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Effects and interaction, of cariporide and preconditioning on cardiac arrhythmias and infarction in rat *in vivo*

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1 Although Na^+-H^+ exchange (NHE) inhibitors are reported to protect the myocardium against ischaemic injury, NHE activation has also been proposed as a potential mechanism of ischaemic preconditioning-induced protection. This study was performed to test any modifiable effect of cariporide, an NHE inhibitor, on cardioprotective effects of preconditioning.

2 Anaesthetized rats were subjected to 30 min of coronary artery occlusion and 150 min of reperfusion. The preconditioning (PC) was induced by 3 min of ischaemia and 10 min of reperfusion (1PC) or three episodes of 3 min ischaemia and 5 min reperfusion (3PC). Cariporide (0.3 mg kg^{-1}) an NHE inhibitor, was administered 30 min (cari(30)) or 45 min (cari(45)) before coronary ligation ($n=8-11$ for each group).

3 Ventricular arrhythmias during 30 min ischaemia and infarct size (measured by triphenyltetrazolium (TTC) and expressed as a per cent area at risk (%AAR)) were determined. Cari(30) reduced ventricular fibrillation (VF) incidence and infarct size (from 45 to 0% and 34 ± 4 to 9 ± 2%; each $P<0.05$), whereas cari(45) did not. Likewise, 3PC reduced these variables (to 0% and 10 ± 2%; $P<0.05$ in each case) whereas 1PC did not. Moreover, subthreshold preconditioning (1PC) and cariporide (cari(45)), when combined, reduced VF incidence and infarct size (to 0% and $15+3$%; each $P<0.05$).

4 In conclusion, changes in NHE activity do not seem to be responsible for the cardioprotective action of ischaemic preconditioning. Protective effects of NHE inhibition and subthreshold preconditioning appear to act additively.

Keywords: Na^+-H^+ exchange; arrhythmias; cariporide; ischaemia; infarction; preconditioning; reperfusion

Abbreviations: α1, alpha 1; %AAR, % area at risk; ANOVA, analysis of variance; ECG, electrocardiogram; HOE642, 4-isopropyl-3-methylsulphonylbenzoyl-guanidine; HOE694, 3-methylsulphonyl-4-piperidinobenzoyl-guanidine; NHE, sodium proton exchange; NHE-1, sodium proton exchanger isoform 1; NIH, National Institutes of Health; PC, preconditioning; pH_i, intracellular pH; TTC, triphenyltetrazolium chloride; VF, ventricular fibrillation; VPB, ventricular premature beat; VT, ventricular tachycardia

Introduction

Ischaemic preconditioning is a phenomenon in which one or more brief periods of ischaemia protects the myocardium against ischaemic injury from a subsequent prolonged period of ischaemia (Murry *et al*., 1986). The protection includes decrease of infarct size, arrhythmias, contractile dysfunction and energy demand in various animal species (Li *et al*., 1990; Murry *et al*., 1990; Liu & Downey, 1992; Vegh *et al*., 1992; Lawson *et al*., 1993; Steenbergen *et al*., 1993; Ytrehus *et al*., 1994). Ischaemic preconditioning has been reported to preserve myocardial high energy stores during ischaemia (Murry *et al*., 1990), stimulate adenosine receptors (Liu *et al*., 1991), and α1-adrenergic receptors (Banerjee *et al*., 1993), activate ATP sensitive K channels (Tan *et al*., 1993; Schulz *et al*., 1994), translocate and activate protein kinase C (Liu *et al*., 1994; Speechly-Dick *et al*., 1994), and reduce acidosis during subsequent prolonged ischaemia (Kida *et al*., 1991).

The sarcolemmal Na^+-H^+ exchange (NHE) is a major acid extrusion system in cardiac myocytes and plays an important role in restoration of intracellular pH following an acid load (Frelin *et al*., 1985; Lazdunsky *et al*., 1985; Kaila & Vaughan-Jones, 1987; Weissberg *et al*., 1989). The exchanger may also play an important role in determining the severity of the unfavourable sequelae of myocardial ischaemia and reperfusion, such as arrhythmias, contractile dysfunction and infarction (Ikeda *et al*., 1988; Karmazyn & Moffat, 1993; Yasutake & Avkiran, 1995; Avkiran, 1996; Avkiran & Yasutake, 1996; Karmazyn, 1996). In cardiac myocytes the activity of NHE is regulated not only by intracellular pH, but also by a number of extracellular stimuli such as exposure to adrenergic agonists (Wallert & Frohlich, 1992), thrombin (Yasutake *et al*., 1996) and angiotensin II (Matsui *et al*., 1995; Libonati *et al*., 1997) through receptor mediated mechanisms. The NHE inhibitors, amiloride and its derivatives such as ethylisopropylamiloride, dimethylamiloride, hexamethylamiloride and methylisobutylamiloride, as well as benzoyl guanidine derivatives such as HOE694 (3-methylsulphonyl-4-piperidinobenzoyl-guanidine) and cariporide (HOE642: 4-isopropyl-3-methylsulphonylbenzoyl-guanidine), protect the myocardium from ischaemia- and/or reperfusion-induced injuries in various animal species (Pierce & Czubryt, 1995; Scholz & Albus, 1995; Scholz *et al*., 1995). We have also shown that inhibition of NHE with cariporide protected hearts from arrhythmias induced by ischaemia and reperfusion in dogs and rats (Xue *et al*., 1996; Aye *et al*., 1997). Garcia-Dorado *et al*. (1997), Miura *et al*. (1997) and Linz *et al*. (1998) have shown that cariporide reduces the infarct size induced by occlusion and reperfusion of the coronary artery of animal hearts.

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Recently Ramasamy *et al.* (1995) reported that ischaemic preconditioning stimulates the NHE and this activated exchanger may contribute to attenuate intracellular acidosis and to reduce ischaemia/reperfusion-induced injury in isolated rat hearts. However Shipolini *et al.* (1997) have shown that cardioprotective effects of ischaemic preconditioning and NHE inhibition, as assessed by recovery of the left ventricular developed pressure and the amount of creatine kinase leakage during reperfusion, are additive rather than counteractive in isolated rat hearts. There is some evidence to suggest that preconditioning may protect against one form of dysfunction (e.g. arrhythmias) by a mechanism different from that mediating protection against another (e.g. infarction) (Gross *et al.*, 1992; Piacentini *et al.*, 1993; Liu & Downey, 1993; Vegh *et al.*, 1993; Ravingerova *et al.*, 1995). The aim of this study was to test for any modifiable effect of the NHE inhibitior, cariporide, on the cardioprotective effect of ischaemic preconditioning by two indices of injuries, arrhythmias and infarct size, in the same *in vivo* anaesthetized rats.

Methods

Production of coronary ischaemia/reperfusion injury in rats

As reported earlier (Selye *et al.*, 1960; Clark *et al.*, 1980) and as modified by Komori *et al.* (1994), male Sprague-Dawley rats (body weight 250–400 g) were anaesthetized with sodium pentobarbitone (60 mg kg^{-1}, i.p.). The femoral vein was cannulated to allow drug administration, and the trachea was cannulated for artificial ventilation. The systemic blood pressure was monitored *via* a catheter inserted into the carotid artery and a standard limb lead I electrocardiogram (ECG) was continuously monitored on a recorder (Nihon Kohden, RM-62001, Tokyo, Japan). The chest was opened by left thoracotomy at approximately 2 mm to the left of the sternum and followed by sectioning the fourth and fifth ribs. Artificial ventilation was immediately started using room air (volume 1.5 ml 100 g^{-1}, rate 54 strokes min^{-1}) to maintain PCO_2, PO_2 and pH within the normal limits. After incising the pericardium, the heart was exteriorized using gentle pressure on the rib cage, and a 6/0 braided silk suture was placed around the left coronary artery. The heart was placed back into the chest and the animal was allowed to stabilize.

Transient regional myocardial ischaemia was induced by passing the threads through a small plastic tube and pressing the tube against the coronary artery, and reperfusion was initiated by releasing the ligature and removing the plastic tube. As for inducing ischaemic preconditioning (PC), 3 min brief occlusion followed by 5 min reperfusion was performed three times (3PC) (Li *et al.*, 1992) or single 3 min brief occlusion followed by 10 min reperfusion (1PC) (Vegh *et al.*, 1992) were used. The coronary artery was finally occluded for 30 min followed by 150 min of reperfusion. The severity of arrhythymias during the 30 min occlusion period was assessed and compared with that of control rats as well as that of cariporide treated rats with or without ischaemic preconditioning. Ischaemia and reperfusion were confirmed as described previously (Lawson *et al.*, 1993). In short, successful occlusion was confirmed by the increase of the height of the R wave voltage during the first few seconds of each occlusion (Carbonin *et al.*, 1980) and a 20–30% reduction in the arterial blood pressure compared to the pre-ischaemic values. Successful reperfusion was confirmed by the return of the height of the R wave voltage and the arterial blood pressure to the pre-ischaemic values.

Animals were obtained through the Animal Laboratory for Research of Yamanashi Medical University. All experiments were carried out according to the Guideline for Animal Experiments of Yamanashi Medical University.

Infarct size (measurement of infarct and risk areas)

At the end of the experiments, the infarct size and the size of the ischaemic risk area were measured as described elsewhere (Donnelly *et al.*, 1992; Wolfe *et al.*, 1993). In short, the heart was excised and the coronary blood was washed out with saline. The left coronary artery was reoccluded and then the heart was mounted on a constant pressure apparatus (ATTO SJ 1220 Peristaltic pump, Tokyo, Japan). Evans Blue dye (0.25%) was perfused for 5 min at a rate of 1.5 ml min^{-1} causing the dye to infuse into the nonischaemic area of the left ventricle, leaving the ischaemic regions unstained. The heart was then removed from the apparatus and rinsed of excess blue dye, and was sliced transversely into 2 mm thick sections. After the right ventricle and atria were dissected away, the left ventricle was incubated (20 min) in a 1% solution of 2,3,5-triphenyltetrazolium chloride (TTC) at 37°C. TTC staining (Nachlas & Shnitka, 1963; Klein *et al.*, 1981) has been shown to demarcate viable tissue by reacting with myocardial dehydrogenase enzymes to form a brick red stain. Necrotic tissue which has lost its dehydrogenase enzymes does not form a red stain and shows up as pale yellow. The left ventricular sections were placed between two glass slides, and immersed for 6–12 h in 10% formalin to enhance the contrast of the stain, and weighed. Color photographs were taken for each slice with a digital camera (Cannon). Areas that were stained with blue dye (non-ischaemic), stained red with TTC (ischaemic but non-infarcted), and unstained (infarcted) were measured by a Macintosh computer using the public domain NIH (National Institutes of Health) image program (Version 1.61). The fraction of the left ventricular area representing nonischaemic, ischaemic, and infarcted tissue were averaged from the photograph of each side of each section and multiplied by the weight of that section to determine the absolute weight of tissue in each region. Ischaemic risk area was defined as the sum of the weights of infarct and non-infarct (TTC red) tissue from all slices. The infarct size was calculated as a percentage of the total left ventricular mass. The risk area was calculated as a percentage of total left ventricular mass and the infarct size was expressed as a percentage of the ischaemic risk area (% area at risk: %AAR).

Definition of arrhythmias and analysis

Definitions of arrhythmias were based on those described in the Lambeth Conventions (Walker *et al.*, 1988). Ectopic activity was categorized as a single ventricular premature beat (VPB), ventricular tachycardia (VT, four or more consecutive VPB) or ventricular fibrillation (VF, inability to distinguish individual QRS complexes and to measure the rate). Complex forms (e.g. bigeminy) were included in the count of VPB and were not analysed separately. Reference was made to the blood pressure tracings to confirm which type of ectopic activity was occurring, particularly to distinguish between polymorphic VT and VF. When the former occurred, the blood pressure was usually still pulsatile whereas with VF the blood pressure fell

rapidly towards zero and was no longer pulsatile. VF may be sustained or may revert spontaneously to a normal sinus rhythm in the rat (Curtis *et al*., 1987). In all experiments the incidence of VT and VF was noted.

Exclusion criteria

Experiments were terminated or excluded from the final data analysis, if any of the following occurred (Shaw & Coker, 1996): arrhythmias prior to coronary artery occlusion; mean arterial pressure less than 60 mmHg prior to drug or vehicle administration and atrioventricular block during the first 5 min of ischaemia (probably caused by ligature occluding the septal branch of the left coronary artery). Seventy-eight rats were entered in this study. Two rats were excluded for absence of sign of ischaemia, and one for atrioventricular block during the first 5 min of ischaemia. Another four rats were excluded for inadequate staining or insufficient quality of the photographic images to assess the infarct size and the risk area.

Experimental protocols

Protocol I The objective of this protocol was to determine the duration of the effective inhibitory actions of i.v. cariporide (0.3 mg kg^{-1}) on prolonged ischaemia/reperfusion-induced arrhythmias and infarct size (Figure 1). This protocol consisted of three groups. In the control group ($n=11$), after an adequate stabilization period, 30 min of ischaemia was followed by 150 min of reperfusion. In the cari(30) ($n=8$) and cari(45) ($n=11$) groups, cariporide 0.3 mg kg^{-1} was administered 30 min or 45 min before the onset of 30 min ischaemia, respectively. We chose a dose of 0.3 mg kg^{-1} because cariporide has already been reported to be specific and selective in its actions on the exchanger at a dose of 0.1–1 μM and 0.1–1 mg kg^{-1} *in vitro* and *in vivo*, respectively, (half-maximal inhibition (IC_{50}) = 0.1–1 μM for the recovery of pH_i after acid load in rat cardiomyocytes, and K_i was 0.05 μM for NHE-1 (exchanger isoform 1) (Scholz *et al*., 1995; Rub *et al*., 1996)). The same dose of cariporide effectively reduced reperfusion-induced arrhythmias in rat (Aye *et al*., 1997), and infarct size in rabbit (Linz *et al*., 1998).

Protocol II The aim of this protocol was to determine whether cariporide interacts with the effects of ischaemic preconditioning on arrhythmias and infarct size. As summarized in Figure 1, there were two different groups ($n=8$ per group). In the three episodes of ischaemic preconditioning (3PC) group, 3 min occlusion followed by 5 min reperfusion was repeated 3 times before 30 min of occlusion followed by

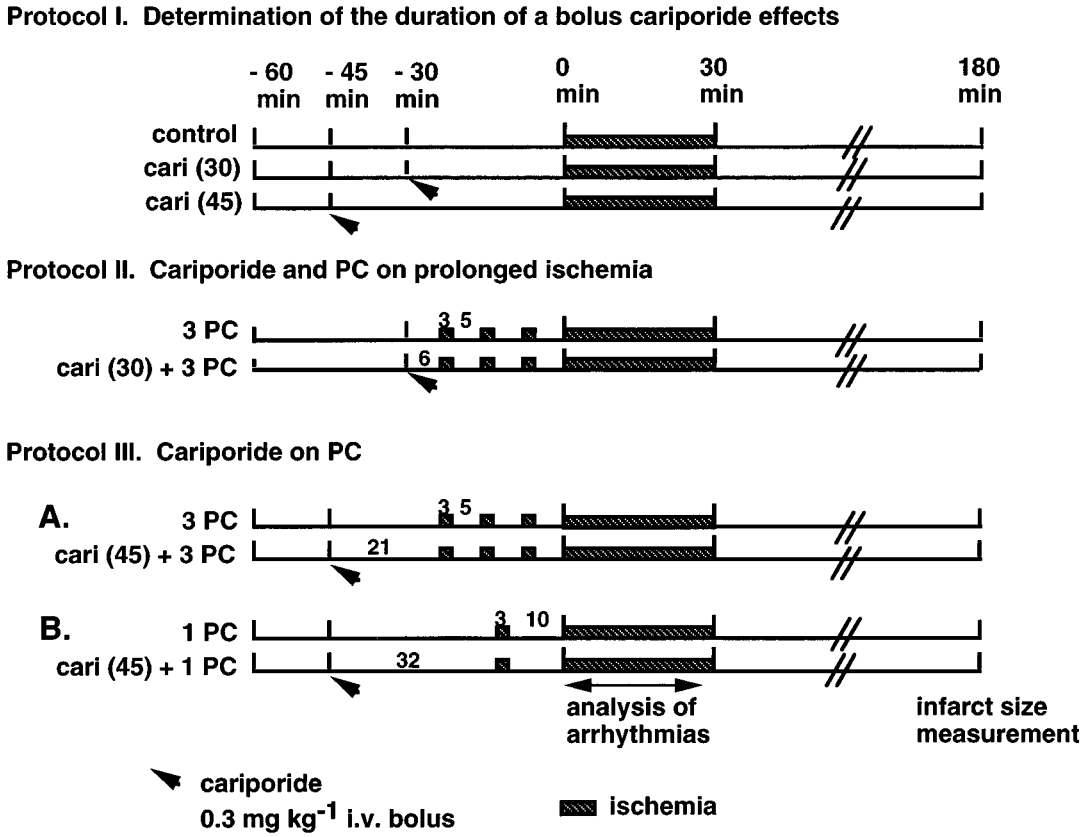

Figure 1 Experimental protocols used. In all experiments, the final 30 min ischaemia was followed by 150 min reperfusion. In Protocol I, 30 min ischaemia was done after the stabilization period, 30 min and 45 min after administration of cariporide in the control, cari(30), and cari(45) groups, respectively. In Protocol II, three episodes of 3 min occlusion followed by 5 min reperfusion (3PC) were done before 30 min ischaemia in 3PC group, and cariporide was administered 6 min before the first (preconditioned) occlusion of 3PC in cari(30) + 3PC group. In part A of the Protocol III, 3PC was performed as above, and cariporide was administered 21 min before the first (preconditioned) occlusion of 3PC in cari(45) + 3PC group. In part B, single 3 min occlusion followed by 10 min reperfusion (1PC) was done before 30 min ischaemia in 1PC group, and cariporide was administered 32 min before 1PC in the cari(45) + 1PC group. Arrows indicate the time of administration of cariporide 0.3 mg kg^{-1} i.v. bolus. Cross hatched columns indicate ischaemia. Arrhythmias were analysed during 30 min ischaemia and infarct sizes were measured at the end of experiments ($n=8-11$ in each group).

150 min of reperfusion. In the other group, cariporide 0.3 mg kg^{-1} bolus i.v. was administered 6 min before the first (preconditioning) occlusion.

Protocol III The aim of this study was to determine whether cariporide influences the beneficial effects of ischaemic preconditioning. As summarized in Figure 1, there were two main protocols in this study. In part A, an ischaemic preconditioning was repeated three times (3PC) ($n=8$) and for part B it was only carried out once (1PC) ($n=8$). Cariporide 0.3 mg kg^{-1} bolus i.v. was administered 21 min or 32 min before the first (preconditioning) occlusion in the cari(45)+3PC ($n=8$) and cari(45)+1PC ($n=9$) groups, respectively.

In all groups, ventricular arrhythmias during the final ischaemic period (30 min) and a risk area (as a percentage of total left ventricular weight) and infarct size (%AAR) were analysed.

Drugs

Drugs used in the present study were cariporide (Hoechst Marion Roussel Japan Limited, Tokyo, Japan), dimethyl sulphoxide (DMSO, Wako, Osaka, Japan) and pentobarbitone sodium (Tokyo Kasei Kogyo, Tokyo, Japan). Cariporide was dissolved in 5% DMSO on the day of the experiments.

Statistics

Statistical analysis was based upon the guidelines for statistics (Wallenstein *et al*., 1980) as modified for the study of arrhythmias using rat hearts (Curtis *et al*., 1987; Curtis & Hearse, 1989). Gaussian-distributed data are expressed as means ± s.e.mean. Non-Gaussian-distributed data were transformed to Gaussian-distributed data by deriving the $\log_{10}$ values. Differences in mean values between experimental groups were analysed by a one-way ANOVA (analysis of variance) followed by Dunnett's multiple comparison test or Fisher's protected least significant difference test. Differences in the incidence of arrhythmias among groups were analysed with Fisher's exact probability test. A P value of less than 0.05 was considered statistically significant.

Results

Determination of the duration of effect of a bolus injection of cariporide (0.3 mg kg-1) (Protocol I)

The administration of the drug had no significant effects on the blood pressure or heart rate (Table 1). As shown in Figure 2, VT duration ($\log_{10}$ s), VF incidence and infarct size as a percentage AAR of the control group were 2.2 ± 0.1, 45% and 34 ± 4%, respectively. In the cari(30) group they were significantly reduced to 1.4 ± 0.2, 0% and 9 ± 2% ($P<0.05$), respectively. In the cari(45) group, the VT duration, VF incidence and percentage AAR were 1.8 ± 0.2, 55% and 29 ± 4%, respectively, (no significant effect versus control). The rather low VF incidence in the control group might be due to a high serum potassium concentration which can often occur after surgery, and which can lead to suppression of ischaemia-induced arrhythmias (Curtis *et al*., 1985). Serum potassium concentration was not measured in the present study. These data indicate that when examing for a possible facilitation of subthreshold preconditioning by cariporide, the cari(45) protocol, itself subthreshold in terms of effects on VF and infarction, would be an appropriate choice for protocol III (see below).

Effects of both the cariporide and preconditioning on prolonged ischaemia (Protocol II)

Total VPB ($\log_{10}$ number of VPB), VT duration ($\log_{10}$ s) and VF incidence were reduced, $\log_{10}$ VPB from 3.24 ± 0.1 to 1.9 ± 0.3 ($P<0.05$), 2.8 ± 0.1 (not significant) and 2.4 ± 0.2 ($P<0.05$), $\log_{10}$ VT duration from 2.2 ± 0.1 to 0.2 ± 0.2, 1.4 ± 0.2 and 1.1 ± 0.3 ($P<0.05$), and VF incidence from 45 to 0, 0 and 0% ($P<0.05$) in the 3PC, cari(30) and cari(30)+3PC groups, respectively. There were no significant differences between the treated groups compared with one another.

There were no significant differences in the risk areas among the 3PC, cari(30) and cari(30)+3PC groups (54 ± 2, 51 ± 3 and 46 ± 4%, respectively). There was a significant reduction of risk area in the cari(30)+3PC group when compared to the control group (61 ± 4 versus 46 ± 4% ($P<0.05$)). As for the infarct size,

Table 1 Changes in the heart rate and blood pressure

		Stabilization		*Pre-ischaemia*		*End of ischaemia*		*60-min reperfusion*		*120-min reperfusion*		*150-min reperfusion*	
Group	n	*HR* (bpm)	*MBP* (mmHg)	*HR* (bpm)	*MBP* (mmHg)	*HR* (bpm)	*MBP* (mmHg)	*HR* (bpm)	*MBP* (mmHg)	*HR* (bpm)	*MBP* (mmHg)	*HR* (bpm)	*MBP* (mmHg)
Control	9	381 ± 16	86 ± 9	380 ± 15	88 ± 8	373 ± 11	94 ± 7	372 ± 12	105 ± 7	359 ± 12	97 ± 6	360 ± 13	102 ± 6
Cari (30)	8	366 ± 19	85 ± 6	363 ± 14	100 ± 6	346 ± 12	100 ± 6	342 ± 15	118 ± 4*	344 ± 14	111 ± 5*	344 ± 13	108 ± 4*
Cari (45)	9	386 ± 20	93 ± 6	378 ± 20	104 ± 3	387 ± 13	105 ± 7	386 ± 11	102 ± 5	379 ± 13	98 ± 5	371 ± 14	97 ± 7
1PC	8	398 ± 11	95 ± 7	397 ± 9	105 ± 5	384 ± 7	92 ± 11	363 ± 13	110 ± 6	362 ± 10	109 ± 6	364 ± 10	109 ± 6
3PC	8	381 ± 18	92 ± 7	381 ± 18	92 ± 7	355 ± 19	95 ± 5	364 ± 12	113 ± 3*	347 ± 13	106 ± 7	347 ± 11	104 ± 14
Cari(30)+3PC	8	387 ± 16	88 ± 5	387 ± 15	89 ± 6	382 ± 16	104 ± 5	380 ± 15	112 ± 7	381 ± 16	102 ± 7	378 ± 15	100 ± 8
Cari(45)+1PC	9	388 ± 11	83 ± 7	396 ± 9	101 ± 5	396 ± 9	110 ± 4*	385 ± 6	108 ± 4*	362 ± 7	107 ± 5*	361 ± 7	100 ± 6
Cari(45)+3PC	8	391 ± 20	86 ± 4	394 ± 13	97 ± 5	389 ± 20	106 ± 4*	358 ± 21	109 ± 5*	342 ± 14	100 ± 4	340 ± 16	98 ± 5

Values are mean ± s.e.mean. Control: no drug treatments nor preconditioning were performed; cari(30): 30 min ischaemia was induced 30 min after i.v. cariporide; cari(45): 30 min ischaemia was induced 45 min after i.v. cariporide; 1PC: one episode (3 min occlusion followed by 10 min reperfusion) of preconditioning; 3PC: three episodes (three times of 3 min occlusion followed by 5 min reperfusion) of preconditioning; cari(30)+3PC: cariporide was administered 6 min before the first (PC) occlusion of 3PC; cari(45)+1PC: cariporide was administered 32 min before 1PC; cari(45)+3PC: cariporide was administered 21 min before the first (PC) occlusion of 3PC; Stabilization: values are immediately before administration of cariporide in drug treated groups; *n*: number of rats in each group; HR: heart rate; MBP: mean blood pressure; bpm: beats per minute; *$P<0.05$ versus stable time.

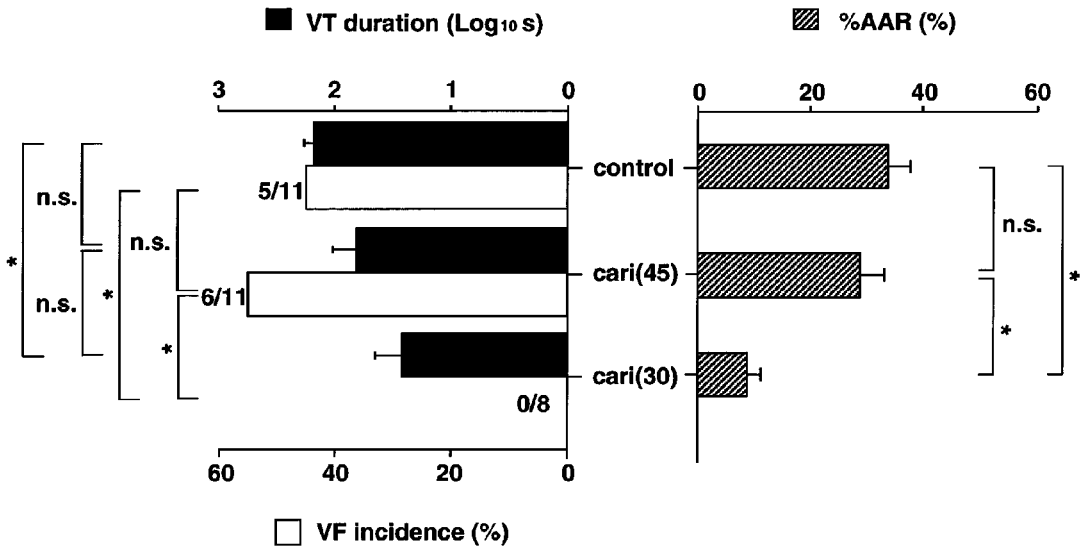

Figure 2 A positive relationship was seen between arrhythmias and infarct size. Cardioprotective effects of cariporide (0.3 mg kg^{-1}) disappeared 45 min after i.v. bolus. There were no drug treatments nor ischaemic preconditioning in the control group ($n=11$). The prolonged ischaemia was induced 30 min after i.v. cariporide in the cari(30) group ($n=8$) and 45 min after i.v. cariporide in the cari(45) group ($n=11$). Numbers beside columns give incidence of VF. Values are mean±s.e.mean. n.s.: not significant; *$P<0.05$, %AAR: percentage area at risk.

there was a significant reduction in all treated groups (34±4% in the control group versus 10±2, 9±2, 10±2% ($P<0.05$) in the 3PC, cari(30) and cari(30)+3PC groups, respectively).

Effects of cariporide on ischaemic preconditioning (Protocol III)

In part A, total VPB ($\log_{10}$ number of VPB), VT duration ($\log_{10}$ s) and VF incidence were significantly reduced in PC treated groups $\log_{10}$ VPB from 3.2±0.1 to 1.9±0.3 and 2.3±0.2 ($P<0.05$), $\log_{10}$ VT duration from 2.2±0.1 to 0.2±0.2 and 0.7±0.2 ($P<0.05$), and VF incidence from 55 to 0 and 0% ($P<0.05$) in the 3PC and cari(45)+3PC group, respectively. There were no significant differences between PC treated groups.

There were no significant differences in the risk areas between groups (59±4, 54±2 and 57±3% in the cari(45), 3PC and cari(45)+3PC group, respectively). As for the infarct size, there was a significant reduction in PC treated groups (29±4% in the cari(45) group versus 10±2 and 8±2% ($P<0.05$) in the 3PC and cari(45)+3PC group, respectively). Again, there were no significant differences among PC treated groups.

In part B, 1PC significantly reduced the $\log_{10}$ VPB and $\log_{10}$ VT duration from 3.2±0.1 and 2.2±0.1 of the control group to 2.3±0.3 and 1.2±0.3 ($P<0.05$), respectively, but it did not significantly reduce the incidence of VF (45% versus 38%). There were no significant differences in the $\log_{10}$ VPB and $\log_{10}$ VT duration among the cari(45), 1PC and cari(45)+1PC groups (3±0.1, 2.3±0.3 and 2.7±0.2, and 1.8±0.2, 1.2±0.3 and 1.4±0.3, respectively). VF incidence was significantly reduced in the cari(45)+1PC group compared with the cari(45) as well as the control group (0% versus 55 and 45% ($P<0.05$), respectively).

There were no significant differences in the risk areas between groups (61±4, 59±4, 61±3 and 56±3% in the control, cari(45), 1PC and cari(45)+1PC, respectively). As for the infarct size, there was no significant difference among the control, cari(45) and 1PC groups (34±4, 29±4 and 30±6% in the control, the cari(45) and 1PC group, respectively) whereas in the cari(45)+1PC a significant reduction (15±3% ($P<0.05$) versus control, cari(45) and 1PC groups) was observed.

Discussion

Our results showed that a bolus injection of cariporide, 0.3 mg kg^{-1}, reduced the incidence of arrhythmias and infarct size in rats *in vivo*. It was also shown that 0.3 mg kg^{-1} dose of cariporide has similar protective effects to that of the three episodes of ischaemic preconditioning against ischaemia-induced arrhythmias and ischaemia and reperfusion-induced infarction in rats. Importantly, cariporide did not impair cardioprotective effects of the three episodes of ischaemic preconditioning. Moreover, suboptimal preconditioning combined with a subthreshold cariporide dosing schedule abolished VF and reduced infarct size, indicative of an additive interaction between cariporide and preconditioning.

Reperfusion-induced VF seldom occurs after prolonged coronary occlusion (Manning & Hearse, 1984; Manning *et al.*, 1989) which inevitably causes myocardial necrosis, but a high incidence of VF occurred after shorter coronary occlusion with small or no necrosis. In this study we observed VF only in 10% of the animals during reperfusion in the control, and both preconditioning and cariporide abolished it (data not shown).

Cariporide is a highly selective NHE subtype-1 inhibitor (Scholz *et al.*, 1995) and protected against ischaemia/reperfusion injury of the myocardium of various animal species (Scholz *et al.*, 1995; Xue *et al.*, 1996; Aye *et al.*, 1997; Chakrabarti *et al.*, 1997; Garcia-Dorado *et al.*, 1997; Klein *et al.*, 1997; Miura *et al.*, 1997; Perchenet *et al.*, 1997; Shipolini *et al.*, 1997; Dhein *et al.*, 1998; Linz *et al.*, 1998). Cariporide in a concentration range of 0.1–1 μM selectively inhibited NHE-1 activity in cardiac cells (Scholz *et al.*, 1995; Shipolini *et al.*, 1997), and effectively improved ischaemia and/or reperfusion-induced contractile dysfunction or depletion of high energy substrates *in vitro* hearts (Scholz *et al.*, 1995; Shipolini *et al.*,

1997; Dhein *et al*., 1998). The *in vivo* dose range of 0.1–1 mg kg^{-1} which effectively suppressed ischaemia and/or reperfusion-induced arrhythmias (Scholz *et al*., 1995; Xue *et al*., 1996; Aye *et al*., 1997) and infarct size (Klein *et al*., 1997; Miura *et al*., 1997; Linz *et al*., 1998) has been shown to give a plasma concentration range of 0.1–1 μM (Xue *et al*., 1996; Klein *et al*., 1997; Linz *et al*., 1998) which is comparable to the effective dose (0.1–1 μM) *in vitro* studies, thus we chose 0.3 mg kg^{-1} as a dose to show specific and selective NHE inhibition in its actions on the exchanger.

The mechanism by which NHE inhibitors protect the myocardium from reperfusion injury may result from a reduction of H^+ extrusion *via* Na^+-H^+ exchange during reperfusion during which the H^+ gradient shifts strongly in favour of H^+ extrusion (Lazdunski *et al*., 1985; Tani & Neely, 1989; Karmazyn & Moffat, 1993). This would limit the sodium influx through the exchanger and subsequently reduce Ca^{2+} 'overload' *via* Na^+-Ca^{2+} exchanger (Tani & Neely, 1989; Pierce & Meng, 1992; Pierce & Czubryt, 1995). The protection achieved during ischaemia may appear paradoxical, since NHE block during ischaemia might be expected to exacerbate ischaemia-induced intracellular acidosis (Khandoudi *et al*., 1990; Ward & Moffat, 1995; Koike *et al*., 1996). However NHE inhibition during ischaemia has also been reported to not enhance intracellular acidosis in the ischaemic myocardium (Pike *et al*., 1993; Hendrikx *et al*., 1994; Schafer *et al*., 1995). This may be due to extrusion of the protons generated within the ischaemic cells by mechanisms unrelated to the NHE, such as the Na^+/HCO_3^- symporter and lactate/H^+ symporter (Grace *et al*., 1993; Vandenberg *et al*., 1993) when the exchanger is inhibited. It has not been reported so far that NHE inhibition during ischaemia causes a substantial difference in the development of intracellular acidosis.

Ischaemic preconditioning is associated with reduce acidosis during ischaemia (Kida *et al*., 1991; Asimakis *et al*., 1992; Steenbergen *et al*., 1993). This may be due to either reduced proton production (for instance by a decrease in glycolysis or glycogenolysis (Steenbergen *et al*., 1993; Wolfe *et al*., 1993; Finegan *et al*., 1995)) or enhanced proton efflux, the latter possibly being due to enchanced NHE activity (Ramasamy *et al*., 1995). If the latter were correct, then the NHE block during ischaemia would be expected to antagonize the protective effect of ischaemic preconditioning contrary to consequences of ischaema and reperfusion. On the other hand, NHE block during ischaemia and reperfusion would be expected to facilitate the protective effects of preconditioning. By examining the outcome when combining preconditioning with an NHE blocker, we attempted to explore which of these possibilities is correct.

We found that subthreshold preconditioning and cariporide interacted to elicit significant protection against VF and infarction. These findings expand those of Shipolini *et al*. (1997) and Bugge & Ytrehus. (1995) who explored the interaction between suprathreshold NHE block and preconditioning, and those of Liu & Downey. (1992) and Vegh *et al*. (1992). Together the data strongly indicate that enhanced proton efflux during ischaemia *via* stimulation of NHE activity is unlikely to contribute to the mechanism of preconditioning since, if it did, an NHE blocker would be expected to block rather than facilitate preconditioning.

The role of NHE in myocardial preconditioning is complex. Attenuation (Steenbergen *et al*., 1993) and activation (Ramasamy *et al*., 1995) of NHE activity have been proposed as mediators of preconditioning. Our results showed that subthreshold preconditioning and NHE blockade facilitate one another's protection, an observation that is consistent with previous reports (Bugge & Ytrehus, 1995; Perchenet *et al*., 1997; Shipolini *et al*., 1997) that NHE blockers do not antagonize the effects of preconditioning. To our knowledge, there has been no report of simultaneous measurement of pH_i, NHE activity, arrhythmias and infarct size in the same heart with or without preconditioning, in which case the present study is the first to examine the effects of cariporide and preconditioning on arrhythmias and infarct size simultaneously. However, the precise relationship between NHE activity and preconditioning requires further investigation.

Study limitation

In this *in vivo* study, intracellular pH and NHE activity could not be measured. Also, the plasma concentration of cariporide was not measured. Nevertheless, effective doses of cariporide against *in vivo* ischaemia/reperfusion injuries in different animal species have been reported as 0.1–1 mg kg^{-1} (Scholz *et al*., 1995; Xue *et al*., 1996; Aye *et al*., 1997; Miura *et al*., 1997; Linz *et al*., 1998). Cariporide, 1 mg kg^{-1}, has been reported to raise the plasma concentration to about 2 and 1.5 μM at 5 and 29 min after i.v. administration in the dog (Xue *et al*., 1996) and to about 1.3 μM at 10 min after i.v. administration in the pig (Klein *et al*., 1997). In rabbit, 0.1 and 0.3 mg kg^{-1} of cariporide has also been reported to raise the plasma concentration up to about 0.2 and 0.1 μM, and 0.6 and 0.4 μM at 5 and 30 min after i.v. administration, respectively, (Linz *et al*., 1998). From those results we expected that plasma concentration of cariporide in the present study reached up to $\sim$1 μM when interacted with preconditioning. The approximate IC_{50} of cariporide on pH_i recovery and NHE activity has been reported to be 1 μM and 0.1 μM, respectively, (Scholz *et al*., 1995; Rub *et al*., 1996; Shipolini *et al*., 1997). Thus we suspect that the effects of cariporide observed in the present study resulted from the specific NHE inhibition that would be anticipated with the doses used.

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