

Enhanced effect of gap junction uncouplers on macroscopic electrical properties of reperfused myocardium

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Transient inhibition of gap junction (GJ)-mediated communication with heptanol during myocardial reperfusion limits infarct size. However, inhibition of cell coupling in normal myocardium may be arrhythmogenic. The purpose of this study was to test the hypothesis that the consequences of GJ inhibition may be magnified in reperfused myocardium compared with normal tissue, thus allowing the inhibition of GJs in reperfused tissue while only minimally modifying overall macroscopic cell coupling in normal myocardium. Concentration–response curves were defined for the effects of heptanol, 18 α -glycyrrhetic acid, halothane, and palmitoleic acid on conduction velocity, tissue electrical impedance, developed tension and lactate dehydrogenase (LDH) release in normoxically perfused rat hearts ($n = 17$). Concentrations lacking significant effects on tissue impedance were added during the initial 15 min of reperfusion in hearts submitted to 60 min ($n = 43$) or 30 min ($n = 35$) of ischaemia. These concentrations markedly increased myocardial electrical impedance (resistivity and phase angle) in myocardium reperfused after either 30 or 60 min of ischaemia, and reduced reperfusion-induced LDH release after 1 h of ischaemia by 83.6, 57.9, 51.7 and 52.5% for heptanol, 18 α -glycyrrhetic acid, halothane and palmitoleic acid, respectively. LDH release was minimal in hearts submitted to 30 min of ischaemia, independently of group allocation. In conclusion, the present results strongly support the hypothesis that intercellular communication in postischaemic myocardium may be effectively reduced by concentrations of GJ inhibitors affecting only minimally overall electrical impedance in normal myocardium. Reduction of cell coupling during initial reperfusion was consistently associated with attenuated lethal reperfusion injury.

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Chemical coupling through gap junctions (GJs) has been proposed to allow the spreading of cell death in different situations, including apoptosis of tumoral cells, either spontaneous or induced by anti-neoplastic treatments (McMasters *et al.* 1998; Krutovskikh *et al.* 2002), and cell death secondary to ischaemia–reperfusion in astrocytes (Rawanduzy *et al.* 1997; Lin *et al.* 1998) and cardiomyocytes (Garcia-Dorado *et al.* 1997; Garcia-Dorado *et al.* 2004). In myocardium, reperfusion-induced hypercontracture can spread due to the passage of Na⁺ through GJs, followed by reverse Na⁺–Ca²⁺ exchange (Ruiz-Meana *et al.* 1999). Heptanol, a standard GJ inhibitor, prevented cell-to-cell progression of hypercontracture and reduced cell necrosis in different models, when given at the time of reperfusion (Garcia-Dorado *et al.* 1997). More recently, it has been shown that GJ-mediated intercellular communication may persist during ischaemia and allow

propagation of rigor contracture (Ruiz-Meana *et al.* 2001). In support of a role of GJ-mediated communication in the progression of ischaemic injury, it has been shown that pretreatment with heptanol (Saltman *et al.* 2002) or underexpression of Cx43 in heterozygous connexin43-deficient mice (Kanno *et al.* 2003) was associated with reduced infarct size after transient or permanent coronary occlusion, respectively. However, other studies have failed to find a protective effect by pretreatment with heptanol (Gysembergh *et al.* 2001) or underexpression of Cx43 (Schwanke *et al.* 2002) in isolated mice hearts submitted to transient coronary occlusion.

The therapeutic exploitation of the protective effect of inhibition of GJ-mediated communication with heptanol against ischaemia–reperfusion injury is limited by its relatively low specificity as a GJ inhibitor (it has dose-dependent effects on Na⁺ channels and other ion transport

systems (Niggli *et al.* 1989; Nelson & Makielski, 1991; Takens-Kwak *et al.* 1992), and by the undesirable effects of inhibition of GJ-mediated communication in normal myocardium, in particular, electrophysiological alterations causing arrhythmogenicity. This limitation is particularly serious because it may apply not only to heptanol but also to any other GJ inhibitor.

Previous studies have shown the existence of a high 'safety factor' in normal myocardium (i.e. that the number of available GJ channels is much higher than those strictly necessary to maintain conduction). This allows important reductions in the number of open junctional channels and/or their average conductivity to have undetectable effects on macroscopic electrical coupling (Jongsma & Wilders, 2000). However, ischaemia and reperfusion have been shown to induce changes in cytoplasmic ionic composition (Maurer & Weingart, 1987; Noma & Tsuboi, 1987; Calero *et al.* 1998), in phosphorylation of connexin43 proteins (Beardslee *et al.* 2000; Schulz *et al.* 2003), and in ATP availability (Sugiura *et al.* 1990). All these changes reduce the number of available channels, which, in turn, may cause the effect of GJ inhibitors on macroscopic electrical properties to be different in normal and ischaemic-reperfused tissue. A previous study has shown that the effects of heptanol on conduction may be different in normal and diseased myocardium (Spear *et al.* 1990). We propose the hypothesis that the different effects on electrical behaviour of GJ uncouplers in normal and ischaemic-reperfused myocardium could allow interference with GJ-mediated communication in reperfused myocardium without significantly altering overall macroscopic electrophysiological behaviour of control tissue. To test this hypothesis, the electrophysiological effects of four widely used GJ inhibitors have been characterized in normoxic and reperfused tissues, and correlated with their possible protective effect on cell death.

Methods

The investigation 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) and was performed in accordance with European legislation. The study was approved by the Ethics Committee of our institution.

Experimental preparation

One hundred adult male Sprague-Dawley rats (250–350 g) were killed by an intraperitoneal overdose of

pentobarbital. The hearts were quickly removed and retrogradely perfused through the aorta with an oxygenated (95% O₂–5% CO₂) Krebs solution at 37°C (mm: NaCl 118, KCl 4.7, MgSO₄ 1.2, CaCl₂ 2.5, NaHCO₃ 25, KH₂PO₄ 1.2 and glucose 11, pH 7.4). After removing both atria, complete atrioventricular conduction block was induced by crushing the His bundle with fine forceps. Immediately, the right ventricle and interventricular septum were opened by a longitudinal incision from the cardiac base to the apex. Opened hearts were pinned to a silicone membrane placed at the bottom of an organ bath, exposing the endocardial surface of the left ventricle (Rodriguez-Sinovas *et al.* 2003). A 2.0 silk-snare was placed in the septum of the hearts and connected to an isometric force transducer (FSG-01, SG-M DC bridge amplifier module, Experimentia, London, UK). Resting tension was 1 g. Preparations were paced from the base of the hearts using rectangular pulses of 2.5 ms duration and 4 V amplitude, at 400 ms basic cycle length.

Transmembrane action potential recordings

Transmembrane action potentials were recorded from the apical region of the heart as previously described (Rodriguez-Sinovas *et al.* 2003). Conduction time was measured as the time between the stimulus artefact and the onset of the rapid depolarization of the action potential. Using this method, the estimated conduction velocity represents an averaged velocity between the stimulus site and the recording site, as the pathway of activation is not known.

Myocardial electrical impedance

Measurement of myocardial electrical impedance and its two components, tissue resistivity (R) and phase angle (θ), was performed using a four-electrode probe placed in the septum, at 7 kHz, as previously described (Rodriguez-Sinovas *et al.* 2003). The impedance probe consisted of a linear array of four platinum electrodes (length: 5 mm, diameter: 0.4 mm), placed at an inter-electrode distance of 2.5 mm. An alternating current (10 μ A) was applied through the outer pair of electrodes, and the in-phase components of voltage were recorded continuously through the inner pair of electrodes (high-input impedance lock-in amplifier, Princeton Applied Research, model 5110, USA).

Lactate dehydrogenase (LDH) release and myocardial water content

Enzyme release was determined in samples taken from the effluent by a spectrophotometric method at 340 nm, as previously described (Rodriguez-Sinovas *et al.* 2003). Water content was measured in hearts submitted to ischaemia–reperfusion at the end of the experiments. Tissue samples were weighed before and after desiccation at 100°C for 24 h, and myocardial water content was determined as the difference between wet and dry weight, and was expressed as millilitres of water per 100 g dry weight.

Experimental protocol

Effects of GJ inhibitors during normoxia. After 30 min of equilibration, cumulative concentration–response curves to heptanol ($n=6$, from 10^{-4} to 1.78×10^{-2} M), 18 α -glycyrrhetic acid ($n=4$, from 10^{-6} to 5.62×10^{-5} M), halothane ($n=3$, from 10^{-4} to 5.62×10^{-2} M), and palmitoleic acid ($n=4$, from 10^{-6} to 10^{-4} M) were performed. Each concentration was applied for 15 min, recording continuously developed tension, myocardial electrical impedance and transmembrane action potentials. LDH release was determined at the end of each concentration. Those concentrations causing LDH release and rigor development were excluded from the analysis. Concentration–response curves to the different GJ inhibitors were adjusted to sigmoid curves to determine the concentration that caused half-maximal effects (EC_{50}). Three additional hearts were used to assess stability of the preparation during 2 h of normoxic perfusion.

Effects of GJ inhibitors after ischaemia–reperfusion. After 30 min of equilibration, 45 hearts were submitted to 1 h of no-flow true global ischaemia followed by reperfusion for 60 min. During the ischaemic period, preparations were immersed in Krebs solution (bubbled with 95% N₂–5% CO₂, pH 7.4, no glucose), in order to maintain temperature (36.5–36.8°C). GJ inhibitors were added to the oxygenated Krebs solution during the first 15 min of reperfusion. The concentrations used were chosen based on their effects during normoxia: well-characterized inhibitory effects on single-channel microscopic GJ conductance in normal myocytes and negligible actions on tissue impedance (myocardial resistivity and phase angle) in normoxically perfused hearts. These concentrations were 10^{-3} M for heptanol ($n=5$), 10^{-5} M for 18 α -glycyrrhetic acid ($n=5$), 10^{-3} M for halothane

($n=7$), and 3×10^{-5} M for palmitoleic acid ($n=6$). Each group of treatment had its paired control group ($n=5–7$). In order to dissociate the effects of GJ inhibitors on electrical impedance during reperfusion from their potential effects on the extent of cell death, a second series of experiments ($n=35$) was performed in which the duration of ischaemia was reduced to 30 min, a protocol that results in only minimal necrosis (heptanol: $n=6$; 18 α -glycyrrhetic acid: $n=4$; halothane: $n=4$; palmitoleic acid: $n=4$, each group with its corresponding control group). In these last hearts, the extent of necrosis was determined after 2 h of reperfusion. Hearts were cut into 2–3 mm slices perpendicular to the longitudinal axis. The slices were weighed and immersed during 15 min in a 1% triphenyltetrazolium chloride (TTC) solution, buffered at pH 7.4, at 37°C, and then imaged using a digital camera under white light. The areas with negative reaction to the TTC were identified as necrotic areas. Infarct mass was determined using the commercially available software MicroImage (Olympus Optical, Hamburg, Germany), as previously described (Garcia-Dorado *et al.* 1997), and expressed as a percentage of the total weight of the heart.

Changes in developed tension, conduction velocity and myocardial electrical resistivity and phase angle were continuously recorded during the whole experiments, and cell injury was evaluated by measurement of LDH release during reperfusion as previously described (Rodriguez-Sinovas *et al.* 2003).

Chemicals

Heptanol (1-heptanol), 18 α -glycyrrhetic acid (3 β -hydroxy-11-oxo-18 α ,20 β -olean-12-en-29-oic acid) and palmitoleic acid (*cis*-9-hexadecenoic acid) were obtained from Sigma Chemical (St Louis, MO, USA). Halothane (Fluothane®, 2-bromo-2-chloro-1,1,1-trifluoroethane) was obtained from Zeneca Farma (Pontevedra, Spain). Palmitoleic acid and 18 α -glycyrrhetic acid were initially dissolved in dimethyl sulfoxide (DMSO), and then dissolved in Krebs solution to the desired concentration. Final DMSO concentration was 0.15 and 0.1%, respectively. Heptanol and halothane were directly dissolved in Krebs solution. All other reagents were commercially obtained.

Statistical analysis

Statistical analysis was performed using commercial available software (SPSS for Windows 8.0). Data are expressed as the mean \pm s.e.m. Repeated measures analysis

of variance (MANOVA) and Dunnett's *post hoc* test were used to assess differences in the time course of the changes in tissue electrical impedance, LDH release, developed tension and conduction velocity during reperfusion in the distinct treatment groups. Changes in tissue electrical impedance were analysed at baseline and at minutes 1, 2, 3, 4 and 5 of reperfusion. In the groups of 30 min of ischaemia, statistics for electrical impedance were one tailed, as the direction of the drug effect was previously demonstrated in hearts submitted to 60 min of ischaemia. Student's *t* test was used to analyse differences in accumulated LDH release, rigor onset and functional recovery. A *P* value lower than 0.05 was considered to be significant.

Results

Effects of GJ inhibitors during normoxia

In the absence of GJ inhibitors, all three hearts perfused under normoxic conditions for 2 h remained stable, without significant changes in contractile function (developed tension at baseline 380.3 ± 137.6 versus 339.6 ± 101.0 mg at 2 h) or electrophysiological variables (resistivity: 122.8 ± 12.9 versus 123.0 ± 12.5 Ω cm; phase angle: -2.4 ± 0.2 versus -2.2 ± 0.2 deg; conduction velocity: 33.2 ± 9.4 versus 30.1 ± 8.5 cm s^{-1}).

As seen in Fig. 1 and Table 1, all GJ inhibitors induced a concentration-dependent reduction in developed tension. Heptanol and 18α -glycyrrhetic acid had also a prominent effect on conduction velocity, whereas the effects of palmitoleic acid and halothane were less pronounced (Fig. 1 and Table 1). In contrast to their effects on developed tension and conduction velocity, all four GJ inhibitors investigated induced only minor changes in myocardial resistivity and phase angle (Fig. 1 and Table 1).

Effects of GJ inhibitors after ischaemia–reperfusion

Changes in tissue electrical impedance during ischaemia–reperfusion. As previously described (Rodriguez-Sinovas *et al.* 2003), 60 min of ischaemia induced a marked increase in myocardial resistivity (from 120.6 ± 2.9 to 369.0 ± 36.2 Ω cm at 60 min of ischaemia, pooled data from all controls, $P < 0.001$) and an accentuated decrease in phase angle (from -1.8 ± 0.1 to -11.1 ± 0.8 deg, $P < 0.001$). Reperfusion caused a quick recovery in both resistivity and phase angle values in all control hearts (Fig. 2). However, hearts treated with any of the GJ inhibitors investigated, heptanol,

Table 1. Concentrations (M) causing half-maximal effects on developed tension and conduction velocity in normoxic isolated rat hearts treated with heptanol, 18α -glycyrrhetic acid (GA), halothane and palmitoleic acid (PA)

	Developed tension	Conduction velocity
Heptanol	$(5.1 \pm 0.7) \times 10^{-4}$	$(1.2 \pm 0.1) \times 10^{-3}$
18α -GA	$(8.4 \pm 1.8) \times 10^{-6}$	$(8.1 \pm 2.9) \times 10^{-6}$
Halothane	$(2.0 \pm 1.0) \times 10^{-2}$	—
PA	$(7.5 \pm 4.3) \times 10^{-5}$	—

18α -glycyrrhetic acid, halothane or palmitoleic acid, showed marked attenuation of the recovery of myocardial electrical impedance during initial reperfusion (Fig. 2). Similar results were observed when duration of ischaemia was set at 30 min, a protocol that induced only minimal cell death (see below) (Fig. 3).

Functional recovery during reperfusion. Developed tension decreased during ischaemia, to reach a minimal value several minutes later. Rigor contracture, detected as an abrupt increase in diastolic tension, occurred between 14 and 16 min after perfusion cessation, with no differences between groups.

In the absence of GJ inhibitors all hearts submitted to 60 min of ischaemia showed only minor recovery of developed tension during the 60-min period of reperfusion. In contrast, all five hearts treated with heptanol in the first 15 min of reperfusion showed a clear recovery ($44.9 \pm 9.7\%$ of baseline tension compared with no functional recovery in their 5 paired controls; Student's *t* test, $P < 0.01$). The other GJ inhibitors investigated failed to induce any significant improvement in functional recovery during reperfusion.

Hearts submitted to 30 min of ischaemia showed marked functional recovery upon reperfusion, with no differences between groups (Fig. 4). Recovery of developed tension occurred at 12.7 ± 1.6 min of reperfusion in control hearts. The time course of recovery was not modified by treatment with palmitoleic acid or halothane, but appeared to be delayed in groups treated with heptanol or 18α -glycyrrhetic acid. The onset of recovery occurred at 24.8 ± 1.4 and 33.7 ± 9.1 min, respectively (ANOVA and Dunnett's test, $P < 0.05$) (Fig. 4).

Conduction velocity. In hearts submitted to 30 min of ischaemia, initial conduction velocity ranged between 30 and 45 cm s^{-1} , with no differences between groups. During ischaemia, conduction velocity decreased markedly and reached zero several minutes later. Upon reperfusion, conduction velocity rapidly recovered in control hearts.

In heptanol-treated hearts, recovery occurred with some delay (Fig. 5). In hearts treated with 18 α -glycyrrhetic acid, palmitoleic acid or halothane, conduction velocity remained reduced throughout the reperfusion period (Fig. 5) (repeated measures ANOVA and Dunnett's test, $P < 0.05$).

Enzyme release. Control isolated rat hearts submitted to 60 min of ischaemia–reperfusion in the absence of GJ inhibitors showed marked LDH release at the time of reperfusion, which was characterized by an early peak occurring in the first 2–4 min of reperfusion, followed by a rapid decay. In contrast, all four GJ inhibitors induced an

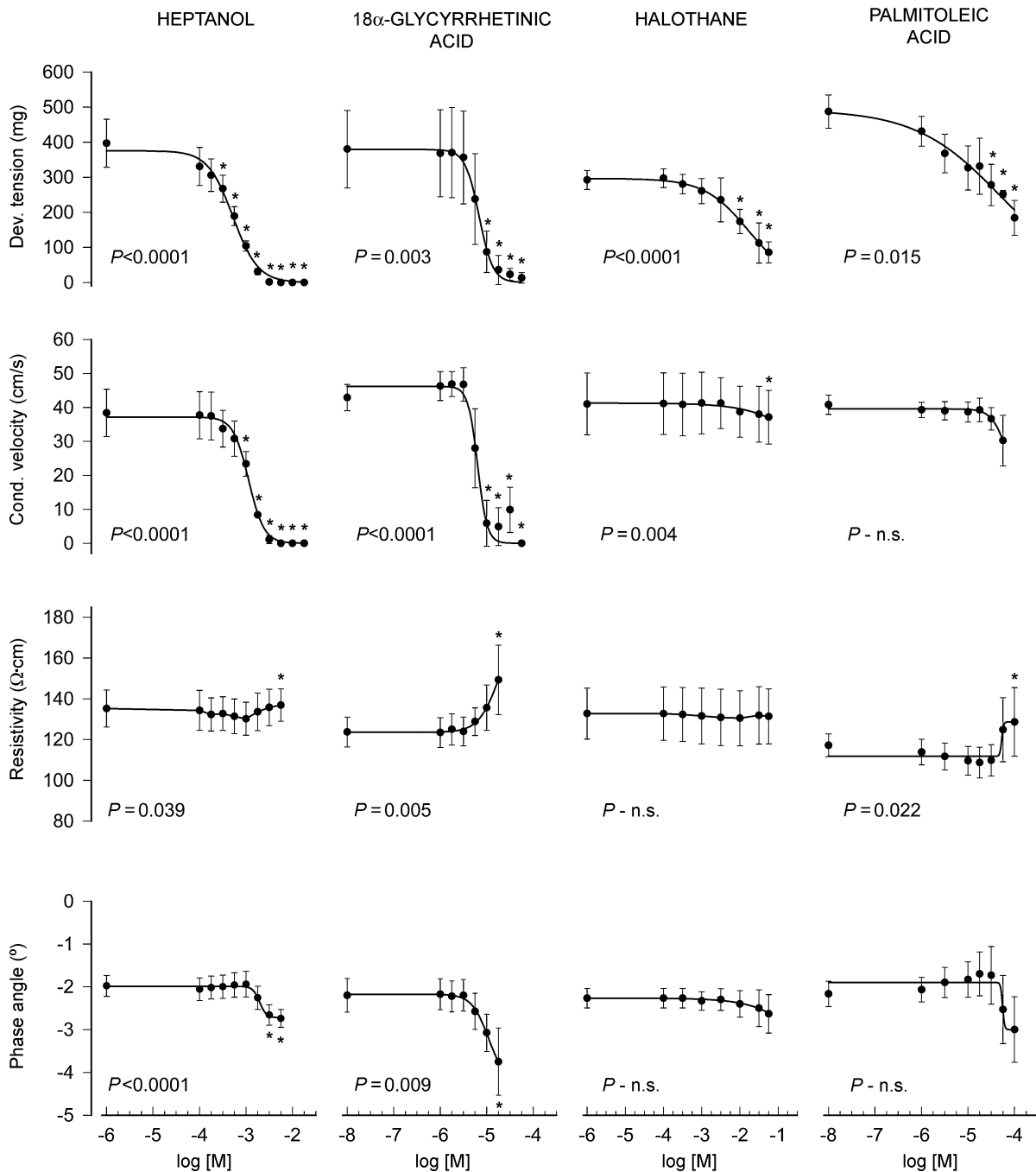


Figure 1. Changes in developed tension (Dev. tension), conduction velocity (Cond. velocity), electrical resistivity and phase angle in normoxic isolated rat hearts treated with heptanol, 18 α -glycyrrhetic acid, halothane and palmitoleic acid

* $P < 0.05$ indicates significant differences with respect to initial values. The P value shown in each graph indicates the MANOVA P value.

attenuated enzyme release during the entire reperfusion period (Figs 2 and 6). No significant correlation was observed between the degree of protection afforded by each GJ inhibitor and the effect of the concentrations used on developed tension during normoxia, suggesting that the protective effect is independent of their actions on contractility (Fig. 7).

Isolated rat hearts submitted to 30 min of ischaemia showed only minimal LDH release during reperfusion, with no significant differences between groups ($\text{U (g dry tissue)}^{-1} (15 \text{ min})^{-1}$: 10.9 ± 1.8 in control hearts, and 9.7 ± 1.3 , 9.3 ± 4.3 , 9.0 ± 3.1 and 4.6 ± 0.7 in heptanol, 18α -glycyrrhetic acid, palmitoleic acid and halothane-treated hearts, respectively). The area of necrosis determined by TTC staining in these hearts was also small and homogeneous among groups ($7.8 \pm 1.0\%$

of total cardiac weight in control hearts, and 5.5 ± 1.2 , 11.5 ± 1.8 , 9.0 ± 2.9 and $6.8 \pm 1.2\%$ in heptanol, 18α -glycyrrhetic acid, palmitoleic acid and halothane-treated hearts, respectively).

Myocardial water content. Both heptanol and 18α -glycyrrhetic acid caused a reduction in myocardial water content in hearts submitted to 60 min of ischaemia followed by reperfusion ($484.0 \pm 18.9 \text{ ml (100 g dry tissue)}^{-1}$ at the end of the reperfusion in heptanol-treated hearts *versus* 588.4 ± 30.2 in control hearts, $P = 0.021$; and $515.7 \pm 15.5 \text{ ml (100 g dry tissue)}^{-1}$ in 18α -glycyrrhetic acid-treated *versus* 597.6 ± 17.3 in controls preparations, $P < 0.01$). In contrast, hearts treated with halothane or palmitoleic acid did not show any significant change in water content at 1 h of

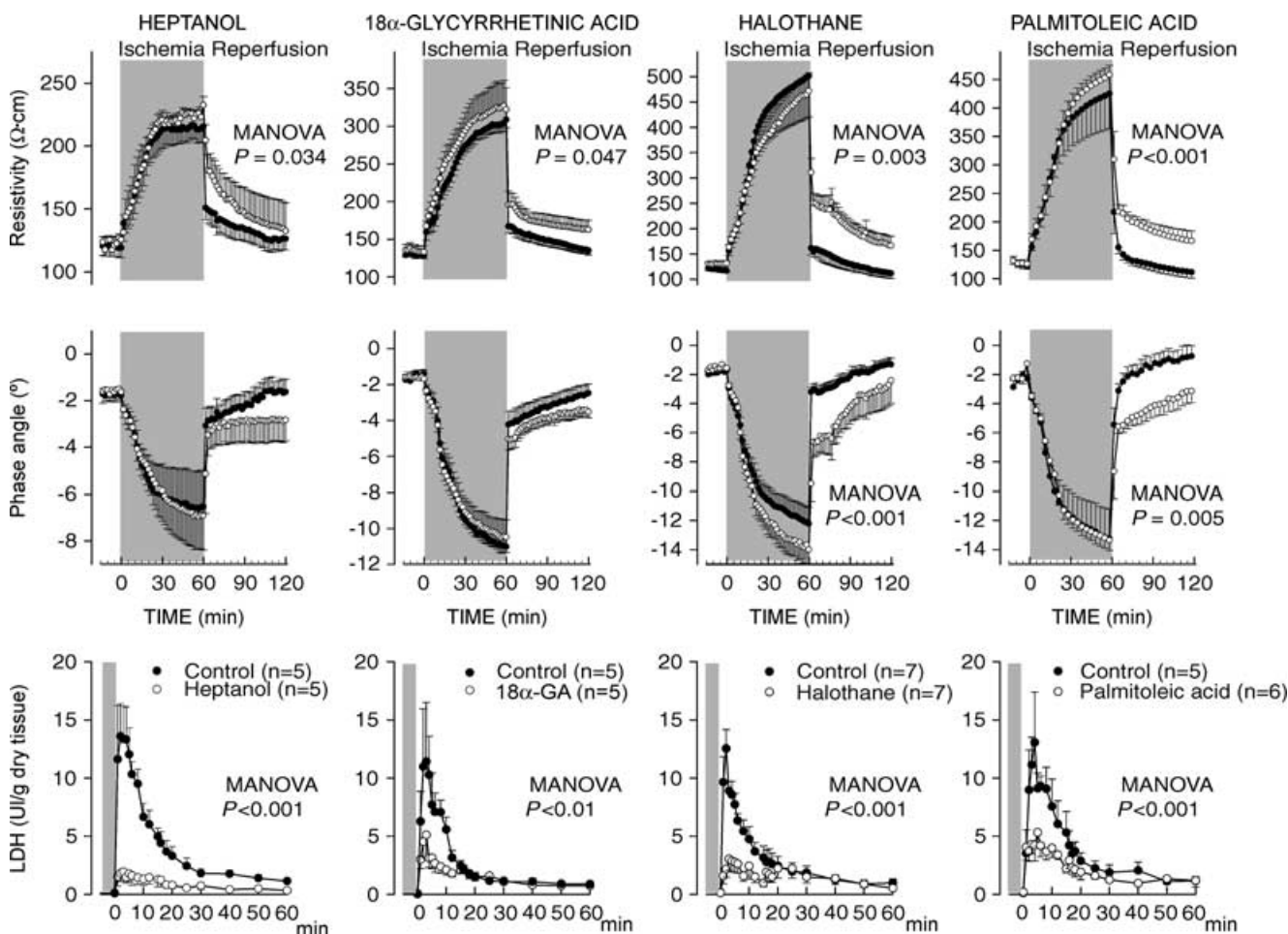


Figure 2. Changes in electrical resistivity and phase angle during ischaemia–reperfusion, and LDH release during reperfusion, in control isolated rat hearts submitted to 60 min of ischaemia–reperfusion and in hearts treated with different inhibitors of GJ mediated communication during the first 15 min of reperfusion

Concentrations of GJ inhibitors were as follows: heptanol: 10^{-3} M; 18α -glycyrrhetic acid: 10^{-5} M; halothane: 10^{-3} M; and palmitoleic acid: $3 \cdot 10^{-5}$ M. The P value shown in each graph indicates the MANOVA P value.

reperfusion. Hearts submitted to 30 min of ischaemia followed by 1 h of reperfusion showed a less severe degree of oedema without differences between groups ($\text{ml (100 g dry tissue)}^{-1}$: 510.4 ± 14.0 in control hearts and 507.9 ± 15.3 , 467.3 ± 29.7 , 504.2 ± 12.0 and 487.6 ± 21.1 in heptanol, 18α -glycyrrhetic acid, palmitoleic acid and halothane-treated hearts, respectively).

Discussion

This study demonstrates that four chemically unrelated GJ inhibitors increase electrical impedance during myocardial reperfusion at concentrations lacking measurable effects on electrical resistivity and phase angle under normal conditions. This effect was consistently associated with a marked attenuation of myocardial necrosis induced by ischaemia–reperfusion. These results extend previous observations showing a protective effect of heptanol against ischaemia–reperfusion injury to other GJ inhibitors, and open the possibility of devising pharmacological strategies to inhibit GJ-mediated communication in reperfused myocardium without

altering overall macroscopic electrical coupling in control tissue.

The present results provide further support for the hypothesis that GJ-mediated spread of cell death significantly contributes to the final extent of necrosis induced by transient myocardial ischaemia. First, the protection observed with heptanol is extended to other chemically unrelated widely used GJ inhibitors such as halothane, 18α -glycyrrhetic acid and palmitoleic acid. This is important because of the relative lack of specificity of heptanol, and to a lesser extent of the other GJ inhibitors. Second, it links for the first time the protective effect to the effects on cell coupling, as assessed by analysis of tissue electrical impedance.

The protective effect of GJ uncoupling against lethal reperfusion injury is not exclusive of heptanol

Heptanol, a polyalcohol, has been so far the most widely used GJ inhibitor. Its mechanism of action seems to be related to the modification of the physical properties of the cholesterol-rich domains of the plasma membrane

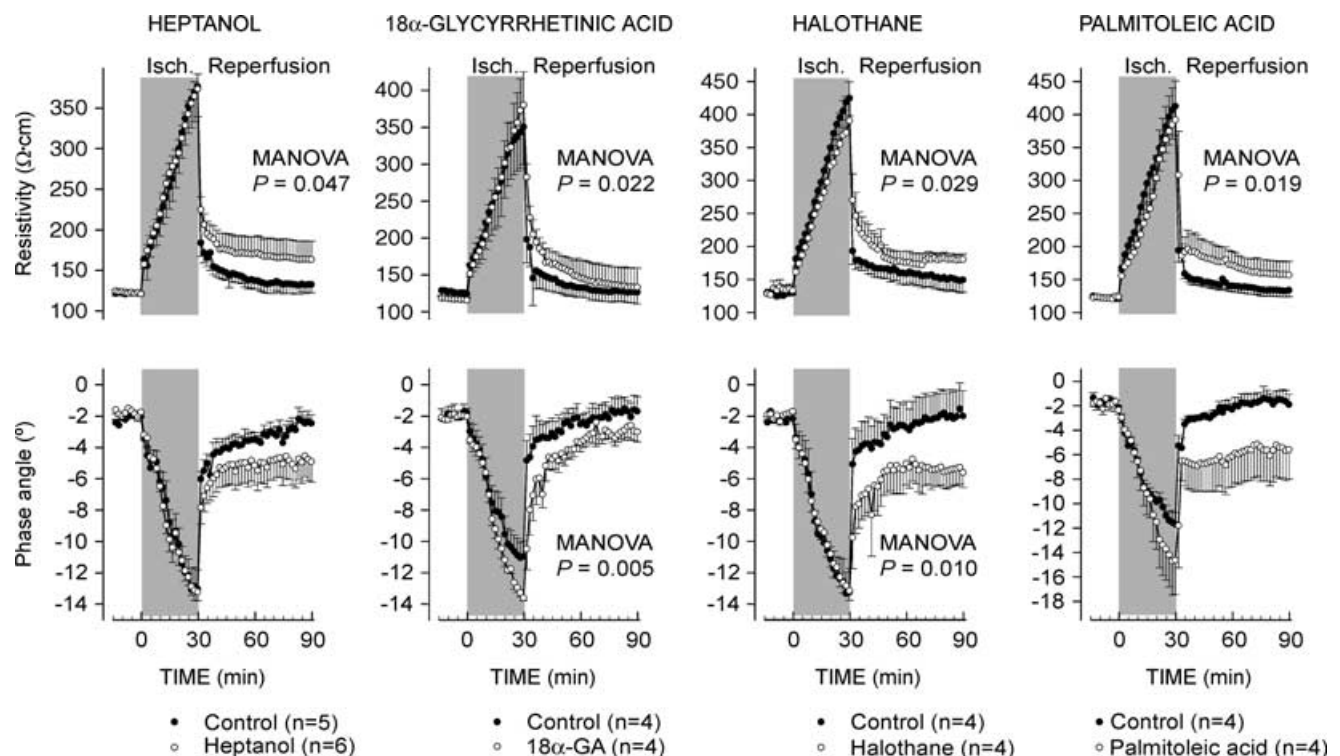


Figure 3. Changes in electrical resistivity and phase angle during ischaemia–reperfusion in control isolated rat hearts submitted to 30 min of ischaemia–reperfusion and in hearts treated with different inhibitors of GJ-mediated communication during the first 15 min of reperfusion

Concentrations of GJ inhibitors were as follows: heptanol: 10^{-3} M; 18α -glycyrrhetic acid: 10^{-5} M; halothane: 10^{-3} M; and palmitoleic acid: 3×10^{-5} M. The *P* value shown in each graph indicates the MANOVA *P* value.

resulting in a reduction of the open probability of junctional channels (Takens-Kwak *et al.* 1992; Bastiaanse *et al.* 1993).

Previous studies demonstrated that heptanol reduces cell-to-cell progression of hypercontracture in isolated cardiomyocytes and LDH release and infarct size in intact hearts when given at the time of reperfusion (Garcia-Dorado *et al.* 1997). Pretreatment with heptanol (Saltman *et al.* 2002) or infusion of this alcohol during ischaemia (Miura *et al.* 2003) has been shown to reduce infarct size in isolated rabbit hearts submitted to coronary occlusion followed by reperfusion. Heptanol and other polyalcohols have been found to be protective against cell death induced by ischaemia in brain and *in vitro* neuronal preparations (Rawanduzy *et al.* 1997; Rami *et al.* 2001). In contrast to the general belief that heptanol is protective in the ischaemia–reperfusion setting, some authors found no significant attenuation of infarct size in isolated rabbit hearts (Gysembergh *et al.* 2001), although this lack of effect might be explained by the lower heptanol concentration used in their study.

In addition to its known effects on GJ (Takens-Kwak *et al.* 1992; Bastiaanse *et al.* 1993), heptanol reduces other non-junctional membrane ionic currents, including Na^+ and Ca^{2+} inward currents (Niggli *et al.* 1989; Nelson &

Makielski, 1991; Takens-Kwak *et al.* 1992). These actions of heptanol may contribute to the observed effects on developed tension and conduction velocity observed in this and other studies (Balke *et al.* 1988; Keevil *et al.* 2000), and could contribute to its protective effect against reperfusion injury by GJ-independent mechanisms. However, the observation that other inhibitors of GJ communication, chemically unrelated to heptanol, share its protective effect during myocardial reperfusion is against this possibility.

The effect of halothane, a halogenated anaesthetic, on GJ-mediated intercellular communication has been characterized in detail (Burt & Spray, 1989; He & Burt, 2000) and seems to be due to a reduction in the open probability of junctional channels without change in their unitary conductance. Halothane also acts on several other cell systems, such as the sarcoplasmic reticulum (Wheeler *et al.* 1997) and different non-junctional currents (Terrenoire *et al.* 2000). As in the case of heptanol, these actions could contribute to the observed protective effect of halothane against ischaemia and reperfusion injury. Previous studies have shown that pretreatment with halothane protects against cell death secondary to transient ischaemia (Cope *et al.* 1997; Coetzee *et al.* 2000). Halothane has been shown to protect also against cell

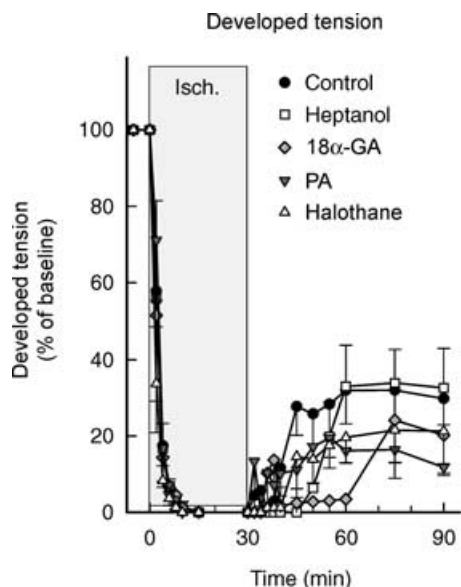


Figure 4. Functional recovery after ischaemia–reperfusion in control isolated rat hearts submitted to 30 min of ischaemia–reperfusion and in hearts treated with different inhibitors of GJ-mediated communication during the first 15 min of reperfusion

Concentrations of GJ inhibitors were as follows: heptanol: 10^{-3} M; 18 α -glycyrrhetic acid: 10^{-5} M; halothane: 10^{-3} M; and palmitoleic acid: 3×10^{-5} M.

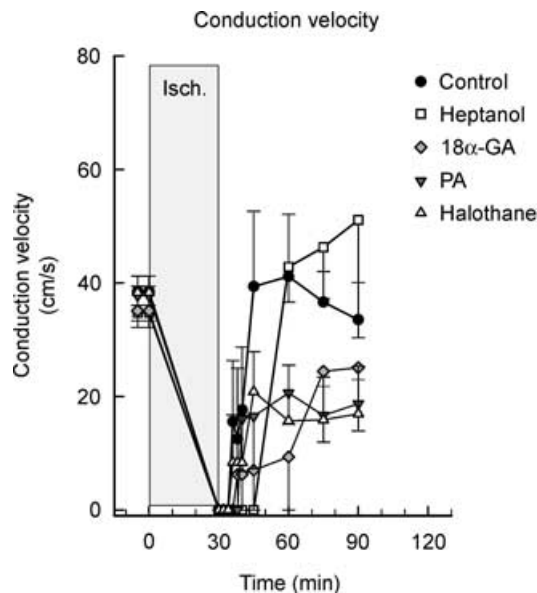


Figure 5. Recovery of conduction velocity after ischaemia–reperfusion in control isolated rat hearts submitted to 30 min of ischaemia–reperfusion ($n = 4$) and in hearts treated with different inhibitors of GJ-mediated communication during the first 15 min of reperfusion

Concentrations of GJ inhibitors were as follows: heptanol: 10^{-3} M ($n = 3$); 18 α -glycyrrhetic acid: 10^{-5} M ($n = 3$); halothane: 10^{-3} M ($n = 3$); and palmitoleic acid: 3×10^{-5} M ($n = 3$).

death when given at the time of reperfusion in the *in situ* rabbit heart (Schlack *et al.* 1997), in isolated rat hearts (Schlack *et al.* 1996) and in isolated rat cardiomyocytes (Siegmund *et al.* 1997). The protective effect of pretreatment with halothane has been related to preconditioning, as it depended on adenosine receptors and protein kinase C (Cope *et al.* 1997) and ATP-dependent potassium channels (Coetzee *et al.* 2000). It has been proposed that the protection afforded during reperfusion is due to attenuation of Ca^{2+} oscillations between cytosol and sarcoplasmic reticulum (Siegmund *et al.* 1997), and had not been previously related to the effect of the drug on GJs.

18α -Glycyrrhetic acid and palmitoleic acid are widely used GJ inhibitors. Both are effective in the micromolar range, and are supposedly more specific than heptanol or halothane, effective only in the millimolar range. 18α -Glycyrrhetic acid seems to act through changes in phosphorylation and/or connexin assembly, while palmitoleic acid is supposed to act, like heptanol, as a lipophilic drug (Burt *et al.* 1991; Guo *et al.* 1999; Dhein, 1998), but both substances have also other effects, such as inhibition of sarcolemmal Ca^{2+} currents and of mRNA synthesis (Shimada & Somlyo, 1992; Guo *et al.* 1999).

In contrast to heptanol and halothane, data on the potential protective effect of 18α -glycyrrhetic acid and palmitoleic acid are scant and contradictory. Fatty acids such as oleic acid have been shown to protect cardiac myocytes when given before simulated ischaemia (Mackay & Mochly Rosen, 2001). However, other studies have suggested that they aggravate ATP loss and mitochondrial injury during low-flow anoxia (Piper & Das, 1987). The effects of 18β -glycyrrhetic acid when infused during ischaemia have been described only very recently in a study by Miura *et al.* (2003) showing a reduction in infarct size in isolated rabbit hearts. Our present data demonstrate that 18α -glycyrrhetic acid and palmitoleic acid, like heptanol and halothane, reduce cell death when given at the time of reperfusion.

The GJ inhibitors used in the present study were given during reperfusion at concentrations that depressed developed tension in normoxic hearts. This fact obscures the ascription of the protective effect of these drugs to their effects on cell coupling and, in addition, limits their potential clinical application. However, there are data in the present study that indicate that the protective effect induced by GJ inhibitors is largely independent of their effects on contractility. First, as shown in Fig. 7,

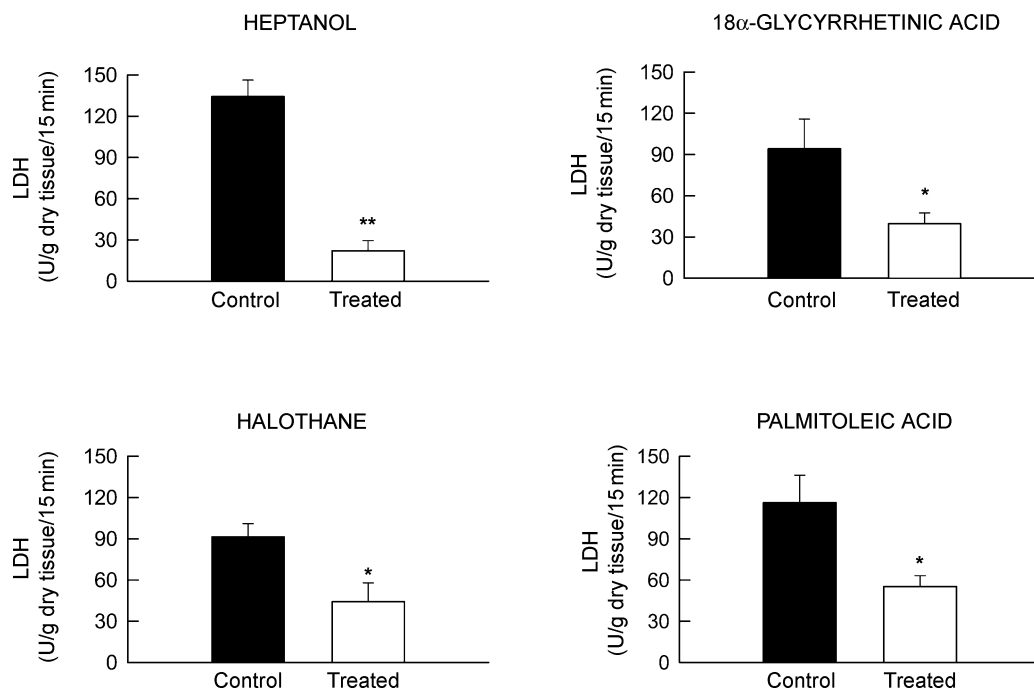


Figure 6. Accumulated LDH release during the first 15 min of reperfusion in groups treated with different inhibitors of GJ-mediated communication and their respective controls

Concentrations of GJ inhibitors were as follows: heptanol: 10^{-3} M; 18α -glycyrrhetic acid: 10^{-5} M; halothane: 10^{-3} M; and palmitoleic acid: 3×10^{-5} M. ** $P < 0.01$ and * $P < 0.05$ indicate significant differences compared to control hearts.

there was no significant correlation between the degree of reduction of developed tension during normoxia and the protective effect on LDH release during reperfusion. Second, halothane at 1 mM had nearly undetectable effects on contractile function yet it was very effective in attenuating LDH release.

Finally, the protective effect shared by all four GJ inhibitors was clearly observable in hearts submitted to prolonged (60 min) ischaemia, but not in those submitted to shorter ischaemic periods (30 min), in which no significant effect of the GJ inhibitors on TTC staining or LDH release could be demonstrated. A possible explanation for this lack of significant protection against cell death after 30 min of ischaemia is the very limited extent of necrosis in these experiments.

Effect of inhibitors of GJ-mediated communication on tissue electrical impedance in reperfused myocardium

All four GJ inhibitors investigated markedly reduced conduction velocity and attenuated the recovery of myocardial electrical impedance during the first minutes of reperfusion. This observation is a prerequisite for any attempt to establish a cause–effect relationship between the actions of these drugs on GJ-mediated communication and their protective effects against myocardial necrosis secondary to ischaemia–reperfusion.

A potential limitation of the use of changes in tissue electrical impedance as markers of changes in gap junctional conductance is that they can be influenced by alterations in intracellular and extracellular resistances, as

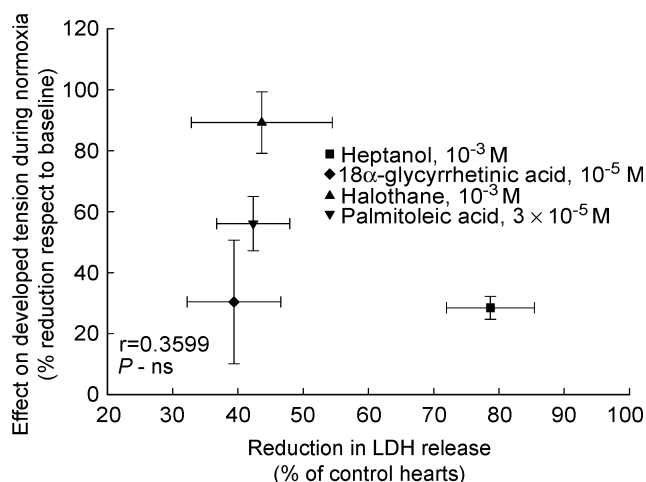


Figure 7. Correlation between the decrease in developed tension induced by the different drugs when administered during normoxia and the reduction in total LDH release induced when given at the onset of reperfusion

No clear relationship between both effects was observed.

well as by membrane resistance and capacitance (Gebhard *et al.* 1987). In the present study, a reduced rupture of cell membranes in hearts receiving GJ inhibitors could have contributed to the attenuated recovery of myocardial resistivity observed in hearts submitted to 60 min of ischaemia followed by reperfusion. However, our data obtained in preparations in which the duration of ischaemia was set at 30 min seem to exclude this possibility as cell death (i.e. LDH release or TTC-negative staining) in these conditions was almost negligible. Moreover, other strongly protective treatments, like cariporide (Rodriguez-Sinovas *et al.* 2003) or ischaemic preconditioning (Padilla *et al.* 2003), did not induce any change in the recovery of myocardial electrical resistivity during reperfusion. Reduced tissue oedema could have also contributed to the attenuated reduction of myocardial resistivity during reperfusion observed in the presence of GJ inhibitors, since oedema increases tissue conductivity. However this possibility seems unlikely: although both heptanol and 18α-glycyrrhetic acid caused a decrease in myocardial water content in hearts submitted to 60 min of ischaemia–reperfusion, the effects of halothane and palmitoleic acid on the recovery of tissue resistivity were not associated with any significant action on myocardial water content. Furthermore, in those hearts submitted to 30 min of ischaemia followed by reperfusion, no difference in tissue water content was observed between control hearts and those treated with GJ inhibitors. Thus, the effects of GJ inhibitors on electrical impedance appear to be dissociated from their effects on tissue oedema.

Another possible limitation of the use of electrical impedance is that, due to the probe's size, it reflects a relatively large area of myocardium around the electrode, which in rat ventricle may include the complete thickness of the ventricular wall. Thus, we cannot exclude the possibility of transmural differences in the response. In addition, our methods do not provide information on changes in the lateral conduction velocity or its dispersion, nor in changes in anisotropy of the tissue. The limited spatial resolution of impedance analysis may, on the other hand, have the advantage of minimizing the influence of small areas of tissue heterogeneities (as in the case of hearts submitted to 30 min of ischaemia showing patchy areas of necrosis) on the final measurement.

Enhanced effect on macroscopic electrical properties of GJ inhibitors in reperfused myocardium

The inhibitory effect of the investigated GJ inhibitors on intercellular communication has been solidly

demonstrated (Burt & Spray, 1989; Burt *et al.* 1991; Takens-Kwak *et al.* 1992; Bastiaanse *et al.* 1993; Dhein, 1998; Guo *et al.* 1999; He & Burt, 2000). The most plausible explanation for the lack of effect of GJ inhibitors on electrical impedance observed in the present study in normoxic myocardium, at concentrations that markedly affect it after ischaemia–reperfusion, is that although the fractional decrease of communication could be the same in normoxic and ischaemic–reperfused myocardium, due to non-linearities in the response, the effects of GJ blockade were more pronounced in reperfused myocardium, due to a strongly reduced number of available GJ channels. This may reflect the high ‘safety factor’ in normal myocardium that allows important reductions in the number of open junctional channels and/or their average conductivity with almost no detectable reduction of electrical coupling (Jongsma & Wilders, 2000). Previous computer simulation studies indicate that even a reduction of 40% in GJ content would induce only moderate effects on conduction velocity in normal tissue, and that a reduction of gap junctional conductance from 7 μS to 3 μS is necessary to induce noticeable effects on gap junctional resistivity (Jongsma & Wilders, 2000). Other authors have suggested that an intervention having physiologically relevant effects on cell-to-cell coupling must result in closure of more than 90% of GJ channels (Christ *et al.* 1996). It is easily conceivable that the alterations in ionic composition (decrease in intracellular pH and increase in free calcium concentration) (Maurer & Weingart, 1987; Noma & Tsuboi, 1987; Calero *et al.* 1998) that occur during ischaemia and persist during the initial minutes of reperfusion, together with the dephosphorylation of connexin43 proteins (Beardslee *et al.* 2000), the reduction in ATP availability (Sugiura *et al.* 1990), and the increased concentrations of amphipathic catabolites (Wu *et al.* 1993), factors all known to cause closure of GJs (Dhein, 1998), contribute to reduce the ‘safety factor’. As a consequence, any additional reduction in the open probability and/or average conductance induced by GJ inhibitors would have more apparent effects on electrical tissue impedance in reperfused than in normoxic conditions. Differential effects between normal and diseased myocardium have also been observed by other authors who demonstrated that heptanol depressed conduction in strips from infarcted canine myocardium at concentrations that were without effect in normal tissues (Spear *et al.* 1990).

The increased effect of GJ inhibitors on macroscopic electrical properties of reperfused myocardium opens the possibility of the development of strategies able to interfere with GJ-mediated spread of necrosis in

myocardium undergoing reperfusion without significant overall macroscopic effects in myocardium at a distance. This may require the search for new, more specific inhibitors of GJ-mediated intercellular communication lacking the depressor effects on contractility associated with inhibition of sarcolemmal Na^+ and Ca^{2+} currents and with altered function of the sarcoplasmic reticulum (Niggli *et al.* 1989; Nelson & Makielski, 1991; Takens-Kwak *et al.* 1992; Shimada & Somlyo, 1992; Wheeler *et al.* 1997; Guo *et al.* 1999; Terrenoire *et al.* 2000).

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