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RESEARCH PAPER

Mast cell degranulation – a mechanism for the anti-arrhythmic effect of endothelin-1?

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Background and purpose: The aim of this study was to investigate whether the previously reported anti-arrhythmic effect of endothelin-1 (ET-1) is mediated by degranulation of cardiac mast cells prior to myocardial ischaemia.
Experimental approach: Male Sprague-Dawley rats received either ET-1 (1.6 nmol·kg^{-1}) in the presence or absence of disodium cromoglycate (DSCG; 20 mg·kg^{-1}·h^{-1}) prior to coronary artery occlusion (CAO). In separate experiments rats were given compound 48/80 (50 μg·kg^{-1}) to compare the effects of ET-1 with those of a known mast cell degranulator. Ischaemia-induced ventricular arrhythmias were detected through continuous monitoring of a lead I electrocardiogram. After 30 min of CAO, the hearts were removed and mast cell degranulation determined by histological analysis. A parallel series of sham groups were performed to determine the direct effects of ET-1 and compound 48/80 on mast cell degranulation in the absence of ischaemia.
Key results: ET-1 and compound 48/80 both exerted profound anti-arrhythmic effects, significantly reducing the total number of ventricular ectopic beats ($P < 0.001$) and the incidence of ventricular fibrillation ($P < 0.05$). These anti-arrhythmic effects were abolished by concomitant DSCG infusion prior to CAO. In sham animals ET-1 and compound 48/80 both induced mast cell degranulation ($P < 0.001$), an effect which was abolished by DSCG, confirming their ability to induce degranulation of mast cells.
Conclusions and implications: These results demonstrate for the first time that when given prior to ischaemia ET-1 mediates its anti-arrhythmic effects, at least in part, via cardiac mast cell degranulation.
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Abbreviations: CAO, coronary artery occlusion; DSCG, disodium cromoglycate; ECG, electrocardiogram; ET-1, endothelin-1; ET_A receptor, endothelin A receptor; ET_B receptor, endothelin B receptor; HR, heart rate; MABP, mean arterial blood pressure; VEB, ventricular ectopic beat; VF, ventricular fibrillation; VT, ventricular tachycardia

Introduction

Increased production of endothelin-1 (ET-1) in response to myocardial ischaemia has been demonstrated in both experimental and clinical settings (Brunner *et al.*, 1992; Lechleitner *et al.*, 1992) and there is abundant evidence to show that ET receptor antagonists are both cardioprotective and anti-arrhythmic (see Wainwright *et al.*, 2005), implying a role for endogenous ET-1 in the consequences of ischaemia and reperfusion. Paradoxically, we demonstrated some time ago that pretreatment (i.e. pharmacological preconditioning) with low doses of ET-1 prior to the onset of ischaemia reduces both the incidence of ventricular arrhythmias (Sharif *et al.*, 1998), and it has been shown by others to be cardioprotective in terms of extent of tissue injury (Hide *et al.*, 1995; Bugge and Ytrehus, 1996). The underlying mechanism for the anti-arrhythmic effects of ET-1 remain elusive, although the ET_B (endothelin B) receptor has been implicated in these anti-arrhythmic effects (Crockett *et al.*, 2000; see Wainwright *et al.*, 2005).

Mast cells are nucleated haematopoietic cells that arise from the bone marrow, and have an almost ubiquitous distribution in the connective tissues of the body. Unlike other immune cells (basophils, neutrophils and eosinophils), mature differentiated mast cells are present within tissues under normal physiological conditions (see Triggiani *et al.*, 2008) and tend to congregate around nerves and blood vessels. Mast cells produce and release a variety of vasoactive mediators (proteolytic enzymes, heparin, histamine and cytokines) and, although generally thought of as pro-inflammatory cells,

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their exact physiological role has not been fully elucidated. A pathophysiological role for mast cells in cardiovascular conditions has been suggested, since increased numbers of mast cells are located around coronary arteries in atherosclerosis (Pouchlev *et al*., 1966) and myocarditis (Estensen, 1984). In addition, myocardial ischaemia is known to cause degranulation of resident cardiac mast cells, leading to the release of cytotoxic mediators that contribute to both ischaemia-induced injury (Kubes and Granger, 1996) and the generation of ventricular arrhythmias (Wolff and Levi, 1988; Mackins *et al*., 2006), while mast cell stabilizers [e.g. disodium cromoglycate (DSCG)] given during ischaemia are anti-arrhythmic (Humphreys *et al*., 1998; Parikh and Singh, 1998; Mackins *et al*., 2006).

As with ET-1, a paradoxical protective role for mast cell degranulation has been suggested in tissues subjected to ischaemia; for example, the mast cell degranulating compound 48/80, when given prior to experimental ischaemia, protects the myocardium (Parikh and Singh, 1997) and the intestine (Boros *et al*., 1999). Moreover, mast cell degranulation during preconditioning ischaemic periods has been proposed to contribute, at least in part, to the anti-arrhythmic effect of ischaemic preconditioning against reperfusion arrhythmias (Parikh and Singh, 1998). Taken together, these observations imply that depletion of cytotoxic mediators from mast cells prior to ischaemia prevents their accumulation in the ischaemic tissue and thus eliminates their pro-arrhythmic effects.

It has been known for some time that certain mast cells possess a single class of specific, high-affinity receptors with distinct selectivity for ET-1 and with a pharmacological profile resembling that of the endothelin A (ET_A) receptor (Ehrenreich *et al*., 1992), but it has only recently been shown that ET-1 can induce degranulation of both mucosal (Boros *et al*., 2002) and connective tissue-type (Murray *et al*., 2004) mast cells. Taken together, with the observation that mast cell degranulation prior to ischaemia can protect against reperfusion arrhythmias, this suggests that the anti-arrhythmic effects of ET-1 could be, at least in part, mediated by ET-1-induced mast cell degranulation. The present study was therefore undertaken to investigate this possibility in an *in vivo* model of myocardial ischaemia.

Methods

Animal sources

All studies were performed under an appropriate Project License authorized under the UK Animals (Scientific Procedures) Act 1986. Male Sprague-Dawley rats, weighing 300–400 g, were bred and housed in the University of Aberdeen Medical Research Facility. Animals were obtained on a daily basis and allowed to acclimatize for 30 min before commencing the study. Animals were maintained at a temperature of 21 ± 2°C, with a 12 h light/dark cycle and with free access to food and tap water.

Preparation for coronary artery occlusion

Animals were anaesthetized with pentobarbital sodium salt (60 mg·kg^{-1} i.p; Sigma Aldrich, Poole, Dorset, UK) and the trachea cannulated to allow artificial respiration when required. The left carotid artery and the right jugular vein were cannulated with Portex polythene tubing (0.58 mm ID × 0.96 mm OD; Smiths Medical International Ltd., Hyde, Kent, UK). Arterial blood pressure was recorded via the left carotid artery using a pressure transducer (MLT844 physiological pressure transducer; AD Instruments, Chalgrove, Oxfordshire, UK). A steel thermistor probe (Fisher Scientific Ltd., Loughborough, Leicestershire, UK) was inserted into the rectum to measure core temperature which was maintained at 37–38°C with the aid of a Vetcare heated pad (Harvard Apparatus Ltd.). The animal was then prepared for *in vivo* occlusion of the left anterior descending coronary artery (Clark *et al*., 1980) through a left thoracotomy and subsequently ventilated on room air (54 strokes min^{-1}; tidal volume, 1.5 mL·100 g^{-1} to maintain PCO_2 at 18–24 mmHg, PO_2 at 100–130 mmHg, and pH at 7.4; Harvard small animal respiration pump; Edenbridge, Kent, UK). Anaesthesia was maintained throughout by administration of pentobarbital sodium salt (3–4 mg·kg^{-1}) via the venous cannula every 30 min or as required. After placement of the ligature, rats were allowed to stabilize for 15 min before commencing any drug infusions. On completion of pre-ischaemic drug protocols, the coronary artery was occluded (CAO) by tightening the ligature to induce regional ischemia for 30 min. A standard limb lead I electrocardiogram (ECG) was monitored continuously throughout the experimental period by insertion of subcutaneous needle electrodes. Both mean arterial blood pressure (MABP) and heart rate (HR), which was calculated from the ECG, were also continuously monitored throughout the experimental period. Data obtained via the transducer and electrodes were transmitted to a Power Lab (AD Instruments) data acquisition system via a Bridge Amplifier (AD Instruments) and Animal Bio Amplifier (AD Instruments), respectively, for storage and analysis. Chart Software (AD Instruments) was then used to analyse the data obtained via the Power lab. Following completion of the *in vivo* protocol, the heart was removed and fixed in 10% buffered formol saline (Thermo Scientific, Runcorn, Cheshire, UK) for histological analysis. Any animals which had a starting MABP of <70 mmHg or developed spontaneous arrhythmias prior to CAO were excluded from the study.

Histological assessment of cardiac mast cell degranulation

Fixed hearts were sliced into 2–3 mm slices from the apex to the base and the slice just below the ligature used for histological analysis. Myocardial tissue slices were then embedded in paraffin wax (Thermo Scientific) and 3 μm sections cut. Staining of the sections was carried out with the use of an autostainer (Varistain 24-4, Thermo Scientific) and involved dehydration of the tissue through a series of Histosolve (Thermo Scientific) and graded alcohols. Sections were incubated manually in 0.1% w/v toluidine blue (Fisher Scientific Ltd.) at 37°C and subsequently rehydrated through graded alcohols and Histosolve. After staining, sections were mounted with a xylene substitute mountant (Thermo Scientific) and coverslipped. Examination of the tissue was carried out with the use of a Leica DMLB light microscope (Leica Microsystems, Milton Keynes, Bucks, UK) at a magnification of ×400. Mast cells were counted manually because of the

relatively small numbers and the count encompassed the entire area of the tissue. Photomicrographs of the sections were taken with a Leica DC150 camera (Leica Microsystems) in conjunction with Leica QWin software (Leica Microsystems). The total area of the myocardial section was measured using computerized planimetry (ImageJ software, NIH) to express mast cell counts as mast cells mm^{-2}. Mast cell degranulation was determined as a loss of mast cell membrane integrity with extrusion of intracellular granules to the extracellular space or mast cells completely lacking in intracellular granules as described previously (Messina *et al.*, 2000).

Immunostaining for endothelin-1 (ET-1)

Immunohistochemical staining for ET-1 was performed in fixed heart sections from all groups. At the end of the *in vivo* protocol, hearts were fixed, then sliced into 2–3 mm slices from the apex to the base and the slice just below the ligature used for immunohistochemical staining. Myocardial tissue slices were then embedded in paraffin wax and 3 μm transverse sections cut and mounted on polysine slides. Following dehydration and deparaffinization, endogenous peroxidase activity was quenched by incubating the sections in 0.3% (v/v) hydrogen peroxide for 30 min, followed by washing in phosphate buffered saline (PBS) for 5 min. Slides were subsequently placed in a pressure cooker containing boiling PBS and further heated for 6 min in a microwave to unmask the antigens. Sections were cooled with tap water and then subjected to the blocking step which involved incubation of the slides with 2.5% normal horse serum for 30 min. Slides were then washed in PBS and incubated with the primary antibody (murine monoclonal anti-ET-1 antibody (Abcam, Cambridgeshire, UK)) at a dilution of 1:250 in a humidifying chamber for 1 h at room temperature. Following a further wash in PBS, sections were subsequently incubated with the ImmPRESS reagent (Vector Laboratories, Peterborough, UK) which consists of a combination of both anti-mouse and anti-rabbit IgG secondary antibodies coupled to peroxidase for 30 min under the same conditions. Sections were then washed in PBS and incubated with Vector VIP substrate until the desired stain intensity was achieved (3–5 min). Sections were then counterstained with Mayer's haematoxylin (RA Lamb Ltd, East Sussex, UK) and finally dehydrated and cleared through a series of graded alcohols and Histosolve. Sections were mounted with Immu-mount (Thermo Shandon) and analysed to detect positive staining for ET-1 (as indicated by intense localised purple staining) using a Leica DMLB light microscope (Leica Microsystems, Bucks, UK) at a magnification of ×400. Photomicrographs of the sections were taken with the use of a Leica DC150 camera (Leica Microsystems, Bucks, UK) in conjunction with the Leica QWin software (Leica Microsystems, Bucks, UK). Each staining run contained a negative control slide in which the primary antibody was replaced with an IgG negative antibody control.

Experimental protocols

Schematic representations of the experimental protocols are shown in Figure 1.

(i) *Comparison of the anti-arrhythmic effects of ET-1 with a known mast cell degranulating agent:* All rats were given an i.v. saline infusion, commencing 30 min prior to CAO and continued throughout the 30 min ischaemic period. The rats were then given a bolus i.v. injection of either saline ($n = 12$), ET-1 (1.6 $nmol \cdot kg^{-1}$; $n = 11$; Sigma Aldrich) or compound 48/80 (50 $\mu g \cdot kg^{-1}$; $n = 8$; Sigma Aldrich), via the right jugular vein, 5 min prior to CAO (Figure 1A).

(ii) *Determination of the effects of mast cell stabilization on the anti-arrhythmic effects of compound 48/80 and ET-1.* To confirm the contribution of mast cell degranulation to the anti-arrhythmic effects of ET-1 and compound 48/80 rats were given an infusion of DSCG (20 $mg \cdot kg^{-1}\ h^{-1}$; 0.05 $mL \cdot min^{-1}$; Sigma Aldrich) for 30 min up to the point of CAO, followed by a saline infusion during CAO (Figure 1B). Intravenous bolus doses of either saline ($n = 12$), ET-1 (1.6 $nmol \cdot kg^{-1}$; $n = 11$) or compound 48/80 (50 $\mu g \cdot kg^{-1}$; $n = 8$) were then administered 5 min prior to CAO (Figure 1B). For both protocols (i) and (ii), ventricular arrhythmias during the 30 min occlusion period were quantified and the percentage of mast cells that had undergone degranulation were assessed histologically.

(iii) *Assessment of the effects of ET-1 and DSCG on cardiac mast cell degranulation in the absence of ischaemia.* Since ischaemia itself induces mast cell degranulation, we undertook a replicate series of experiments for protocols (i) and (ii) in sham-operated time controls (in which the ligature was placed around the left coronary artery but not tightened) to visually confirm degranulation/

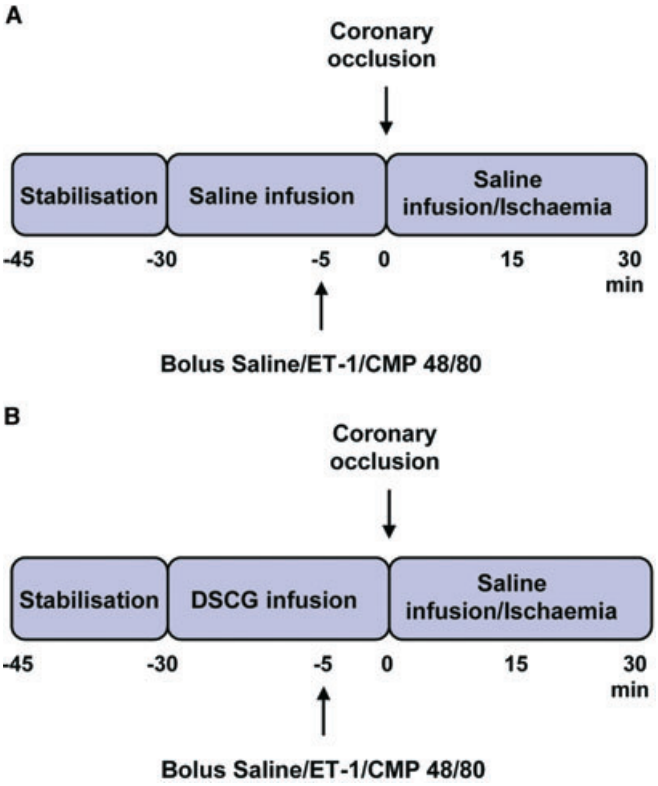

Figure 1 Experimental protocols used to investigate (A) the effects of ET-1 and compound 48/80 (CMP 48/80) on ischaemia-induced ventricular arrhythmias and (B) the effects of ET-1 and compound 48/80 on ischaemia-induced ventricular arrhythmias in the presence of DSCG.

stabilization in direct response to ET-1 ($n = 6$), compound 48/80 ($n = 4$) and DSCG ($n = 5$) by histological analysis of heart sections.

Statistical analysis

For the haemodynamic data (expressed as mean ± SEM) a Student's *t*-test was used to compare pre-injection and post-injection MABP/HR values. A one-way ANOVA with Dunnett's *post hoc* test was used to compare pre-occlusion and post-occlusion MABP/HR. Post-occlusion MABP/HR comparisons between the control and drug treated groups were made using a two-way ANOVA with Bonferroni *post hoc* test. Ventricular arrhythmias were determined from the ECG trace and classified according to the Lambeth Conventions (Walker *et al.*, 1988). The effect of agents on the number of ischaemia-induced ventricular ectopic beats [VEBs; reported as singles, salvos, ventricular tachycardia (VT) and total VEB count and values expressed as mean ± SEM] was analysed using a one-way ANOVA with Dunnett's *post hoc* test. The effect of agents on the incidences of VT, reversible and irreversible ventricular fibrillation (VF) and on mortality were analysed using a Fisher's exact test. The effects of ischaemia and agents on the incidence of cardiac mast cell degranulation were analysed using both a Student's *t*-test and one-way ANOVA with Dunnett's *post hoc* test.

Drugs and reagents

All chemicals were purchased from Sigma-Aldrich (Dorset, UK) with the exception of toluidine blue, paraffin wax and PBS tablets which were from Fisher Scientific (Leicestershire, UK), heparin from Leo Laboratories Ltd. (Bucks, UK) and those otherwise stated. All drug/molecular target nomenclature for receptors and ion channels conform to the British Journal of Pharmacology's Guide to Receptors and Channels (Alexander *et al.*, 2008).

Results

Effects of drug interventions on haemodynamic variables

The effects of the various drug interventions prior to CAO on MABP and HR are summarized in Table 1. Administration of ET-1 induced a significant but transient fall in MABP prior to coronary occlusion ($P < 0.01$), which was unaffected by DSCG infusion. Treatment with compound 48/80 similarly induced a significant but transient fall in MABP prior to coronary occlusion ($P < 0.001$); however, this hypotensive response was abolished when compound 48/80 administration was combined with DSCG infusion. All groups exhibited the characteristic fall in MABP upon occlusion of the coronary artery ($P < 0.001$). Both ET-1 and compound 48/80 given prior to CAO improved the recovery of MABP at 3, 15 and 30 min post occlusion when compared with control animals ($P < 0.001$). DSCG had no effect on either the ET-1 or compound 48/80 induced recovery of MABP after coronary occlusion. None of the drug treatments or infusions affected HR (Table 1).

Influence of drug intervention on ventricular arrhythmias

Induction of myocardial ischaemia resulted in the generation of a significant number of ventricular arrhythmias in the control group (Figure 2A). In most cases arrhythmias commenced 9–10 min post coronary occlusion (data not shown) and the majority occurred as VT (Figure 2A). Treatment with ET-1 prior to coronary occlusion significantly reduced the incidence of all types of ischaemia-induced VEBs [single VEBs ($P < 0.05$), salvos ($P < 0.01$), VT ($P < 0.01$) and the total number of VEBs ($P < 0.001$; Figure 2A)]. However, when ET-1 administration was combined with DSCG infusion this anti-arrhythmic effect was abolished. Treatment with compound 48/80 prior to CAO similarly reduced arrhythmias (Figure 2A), an effect that was also abolished by concomitant DSCG infusion. In control animals, the total

Table 1 Summary of mean arterial blood pressure (MABP) and heart rat (HR) in rats given saline, ET-1 and CMP 48/80 in the presence or absence of DSCG

Time (min)	*Saline*	*ET-1*	*ET-1 & DSCG*	*CMP 48/80*	*CMP 48/80 & DSCG*
MABP (mmHg)					
–15	104 ± 9	125 ± 4	120 ± 7	126 ± 2	127 ± 4
–5	106 ± 8	126 ± 4	125 ± 6	126 ± 2	124 ± 4
–4	104 ± 4	94 ± 9††	86 ± 7††	63 ± 2†††	123 ± 4
0	105 ± 3	122 ± 6	123 ± 5	123 ± 2	127 ± 3
1	62 ± 4***	81 ± 4***	76 ± 5***	89 ± 2***	77 ± 5***
3	65 ± 6***	97 ± 4$^{\#\#\#}$	88 ± 8	102 ± 3$^{\#\#\#}$	93 ± 7$^{\#\#}$
15	82 ± 5***	116 ± 6$^{\#\#\#}$	99 ± 2	111 ± 4$^{\#\#\#}$	115 ± 5$^{\#\#\#}$
30	85 ± 6***	97 ± 9	92 ± 1	112 ± 2$^{\#\#\#}$	103 ± 3
HR (bpm)					
–15	434 ± 15	475 ± 17	472 ± 19	467 ± 5	480 ± 21
–5	426 ± 12	457 ± 12	457 ± 11	449 ± 10	476 ± 23
–4	422 ± 11	442 ± 9	450 ± 8	449 ± 7	460 ± 17
0	425 ± 12	451 ± 9	468 ± 12	443 ± 7	474 ± 13
1	426 ± 10	453 ± 13	459 ± 12	458 ± 5	478 ± 13
3	420 ± 15	449 ± 16	453 ± 13	457 ± 6	473 ± 13
15	394 ± 20	399 ± 23	457 ± 14	420 ± 10	467 ± 20
30	389 ± 19	387 ± 23	454 ± 16	417 ± 11	464 ± 18

Values are given as mean ± SEM ***$P < 0.001$ versus pre-occlusion; $^{\dagger\dagger}P < 0.01$ & $^{\dagger\dagger\dagger}P < 0.001$ versus pre-bolus injection values; $^{\#\#}P < 0.01$ & $^{\#\#\#}P < 0.001$ versus saline.

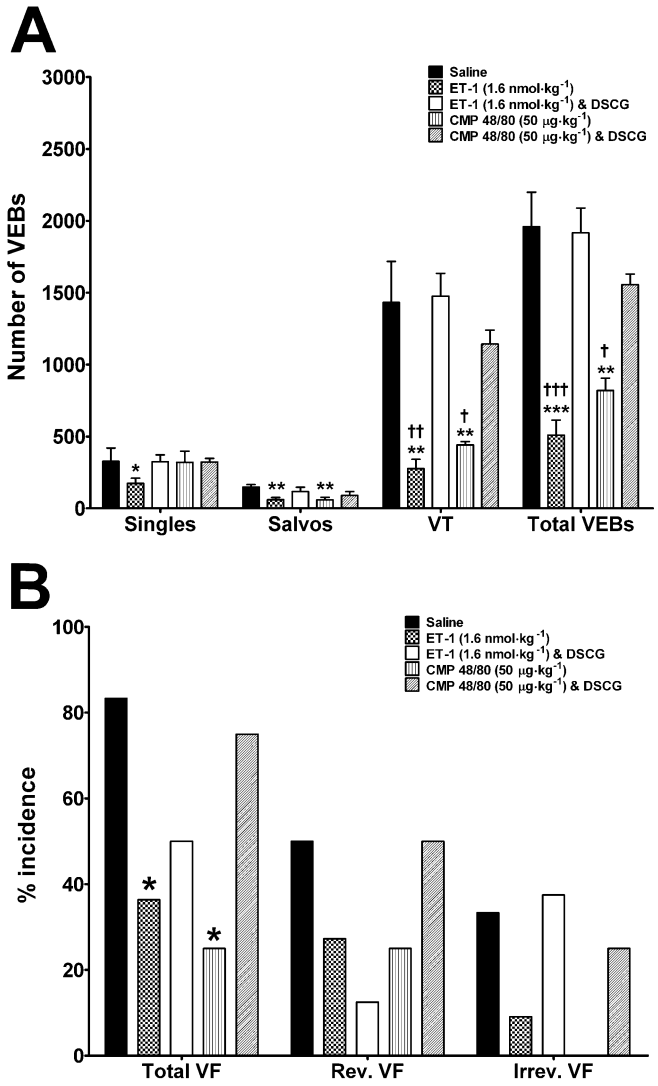

Figure 2 Effect of saline/ET-1/ compound 48/80 (CMP 48/80) alone and in the presence of DSCG on both ischaemia-induced arrhythmias and the incidence of VF. The effects of a bolus administration of either saline, ET-1 or compound 48/80 (5 min prior to coronary occlusion) in the absence or presence of DSCG infusion (30 min prior to coronary occlusion) on both (A) the incidence of each type of arrhythmia and (B) the incidence of each type of VF was investigated. For the incidence of all types of ischaemia-induced arrhythmias, data were recorded and expressed as mean ± SEM (n = 5–10) *P < 0.05, **P < 0.01 & ***P < 0.001 versus saline, $^{\dagger\dagger}P$ < 0.01 & $^{\dagger\dagger\dagger}P$ < 0.001 versus ET-1 & DSCG and $^{\dagger}P$ < 0.05 versus compound 48/80 & DSCG. The percentage incidence of both reversible and irreversible VF was recorded and the data expressed as mean (n = 8–12). *P < 0.05 versus saline.

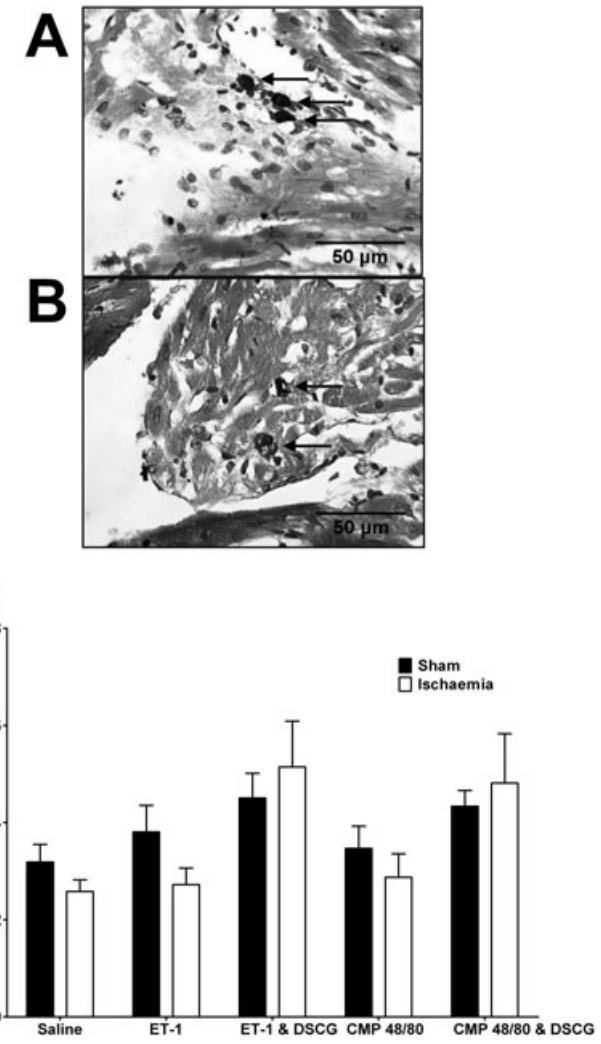

Figure 3 Effects of ET-1 on the percentage of mast cells degranulated in the rat myocardium. Light photomicrographs (×400) of (A) myocardial tissue from saline treated sham animals displaying intact mast cells (indicated by arrows) and (B) myocardial tissue from ET-1 treated sham animals displaying degranulated mast cells (indicated by arrows), stained with toluidine blue. (C) The number of mast cells per section of ventricular tissue expressed as mast cells mm^{-2} in all treatment groups.

incidence of VF was 83% (50% reversible; 33% irreversible) (Figure 2B). Administration of both ET-1 and compound 48/80 significantly reduced total VF (P < 0.05) and the concomitant DSCG infusion attenuated this anti-fibrillatory effect (Figure 2B). DSCG infusion in rats given a subsequent bolus injection of saline did not exert an anti-arrhythmic effect [1959 ± 473 vs. 1959 ± 240 (total VEBs); 63% vs. 83% (total VF); data not shown] when compared with the control.

Mast cell degranulation and ET-1 immunostaining

Figure 3 illustrates photomicrographs showing the typical appearance of both intact (Figure 3A) and degranulated mast cells (Figure 3B) in myocardial sections. None of the treatments significantly affected mast cell numbers in ventricular tissue (Figure 3C). In sham-operated animals (Figure 4, solid bars) both ET-1 and compound 48/80 induced a significant increase in cardiac mast cell degranulation compared with sham animals receiving only saline (P < 0.001); in both cases this response was prevented by co-administration with DSCG. Myocardial ischaemia (Figure 4), induced significant mast cell degranulation in saline treated control rats, when compared with the saline sham group (P < 0.001), and to a similar degree as that seen with ET-1 and compound 48/80 in sham animals. Administration of ET-1 or compound 48/80 prior to CAO did not alter the extent of mast cell degranulation over and above

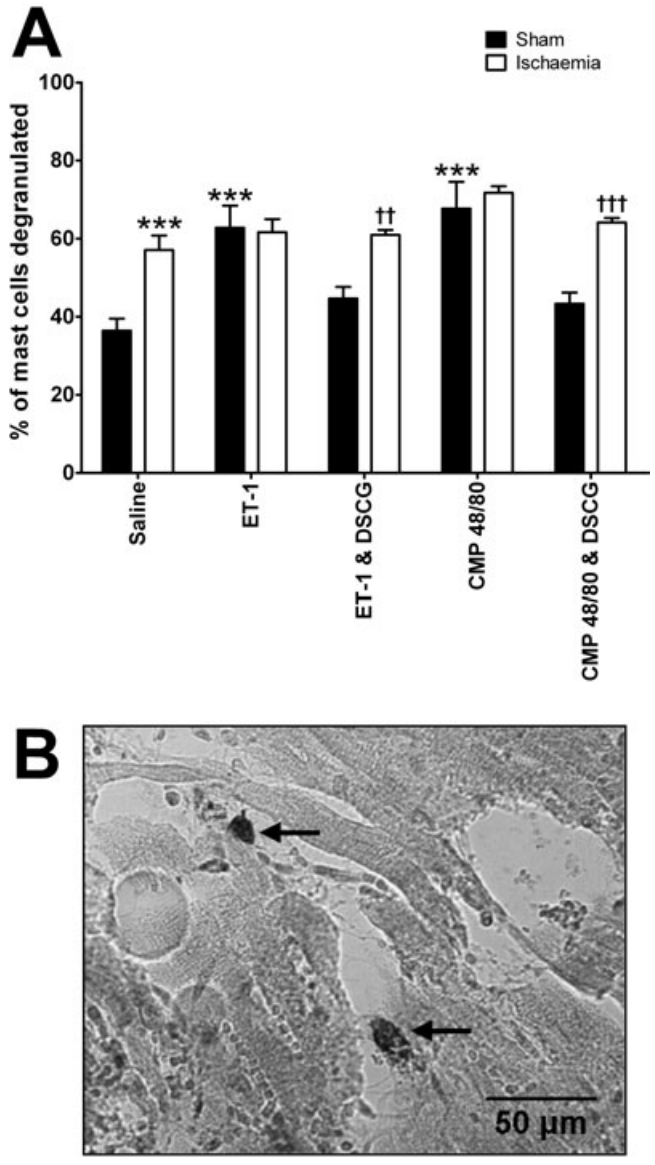

Figure 4 Effects of ischaemia, ET-1 and compound 48/80 (CMP 48/80) both alone and in the presence of DSCG on the percentage of mast cells degranulated in the rat myocardium. (A) Mast cell degranulation was measured as the percentage of the total number of mast cells present that had undergone degranulation and was expressed as the mean ± SEM (n = 3–10). The percentage incidence of mast cell degranulation was determined at a magnification of ×400 and encompassed an entire cross-section of ventricular tissue. Both sham and ischaemic animals were treated with a bolus dose of either saline, ET-1 or compound 48/80 in the absence or presence of DSCG (infused for 30 min prior to coronary occlusion). The effect of ischaemia alone on mast cell degranulation was determined via a comparison of saline-treated ischaemic animals with saline-treated sham animals. ***$P < 0.001$ versus saline sham, $^{\dagger\dagger}P < 0.01$ versus ET-1 & DSCG sham and $^{\dagger\dagger\dagger}P < 0.001$ versus compound 48/80 & DSCG sham. (B) Light photomicrograph (×400) shows mast cells positively stained for ET-1 in close proximity to coronary vessels and demonstrates both intact and degranulated mast cells (induced by ET-1) and the accompanying diffusion of ET-1-rich granules from the degranulated mast cell (indicated by arrows). This myocardial section was taken from an ET-1 treated sham-operated time control rat which was subjected to the same *in vivo* protocol as the ischaemic rats with the exception that the ligature placed around the left coronary artery was not tightened during the 'ischaemic' period.

that induced by ischaemia. As DSCG infusion was stopped prior to induction of ischaemia, it did not prevent the ischaemia-induced mast cell degranulation in either saline controls or in ET-1 or compound 48/80 treated ischaemic animals (Figure 4A). Similarly, DSCG infusion in rats given a subsequent bolus injection of saline did not attenuate the subsequent ischaemia-induced mast cell degranulation (66 ± 2% vs. 31 ± 4%; data not shown) when compared with the DSCG-treated sham heart. Immunostaining for ET-1 in rat myocardial tissue revealed that ET-1 was co-localized with mast cells (Figure 4B) located in the endocardium and in close proximity to the coronary vasculature. Positive staining for ET-1 was also detected in and around mast cell granules following degranulation, suggesting that cardiac mast cells store and release ET-1 (Figure 4B).

Discussion

The main focus of the present study was to explore whether degranulation of mast cells prior to the onset of ischaemia (rather than during the ischaemic period) can explain the previous observations from our group that ET-1 can act as a pharmacological preconditioning agent against life-threatening arrhythmias during the early stages of acute myocardial infarction (see Wainwright *et al.*, 2005). Our present data show that degranulation of mast cells prior to the onset of ischaemia with a known degranulating agent (CMP 48/80) is indeed anti-arrhythmic during the first 30 min of ischaemia, and that this effect is mimicked by ET-1. Moreover, the anti-arrhythmic effects of both compound 48/80 and ET-1 were abolished by concomitant administration of the mast cell stabilizer, DSCG, during the pre-occlusion period. As yet the mechanism(s) via which DSCG stabilizes mast cells are not fully understood. However, studies have shown that DSCG can inhibit Ca^{2+} uptake by mast cells (Foreman *et al.*, 1977) and block both Ca^{2+} and Cl^{-} channels on mast cell membranes which determine the extent of Ca^{2+} influx and consequently intracellular Ca^{2+} concentration (Franzius *et al.*, 1994). Receptor activation by either ET-1 or compound 48/80 leads to an increase in intracellular Ca^{2+} concentration (Mori *et al.*, 2000; Kawanabe and Nauli, 2005), a process which is a prerequisite for mast cell degranulation (see Peavy and Metcalfe, 2008). Thus, it may be possible that DSCG prevents both ET-1 and compound 48/80-mediated mast cell degranulation via a common mechanism, that is, blocking the increase in intracellular Ca^{2+} concentration. Finally we have demonstrated by histological analysis of hearts from sham-operated rats that both agents induce cardiac mast cell degranulation.

While cardiac mast cells have classically been regarded as playing a deleterious role in myocardial ischaemia and reperfusion through their pro-inflammatory, pro-oxidant and pro-arrhythmic effects, our findings are in support of other studies that have suggested that mast cell degranulation may also be protective, in the setting of ischaemia/reperfusion, depending upon when it occurs (see Singh and Saini, 2003). Pre-emptive treatment with compound 48/80 prior to ischaemia has previously been shown to induce protection against tissue injury in the intestine (Boros *et al.*, 1999) and, more recently in the

myocardium (Jaggi *et al.*, 2007). While compound 48/80 given prior to ischaemia has previously been demonstrated to reduce the incidence of reperfusion arrhythmias in rat isolated hearts (Parikh and Singh, 1997; 1998), the present *in vivo* study is the first to demonstrate that it suppresses ischaemia-induced ventricular arrhythmias.

Pretreatment with DSCG prevented both ET-1 and compound 48/80-induced cardiac mast cell degranulation and prevented their anti-arrhythmic effects, implying a common mechanism for both. It is unlikely that this reversal is due to any pro-arrhythmic effect of DSCG on the myocardium that opposes the anti-arrhythmic effects of compound 48/80 and ET-1, as mast cell stabilisation during ischaemia is known to be anti-arrhythmic (Humphreys *et al.*, 1998). We are confident that the effect of DSCG was through inhibition of compound 48/80 and ET-1-induced degranulation of mast cells, prior to ischaemia, for several reasons. First, the infusion of DSCG (which has a very short plasma half-life) was discontinued prior to induction of ischaemia and therefore was not present in sufficient concentrations to stabilize mast cells during ischaemia. This was supported by the observation that DSCG infused only pre-ischaemia did not prevent the ischaemia-induced degranulation of mast cells seen in the hearts of control rats nor did it have any anti-arrhythmic effect. Moreover, in additional studies we have found that when DSCG infusion is continued throughout the period of ischaemia there is a marked anti-arrhythmic effect (data not shown), again supporting the claim that in the present experiments DSCG was not present in the heart at effective concentrations during ischaemia. Second, DSCG prevented mast cell degranulation induced in the hearts of sham-operated rats by both ET-1 and compound 48/80. While this does not directly demonstrate that mast cell degranulation *per se* is responsible for the anti-arrhythmic effects of ET-1 and compound 48/80, it does support the notion of a common mechanism. Third, DSCG did not prevent the hypotension induced by ET-1. This is most likely due to the significant contribution of endothelial ET_B receptor activation and subsequent NO release (Gray and Webb, 1996) to the vasodilator response to ET-1. What this observation does tell us is that this transient vasodilatation is not involved in ET-1's ability to protect against ischaemia-induced arrhythmias. Taken together, our observations suggest that ET-1 and compound 48/80 are both anti-arrhythmic through mast cell degranulation.

Considering the popular concept that mast cell degranulation is associated with arrhythmogenesis, our paradoxical observations implying ET-1-induced mast cell degranulation as an anti-arrhythmic strategy is worthy of discussion and there are indeed several possible explanations. The first of these is that, through degranulation prior to ischaemia, the mast cell-derived cytotoxic mediators that contribute to arrhythmias during ischaemia have been depleted (principally histamine; Wolff and Levi, 1986). Histological analysis of the anatomical distribution of the mast cells within cardiac tissue shows that they are largely located in the perivascular region. Thus, while degranulation during periods of low blood flow (i.e. ischaemia) would ensure high local concentrations of these mediators, under conditions of normal blood flow, these mediators are well placed to enter the vasculature for removal via the blood. Consequently, by undergoing degranulation before ischaemia the released contents are washed out of the myocardial tissue and the depleted mast cells do not then have anything to release in response to ischaemia. A second explanation is that degranulation causes the release of cardioprotective mast cell-derived mediators such as tryptase [a ligand for the cardioprotective proteinase activated receptor-2 (Lim *et al.*, 2007)], heparin (Kilgore *et al.*, 1999) and NO (Iliodromitis *et al.*, 2006), or more likely a combination of several of these. However, if pro-arrhythmic mediators released by ET-1 are washed away as suggested above, then the same would probably apply to anti-arrhythmic mediators.

A third intriguing possibility focuses on recent findings that suggest ET-1-induced mast cell degranulation may reduce concentrations of the endogenous peptide through an autoregulatory mechanism. Maurer *et al.* (2004) demonstrated that in mucosal mast cells ET-1 can control local endogenous ET-1 levels through a mechanism involving the activation of ET_A receptors and subsequent release of mast cell-derived enzymes (chymase and carboxypeptidase A) which degrade ET-1. It is plausible therefore, that in the setting of myocardial ischaemia, which has long been associated with elevated levels of pro-arrhythmic endogenous ET-1 (Brunner *et al.*, 1992; Garjani *et al.*, 1995), ET-1-induced degranulation of mast cells may contribute to cardioprotection through a compensatory homeostatic mechanism (i.e. release of ET-1 degrading enzymes) designed to control endogenous levels of the peptide and reduce its bioavailability. In order to explore this possibility we undertook immunostaining for ET-1 to determine whether or not there was less present in the tissues that had been challenged with exogenous ET-1. What in fact this revealed was that ET-1 was co-localized with both intact and degranulated mast cells, with very little staining elsewhere in the tissue, demonstrating a close association between the peptide and the mast cell. This co-localization has been reported in previous studies in both mucosal and connective tissue-type mast cells in human tonsil (Li *et al.*, 1999) and rodent lungs and gastrointestinal tract (Liu *et al.*, 1998), where ET-1 was mainly localized to the mast cell granules. This suggests that mast cells store ET-1 that is either synthesized by the cell or accumulated via uptake of extracellular ET-1 via mast cell bound ET_B receptors (Liu *et al.*, 1998). Indeed, mouse bone marrow-derived mast cells have been shown to synthesize and release ET-1 in response to phorbol myristate acetate and calcimycin (Ehrenreich *et al.*, 1992). These findings suggest that a paradoxical relationship may exist between mast cells and ET-1; while ET-1-induced mast cell degranulation releases both carboxypeptidase A and chymase which consequently degrade extracellular ET-1 (thus reducing its concentration), mast cells concomitantly release ET-1 resulting in elevated extracellular concentrations of the peptide. The exact role (if any) of mast cells in regulating endogenous ET-1 concentrations during ischaemia, and the consequence of inducing mast cell degranulation prior to ischaemia on this regulatory balance, therefore warrants further investigation.

Conflicts of interest

None.

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