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Effects of the prostanoid EP_3-receptor agonists M&B 28767 and GR 63799X on infarct size caused by regional myocardial ischaemia in the anaesthetized rat

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1 This study investigates the effects of two agonists of the prostanoid EP_3-receptor (M&B 28767 and GR 63799X) on the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetized rat.

2 One hundred and sixty-seven, male Wistar rats were anaesthetized (thiopentone, 120 mg kg^{-1}, i.p.), ventilated (8–10 ml kg^{-1}, 70 strokes min^{-1}, inspiratory oxygen concentration: 30%; PEEP: 1–2 mmHg) and subjected to occlusion of the left anterior descending coronary artery (LAD, for 7.5, 15, 25, 35, 45 or 60 min) followed by reperfusion (2 h). Infarct size was determined by staining of viable myocardium with a tetrazolium stain (NBT), histological evaluation by light and electron microscopy and determination of the plasma levels of cardiac troponin T.

3 M&B 28767 (0.5 μg kg^{-1} min^{-1}, i.v., $n=7$) or GR 63799X (3 μg kg^{-1} min^{-1}, i.v., $n=7$) caused significant reductions in infarct size from 60 ± 3% (25 min ischaemia and 2 h reperfusion; saline-control, $n=8$) to 39 ± 6 and 38 ± 4% of the area at risk, without causing a significant fall in blood pressure. Pretreatment of rats with 5-hydroxydecanoate (5-HD), an inhibitor of ATP-sensitive potassium channels, attenuated the cardioprotective effects of both EP_3-receptor agonists. The reduction in infarct size afforded by M&B 28767 was also abolished by glibenclamide and the protein kinase C (PKC) inhibitors staurosporine and chelerythrine.

4 Thus, M&B 28767 and GR 63799X reduce myocardial infarct size in the rat by a mechanism(s) which involves the activation of PKC and the opening of ATP-sensitive potassium channels.

Keywords: Cardioprotection; EP-receptors; E-type prostaglandins; GR 63799X; M&B 28767; myocardial ischaemia; myocardial infarction; protein kinase C; troponin T

Abbreviations: EP-receptor, E-type prostanoid receptor; HR, heart rate; LDH, lactate dehydrogenase; MAP, mean arterial blood pressure; PGE_1, prostaglandin E_1; PKC, protein kinase C; PRI, pressure-rate index; TnT, cardiac troponin T; PEEP, positive endexspiratory pressure

Introduction

E-type prostaglandins including prostaglandin E_1 (PGE_1) exert beneficial effects on biochemical, electrocardiographic and functional indices of myocardial ischaemia-reperfusion injury (Judgutt *et al*., 1981; Schrör *et al*., 1988; Simpson *et al*., 1988) and reduce myocardial infarct size (Hide *et al*., 1995). The effects of E-type prostaglandins are mediated by specific G-protein coupled receptors (EP-receptors) which have been classified into four subtypes, EP_1, EP_2, EP_3 and EP_4 (Coleman *et al*., 1990). The cardioprotective effects of these eicosanoids may be secondary to a reduction in afterload, an increase in coronary blood flow, inhibition of platelet function and/or inhibition of the activation and extravasation of polymorphonuclear (PMNs) cells (Lucchesi & Mullane, 1986), all of which are secondary to the activation of EP_2-receptors, which activate G_s and cause an activation of adenylate cyclase (Coleman *et al*., 1990). In addition, the protection of isolated cells or organs by prostaglandins has been attributed to an ill-defined 'cytoprotective' or 'cardioprotective' effect of these agents. The mechanism(s) or the prostanoid receptor(s) mediating this effect is unknown (Schrör, 1987).

In 1995/96, we have discovered that the cardioprotective effects of E-type prostaglandins are (at least in part) due to activation of EP_1 or EP_3-receptors which, in turn, leads to the opening of ATP-sensitive potassium (K_{ATP}) channels (Hide *et al*., 1995; Hide & Thiemermann, 1996). This hypothesis is supported by the following findings: (1) The cardioprotective effects of PGE_1 (non-selective agonist for all EP-receptors) and sulprostone (selective agonist of EP_1 and EP_3-receptors) are abolished by inhibition of K_{ATP}-channels with glibenclamide or 5-hydroxydecanoate (Hide *et al*., 1995; Hide & Thiemermann, 1996). (2) Sulprostone causes cardioprotection without having any haemodynamic (EP_2-mediated) effects (Hide & Thiemermann, 1996). (3) Activation of EP_1 and EP_3-receptors may result in activation of protein kinase C (PKC) (Coleman *et al*., 1990; 1994). As EP_3-receptors are expressed on cardiomyocytes and are up-regulated following ischaemia of the heart (Hohlfeld, 1995; Hohlfeld *et al*., 1997), we hypothesized that it is the activation of EP_3-receptors which accounts for the 'cardioprotective' and or 'cytoprotective' effects of E-type prostaglandins.

The overall aim of this study was to elucidate the effects of two prostanoid EP_3-receptor agonists, namely M&B 28767 and GR 63799X (see Coleman *et al*., 1990), on the infarct size caused by regional myocardial ischaemia and reperfusion in the anaesthetized rat. Having found that these agents do

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indeed reduce myocardial infarct size in the rat, we have subsequently investigated the role of protein kinase C (PKC) and of K_{ATP}-channels in the cardioprotective effects of these agents.

Methods

Coronary artery ligation in the rat in vivo

One hundred and sixty-seven male Wistar rats (240–350 g, Tucks, Reyleigh, Essex, U.K.) were anaesthetized with thiopentone sodium (120 mg kg^{-1}, i.p.). The rats were tracheotomized, intubated and ventilated with a Harvard ventilator (70 strokes min^{-1}, tidal volume: 8–10 ml kg^{-1}, inspiratory oxygen concentration: 30%; PEEP: 1–2 mmHg resulting in pCO_2 values of 36–44 mmHg and pO_2 values over 150 mmHg). Body temperature was maintained at $38 \pm 1^{\circ}C$. The right carotid artery was cannulated and connected to a pressure transducer to monitor mean arterial blood pressure (MAP). The right jugular vein was cannulated for the administration of drugs. The chest was opened by a mid-sternal thoracotomy, the pericardium incised and an atraumatic needle was placed with an occluder around the left anterior descending coronary artery (LAD). Subdermal platinum electrodes were placed to allow the determination of a lead II electrocardiogram (ECG). After completion of the surgical procedure the animals were allowed to stabilize for 30 min before infusion of drugs and LAD ligation. The coronary artery was occluded at time 0 by tightening of the occluder. This was associated with the typical electrocardiographic (ST-segment elevation and increase in R-wave amplitude) and haemodynamic changes (fall in MAP) of myocardial ischaemia. After 7.5, 15, 25, 35, 45 or 60 min of acute myocardial ischaemia, the occluder was re-opened to allow the reperfusion of the previously ischaemic myocardium for 2, 4 or 8 h. Heart rate (HR) and MAP were continuously recorded on a 4-channel Grass 7D polygraph recorder. The HR was automatically calculated from systolic pulse curves by means of a Grass 7P4H tachograph. The pressure rate index (PRI), a relative indicator of myocardial oxygen consumption (Baller *et al*., 1981), was calculated as the product of MAP and HR, and expressed in mmHg min^{-1} 10^3. After re-occluding the LAD and i.v. injection of Evans blue dye (1 ml of 2% w v^{-1}) to stain the area at risk (AR), the heart was removed and cut into four to five horizontal slices. The Evans blue solution stains the perfused myocardium, while the occluded vascular bed remains uncoloured. After removing the right ventricular wall, the AR and non-ischaemic myocardium were separated by following the line of demarcation between blue stained and unstained (pink/red) tissue. To distinguish between ischaemic and infarcted tissue, the AR was cut into small pieces and incubated with *p*-nitro-blue tetrazolium (NBT, 0.5 mg ml^{-1}, 20 min at 37°C). In the presence of intact dehydrogenase enzyme systems (normal myocardium), NBT forms a dark blue formazan, while areas of necrosis lack dehydrogenase activity and therefore do not stain (Nachlas & Shnitka, 1963). The AR and infarct size were calculated and expressed as per cent of the AR.

*Experimental design (*in vivo *studies)*

Study I The following seven experimental groups were studied to elucidate the effects of varying periods of regional myocardial ischaemia on infarct size and on the plasma levels of cardiac troponin T: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle (saline, 2.4 ml kg^{-1} h^{-1}), starting 10 min prior to LAD-occlusion and maintained throughout the experiment ($n=3$). (2) LAD-occlusion (7.5 min) and reperfusion (2 h) plus infusion of vehicle ($n=5$). (3) LAD-occlusion (15 min) and reperfusion plus infusion of vehicle ($n=5$). (4) LAD-occlusion (25 min) and reperfusion plus infusion of vehicle ($n=8$). (5) LAD-occlusion (35 min) and reperfusion plus infusion of vehicle ($n=5$). (6) LAD-occlusion (45 min) and reperfusion plus infusion of vehicle ($n=11$). (7) LAD-occlusion (60 min) and reperfusion plus infusion of vehicle ($n=6$).

Study II The following two experimental groups were studied to elucidate the effects of increasing periods of reperfusion after 25 min of myocardial ischaemia on infarct size: (1) LAD-occlusion (25 min) and reperfusion (4 h) plus infusion of vehicle (saline, 2.4 ml kg^{-1} h^{-1}), starting 10 min prior to LAD-occlusion and maintained throughout the experiment ($n=5$). (2) LAD-occlusion (25 min) and reperfusion (8 h) plus infusion of vehicle ($n=5$).

Study III This study was designed to elucidate the effect of the EP_3-receptor agonists M&B 28767 and GR 63799X on the infarct size caused by regional myocardial ischaemia (25 min) and reperfusion (2 h). We have subsequently investigated the role of the activation of K_{ATP}-channels and/or PKC in the cardioprotective effects of M&B 28767 and GR 63799X. To do this, the following further 19 experimental groups were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle (identical to group 1 in study 1, $n=3$). (2) LAD-occlusion (25 min) and reperfusion (2 h) plus infusion of vehicle (identical to group 4 in study I, $n=8$). (3) LAD-occlusion and reperfusion plus infusion of M&B 28767 (0.5 μg kg^{-1} min^{-1}, i.v. starting 10 min prior to coronary artery occlusion, $n=7$). (4) No occlusion of the LAD (sham-operation) plus infusion of M&B 28767 ($n=3$). (5) LAD-occlusion and reperfusion plus infusion of GR 63799X (3 μg kg^{-1} min^{-1}, i.v. starting 10 min prior to coronary artery occlusion, $n=7$). (6) No occlusion of the LAD (sham-operation) plus infusion of GR 63799X ($n=3$). (7) LAD-occlusion and reperfusion plus injection of 5-HD (5 mg kg^{-1}, i.v. at 10 min prior to coronary artery occlusion, $n=6$). (8) No occlusion of the LAD (sham-operation) plus injection of 5-HD ($n=3$). (9) LAD-occlusion and reperfusion plus administration of 5-HD 10 min prior the infusion of M&B 28767 ($n=6$). (10) LAD-occlusion and reperfusion plus administration of 5-HD 10 min prior the infusion of GR 63799X ($n=6$). (11) LAD-occlusion and reperfusion plus injection of glibenclamide (0.3 mg kg^{-1}, i.v. at 10 min prior to coronary artery occlusion, $n=6$). (12) No occlusion of the LAD (sham-operation) plus injection of glibenclamide ($n=3$). (13) LAD-occlusion and reperfusion plus administration of glibenclamide 10 min prior the infusion of M&B 28767 ($n=4$). (14) LAD-occlusion and reperfusion plus injection of staurosporine (1 μg kg^{-1}, i.v. 10 min prior to LAD-occlusion, $n=6$). (15) No occlusion of the LAD (sham-operation) and injection of staurosporine ($n=3$). (16) LAD-occlusion and reperfusion plus administration of staurosporine 10 min prior the infusion of M&B 28767 ($n=6$). (17) LAD-occlusion and reperfusion plus injection of chelerythrine (0.7 mg kg^{-1}, i.v. 10 min prior to LAD-occlusion, $n=6$). (18) No occlusion of the LAD (sham-operation) and injection of chelerythrine ($n=3$). (19) LAD-occlusion and reperfusion plus administration of chelerythrine 10 min prior the infusion of M&B 28767 ($n=6$).

The *n*-numbers in the above experimental groups refer to animals, which survived until the end of the experiment. The

average mortality was approximately 13%. The number of animals which died in the individual groups of animals studied were the following: *Study I*: group 1, 0; group 2, 0; group 3, 0; group 4, 1; group 5, 1; group 6, 2; group 7, 2; *Study II*: group 1, 0; groups 2, 0; *Study III*: group 1, 0; group 2, 1 (identical to group 4, study I); group 3, 1; group 4, 0; group 5, 1; group 6, 0; group 7, 1; group 8, 0; group 9, 1; group 10, 1; group 11, 2; group 12, 0; group 13, 5; group 14, 1; group 15, 0; group 16, 1; group 17, 1; group 18, 0; group 19, 0.

Measurement of the plasma levels of cardiac troponin T in the rat

At the end of the experiment a blood sample (1 ml) was obtained from the carotid cannula and centrifuged to obtain plasma. To do this the following experimental groups were studied: (1) No occlusion of the LAD (sham-operation) plus infusion of vehicle ($n=3$). (2) LAD-occlusion (7.5 min) and reperfusion (2 h) plus infusion of vehicle (identical to group 2 in study I, $n=3$). (3) LAD-occlusion (15 min) and reperfusion plus infusion of vehicle (identical to group 3 in study I, $n=3$). (4) LAD-occlusion (25 min) and reperfusion plus infusion of vehicle ($n=6$). (5) LAD-occlusion (35 min) and reperfusion plus infusion of vehicle (identical to group 5 in study I, $n=3$). The concentration of cardiac troponin T was determined by the STAT (short-turn-around-time) assay (provided by Boehringer Mannheim, Germany) using an Elecixs® System 2010.

Determination of the degree of myocardial tissue injury by lightmicroscopy

Biopsies of all sections of the heart (non-ischaemic, ischaemic and infarcted) were fixed in paraformaldehyde (4% w v^{-1}), embedded in paraffin, cut into section (4 μm), de-waxed and stained with Fuchsin and Luxol-Fast-Blue according to Bancroft & Stevens (1995).

Determination of the degree of myocardial tissue injury by electronmicroscopy

Biopsies of all sections of the heart (non-ischaemic, ischaemic and infarcted) were fixed in paraformaldehyde (4% w v^{-1}), embedded in paraffin. All biopsies were de-paraffinized (washed twice for 5 min in xylol and then in decreasing concentrations of ethanol, 100–50%). The sample was subsequently fixed again in osmium tetroxid (1% w v^{-1}) and incubated in increasing concentrations of ethanol (50–100%) to remove any excess water. The biopsies were then embedded in Agar 100 Resin (Plano, Marburg, Germany) and cut into section of 1 μm (Ultracut R, Leica, Cologne, Germany) and stained according to Richardson. Selected samples were then cut into ultrathin slices (90 nm) (Ultracut R, Leica, Cologne, Germany). The samples were then stained with lead acetate and analysed with an EM410 electronmicroscope (Phillips, Germany). All tissue biopsies were evaluated by an experienced cardiovascular pathologist in double-blind fashion (M.O.).

Drugs and materials

Unless otherwise stated all compounds were obtained from Sigma Chemical Co. (Poole, Dorset, U.K.). Thiopentone sodium (Intraval®) was obtained from May & Baker Ltd. (Dagenham, U.K.). Chelerythrine and staurosporine were from Calbiochem (Nottingham, U.K.). We thank Dr Simon Lister (GlaxoWellcome, Research and Development, Stevenage, Herts, U.K.) for the generous supply of GR 63799X and Dr Jean Hough (Rhône-Poulenc Rorer, Research and Development, Dagenham, U.K.) for the generous supply of M&B 28767.

Statistical analysis

All values in the text, figures and tables are expressed as the means ± s.e.mean of n observations. Statistical analysis was performed (on absolute values) by one-way analysis of variance (ANOVA) followed, if appropriate, by a Bonferroni's test for multiple comparisons. A P value of less than 0.05 was considered statistically significant.

Results

Effects of varying lengths of regional myocardial ischaemia followed by reperfusion (2 h) on myocardial infarct size and plasma levels of cardiac troponin T in the rat

In rats which were subjected to the surgical procedure, but not to LAD-occlusion (sham-operation), there was no significant degree of myocardial necrosis as determined by NBT staining (Figure 1a). Occlusion of the LAD for 7.5 min followed by reperfusion for 2 h resulted in an infarct size of ~33% of the area at risk. Increasing the length of the period of regional ischaemia resulted in a time-related increase in infarct size (Figure 1a). A maximal degree of infarction of ~70–75% of the area at risk was observed after 35 min.

In rats which were subjected to the surgical procedure, but not to LAD-occlusion (sham-operation), there was no significant increase in the plasma levels of the cardiac-specific marker troponin T (Figure 1b). Occlusion of the LAD for 7.5 min followed by reperfusion for 2 h resulted in a significant increase in the plasma levels of cardiac troponin T (to ~14 ng ml^{-1}). Increasing the length of the period of regional ischaemia resulted in a time-related, further increase in the plasma levels of cardiac troponin T (Figure 1b).

Morphological alterations caused by regional myocardial ischaemia (25 min) and reperfusion (2 h) in the anaesthetized rat

Histological evaluation (by light microscopy) of biopsies of the area at risk (which failed to stain with NBT) of hearts subjected to regional ischaemia (25 min) and reperfusion (2 h) demonstrated the occurrence of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, which demonstrates a strong staining with Fuchsin (Figure 2a) as well as submaximal staining with Luxol-Fast Blue (Figure 2b) in the peripheral myocytes, whereas the central zone is negative. All nuclear details have disappeared. The section also demonstrates a substantial degree of extravasation of red blood cells.

Evaluation by electron microscopy of biopsies of the area at risk (which failed to stain with NBT) of hearts subjected to regional ischaemia (25 min) and reperfusion (2 h) demonstrated nuclei with margination and clumping of chromatin with intervening areas of pale nucleoplasm. A granular disruption of the I-bands and Z-lines compared to a normal A-band was also demonstrated (Figure 3a). The mitochondria

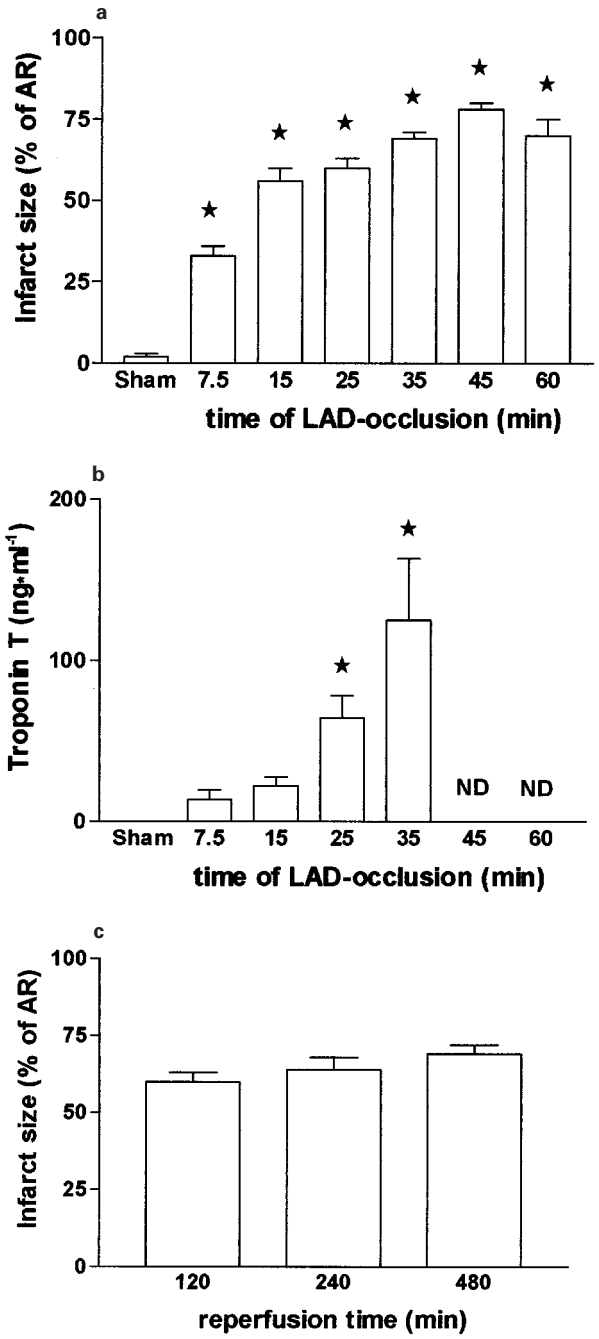

Figure 1 (a) Infarct size (expressed as per cent of the area at risk, AR) caused by occlusion 7.5, 15, 25, 35, 45 or 60 min and followed by reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. (b) Alterations in the plasma levels of cardiac troponin T caused by occlusion for 7.5 15, 25 or 35 min and followed by reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. $*P<0.05$ when compared to sham-operated control. ND, not determined. (C) Infarct size (expressed as per cent of the area at risk, AR) caused by occlusion for 25 min and reperfusion for 2, 4 or 8 h of the left anterior descending coronary artery (LAD) in the anaesthetized rat.

were markedly swollen, the inner compartment was electron translucent and expanded. Contraction bands (Figure 3b and c) as well as oedema was widely present. In several places, osmophilic degeneration (Figure 3b) of mitochondria as well as flocculent densities (Figure 3c) and intramitochondrial calcifications (Figure 3b and c) were present. In some places, the T tubules were swollen and partially disrupted.

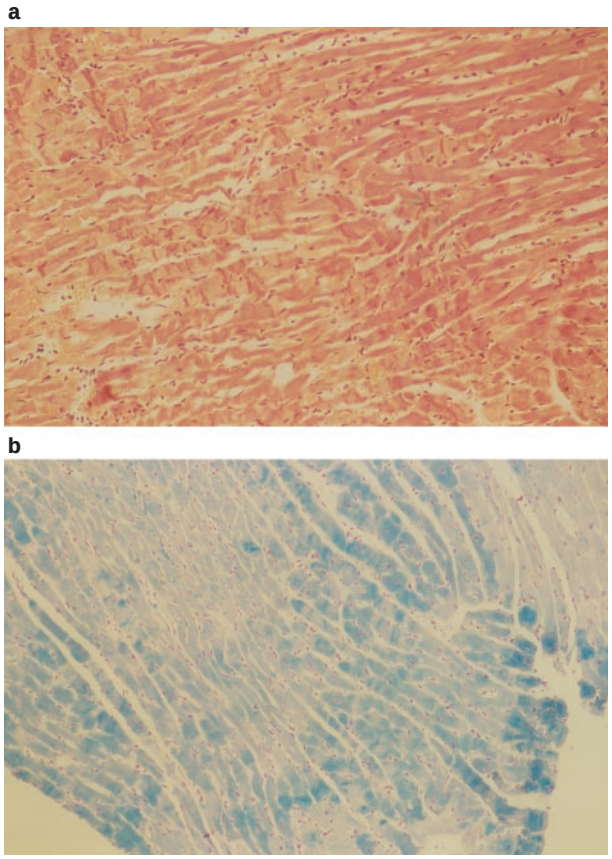

Figure 2 (a) Histological section after Fuchsin staining of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. Depicted is an example of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, which demonstrates a strong staining with Fuchsin. (b) Histological section after Luxol-Fast-Blue staining of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. Depicted is a further example of complete coagulation necrosis with eosinophilic cytoplasm of myocytes which demonstrates a submaximal staining with Luxol-Fast-Blue in the peripheral myocytes.

Effects of varying lengths of reperfusion following 25 min of regional myocardial ischaemia on myocardial infarct size in the rat

Occlusion of the LAD for 25 min followed by reperfusion for 2 h resulted in an infarct size of ~60% of the area at risk. Increasing the length of the reperfusion period to either 4 or 8 h did not result in a further, significant increase in infarct size (Figure 1c).

Effects of M&B 28767 or GR 63799X on the infarct size caused by regional myocardial ischaemia (25 min) and reperfusion (2 h) in the rat in vivo

The mean values for the areas at risk ranged from 41 ± 4, to 52 ± 3% and, hence, were similar in all animal groups studied ($P>0.05$, data not shown). In rats which had received an infusion of the vehicle (saline) for the EP_3-receptor agonists, occlusion of the LAD (for 25 min) followed by reperfusion (for 2 h) resulted in an infarct size of 60 ± 3% of the area at risk (control, $n=8$). When compared to vehicle, infusion of either M&B 28767 ($n=7$) or GR 63799X ($n=7$) caused a significant reduction in infarct size of approximately 35% (Figure 4).

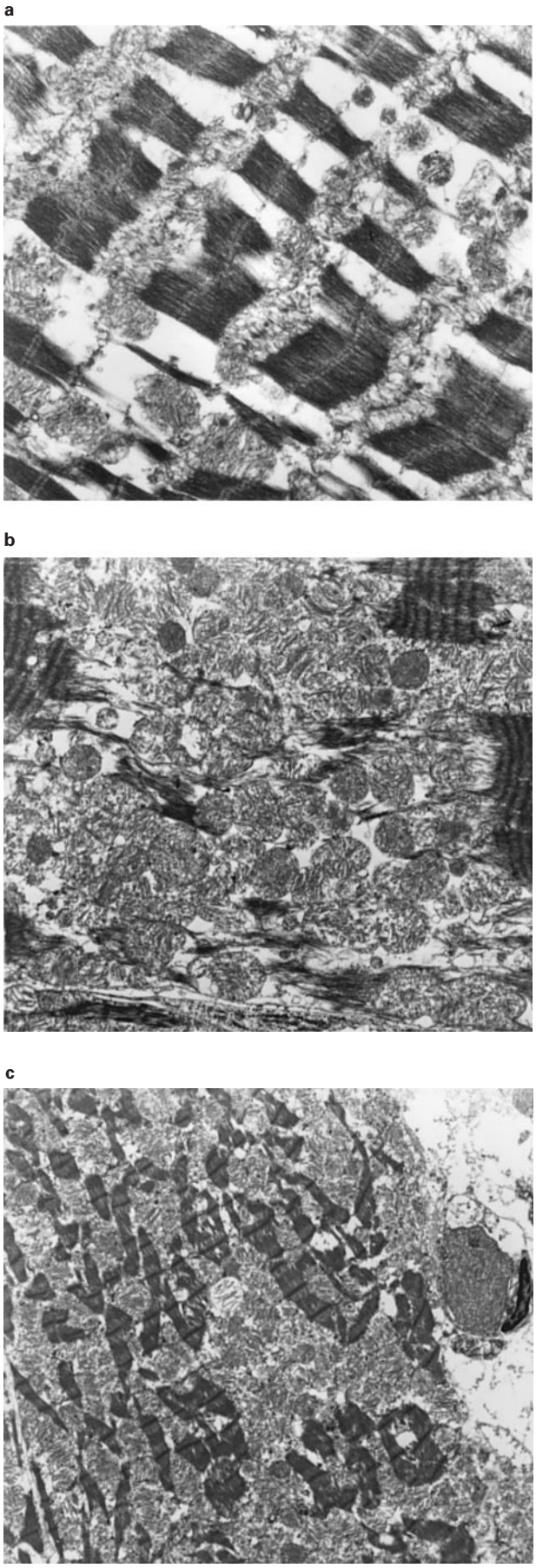

Figure 3 Electron microscopical section of the area at risk of a rat heart subjected to 25 min of myocardial ischaemia followed by 2 h of reperfusion. (a) Depicted are typical examples of granular disruption of the I-bands and the Z-lines compared to a normal A-band. (b) Depicted is an example of early contraction bands, osmiophilic degradation of mitochondria and intramitochondrial calcifications. (c) Depicted are typical examples of swollen or osmophilic degraded mitochondria as well as vacuolization of the wall of small vessels. These findings are similar to those exhibited by sections of human heart at 12 h after transmural myocardial infarction.

Pretreatment of rats with the K_{ATP}-channel blocker 5-HD abolished the reduction in infarct size afforded by the subsequent infusion of either M&B 28767 (Figure 4a, $n=6$) or GR 63799X (Figure 4b, $n=6$, $P>0.05$ when compared to control). However, pretreatment of rats with 5-HD prior to commencing the infusion of vehicle for the EP_3-receptor agonists (saline) did not result in a significant reduction in myocardial infarct size ($P>0.05$ when compared to vehicle-control) (Figure 4). Pretreatment of rats with glibenclamide also abolished the reduction in infarct size afforded by M&B 28767 (to $68\pm2\%$, $n=4$), while glibenclamide did not affect infarct size when given to rats treated with vehicle for M&B 28767 (infarct size: $64\pm6\%$, $n=6$). Similarly, pretreatment of rats with the PKC inhibitor staurosporine also abolished the reduction in infarct size afforded by M&B 28767 (Figure 5a), while staurosporine did not affect infarct size when given to rats treated with vehicle for M&B 28767 (Figure 5a). Pretreatment of rats with the PKC inhibitor chelerythrine also abolished the reduction in infarct size afforded by M&B 28767

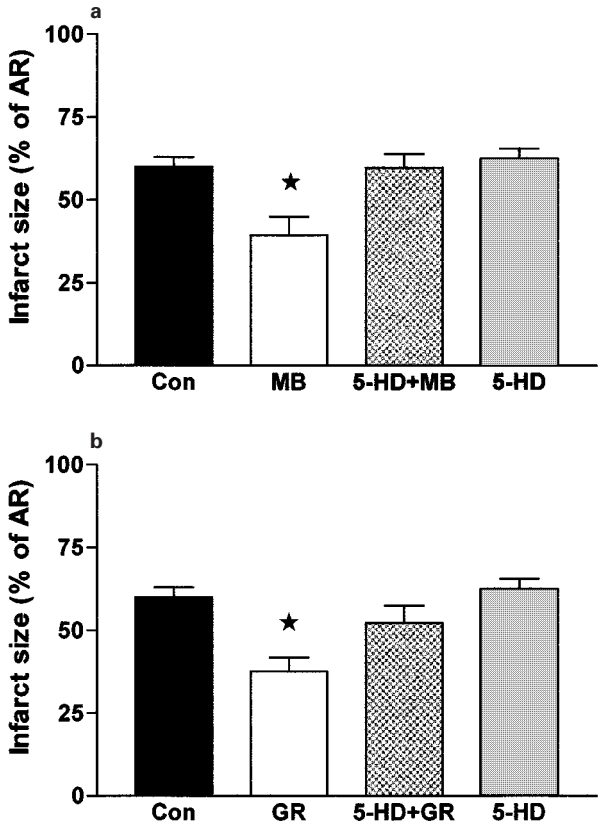

Figure 4 Infarct size caused by occlusion (25 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. Different groups of animals were treated with (a) vehicle (control, $n=8$), M&B 28767 (0.5 μg kg^{-1} h^{-1}, i.v., $n=7$), 5-hydroxydecanoate (5-HD, 5 mg kg^{-1}, i.v., $n=6$), 5-HD plus M&B 28767 ($n=6$) and (b) vehicle (control, $n=8$), GR 63799X (3.0 μg kg^{-1}, h^{-1}, i.v., $n=7$), 5-HD (5 mg kg^{-1}, i.v., $n=6$), 5-HD plus GR 63799X ($n=6$). *$P<0.05$ when compared to control.

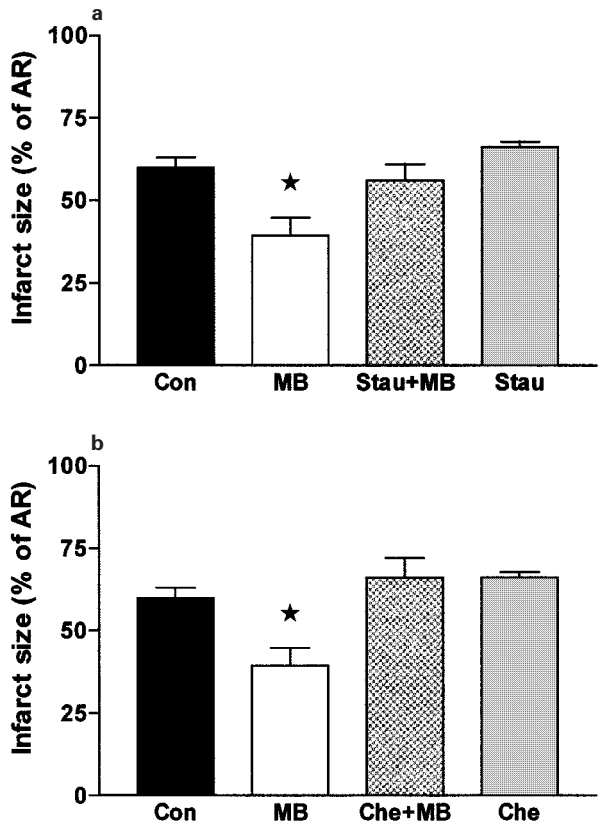

Figure 5 Infarct size caused by occlusion (25 min) and reperfusion (2 h) of the left anterior descending coronary artery (LAD) in the anaesthetized rat. Different groups of animals were treated with (a) vehicle (control, $n=8$), M&B 28767 (0.5 μg kg^{-1} h^{-1}, i.v., $n=7$), staurosporine (1 μg kg^{-1}, i.v., $n=6$), staurosporine plus M&B 28767 ($n=6$) and (b) vehicle (control, $n=8$), M&B 28767 (0.5 μg kg^{-1} h^{-1}, i.v., $n=7$), chelerythrine (0.7 mg kg^{-1}, i.v., $n=6$), chelerythrine plus M&B 28767 ($n=6$) *$P<0.05$ when compared to control.

(Figure 5b), while chelerythrine did not affect infarct size when given to rats treated with vehicle for M&B 28767 (Figure 5b).

Sham-operation did not result in a significant degree of infarction in any of the groups studied (less than 3% of the AR, data not shown).

Haemodynamic effects of the EP3-receptor agonists, M&B 28767 or GR 63799X, in rats subjected to regional myocardial ischaemia and reperfusion

Values for MAP, HR and PRI measured during the course of the experiments are given in Tables 1 and 2. Baseline haemodynamic data (e.g. MAP, HR and PRI) were similar ($P>0.05$) in all groups studied. In sham-operated rats (no LAD occlusion), infusion of vehicle (saline), 5-HD, M&B 28767 or GR 63799X, staurosporine or chelerythrine did not (within 60 min) cause any significant effects on any of the parameters measured. In some of the groups studied, the mean values for heart rate were, however, lower than in the control group (Tables 1 and 2). In rats subjected to LAD-occlusion and reperfusion which received an infusion of saline (control, $n=8$), mean values for MAP and PRI fell throughout the experimental period, but there was no alteration in heart rate (Table 1). In rats subjected to LAD-occlusion and reperfusion and treated with GR 63799X, the heart rate was lower than in the respective control group ($P<0.05$ at times -10 to 85 min), while there was no difference in either MAP or PRI ($P>0.05$). In rats subjected to LAD-occlusion and reperfusion and treated with M&B 28767, the heart rate was lower than in the respective control group at one time point in the reperfusion period ($P<0.05$ at 85 min). However, M&B 28767 did not affect MAP or PRI in rats subjected to LAD-occlusion and reperfusion (Table 1). Pretreatment of rats subjected to LAD-occlusion and reperfusion with 5-HD, staurosporine or chelerythrine did also not result in any significant haemodynamic effects (Table 1). In rats subjected to LAD-occlusion and reperfusion and treated with 5-HD and GR 63799X, the heart rate was lower than in the respective control group at times 0 and 15 min ($P<0.05$). In rats subjected to LAD-occlusion and reperfusion and treated with staurosporine plus M&B 28767, the heart rate was lower than in the respective control group ($P<0.05$ at times -10 to 145 min). Similarly, in rats subjected to LAD-occlusion and reperfusion and treated with chelerythrine plus M&B 28767, the heart rate was lower than in the respective control group ($P<0.05$ at time points 0, 15, 85 and 145 min) (Tables 1 and 2). None of the above interventions, however, had any effect on MAP or PRI.

Discussion

This study demonstrates that two agonists of the prostanoid EP$_3$-receptor (M&B 28767 and GR 63799X) reduce the myocardial infarct size caused by coronary artery occlusion and reperfusion in the anaesthetized rat. As in previous studies (Hide *et al*., 1995; Hide & Thiemermann, 1996), we have determined the degree of necrosis caused by regional ischaemia and reperfusion of the heart by staining of viable tissue within the area at risk (non-perfused myocardium) with the tetrazolium dye NBT. In the presence of intact dehydrogenase enzyme systems (viable myocardium), NBT forms a dark blue formazan, whilst areas of necrosis lack dehydrogenase activity and therefore do not stain (Nachlas & Shnitka, 1963). To ensure that those sections of the area at risk which failed to stain with NBT do indeed represent 'necrotic' myocardium, we have subsequently subjected biopsies of these sections to a histological analysis. Evaluation of these sections by light microscopy demonstrated the occurrence of complete coagulation necrosis with deeply eosinophilic cytoplasm of myocytes, the absence of any nuclear details as well as a substantial degree of extravasation of erythrocytes. The additional evaluation by electron microscopy of these biopsies also demonstrated (i) nuclei with margination and clumping of chromatin with intervening areas of pale nucleoplasm, (ii) granular disruption of the I-bands and Z-lines compared to a normal A-band, (iii) swollen mitochondria with intra-mitochondrial calcifications, (iv) contraction band necrosis (Reichenbach & Bendit, 1968; Baroldi, 1975) as well as (v) oedema. These data confirm that regional ischaemia (for 25 min) followed by reperfusion (for 2 h) does indeed lead to the development of a myocardial infarction, which can be detected by light microscopy, electron microscopy and the absence of staining with NBT. In order to further characterize the time-course of the development of myocardial infarction in the rat, we have elucidated the effects of increasing periods of regional myocardial ischaemia (from 7.5–60 min) followed by 2 h of reperfusion on infarct size. Clearly, there was a time-dependent increase in myocardial infarct size, which was maximal after 45 min. In subsequent studies, we have documented that an increase of the reperfusion period from 2 to 8 h does not lead to a further, significant increase in infarct size in the rat. This is

Table 1 Mean arterial pressure (MAP), heart rate (HR) and pressure rate index (PRI) in rats subjected to 25 min of LAD-occlusion and 2 h reperfusion

	Time (min)			*Occlusion*		*Reperfusion*	
Group	*−20*	*−10*	*0*	*15*	*25*	*85*	*145*
Sham vehicle (n=3)							
MAP (mmHg)	127±29	120±23	117±20	115±17	103±13	98±5	102±9
HR (beats min^{-1})	420±0	410±10	400±10	400±10	380±20	340±20*	370±26
PRI (mmHg min^{-1} 10^{-3})	53±12	49±10	46±7	46±6	39±4	33±1	37±1
Vehicle control (n=8)							
MAP (mmHg)	123±9	122±7	122±8	122±8	116±6	93±6	87±4
HR (beats min^{-1})	458±11	458±11	454±11	454±11	450±11	454±12	445±14
PRI (mmHg min^{-1} 10^{-3})	56±5	56±4	56±4	56±5	52±4	42±2	39±3
MB 28767 (n=7)							
MAP (mmHg)	125±7	120±5	112±6	110±8	109±6	111±7	101±8
HR (beats min^{-1})	420±19	411±14	403±11	390±15	386±18	373±16*	394±21
PRI (mmHg min^{-1} 10^{-3})	53±4	49±3	45±3	43±3	42±2	41±3	40±4
Sham MB 28767 (n=3)							
MAP (mmHg)	135±10	120±6	113±9	109±6	108±7	100±6	95±3
HR (beats min^{-1})	450±0	440±10	440±10	420±17	410±10	400±20	390±17
PRI (mmHg min^{-1} 10^{-3})	61±5	53±4	50±4	46±1	44±2	40±1	37±2
GR 63799X (n=7)							
MAP (mmHg)	134±2	126±4	113±8	109±6	119±6	124±8	115±9
HR (beats min^{-1})	399±9	386±10*	364±13*	351±19*	351±19*	356±10*	356±11
PRI (mmHg min^{-1} 10^{-3})	53±1	49±3	41±4	38±4	42±3	44±3	41±4
Sham GR 63799X (n=3)							
MAP (mmHg)	124±8	117±9	105±8	113±12	113±12	123±12	122±16
HR (beats min^{-1})	410±26	400±20	400±20	370±36	370±36	350±56*	370±70
PRI (mmHg min^{-1} 10^{-3})	51±2	47±2	42±3	42±6	42±6	43±6	45±7
5-HD (n=6)							
MAP (mmHg)	127±10	124±10	119±9	112±9	102±10	98±8	96±5
HR (beats min^{-1})	425±20	425±20	425±20	430±23	430±20	415±20	435±24
PRI (mmHg min^{-1} 10^{-3})	54±5	53±6	51±5	48±3	43±4	41±4	41±2
Sham 5-HD (n=3)							
MAP (mmHg)	120±12	113±9	113±9	99±6	93±3	95±3	97±4
HR (beats min^{-1})	450±17	450±17	460±10	440±20	430±10	410±10	410±10
PRI (mmHg min^{-1} 10^{-3})	54±7	51±6	52±5	44±5	40±2	39±1	40±2
5-HD+MB 28767 (n=6)							
MAP (mmHg)	141±7	139±8	134±8	129±7	121±7	113±4	113±3
HR (beats min^{-1})	415±12	410±13	385±20	400±13	385±20	385±12	405±13
PRI (mmHg min^{-1} 10^{-3})	59±3	57±3	52±4	52±3	47±4	44±1	46±0
5-HD+GR 63799X (n=6)							
MAP (mmHg)	133±10	132±11	119±10	116±4	110±5	120±10	113±10
HR (beats min^{-1})	395±27	395±22	360±22*	370±24*	365±26	405±19	385±20
PRI (mmHg min^{-1} 10^{-3})	53±5	52±5	43±2	43±3	40±3	49±4	44±3

M&B 28767 or GR 63799X were administered as a continuous infusion (0.5 μg kg^{-1} h^{-1}, i.v. or 3.0 μg kg^{-1} h^{-1}, i.v.) starting 10 min prior to coronary artery occlusion and continued until the end of reperfusion. 5-hydroxydecanoate (5-HD) was administered as an i.v. bolus (5 mg kg^{-1}, i.v.) 10 min before onset of the M&B 28767 or GR 63799X infusion. In addition, sham-operation (no coronary artery-occlusion) and administration of saline, M&B 28767, GR 63799X or 5-HD was carried out. *$P<0.05$ when compared to vehicle control.

not entirely surprising, as in rabbits subjected to regional myocardial ischaemia, an increase in the length of the reperfusion period from 2 to 8 h does not result in a significant further increase in infarct site in the rat. This is not entirely surprising as in rabbits subjected to regional myocardial ischaemia, an increase in the length of the reperfusion period from 2 to 8 h does also not result in a significant further increase in infarct size (McMurdo *et al*., 1994). In addition to measuring myocardial cell necrosis by NBT-staining or histology, the determination of the plasma levels of enzymes which are released by cardiac myocytes may also be used as a reliable indicator of tissue necrosis. The most specific, biochemical marker of cardiac myocyte necrosis (which can be measured in plasma) is cardiac troponin T (Adams *et al*., 1993). Cardiac troponin T is a structural, regulatory protein which is only present in cardiac myocytes so that an increase in the plasma levels of this marker is synonymous with myocardial cell necrosis (Adams *et al*., 1993). Unlike plasma levels of creatine phosphokinase or lactate dehydrogenase, which are relatively non-specific and, hence, are elevated in open-chest models of myocardial infarction due to the surgical procedure (Adams *et al*., 1993; Zacharowski & Thiemermann, unpublished data), the thoracotomy employed here did not result in a detectable rise in the plasma levels of cardiac troponin T. Increasing periods of regional myocardial ischaemia, however, resulted in time-dependent increases in the plasma levels of cardiac troponin T. This finding also supports our conclusion (see above) that 25 min of regional myocardial ischaemia followed by 2 h of reperfusion leads to a significant degree of myocardial necrosis.

Table 2 Mean arterial pressure (MAP), heart rate (HR) and pressure rate index (PRI) in rats subjected to 25 min of LAD-occlusion and 2 h reperfusion

	Time (min)			*Occlusion*		*Reperfusion*	
Group	*−20*	*−10*	*0*	*15*	*25*	*85*	*145*
Sham vehicle (n=*3*)							
MAP (mmHg)	127±29	120±23	117±20	115±17	103±13	98±5	102±9
HR (beats min^{-1})	420±0	410±10	400±10	400±10	380±20	340±20*	370±26
PRI (mmHg min^{-1} 10^{-3})	53±12	49±10	46±7	46±6	39±4	33±1	37±1
Vehicle control (n=*8*)							
MAP (mmHg)	123±9	122±7	122±8	122±8	116±6	93±6	87±4
HR (beats min^{-1})	458±11	458±11	454±11	454±11	450±11	454±12	445±14
PRI (mmHg min^{-1} 10^{-3})	56±5	56±4	56±4	56±5	52±4	42±2	39±3
Staurosporin (n=*6*)							
MAP (mmHg)	103±6	99±6	97±5	94±4	88±2	89±5	91±4
HR (beats min^{-1})	425±12	420±13	425±12	430±17	430±17	420±20	470±10
PRI (mmHg min^{-1} 10^{-3})	44±2	42±3	41±3	40±2	38±2	38±3	42±1
Sham Stau (n=*3*)							
MAP (mmHg)	103±7	101±10	98±8	91±5	93±9	87±3	90±0
HR (beats min^{-1})	410±36	410±36	410±36	400±40	420±35	410±36	410±36
PRI (mmHg min^{-1} 10^{-3})	43±6	42±8	41±7	37±6	39±4	35±2	37±3
Stau+MB 28767 (n=*6*)							
MAP (mmHg)	111±3	114±5	113±4	107±8	104±8	94±7	86±5
HR (beats min^{-1})	395±14	385±18*	380±17*	375±13*	370±10*	365±8*	370±13*
PRI (mmHg min^{-1} 10^{-3})	44±3	44±3	43±4	40±3	38±4	34±1	32±0
Chelerythrine (n=*6*)							
MAP (mmHg)	101±7	102±8	102±8	89±9	83±7	88±5	83±4
HR (beats min^{-1})	435±17	430±13	430±13	435±10	430±13	440±6	435±7
PRI (mmHg min^{-1} 10^{-3})	44±2	44±3	44±3	39±3	36±3	39±2	36±2
Sham Chel (n=*3*)							
MAP (mmHg)	138±15	133±12	133±12	127±13	121±12	108±15	101±12
HR (beats min^{-1})	440±26	430±36	430±36	420±35	420±35	420±17	420±30
PRI (mmHg min^{-1} 10^{-3})	61±10	57±10	57±10	53±10	51±9	45±8	42±8
Chel+MB 28767 (n=*6*)							
MAP (mmHg)	121±9	119±10	117±11	114±12	108±11	93±11	89±11
HR (beats min^{-1})	440±13	420±11	380±15*	383±13*	385±12	365±9*	375±13*
PRI (mmHg min^{-1} 10^{-3})	53±3	50±3	44±4	44±4	42±2	34±3	33±2

M&B 28767 was administered as a continuous infusion (0.5 $\mu g\ kg^{-1}\ h^{-1}$, i.v. or 3.0 $\mu g\ kg^{-1}\ h^{-1}$, i.v.) starting 10 min prior to coronary artery occlusion and continued until the end of reperfusion. The PKC inhibitors staurosporine (1 $\mu g\ kg^{-1}$) or chelerythrine (0.7 $mg\ kg^{-1}$) were administered as an i.v. bolus 10 min before onset of the M&B 28767 infusion. In addition, sham-operation (no coronary artery-occlusion) and administration of staurosporine or chelerythrine was carried out. *$P<0.05$ when compared to vehicle control.

The observed (moderate) reduction in infarct size amounted to 35% and, hence, was similar to the reductions in infarct size caused by either PGE_1 (non-selective EP_3-receptor agonist) or sulprostone (selective EP_1/EP_3-receptor agonist) in the anaesthetized rabbit (~40%, see Hide *et al*., 1995; Hide & Thiemermann, 1996). A reduction in infarct size of this magnitude may well be biologically important, as the reduction in left ventricular function (Field *et al*., 1972) as well as the incidence of arrhythmias (Roberts *et al*., 1975) occurring as a consequence of myocardial ischaemia are directly proportional to the size of the infarcted myocardium.

Mechanism(s) of the cardioprotective effect of EP_3-receptor agonists in the rat

What, then, is the mechanism(s) by which agonists of the EP_3-receptor reduce myocardial infarct size? Clearly, a reduction in blood pressure and, hence, myocardial oxygen consumption does not account for the cardioprotective effects of either M&B 28767 or GR 63799X, as neither of these EP_3-receptor agonists caused a significant reduction in blood pressure or pressure-rate index. There is a strong positive correlation between myocardial oxygen consumption and pressure-rate index (Baller *et al*., 1981) and, hence, the observed cardioprotective effects of M&B 28767 or GR 63799X are not likely to be secondary to a reduction in myocardial oxygen demand. Although the heart rate of rats treated with M&B 28767 or GR 63799X was (at some time points) lower than in the respective control group, it is unlikely that a moderate reduction in heart rate (which was not sufficient to cause a significant reduction in PRI) accounts for the cardioprotective effects of these EP_3-receptor agonists. This conclusion is also supported by our finding that a similar reduction in heart rate in rats subjected to myocardial ischaemia-reperfusion injury and treated with staurosporine and M&B 28767 was not associated with a significant reduction in myocardial infarct size.

Our finding that neither M&B 28767 nor GR 63799X reduced blood pressure in the rat also confirms that (at the doses used) neither M&B 28767 nor GR 63799X activate EP_2-receptors *in vivo*, activation which results in stimulation of G_s, an increase in cyclic AMP and vasodilatation. Thus, it is very unlikely that the doses of M&B 28767 or GR 63799X used in this study are sufficient to activate EP_2-receptors *in vivo*. We, therefore, propose that the observed cardioprotective effects are due to the activation of EP_3-receptors. In order to gain a better insight into the mechanism(s) by which the EP_3-receptor agonists M&B 28767 or GR 63799X reduce myocardial infarct

size in the rat, we carried out a number of further experiments to elucidate the signal transduction events underlying the observed cardioprotective effects.

Role of ATP-sensitive potassium channels in the cardioprotective effects of M&B 28767 or GR 63799X

The reduction in infarct size caused by either PGE_1 or by the EP_1/EP_3-receptor agonist sulprostone is (at least in part) due to the activation and opening of K_{ATP}-channels (Hide *et al*., 1995; Hide & Thiemermann, 1996). Here we report that the cardioprotective effect of M&B 28767 and GR 63799X are abolished by pretreatment of the animals with 5-HD, a selective blocker of K_{ATP}-channels (Garlid *et al*., 1997). Although glibenclamide is not a specific inhibitor of cardiac K_{ATP}-channels, both glibenclamide and 5-HD have been used to document that the cardioprotective effects of ischaemic preconditioning involve the activation of K_{ATP}-channels (Hide & Thiemermann, 1996). We confirm here that the reduction in infarct size afforded by M&B 28767 is also abolished by a dose of glibenclamide, which has previously been shown to abolish the cardioprotective effects of 'ischaemic preconditioning' (Hide & Thiemermann, 1996). These findings suggest that M&B 28767 and GR 63799X (and presumably the activation of EP_3-receptors) leads to opening of K_{ATP}-channels which, in turn, results in cardioprotection. The mechanism by which opening of K_{ATP}-channels protects the myocardium against ischaemic injury is not clear.

Role of protein kinase C in the cardioprotective effects of M&B 28767

The signal transduction events involved in the cardioprotective effects of M&B 28767 or GR 63799X are reminiscent of those that mediate the potent anti-ischaemic effects of 'ischaemic preconditioning' (Downey & Cohen, 1995). There is evidence that 'preconditioning of the myocardium' which ischaemia results in the release of adenosine and other mediators which activate G-protein coupled receptors resulting in activation of protein kinase C, opening of K_{ATP}-channels and ultimately cardioprotection (Downey & Cohen, 1995; Millar *et al*., 1996). Having demonstrated that the cardioprotective effects of M&B 28767 are abolished by glibenclamide and 5-HD, we have investigated the potential role of PKC in the observed cardioprotective effects of this EP_3-receptor agonist. We demonstrate here that the reduction in infarct size afforded by M&B 28767 in the rat is abolished by two inhibitors of PKC, namely staurosporine and chelerythrine. It should be noted that staurosporine and chelerythrine inhibit the activation of PKC *in vivo* and *ex vivo* (Speechly-Dick *et al*., 1994; Kaye *et al*., 1995; Kozak *et al*., 1997) and also abolish the cardioprotective effects of 'ischaemic preconditioning' in rodents (Speechly-Dick *et al*., 1994; Yoshida *et al*., 1997). These findings suggest that the signal transduction events leading to a reduction in infarct size caused by M&B 28767 involve the activation of PKC.

Conclusion

In conclusion, this study demonstrates that two groups of the prostanoid EP_3-receptor (M&B 28767 and GR 63799X) reduce infarct size in rats subjected to regional myocardial ischaemia and reperfusion. The mechanism(s) of the cardioprotective effects of these agents are not entirely clear, but may involve the activation of PKC and the opening of ATP-sensitive potassium channels. We also propose that selective agonists of the prostanoid EP_3-receptor may be useful to protect the heart against ischaemia-reperfusion injury without causing significant haemodynamic (side-) effects.

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