone during the preconditioning stimulus could attenuate the beneficial effects of preconditioning since the severity of arrhythmias, the inhomogeneity of electrical activation and measures of gap junction function all indicated a reduced or no protection. This finding was in accordance with others reporting a similar diminution of protection if gap junctional communication was impaired during the induction of preconditioning [57, 58].

Interestingly, the application of carbenoxolone by itself as a drug control resulted in a protective effect. This protection was similar to preconditioning against arrhythmias, conduction abnormalities and gap junctional uncoupling. The fact that a gap junction uncoupler can mimic preconditioning has suggested that gap junctional uncoupling may serve as a trigger for preconditioning. According to this hypothesis, a slight gap junctional uncoupling induced by the preconditioning ischaemia, although it is not sufficient to create arrhythmias but may already start in this early phase, triggers preconditioning. However, if this uncoupling is stronger than the adequate stimulus (as it may happen in the CBX + PC

group), it is rather adverse than protective since the protection afforded by preconditioning is known to require an optimal duration and number of the preconditioning stimuli [78, 79].

In order to test this hypothesis, the gap junction opener rotigaptide was administered alone and together with preconditioning. Rotigaptide by itself did not trigger any protection, on the opposite, it seemed to slightly (although not significantly) increase both the severity of arrhythmias and mortality due to VF during ischaemia. Combination of the peptide with preconditioning, however, did not modify the protective effect of preconditioning from any of the aspects studied, thus failed to provide a further evidence for the trigger role of gap junctional uncoupling.

As an alternative explanation, carbenoxolone could have induced a protective effect through a mechanism other than its uncoupler effect. Although carbenoxolone is proved to be ineffective on other sarcolemmal ion currents [23, 24], it is known to have corticoid-like effects and to induce the expression of the stress protein Hsp 70 and Cx43 as well [80, 81]. Nevertheless, the relatively short duration of these experiments and the local administration of the drug minimize the possibility of these non-specific actions.

On the other hand, connexin proteins may have other functions in myocytes as well. Hemichannels, if not integrated into gap junctions, may allow the movement of ions and molecules between the intra- and extracellular space and are reported to open during metabolic inhibition and contribute to cell death [82, 83]. They are also suggested to play a role in cellular osmoregulation [84]. Furthermore, beside their pore-forming activity, there is a growing body of evidence for the signalling role of connexins. These proteins, due to the numerous phosphorylation sites on their C-terminals, can interact with many kinases and phosphatases [14, 15, 16] and due to their membrane localization, with cytoskeletal and cell adhesion proteins as well [15]. They also have been found in the nucleus [85, 86] and in the mitochondrial membrane [87]. These alternative functions of connexins have been suggested to play a role in preconditioning, since whereas preconditioning could not be induced in connexin43-deficient mice [88, 89], its protective effect was manifest in isolated myocytes with intact Cx43 expression but no gap junctional intercellular communication [90]. Co-localization of sarcolemmal Cx43 with PKCα and p38MAPKα and β during ischaemia and its enhancement by preconditioning [91], as well as translocation of sarcolemmal Cx43 to the mitochondrial membrane during preconditioning has also been reported [87, 92].

To summarize our results, gap junctions are likely to play a role already in the induction of preconditioning by allowing the cell-to-cell transfer of those endogenous substances released by the preconditioning stimulus. However, since modification of gap junctional coupling may influence the outcome of a subsequent ischaemic period, the trigger role of gap junctional uncoupling or other, non-gap-junction-related functions of connexin 43 also have to be considered.

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7. ANNEX

Full papers

I. **Papp R,** Gönczi M, Kovács M, Seprényi G, Végh Á. Gap junctional uncoupling plays a trigger role in the antiarrhythmic effect of ischaemic preconditioning. Cardiovasc Res 2007; 74: 396-405.

II. **Papp R**, Gönczi M, Végh Á. A réskapcsolatok szerepe akut miokardiális iszkémia okozta aritmiákban. Cardiol Hung 2008; 38: 109-15.

Full English version:

Papp R, Gönczi M, Végh Á. Role of gap junctions in arrhythmias induced by acute myocardial ischaemia. Cardiol Hung 2008; 38: 116-22.