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An endogenous protectant effect of cardiac cyclic GMP against reperfusion-induced ventricular fibrillation in the rat heart

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1 After a period of myocardial ischaemia, reperfusion of the myocardium can elicit cardiac arrhythmias. Susceptibility to these arrhythmias declines with time, such that a preceding period of more than approximately 40 min ischaemia is associated with few reperfusion-induced arrhythmias. We have tested the hypothesis that this decline in susceptibility occurs, in part, because of protection by endogenous guanosine 3':5'-cyclic monophosphate (cyclic GMP).

2 Rat isolated hearts were subjected to 60 min left regional ischaemia followed by reperfusion ($n=10$ per group). Methylene blue (20 μM), a soluble guanylate cyclase inhibitor, raised the incidence of reperfusion-induced ventricular fibrillation (VF) from 10% in control hearts to 80% ($P<0.05$). This effect of methylene blue was abolished by co-perfusion with zaprinast (100 μM), a phosphodiesterase inhibitor which, in the rat heart, is cyclic GMP-specific (specific for the type-V phosphodiesterase isozyme).

3 Methylene blue reduced cyclic GMP levels in the ischaemic, non-ischaemic and reperfused myocardium ($P<0.05$) to 50 ± 10, 52 ± 12 and 70 ± 7 fmol mg^{-1} tissue wet weight, respectively from control values of 143 ± 38, 147 ± 43 and 156 ± 15 fmol mg^{-1}. Co-perfusion with zaprinast prevented this effect, and cyclic GMP levels were actually elevated ($P<0.05$) to 366 ± 102, 396 ± 130 and 293 ± 22 fmol mg^{-1} in ischaemic, non-ischaemic and reperfused myocardium, respectively. Zaprinast by itself also elevated cyclic GMP content. Cyclic AMP levels were not affected by zaprinast or methylene blue.

4 In conclusion, when endogenous cardiac cyclic GMP synthesis is reduced, susceptibility to reperfusion-induced VF after sustained ischaemia is substantially increased. The effect is prevented by inhibiting cyclic GMP degradation. Therefore cyclic GMP appears to be an endogenous intracellular cardioprotectant, and its actions may account for the low susceptibility to VF normally encountered in hearts reperfused after sustained ischaemia.

Keywords: Cyclic AMP; cyclic GMP; methylene blue; zaprinast; ischaemia; reperfusion; ventricular arrhythmias; heart

Introduction

Elective coronary reperfusion is a strategy for limiting the extent of myocardial necrosis in ischaemic heart disease in man. The timing of reperfusion is critical for this strategy to be effective (Hearse, 1990). However, reperfusion is also a potent stimulus for triggering ventricular fibrillation (VF) in every species in which it has been studied: rat, cat, dog, pig, rabbit, cavies and primate (Manning & Hearse, 1984; Rees & Curtis, 1993). These animal studies have shown that reperfusion is highly arrhythmogenic when ischaemia is brief, but is much less arrhythmogenic if ischaemia is sustained beyond 30-40 min (Manning & Hearse, 1984). Presently, elective reperfusion in man is rarely achieved within 40 min of the onset of symptoms of ischaemia (Curtis & Hearse, 1987). Partly because of this, reperfusion-induced VF is rare during elective reperfusion in man and is not regarded as being a clinically important problem (Curtis & Hearse, 1987). However, it is now recognised that in order for the myocardium to benefit from the salvage achievable by reperfusion, blood supply to the ischaemic tissue must be restored as early as possible after the onset of symptoms (Hearse, 1990). When ischaemia is brief and transient, case reports suggest that reperfusion can induce VF in man (Tzivoni *et al.*, 1983). Thus, if achievement of the goal of early reperfusion becomes more widespread, reperfusion-induced VF in man is likely to be encountered more commonly. Accordingly, as the anticipated emergence of reperfusion-induced VF becomes a perceived clinical problem, a better understanding of the pathophysiological factors that regulate its susceptibility may assist in the development of ways of preventing it.

Antiarrhythmic drugs are largely ineffective in prevention of reperfusion-induced VF in animal studies (Manning & Hearse, 1984). In view of the pessimism in general regarding prevention of VF in patients likely to develop it, whether as a consequence of ischaemia, infarction or reperfusion (Akhtar *et al.*, 1990; Waldo *et al.*, 1994), approaches to the understanding of arrhythmogenesis and its control that do not rely on a conventional electrophysiological model may be warranted (Curtis *et al.*, 1993b). One such approach involves the concept of endogenous cardioprotective substances (Parratt, 1993).

Numerous substances have been proposed as endogenous mediators of protection against VF elicited by ischaemia and by reperfusion. These include bradykinin, acetylcholine, nitric oxide, prostacyclin and adenosine (Parratt, 1993). The concept of endogenous cardioprotection depends upon the existence of a situation where the adverse consequences of ischaemia and reperfusion are attenuated by one or more endogenous protectants. In the case of reperfusion-induced VF, animal studies clearly show that endogenous protection is not operating effectively in hearts reperfused after brief (<40 min) ischaemia since, provided the volume of involved zone is sufficiently large (Curtis & Hearse, 1989a,b), VF is commonly encountered. It has been proposed that a pathophysiological reserve of arrhythmogenic factors operates under these circumstances (Yamada *et al.*, 1990; Curtis *et al.*, 1993b). However, as mentioned above, reperfusion is not a potent trigger for VF in hearts reperfused after more sustained ischaemia. Therefore, it

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has been hypothesized that the diminished susceptibility to reperfusion VF in hearts subjected to sustained ischaemia is due, in part, to the action of endogenous cardioprotective substances (Pabla & Curtis, 1995).

In order to demonstrate that this hypothesis is correct it would be necessary to reduce levels of the proposed protective substance and unmask the underlying tendency for reperfusion to elicit VF. This has recently been achieved in the case of one putative mediator of endogenous cardioprotection, nitric oxide (NO). In rat hearts reperfused after 60 min ischaemia, the incidence of reperfusion-induced VF was quadrupled by the NO synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), an effect that was prevented by L- but not D-arginine (Pabla & Curtis, 1995). Thus basal NO protects hearts against VF when reperfusion occurs after a sustained period of ischaemia. NO shows in common with several other proposed endogenous cardioprotectants the ability to increase guanosine 3':5'-cyclic monophosphate (cyclic GMP) formation (Moncada *et al.*, 1991).

In view of these considerations, we hypothesize that endogenous cardiac cyclic GMP contributes to the low susceptibility to VF in hearts reperfused after sustained (60 min) ischaemia. In the present study we have tested this hypothesis by perfusing hearts with drugs to inhibit cyclic GMP synthesis or inhibit cyclic GMP degradation. If our hypothesis is correct, reduction of basal cardiac cyclic GMP synthesis will unmask (i.e., increase the incidence of) reperfusion-induced VF, whereas inhibition of cyclic GMP metabolism to restore cyclic GMP content to normal (or supranormal) will prevent the unmasking of VF. This, indeed, was what we found.

These findings have been presented, in part, to the British Pharmacological Society (Oxford, 1995).

Methods

All experiments were performed in accordance with the United Kingdom Home Office 'Guide on the Operation of the Animals (Scientific Procedures) Act 1986.' Methods for the study of arrhythmias and ancillary variables in the rat isolated heart, together with their limitation and their advantages have been described previously (Curtis & Hearse, 1989a,b).

Animals and heart perfusion

Male Wistar rats (Tucks, UK; 240–260 g) were anaesthetized with pentobarbitone (60 mg kg^{-1} i.p.) and given heparin sodium 25 i.u. (i.p.). Hearts were excised and placed in ice-cold control perfusion solution containing (in mM): NaCl 118.5, $NaHCO_3$ 25.0, $MgSO_4$ 1.2, NaH_2PO_4 1.2, $CaCl_2$ 1.4, KCl 4.0 and glucose 11.1. All solutions were filtered (5 μm pore size) before use to remove particulate matter. Hearts were perfused according to Langendorff (1895), with perfusion solution delivered at 37°C and pH 7.4. Perfusion pressure was constant and equivalent to 100 cmH_2O.

Electrogram (ECG) recording and induction of ischaemia and reperfusion

A unipolar electrogram (ECG) was recorded by implanting one stainless-steel wire electrode into the centre of the region to become ischaemic with a second connected to the aorta. A traction-type coronary occluder consisting of a silk suture (Mersilk, 4/0) threaded through a polythene guide was used for coronary occlusion. The suture was positioned loosely around the left main coronary artery beneath the left atrial appendage. Regional ischaemia was induced by tightening the occluder, and reperfusion achieved by releasing the occluder.

Experimental protocol

Hearts were perfused for an initial 5 min with control solution, then the solution was switched in a randomised, blinded fashion to one of 4 solutions ($n = 10$ per group) prepared by dissolving the following substances in standard perfusion solution: control (no drug), 20 μM methylene blue, 100 μM zaprinast, or 20 μM methylene blue in combination with 100 μM zaprinast. All drugs were dissolved in NaOH, giving a final concentration of NaOH of 0.05 M in the test solution. The control (no drug) solution contained NaOH at this concentration. After a further 10 min perfusion, the left coronary artery was occluded to induce regional ischaemia. After 60 min ischaemia the occluder was released to allow reperfusion (Figure 1). A duration of 60 min ischaemia was chosen in accordance with the considerations outlined in the introduction.

Basis for choice of drugs

Methylene blue is used routinely as a pharmacological tool to reduce cyclic GMP synthesis; we used 20 μM, a concentration sufficient to inhibit guanylate cyclase activity (Garthwaite *et al.*, 1988). Zaprinast is a specific and relatively selective inhibitor of the V isozyme of phosphodiesterase, and at low (μM) concentrations can block cyclic GMP metabolism by phosphodiesterase purified from rat ventricle (Bode *et al.*, 1991). We chose to use a high concentration of zaprinast (100 μM) in order to accomodate penetration of the drug into cardiac cells. We could not guarantee selective activity of zaprinast on the V isozyme of phosphodiesterase versus other isozymes *a priori* in the present study, so the cardiac content of both cyclic GMP and adenosine 3':5'-cyclic monophosphate (cyclic AMP) was measured to verify the selectivity of zaprinast on cyclic GMP metabolism (see below for method).

Arrhythmia diagnosis and ECG analysis

A digital storage type oscilloscope (Gould, Essex, U.K.; model DSO400) and a Gould chart recorder (model RS3200) were used in the identification and analysis of the ECG complexes and the diagnosis of arrhythmias. Arrhythmias were defined according to the Lambeth Conventions (Walker *et al.*, 1988). Ventricular premature beats (VPB) were defined as discrete and identifiable premature QRS complexes; bigeminy was defined as a rhythm in which a normal sinus beat is followed by a VPB, followed by another normal beat and continuing thus; a run of two or three consecutive VPBs was defined as a salvo; a run of 4 or more VPBs was defined as ventricular tachycardia (VT). Ventricular fibrillation (VF) was defined as a signal from which individual QRS deflections vary in amplitude and coupling interval on a cycle-to-cycle basis. The definition of VF is a slight modification of that described in the Lambeth Conventions, and is used because it lends greater accuracy to diagnosis (Tsuchihashi & Curtis, 1991). From the ECG the incidence of arrhythmias, the RR interval and the QT interval (measured at the point of 100% repolarization with on screen

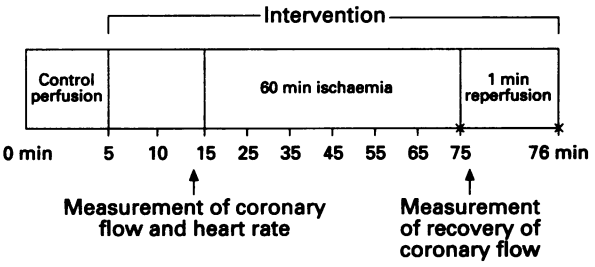

Figure 1 Schematic diagram of experimental time course. After 5 min control perfusion solution was switched to test solution for 10 min. The left coronary artery was then occluded for 60 min. This was followed by reperfusion for 1 min. Coronary flow and heart rate were measured 1 min before ischaemia and samples for measuring recovery of flow were taken during the first min of reperfusion. Samples of heart tissue were taken for cyclic GMP analysis as indicated (X).

cursors) were obtained as described previously (Rees & Curtis, 1993).

Stock solutions were prepared by the experimenter, then coded by another person. Data were analysed blind and codes were revealed after data analysis.

Measurement of occluded zone size and regional coronary flow

Coronary flow was measured by weighing coronary effluent collected over set time intervals (1 ml of effluent weighs 1 g). An Ohaus balance (Ohaus Corporation, Cambridge, U.K.) which is accurate to ± 1 mg (approximately 0.5% of the minimum volume collected) was used. Occlusion was verified by comparing flow at 1 min before tightening the occluder with flow 2 min later. At the end of reperfusion, readmission of flow was verified and the size of the involved zone was quantified by the disulphine blue dye exclusion method (Curtis & Hearse, 1989a). Occluded zone size was expressed as % of total ventricular weight. Values of coronary flow in the uninvolved zone and the reperfused zone were calculated from the total coronary flow and the weight of the involved zone and the uninvolved zone, as described previously (Curtis & Hearse, 1989b).

Cyclic GMP measurement

One min before the start of reperfusion, myocardial biopsies were taken from the centre of the ischaemic zone (visually identifiable from its dark colour and akinesis) and from the non-ischaemic zone. In separate groups of hearts subjected to regional ischaemia followed by reperfusion, a biopsy was taken from the reperfused zone 1 min after the start of reperfusion. All samples were rapidly (within 10 s) frozen in liquid nitrogen and stored at -70°C. The frozen samples of myocardium were weighed (20–30 mg) and homogenized in 1 ml of ice-cold 6% trichloroacetic acid with a cell disrupter (ultra-turrax; 18N shaft) for 90 s (9 x 10 s bursts with a 20 s delay between bursts) at 4°C. Following centrifugation at 10,000 *g* for 10 min at 4°C, the supernatant was extracted 5 times with 2.5 ml of water-saturated ether. Residual traces of ether were evaporated by heat (60°C for 5 min) and the samples stored at -20°C (Kane *et al.*, 1984). Aliquots (100 μl) were assayed in duplicate by use of a modified cyclic GMP assay kit purchased from Amersham (TRK 500). Additional aliquots were used in cyclic AMP analysis. Before being assayed for cyclic GMP content, the samples were diluted 1 in 50 with 50 mM sodium acetate buffer, pH 6.2. Preliminary measurements had shown that the cyclic GMP content of the samples would be sufficiently high to warrant dilution prior to detection and quantification. Cross-reactivity with cyclic AMP is less than 0.001%.

Cyclic AMP measurement

Tissue was prepared exactly as for cyclic GMP measurement. Before being assayed for cyclic AMP content, the samples were diluted 1 in 50 with 0.05 M Tris EDTA buffer, pH 7.5 containing 4 mM EDTA. Aliquots (50 μl) were assayed in duplicate with a cyclic AMP assay kit purchased from Amersham (TRK 432).

Exclusion criteria

Any heart with a sinus rate less than 250 beats min^{-1} or a coronary flow more than 18 ml min^{-1} or less than 8 ml min^{-1} 5 min before the onset of ischaemia was excluded. Any heart not in sinus rhythm during the 2 s before reperfusion was excluded from the study. Furthermore, any heart with an occluded zone size of less than 30% or greater than 55% was discarded. Each excluded heart was replaced by the next heart entered into the study. Exclusion criteria, and the basis for their use, have been described previously (Rees & Curtis, 1993).

Drugs and materials

All compounds were obtained from Sigma Chemicals (Dorset, U.K.) and stored as stock solutions in de-ionised water. Water for preparing perfusion solution was obtained from a reverse osmosis system (Milli-RO 10 and Milli-Q 50, Millipore Ltd., Hertfordshire, U.K.) which provides water of >18 MΩ resistivity.

Statistics

Measurement of all variables was performed in a blinded manner. Gaussian distributed variables were expressed as mean $\pm$ s.e.mean. If treatment constituted a significant source of variance, each group was compared with the control using Dunnett's test for multiple comparisons. A P value <0.05 was taken as significant. Mainland's contingency tables (Mainland *et al.*, 1956) were used for non-parametric analyses (e.g. of VF incidence).

Results

Perfusion with NaOH-containing control solution had no effect on any baseline variable (data not shown). The arrhythmia susceptibility and changes in ancillary variables in the control group, described below, were indistinguishable from values found for published control data from hearts that did not have NaOH added to the start solution (Curtis & Hearse, 1989a; Rees & Curtis, 1993).

Ischaemia-induced arrhythmias

Although the primary focus of the present study was reperfusion-induced arrhythmias, we were able to measure ischaemia-induced arrhythmias too. Hearts perfused with methylene blue exhibited an arrhythmia profile which was very similar to that in control hearts (Figure 2). All hearts developed VPBs, bigeminy, salvos and ventricular tachycardia (VT). Ischaemia-induced VF incidence in hearts perfused with methylene blue (40%) was also similar to that in control hearts (50%). In contrast, zaprinast exerted a cardioprotective effect, reducing the incidences of bigeminy, salvos and VT by 50%

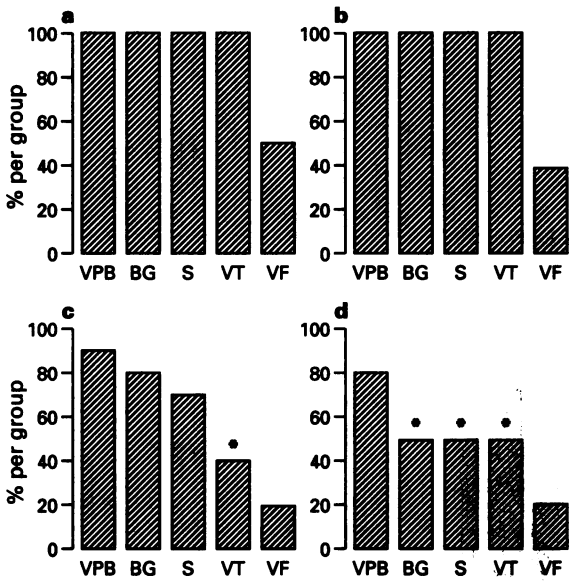

Figure 2 The group incidences (%) of ischaemia-induced ventricular premature beats (VPB), bigeminy (BG), salvos (S), ventricular tachycardia (VT), and ventricular fibrillation (VF) in control hearts (a), hearts perfused with methylene blue (b), methylene blue plus zaprinast (c) and zaprinast alone (d); $n=10$ per group. *$P<0.05$ vs. controls.

($P<0.05$ vs. controls). The incidence of VF appeared also to be reduced by zaprinast (to 20% vs. 50% in control hearts) although this did not reach statistical significance (Figure 2). Since our primary focus was reperfusion-induced arrhythmias (data below) we did not consider it worthwhile to increase group sizes in order to see whether this effect on VF might become significant. Hearts perfused with both methylene blue and zaprinast demonstrated a tendency toward a reduction in ischaemia-induced arrhythmias, although the reduction in the incidence of VT only was statistically significant (Figure 2). Thus, the antiarrhythmic effects of zaprinast were largely overcome by co-perfusion with methylene blue.

Reperfusion-induced arrhythmias

After 60 min of ischaemia the occluders were released to permit reperfusion. This gave rise to VF in only 10% of control hearts. In contrast, 80% of hearts perfused with methylene blue developed reperfusion-induced VF ($P<0.05$). There were no significant effects of methylene blue on the incidences of the other types of ventricular arrhythmia (Figure 3). Hearts perfused with zaprinast showed an overall tendency toward a reduced susceptibility to reperfusion-induced arrhythmias compared with controls, but this did not reach statistical significance, except in the case of VT (Figure 3). However, in hearts perfused with methylene blue and zaprinast the arrhythmia profile was also very similar to the profile in control hearts. Thus, zaprinast completely abolished the ability of methylene blue to increase the incidence of reperfusion-induced VF ($P<0.05$), and methylene blue prevented zaprinast from reducing the incidence of VT.

Haemodynamic changes

Methylene blue had no significant effect on coronary flow either before ischaemia or during reperfusion (Table 1). In contrast, zaprinast increased coronary flow dramatically both before ischaemia and during reperfusion (Table 1). This effect was attentuated by co-perfusion with methylene blue (Table 1). Methylene blue had a bradycardic effect reducing heart rate by approximately 90 beats min^{-1} before the onset of ischaemia compared with controls (Table 1). Zaprinast had no significant effect on heart rate. However, zaprinast attenuated the bradycardic effects of methylene blue, such that heart rate was not significantly different between the controls and the hearts perfused with the combination of zaprinast and methylene blue (Table 1).

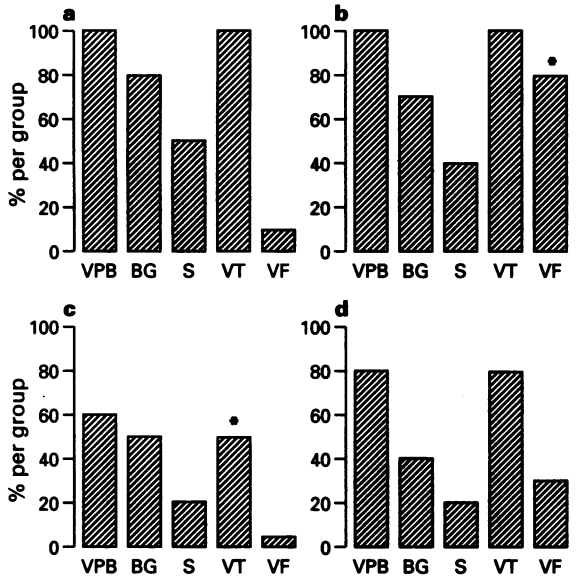

Figure 3 The group incidences (%) of reperfusion-induced ventricular premature beats (VPB), bigeminy (BG), salvos (S), ventricular tachycardia (VT), and ventricular fibrillation (VF) in control hearts (a), hearts perfused with methylene blue (b), methylene blue plus zaprinast (c) and zaprinast alone (d); $n=10$ per group. $*P<0.05$ vs. controls.

Table 1 Effect of methylene blue and zaprinast on heart rate and coronary flow before ischaemia and on recovery of flow during reperfusion

	Heart rate (beats min^{-1})	*Coronary flow* (ml min^{-1} g^{-1})	*Recovery of flow* (ml min^{-1} g^{-1})
Control	340±14	8.5±0.8	8.7±1.9
Methylene blue	251±18*	9.3±0.9	8.9±1.0
Methylene blue plus zaprinast	317±10	14.2±0.8*	18.0±2.1*
Zaprinast	378±12	18.0±1.1*	28.7±2.4*

Data for heart rate and coronary flow were recorded 1 min before ischaemia.
Data for recovery of flow was recorded during this first min of reperfusion. Values are mean±s.e.mean. $n=10$ per group. $*P<0.05$ vs. control.

Effect on QT interval

QT interval measured at 100% repolarization before the onset of ischaemia, was not different between groups (Table 2). During the first few minutes of ischaemia, the QT interval widened in all groups. However the QT interval gradually returned to baseline values by the end of the ischaemic period. These are typical QT interval changes for the regionally-ischaemic rat isolated heart preparation (Ridley & Curtis, 1992; Rees & Curtis 1993). Neither zaprinast nor methylene blue affected the QT interval significantly at any time point (Table 2).

Effects on cyclic nucleotide content

Sham hearts, subjected to no ischaemia, but perfused for 76 min with Krebs solution to time-match the other groups (76 min) had a mean cardiac cyclic AMP content and mean cyclic GMP content of 18.9±5.2 pmol mg^{-1} wet weight of tissue and 187.4±42.1 fmol mg^{-1} wet weight of tissue, respectively. The effects of methylene blue and zaprinast on cyclic GMP content are shown in Figure 4. Neither reperfusion nor drug treatment had any effect on cyclic AMP content compared to untreated sham-ligated hearts (data not shown). The values of cyclic AMP were lowest in the sham group, but values never exceeded 15% of this in any of the other groups, and s.e.mean values overlapped in all groups. In the control group there was no significant change in cyclic GMP during

Table 2 Effect of methylene blue and zaprinast on QT interval (ms)

	I–1 min	*I+1 min*	*I+5 min*	*I+10 min*	*R–1 min*
Control	74±2	86±3	82±3	77±6	73±2
Methylene blue	78±3	84±3	86±5	84±4	75±2
Methylene blue plus zaprinast	75±2	79±4	78±4	75±3	76±2
Zaprinast	68±1	75±4	76±2	81±3	77±2

Data represent QT intervals at 100% repolarization (ms) 1 min before the onset of ischaemia (I–1 min), during ischaemia (I+1 min, I+5 and I+10 min) and 1 min before the onset of reperfusion (R–1 min). Values are means±s.e. mean. $n=10$ per group. $*P<0.05$ vs. control.

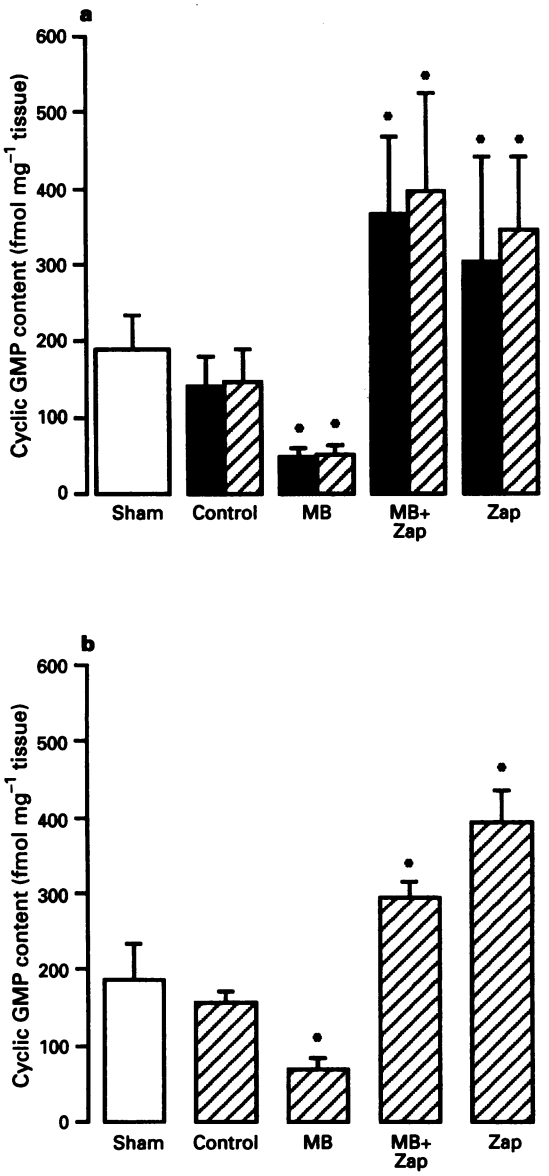

Figure 4 Cyclic GMP content in fmol mg^{-1} tissue. In part (a), myocardium was biopsied from ischaemic (solid columns) and non-ischaemic regions (hatched columns). A separate group of hearts ($n=4$) was sham ligated and perfused for 76 min without regional ischaemia (open columns). Data are mean ± s.e. mean. *$P<0.05$ vs. sham. In (b), hearts were made regionally ischaemic for 60 min and then reperfused. Reperfused tissue was biopsied 1 min after the start of reperfusion (hatched columns). These values were compared with those of the sham group (open column) in which the heart was not rendered ischaemic. Data are mean ± s.e. mean.

ischaemia (in either the ischaemic region or in the adjacent uninvolved region) or during reperfusion compared with sham-ligated time-matched hearts. Methylene blue, however, reduced cyclic GMP content ($P<0.05$) in uninvolved, ischaemic and reperfused tissue by more than 50% compared with the drug-free control group. Co-perfusion with zaprinast completely prevented these effects of methylene blue and cyclic GMP content became elevated ($P<0.05$) compared with the control group. Likewise, zaprinast alone elevated cyclic GMP content in uninvolved, ischaemic and reperfused tissue (Figure 4).

Relationship between cyclic GMP levels and VF susceptibility

The relationship between incidence of reperfusion-induced VF and cyclic GMP measured one min after the start of reperfusion (at the time of peak susceptibility to reperfusion-induced VF) is shown in Figure 5. Hearts that developed reperfusion-induced VF had significantly lower cyclic GMP levels than hearts without VF. It is noteworthy that hearts developing VF included the majority of hearts perfused with methylene blue alone, whereas the majority of hearts perfused with zaprinast are included in the group that did not develop VF.

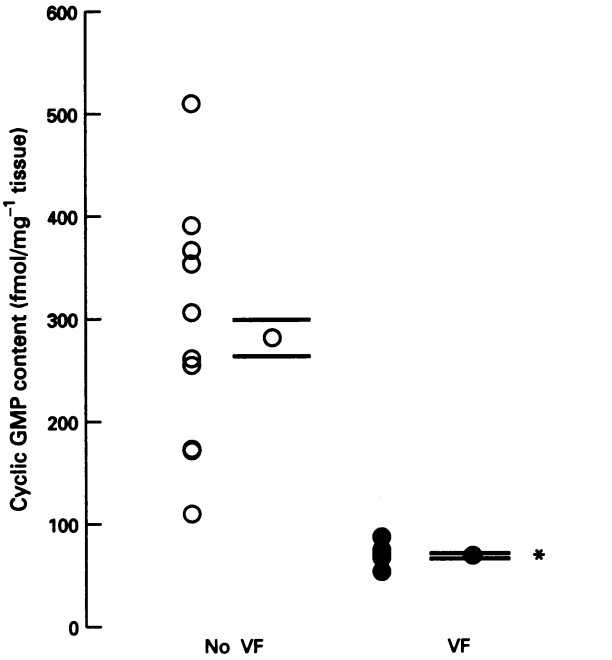

Figure 5 The association between cyclic GMP content, in the reperfused tissue, and the susceptibility to reperfusion-induced VF for the four groups in the study (control, methylene blue, zaprinast, methylene blue plus zaprinast). The point to the right of each set of scatter points is the mean ± s.e. mean. *$P<0.05$.

Discussion

Effects of ischaemia and reperfusion on cardiac cyclic GMP and its role in arrhythmogenesis

There is little available data regarding the effects of ischaemia and reperfusion on cardiac cyclic GMP content (in the absence of drugs) and the relationship between this and susceptibility to reperfusion-induced arrhythmias. It has been shown that transient elevations in cyclic GMP levels occur in rat hearts as a consequence of myocardial ischaemia (Nesher *et al.*, 1977; Busuttil *et al.*, 1978), with levels rapidly declining to below control levels as ischaemia is sustained for 30 min. However, in a more recent study, Kane *et al.* (1984) observed changes in cyclic GMP content (which occurred in uninvolved as well as involved tissue), but were not able to relate the changes to a reduced susceptibility to arrhythmias. In the present study, in drug-free controls, we did not observe any significant changes in cyclic GMP content either following a longer duration (60 min) of ischaemia, or during reperfusion. Likewise, in a previous study using a briefer duration of ischaemia (15 min), we found no significant alteration in cardiac cyclic GMP content, although a non-significant trend to an increase was observed (Curtis *et al.*, 1993a). All in all, it appears that in the absence of drug treatment, cardiac cyclic GMP content is little affected by ischaemia or reperfusion. However, the presence of undiminished levels of cyclic GMP in hearts reperfused after 60 min of ischaemia allows the possibility that cyclic GMP may function as an endogenous cardioprotectant at this time.

Drug-induced changes in cardiac cyclic GMP content, susceptibility to reperfusion-induced VF, and pathophysiological reserve

Our findings show that drugs which alter cardiac cyclic GMP content affect susceptibility to reperfusion-induced VF following sustained (60 min) ischaemia, and thereby support the hypothesis that cyclic GMP functions as an endogenous cardioprotectant against VF. When cyclic GMP content was lowered to below basal levels this unmasked a latent susceptibility to VF. A quantitative relationship existed between cardiac cyclic GMP and VF in the presence and absence of methylene blue, zaprinast and the drug combination. This represents compelling evidence for a cause-effect relationship between cyclic GMP content and a reduced susceptibility to reperfusion-induced VF.

The effects of methylene blue on cyclic GMP content may be direct or indirect, since methylene blue is an inhibitor of NO synthase (Mayer *et al.*, 1993) and may thus reduce cyclic GMP content in part as a secondary consequence of reduced NO content.

Work by Kane *et al.* (1984) has indicated that administration of the non-hydrolyzable cyclic GMP analogue, dibutyryl cyclic GMP may increase susceptibility to ischaemia-induced arrhythmias in hearts subjected to 30 min of left regional ischaemia *in vivo*. However, in a model of ischaemic preconditioning evidence suggests that cyclic GMP may contribute to endogenous protection against arrhythmias (Vegh *et al.*, 1992). Furthermore, Billman (1990) has demonstrated in the conscious dog a powerful anti-fibrillatory effect of dibutryl cyclic GMP during exercise and left circumflex coronary artery occlusion. More recently, Mizumura & Gross (1995) have shown that the cardioprotective effects of nitroglycerin are prevented by methylene blue in the anaesthetized dog. In contrast, Holbrook & Coker (1991) and recently Barnes & Coker (1995) have used phosphodiesterase inhibitors and NO donors to increase cardiac cyclic GMP levels selectively in anaesthetized rats. However, neither group of drugs had any effect on reperfusion-induced arrhythmias following 25 min of coronary artery occlusion. The NO donors, SIN-1 and sodium nitroprusside had no effect on cyclic GMP levels in this study; zaprinast did increase cardiac cyclic GMP levels but it also raised cyclic AMP levels as well. Although our data may seem surprising in the light of the study by Kane *et al.* (1984), Holbrook & Coker (1991) and Barnes & Coker (1995), they are qualitatively consistent with results from other studies which suggest that cyclic GMP is broadly protective to hearts subjected to ischaemia and reperfusion.

One reason for the apparent discrepancy between studies may be that outcome depends on the duration of preceding ischaemia. As noted earlier (and this was the justification for our use of 60 min ischaemia), the cardioprotective effects of NO donors (which elevate cyclic GMP) was time-dependent in the rat heart, whereby a significant anti-arrhythmic effect (in hearts in which endogenous NO production had been suppressed) was observed following 60 min ischaemia only, and not with ischaemic durations of less than 60 min (Pabla & Curtis, 1995). Thus the lack of protection observed with dibutyryl cyclic GMP and agents that elevate endogenous cyclic GMP in some published studies may result from the brief (up to 30 min) duration of ischaemia that preceded reperfusion. Presumably, any protective activity that cyclic GMP may exert is swamped by the multiple mechanisms that can cause VF in hearts reperfused after brief durations of ischaemia via the process of pathophysiological reserve (Curtis *et al.*, 1993a,b). In a previous study (Pabla & Curtis, 1995) the observation that endogenous NO protects against reperfusion-induced VF only following sustained (60 min) ischaemia, was also explained by invoking the concept of pathophysiological reserve for VF. It has been recognised that the mechanisms underlying pathophysiological reserve diminish in importance if hearts are reperfused after more sustained ischaemia (Curtis *et al.*, 1993a,b). Thus, our data suggest that in hearts that are weakly susceptible to reperfusion-induced VF, basal endogenous cyclic GMP exerts a tangible antifibrillatory influence on cardiac rhythm, and that this influence can be suppressed so as to unmask a greater susceptibility to VF by agents such as methylene blue.

Can the drug effects on VF be attributed to cyclic GMP-independent cellular actions?

Since the intention of the present study was to modulate cardiac cyclic GMP selectively by drugs and relate changes to VF susceptibility we were concerned that any lack of drug selectivity would diminish the clarity of our findings.

The possibility of changes in cardiac cyclic AMP content would have rendered our data difficult to interpret. Fortunately, this was found not to be the case. There were no significant differences in cyclic AMP content between groups and neither methylene blue nor zaprinast affected cardiac cyclic AMP content. This indicates that pharmacological selectivity of action in terms of effects on cardiac cyclic GMP versus cyclic AMP was achieved. This also indicates that changes in cyclic AMP did not contribute to the substantial alterations in susceptibility to reperfusion-induced VF produced by the drugs. Previous studies have not shown consistent alterations in cardiac cyclic AMP during myocardial ischaemia and reperfusion, with some studies demonstrating an increase in cardiac cyclic AMP levels (Podzuweit *et al.*, 1976), and others demonstrating variable changes (see Curtis *et al.*, 1993a). The present results show that cyclic AMP content in hearts reperfused after 60 min of ischaemia is no different from control (sham) values.

Methylene blue has been shown to react with molecular oxygen to form superoxide radicals (McCord & Fridovich, 1970), effects of which are prevented by simultaneous administration of superoxide dismutase (Marshall *et al.*, 1989; Wolin *et al.*, 1990). Superoxide has been implicated as a mediator of reperfusion-induced VF (Woodward & Zakaria, 1985). We did not measure superoxide production in the present study. However, it is difficult to explain the antagonism by zaprinast of the pro-arrhythmic effects of methylene blue if methylene blue exacerbates reperfusion-induced VF, as a consequence of an action on superoxide production that is independent of changes in cyclic GMP.

The absence of effect of either zaprinast or methylene blue on QT interval indicates that actions on potassium channels and repolarization are unlikely to have contributed to the changes in susceptibility to VF.

Thus, non-specific cellular effects of methylene blue and zaprinast are unlikely to have contributed to the changes in VF susceptibility that were observed.

Role of coronary flow changes in determining the changes in susceptibility to VF

The possibility that cyclic GMP (and NO; Pabla & Curtis, 1995) protects against reperfusion-induced VF by altering coronary flow requires consideration, since cyclic GMP mediates coronary vasodilatation (Murad, 1986). Martorana and colleagues (1983) and Cano and colleagues (1986) have attributed anti-arrhythmic effects of the NO donor, molsidomine, in the dog, during reperfusion following sustained ischaemia (90 min) to an improvement in myocardial blood flow in the region involved. The dog heart is highly collateralised (Maxwell *et al.*, 1987). However, collateral flow is so low ($<5\%$) in the rat heart (Maxwell *et al.*, 1987) that it is not possible to ameliorate ischaemia by the use of vasoactive drugs in this species.

Role of bradycardia in determining the changes in susceptibility to VF

Methylene blue causes sinus bradycardia. Heart rate has a well-established influence on susceptibility to reperfusion-in-

duced VF. However, bradycardia characteristically lowers VF incidence (Bernier *et al.*, 1989), so it is highly unlikely that methylene blue increased VF incidence as a consequence of its bradycardic action.

Cellular mechanism of action of endogenous cyclic GMP

Determining the cellular mechanism of action of cyclic GMP was beyond the scope of our present objectives. Cyclic GMP may inhibit the slow inward current (I_{Ca}) in cardiac muscle (Kohlhardt & Haap, 1978; Trautwein *et al.*, 1982; Tohse & Sperelakis 1991). However, the role of I_{Ca} in mediating reperfusion arrhythmias is controversial since, although calcium antagonists such as diltiazem attenuate reperfusion-induced VF the effect is attributable to the severe bradycardia that the drugs may induce (Hearse & Tosaki, 1988). In the present study, methylene blue caused bradycardia but actually increased the incidence of reperfusion-induced VF. Further work would be required to establish the cellular protective mechanism of cyclic GMP.

Conclusion

We examined the effects of interventions designed to modulate endogenous cardiac cyclic GMP content on ventricular arrhythmias in the rat isolated heart. As expected, cardiac cyclic GMP content was reduced by methylene blue, and this effect was prevented by co-perfusion with zaprinast. Correspondingly, methylene blue substantially increased (eight fold) the incidence of VF in hearts reperfused after 60 min of ischaemia. This was prevented by co-perfusion with zaprinast. These findings suggest that cardiac cyclic GMP functions as an endogenous protectant against reperfusion-induced VF.

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