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Effects of calcium antagonists on endothelin-1-induced myocardial ischaemia and oedema in the rat

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1 The effects of the calcium channel blockers, verapamil and nifedipine on myocardial ischaemia and oedema evoked by endothelin-1 (ET-1) or IRL 1620, an ET_B receptor-selective agonist were studied in anaesthetized and conscious rats.

2 Bolus injection of ET-1 (1 nmol kg^{-1}, i.v.) or IRL 1620 (1 nmol kg^{-1}, i.v.) to conscious chronically catheterized rats evoked a transient depressor response followed by a prolonged pressor effect. Corresponding to changes in blood pressure, a transient tachycardia and a sustained bradycardia were observed. Pretreatment of the animals with verapamil (1 mg kg^{-1}, i.v.) or nifedipine (200 μg kg^{-1}, i.v.) produced on average 5 mmHg decrease in mean arterial blood pressure. Both verapamil and nifedipine inhibited by 63 and 44% the pressor actions of ET-1 or IRL 1620 (1 nmol kg^{-1}), respectively, and the accompanying bradycardia. Both verapamil and nifedipine potentiated the magnitude of the depressor action of ET-1 and IRL 1620 without affecting the accompanying tachycardia. Decreasing mean arterial blood pressure with hydralazine (0.2-0.3 μmol kg^{-1}, i.v.) to levels comparable to those observed after verapamil or nifedipine had no significant effects on the haemodynamic responses to ET-1 or IRL-1620.

3 Intravenous bolus injection of ET-1 or IRL 1620 (0.1-2 nmol kg^{-1}) into anaesthetized rats produced dose-dependent ST segment elevation of the electrocardiogram without causing arrhythmias. ST segment elevation developed within 30-50 s and persisted for at least 10-20 min following injection of the peptides.

4 Pretreatment of the animals with verapamil (1 mg kg^{-1}, i.v.) or nifedipine (200 μg kg^{-1}, i.v.) inhibited on average by 79 and 76% the ST segment elevation elicited by ET-1 (1 nmol kg^{-1}), respectively. Verapamil and nifedipine also attenuated IRL 1620 (1 nmol kg^{-1})-induced ST segment elevation on average by 71 and 74%, respectively. In contrast, no significant inhibition was observed with hydralazine (0.2-0.3 μmol kg^{-1}).

5 Both ET-1 and, to a lesser extent, IRL 1620 (0.1-2 nmol kg^{-1}) evoked albumin accumulation in cardiac tissues in a dose-dependent fashion as measured by the local extravascular accumulation of Evans blue dye in conscious rats. ET-1 and IRL 1620 (1 nmol kg^{-1}) enhanced albumin extravasation by 109 and 82%, and 34 and 44% in the left ventricle and right atrium, respectively. ET-1 or IRL 1620-induced albumin extravasation was completely prevented by verapamil (1 mg kg^{-1}) or nifedipine (200 μg kg^{-1}) in these vascular beds. In contrast, hydralazine (0.2-0.3 μmol kg^{-1}) failed to modify the effects of ET-1 or IRL 1620 on albumin extravasation.

6 These results show that verapamil and nifedipine are highly effective in protecting the myocardium against the pro-ischaemic and microvascular permeability enhancing effects of ET-1 and suggest that ET_A and constrictor ET_B (tentatively termed ET_{B2}) receptors mediating these actions of ET-1 are coupled to calcium influx through dihydropyridine-sensitive calcium channels.

Keywords: Endothelin; IRL 1620; ET_A and ET_B receptors; calcium; verapamil; nifedipine; myocardial ischaemia; vascular permeability; rat heart

Introduction

Endothelin-1 (ET-1) has recently been recognized as one of the key mediators of myocardial ischaemia (Lüscher, 1991). Elevated plasma ET-1 levels have been detected in the coronary sinus under ischaemic conditions both in patients and laboratory animals (for recent review see Rubanyi & Polokoff, 1994). ET-1 is a potent constrictor of coronary arteries *in vivo* and *in vitro* (see Rubanyi & Polokoff, 1994). Furthermore, intracoronary or intravenous administration of ET-1 evokes ST segment elevations of the electrocardiogram, similar to the clinical phenomenon of variant angina (Nichols *et al.*, 1990; Harada *et al.*, 1993; Filep *et al.*, 1994). ET-1 also enhances albumin extravasation in the rat coronary circulation (Filep *et al.*, 1992; 1994) and therefore contributes to oedema formation, a hallmark of the inflammatory reaction associated with acute myocardial ischaemia (Entman *et al.*, 1991). In addition, anti-ET-1 antibodies were found to protect ischaemic rat hearts (Watanabe *et al.*, 1991).

Cardiac tissues express both known endothelin receptor subtypes, ET_A (which is highly selective for ET-1) and ET_B (non-isopeptide selective) (Arai *et al.*, 1990; Sakurai *et al.*, 1990; Molenaar *et al.*, 1993; Davenport *et al.*, 1995) and possibly a third, yet unidentified (non ET_A/ET_B) receptor subtype (Harrison *et al.*, 1992). Activation of all these three receptor subtypes is thought to mediate the ET-1-induced contraction of coronary arteries (see Rubanyi & Polokoff, 1994). Previous results from our laboratory showed that ET_A and to a lesser extent ET_B receptors are involved in mediating ET-1-induced ischaemia and oedema in the rat coronary circulation (Filep *et al.*, 1992; 1994; 1995). In the arterial smooth muscle, both ET_A and ET_B receptors are coupled to mobilization of Ca^{2+} from

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intracellular stores and enhanced Ca^{2+} influx through voltage-gated calcium channels or through dihydropyridine-insensitive calcium channels (see Highsmith *et al.*, 1992; Rubanyi & Polokoff, 1994). Although in various isolated arteries calcium antagonists are unable to interfere with ET-1-induced contractions (see Rubanyi & Polokoff, 1994), they do reverse the effects of ET-1 in the human, rat and porcine coronary artery (Goto *et al.*, 1989; Franco-Cereceda, 1989; Harada *et al.*, 1993). In the present experiments, we have examined the effects of calcium channel antagonists, verapamil and nifedipine on myocardial ischaemia and oedema elicited by ET-1 or by IRL 1620, an ET_B receptor-selective agonist (Takai *et al.*, 1992).

Methods

Vascular permeability measurements

The experiments were performed on conscious, chronically catheterized male Wistar rats weighing 220–260 g. The animals were housed in individual metabolic cages and catheters were implanted into the abdominal aorta and vena cava as described previously (Filep & Fejes-Tóth, 1986). During the experiments the animals could move freely and had free access to food and water. Mean arterial blood pressure (MABP) and heart rate were monitored continuously by a blood pressure analyzer (Micro-Med, Louisville, KY, USA) using a COBE CDX III pressure transducer.

On the day of the experiment, following an equilibration period of 1 h, basal cardiovascular parameters were measured for 20 min before drug administration. To measure protein extravasation, Evans blue dye (20 mg kg^{-1}) which binds to plasma albumin, was injected i.v. together with ET-1 (0.1–2 nmol kg^{-1}) or the endothelin ET_B receptor-selective agonist, IRL 1620 (0.1–2 nmol kg^{-1}, i.v.). Higher doses of ET-1 were not tested because bleeding from the orbital sinus occurred in conscious animals receiving 2 nmol kg^{-1} ET-1. Another group of animals were given verapamil (1 mg kg^{-1}, i.v.), nifedipine (200 μg kg^{-1}, i.v.) or their vehicle 20 min before injection of ET-1 (1 nmol kg^{-1}) or IRL 1620 (1 nmol kg^{-1}). These doses of verapamil and nifedipine have been proven to be highly effective in the treatment of patients with coronary vasospasm (Antman *et al.*, 1980; Follath, 1989). In some animals, MABP was lowered with hydralazine (0.2–0.3 μmol kg^{-1}, i.v.) to levels observed following verapamil and nifedipine before injection of ET-1 or IRL 1620. Ten min after injection of ET-1 or IRL 1620, the animals were anesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.v.) and the heart was perfused with 40 ml 0.9% NaCl solution through a catheter inserted into the abdominal aorta. Portions of the anterior wall of the left ventricle and right atrium were then excised and tissue Evans blue dye content was measured by spectrophotometry following extraction by formamide as described previously (Filep *et al.*, 1992).

Electrocardiogram measurements

Male Wistar rats (225–250 g) were anaesthetized with sodium pentobarbitone (50 mg kg^{-1}, i.p.). Catheters were inserted into the left femoral artery and vein and electrodes were placed on the left and right forelegs and left hindleg. The change in the ST segment of the lead II electrocardiogram (ECG) was used to monitor coronary ischaemia. Following control cardiovascular and ECG measurements, the animals were pretreated with verapamil (1 mg kg^{-1}, i.v.), nifedipine (200 μg kg^{-1}, i.v.) or their vehicle before injection of ET-1 (0.1–2 nmol kg^{-1}) or IRL 1620 (0.1–2 nmol kg^{-1}) as described above. The animals were monitored for an additional 20 min. Each animal received only one injection of ET-1 or IRL 1620 and one type of calcium antagonist. Lead II ECG was recorded on a Siemens Sirecust 341 electrocardiograph (Germany).

All procedures were in accordance with the Guidelines of the Canadian Council of Animal Care and were approved by the local Animal Care Committee.

Drugs and chemicals

ET-1 and IRL 1620 (Suc-[Glu^9, $Ala^{11,15}$]endothelin-1(18-21)) were synthesized in our laboratories by solid-phase methodology. The purity of the preparations was greater than 97% as measured by high performance liquid chromatography. ET-1 and IRL 1620 were dissolved in distilled water and stored at −20°C. On the day of the experiments an aliquot was removed and diluted further in 0.9% NaCl solution. Hydralazine hydrochloride and verapamil hydrochloride (Sigma Chemical Co., St. Louis, MO, U.S.A.) were dissolved in distilled water containing 300 mM glucose, nifedipine (Sigma Chemical Co.) was dissolved in dimethyl-sulphoxide at a concentration of 20 mg ml^{-1}, and were diluted further with 0.9% NaCl solution.

Statistical analysis

Results are expressed as means ± s.e.mean. Statistical analysis of the data was performed by one-way analysis of variance using ranks (Kruskal-Wallis test) followed by Dunn's multiple contrast hypothesis test when various treatments were compared to the same control group, or by the Wilcoxon test or the Mann-Whitney U test for paired and unpaired observations, respectively. A level of $P<0.05$ was considered significant for all tests.

Results

Effects of endothelin-1 or IRL 1620-induced changes in blood pressure and heart rate

Baseline values for MABP and heart rate were significantly lower in conscious (105 ± 1 mmHg and 327 ± 5 beats min^{-1}, respectively, $n=30$) than in anaesthetized rats (126 ± 1 mmHg and 429 ± 5 beats min^{-1}, respectively, $n=26$, $P<0.001$).

As well established, ET-1 (1 nmol kg^{-1}) evoked biphasic changes in MABP (i.e. an initial transient decrease followed by a sustained elevation) in both conscious and anaesthetized animals (Figure 1). However, the peak depressor response to ET-1 was more pronounced in anaesthetized than in conscious rats, whereas the peak pressor response to ET-1 was significantly greater in conscious than in anaesthetized animals (Figure 1). Qualitatively similar changes were observed with IRL 1620 (1 nmol kg^{-1}), which, on a molar basis, appeared to be an approximately 3 times less potent pressor agent than ET-1 (Figure 2). Corresponding to the changes in MABP, the depressor response was accompanied by a transient tachycardia, whereas prolonged increases in MABP were associated with marked decreases in heart rate (Figures 1 and 2). Verapamil (1 mg kg^{-1}) and nifedipine (200 μg kg^{-1}) produced decreases in MABP in both conscious and anaesthetized rats. MABP decreased from 103 ± 2 mmHg to 98 ± 2 mmHg ($n=10$, $P<0.01$) whereas heart rate increased from 314 ± 8 beats min^{-1} to 321 ± 8 beats min^{-1} ($n=10$, $P<0.01$) following verapamil treatment in conscious animals. In the anaesthetized rat, verapamil decreased MABP and heart rate from 128 ± 2 mmHg and 425 ± 9 beats min^{-1} to 114 ± 2 mmHg ($n=9$, $P<0.01$) and 381 ± 8 beats min^{-1} ($P<0.01$), respectively. Like verapamil, nifedipine also lowered MABP and caused a slight increase in heart rate in conscious rats (99 ± 1 mmHg and 337 ± 8 beats min^{-1} compared to the baseline values of 104 ± 2 mmHg and 330 ± 9 beats min^{-1}, respectively, $n=10$, $P<0.01$), whereas it decreased both MABP and heart rate in anaesthetized animals (MABP and heart rate were 111 ± 1 mmHg and 381 ± 10 beats min^{-1} compared to the baseline values of 125 ± 2 mmHg and 424 ± 9 beats min^{-1}, respectively, $n=8$, $P<0.01$). Verapamil atte-

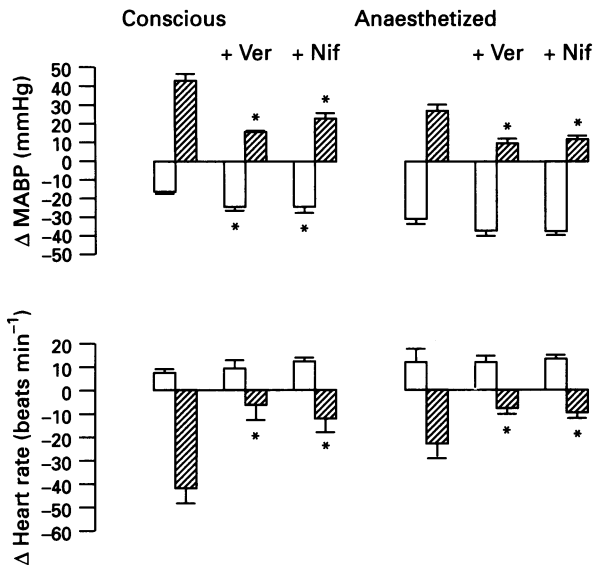

Figure 1 Effects of verapamil (Ver, 1 mg kg^{-1}, i.v.) and nifedipine (Nif, 200 $\mu g\ kg^{-1}$, i.v.) on the peak depressor (open columns) and pressor (hatched columns) responses evoked by 1 nmol kg^{-1} endothelin-1 (ET-1) and accompanying changes in heart rate in conscious and anaesthetized rats. The values for mean arterial blood pressure (MABP) and heart rate before injection of ET-1 in conscious untreated, verapamil-treated and nifedipine-treated rats were 106 ± 3 mmHg and 338 ± 12 beats min^{-1} ($n=6$), 97 ± 2 mmHg and 322 ± 13 beats min^{-1} ($n=6$), and 99 ± 2 mmHg and 339 ± 11 beats min^{-1} ($n=6$), respectively; and in anaesthetized untreated, verapamil-treated and nifedipine-treated animals were 124 ± 2 mmHg and 442 ± 15 beats min^{-1} ($n=5$), 113 ± 3 mmHg and 371 ± 11 beats min^{-1} ($n=5$), and 111 ± 2 mmHg and 374 ± 12 beats min^{-1} ($n=4$), respectively. Values are means with s.e.mean. $^*P<0.05$ (compared to endothelin-1 by Dunn's multiple contrast hypothesis test).

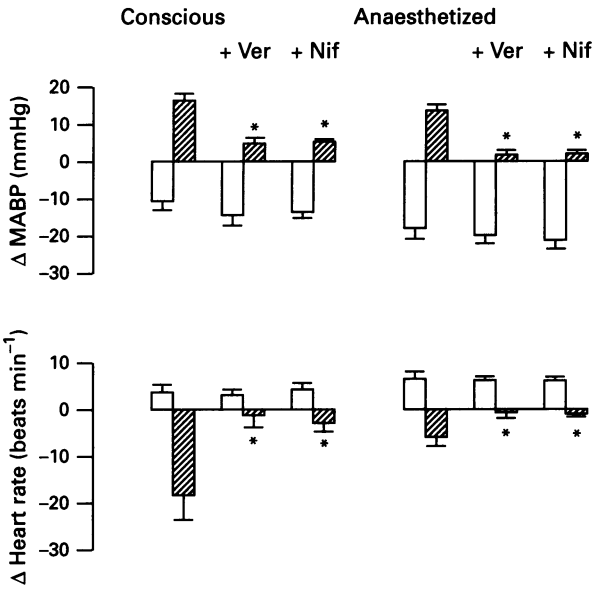

Figure 2 Effects of verapamil (Ver, 1 mg kg^{-1}, i.v.) and nifedipine (Nif, 200 $\mu g\ kg^{-1}$, i.v.) on the peak depressor (open columns) and pressor (hatched columns) responses elicited by the ET_B receptor-selective agonist, IRL 1620 (1 nmol kg^{-1}) and accompanying changes in heart rate in conscious and anaesthetized rats. The values for MABP and heart rate before injection of IRL 1620 in untreated, verapamil-treated and nifedipine-treated conscious animals were 106 ± 2 mmHg and 330 ± 14 beats min^{-1}, 100 ± 1 mmHg and 320 ± 7 beats min^{-1}, and 99 ± 2 mmHg and 334 ± 14 beats min^{-1}, respectively, and in anaesthetized untreated, verapamil-treated and nifedipine-treated rats were 127 ± 3 mmHg and 434 ± 15 beats min^{-1}, 115 ± 2 mmHg and 394 ± 12 beats min^{-1}, and 111 ± 1 mmHg and 388 ± 16 beats min^{-1}, respectively. Values are means with s.e.mean for 4 experiments. $^*P<0.05$ (compared to IRL 1620 by Dunn's multiple contrast hypothesis test).

nuated on average by about 60% the pressor response and concomitant bradycardia elicited by 1 nmol kg^{-1} ET-1 both in conscious and anaesthetized rats (Figure 1). On the other hand, the maximum decreases in MABP elicited by ET-1 were significantly greater in conscious, but not in anaesthetized, rats treated with verapamil than in control animals (Figure 1). As with ET-1, the pressor effect of IRL 1620 (1 nmol kg^{-1}) and the accompanying tachycardia were markedly attenuated by verapamil both in conscious and anaesthetized animals (Figure 2). Pretreatment of the animals with nifedipine resulted in similar inhibition of the haemodynamic responses to ET-1 or IRL 1620 to those observed with verapamil (Figures 1 and 2).

Treatment of conscious rats with hydralazine (0.2–0.3 μmol kg^{-1}) decreased basal MABP from 105 ± 2 mmHg to 99 ± 2 mmHg ($n=9$, $P<0.01$) and increased heart rate from 323 ± 7 beats min^{-1} to 331 ± 6 beats min^{-1} ($n=9$, $P<0.05$). Hydralazine had no affect on the pressor and depressor responses to ET-1 (1 nmol kg^{-1}) and IRL-1620 (1 nmol kg^{-1}). Maximum decreases in MABP evoked by ET-1 were 16 ± 1 mmHg ($n=6$) and 15 ± 1 mmHg ($n=5$) with concomitant increases of 7 ± 2 beats min^{-1} and 8 ± 2 beats min^{-1} in heart rate in the absence and presence of hydralazine, respectively ($P>0.1$). ET-1 induced maximum increases in MABP and accompanying decreases in heart rate were 43 ± 3 mmHg and 37 ± 3 mmHg, respectively and 42 ± 6 beats min^{-1} and 37 ± 4 beats min^{-1} in the absence and presence of hydralazine, respectively ($P>0.1$). IRL-1620 produced 11 ± 2 mmHg and 10 ± 1 mmHg maximum decreases in MABP in the absence and presence of hydralazine, respectively ($n=4$, $P>0.1$) which were accompanied by 3 ± 2 beats min^{-1} and 2 ± 1 beats min^{-1} increases in heart rate, respectively ($n=4$, $P>0.1$). The IRL-1620-induced maximum increases in MABP and the accompanying decreases in heart rate were 16 ± 2 mmHg and 14 ± 1 mmHg, and 19 ± 5 beats min^{-1} and 15 ± 3 beats min^{-1} in control and hydralazine-treated animals, respectively ($n=4$, $P>0.1$).

Effects on endothelin-1 or IRL 1620-induced albumin extravasation

In agreement with out previous observations, both ET-1 and IRL 1620 promoted albumin extravasation in the coronary vascular bed of the conscious rat in a dose-dependent manner (Table 1). ET-1 at 1 nmol kg^{-1} increased albumin accumulation in the left ventricle and right atrium on average by 109 and 82%, respectively (Figure 3). Pretreatment of the animals with either verapamil (1 mg kg^{-1}) or nifedipine (200 $\mu g\ kg^{-1}$) prevented ET-1-induced albumin extravasation in these tissues (Figure 3), whereas none of the calcium channel antagonists alone affected tissue Evans blue dye content (data not shown). IRL 1620 at 1 nmol kg^{-1} produced on average 34 and 44% increases in albumin accumulation in the right atrium and left ventricle, respectively (Figure 4). As with ET-1, these increases were prevented by verapamil or nifedipine (Figure 4). In contrast, albumin accumulation evoked by either ET-1 or IRL 1620 (1 nmol kg^{-1}) did not differ significantly in untreated and hydralazine (0.2–0.3 μmol kg^{-1})-treated animals. Tissue Evans blue dye content in the left ventricle was 71 ± 4 $\mu g\ g^{-1}$ dry weight ($n=6$) and 69 ± 5 $\mu g\ g^{-1}$ dry weight ($n=5$) following ET-1 (1 nmol kg^{-1}) injection in the absence and presence of hydralazine, respectively ($P>0.1$) and 136 ± 12 $\mu g\ g^{-1}$ dry weight versus 142 ± 11 $\mu g\ g^{-1}$ dry weight in the right atrium, respectively ($P>0.1$). Similarly, there were no significant differences in tissue Evans blue dye content in the left ventricle (52 ± 4 $\mu g\ g^{-1}$ dry weight versus 56 ± 4 $\mu g\ g^{-1}$ dry weight, $n=4$, $P>0.1$) and right atrium (75 ± 4 $\mu g\ g^{-1}$ dry

Table 1 Endothelin-1 and IRL-1620-induced albumin extravasation and ST segment elevation in the rat heart

	n	*Albumin accumulation in* *Left ventricle* (μg Evans blue dye g^{-1} dry weight)	*Right atrium*	n	*ST segment elevation* (mV)
Control	6	37±2	65±4	6	0
Endothelin-1					
0.1 nmol kg^{-1}	3	46±3	71±4	3	0.020±0.001
0.3 nmol kg^{-1}	5	62±3*	89±5*	4	0.060±0.010*
1 nmol kg^{-1}	6	71±4**	136±12**	6	0.089±0.010**
2 nmol kg^{-1}	3	82±6*	143±10*	5	0.205±0.070**
IRL 1620					
0.1 nmol kg^{-1}	3	38±3	59±4	3	0.006±0.002
0.3 nmol kg^{-1}	4	44±3	63±4	3	0.015±0.005
1 nmol kg^{-1}	4	52±4*	75±4*	4	0.034±0.002*
2 nmol kg^{-1}	3	57±5*	81±6*	4	0.052±0.003*

For determination of albumin extravasation, ET-1, IRL 1620 or their vehicle (0.9% NaCl, control) were injected i.v. together with Evans blue dye (20 mg kg^{-1}) into conscious rats. Ten min later, the animals were anaesthetized and the heart was perfused with 0.9% NaCl. The permeability measurements were made 15 min after the injection of the dye. ST segment elevation was evaluated in anaesthetized rats by recording the lead II electrocardiogram. Values are means with s.e.mean. *n*, number of animals. $*P<0.05$, $**P<0.01$ (compared to control by Dunn's multiple contrast hypothesis test).

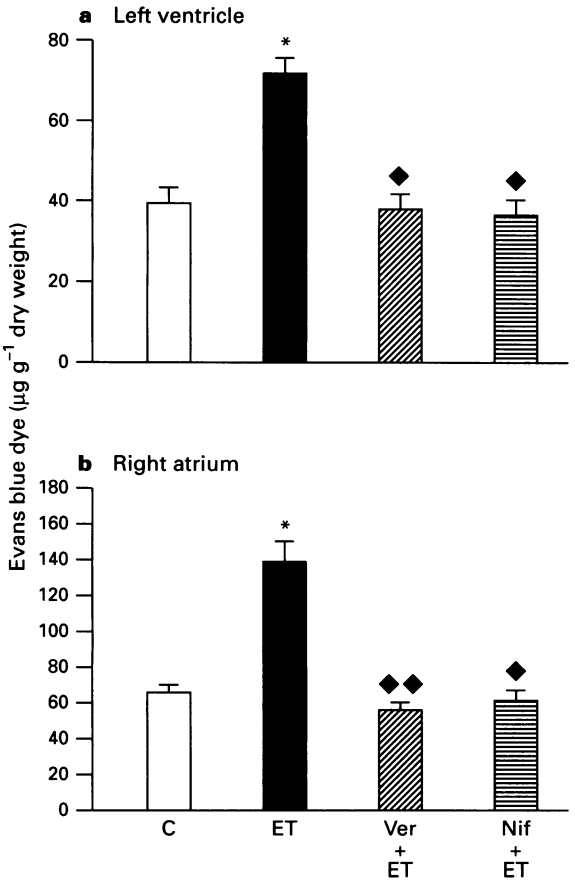

Figure 3 Effects of verapamil and nifedipine on endothelin-1-induced albumin extravasation in the coronary circulation of conscious rats. The animals were pretreated with vehicle (C, control), verapamil (Ver, 1 mg kg^{-1}, i.v.) or nifedipine (Nif, 200 μg kg^{-1}, i.v.) for 20 min before i.v. bolus injection of endothelin-1 (ET, 1 nmol kg^{-1}) or 0.9% NaCl solution (control, C) plus Evans blue dye (20 mg kg^{-1}). Ten min later, the rats were anaesthetized and the heart was perfused with 0.9% NaCl solution. The permeability measurements were made 15 min after the injection of the dye. Values are means with s.e.mean of six experiments. $*P<0.05$ (compared to control by Dunn's multiple contrast hypothesis test); ◆$P<0.05$; ◆◆$P<0.01$ (compared to ET-1).

weight versus 73±3 μg g^{-1} dry weight, $n=4$, $P>0.1$) in response to IRL 1620 (1 nmol kg^{-1}) in untreated control and hydralazine-treated animals, respectively.

Effects on endothelin-1 or IRL 1620-induced ST segment elevation

Bolus i.v. injections of ET-1 or IRL 1620 (0.1–2 nmol kg^{-1}) to anaesthetized rats produced ST segment elevation in a dose-dependent fashion (Table 1). ST segment elevations were detected within 30–50 s, and no complete recovery to control levels was observed within 20 min following injection of 1 nmol kg^{-1} ET-1 or IRL 1620 (Figure 5). At the dose employed, neither ET-1 nor IRL 1620 produced arrhythmias. Verapamil (1 mg kg^{-1}) and nifedipine (200 μg kg^{-1}) attenuated the maximum ST segment elevation evoked by ET-1 (1 nmol kg^{-1}) on average by 79 and 76%, respectively (Figure 5a). Similarly, verapamil and nifedipine inhibited IRL-1620-induced ST segment elevation on average by 71 and 74%, respectively (Figure 5b). None of the calcium channel antagonists alone caused significant changes in ST segment (data not shown). Hydralazine (0.2–0.3 μmol kg^{-1}) failed to protect against ET-1 and IRL 1620 (1 nmol kg^{-1})-induced ST segment elevation (0.080±0.021 mV, $n=5$, versus 0.073±0.011 mV, $n=4$, in response to ET-1, $P>0.1$; and 0.034±0.002 mV, $n=4$, versus 0.029±0.003 mV, $n=4$, in response to IRL 1620 in the absence and presence of hydralazine, respectively, $P>0.1$).

Discussion

The present results showed that the calcium channel antagonists, verapamil and nifedipine cause a marked attenuation of ET-1 or IRL 1620-induced ST segment elevation and myocardial oedema in the rat.

Since the ECG and permeability measurements were performed in anaesthetized and conscious animals, respectively, we compared the effects of verapamil and nifedipine on the haemodynamic effects of ET-1 and IRL 1620 in conscious and anaesthetized rats. Despite the differences in the magnitude of ET-1 or IRL 1620-induced changes in MABP and heart rate in anaesthetized and conscious rats, the calcium antagonists produced similar inhibition of the pressor response and accompanying bradycardia. These results indicate that the ET_A

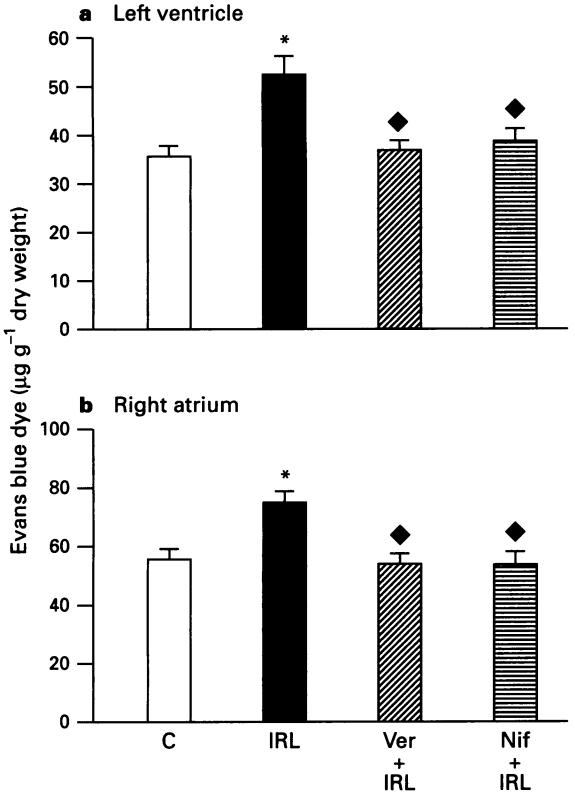

Figure 4 Effects of verapamil and nifedipine on IRL-1620-induced albumin extravasation in the coronary circulation of conscious rats. The animals were pretreated with vehicle (C, control), verapamil (Ver, 1 mg kg^{-1}, i.v.) or nifedipine (Nif, 200 μg kg^{-1}, i.v.) for 20 min before i.v. bolus injection of IRL 1620 (IRL, 1 nmol kg^{-1}) or 0.9% NaCl solution (control, C) plus Evans blue dye (20 mg kg^{-1}). Ten min later, the animals were anaesthetized and the heart was perfused with 0.9% NaCl solution. The permeability measurements were made 15 min after the injection of the dye. Values are means with s.e.mean. $n=6$ for control and $n=4$ for all other treatments. *$P<0.05$ (compared to control by Dunn's multiple contrast hypothesis test); ◆$P<0.05$ (compared to IRL-1620).

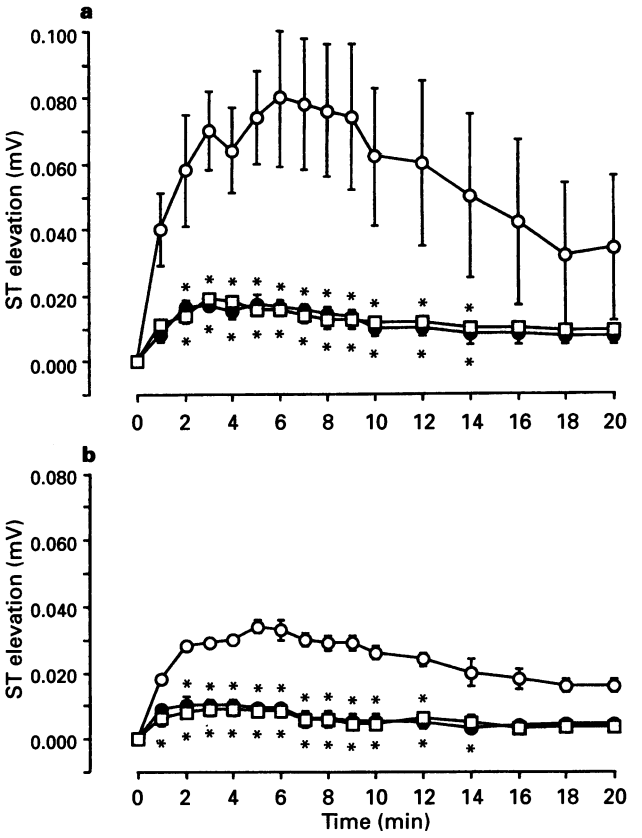

Figure 5 Effects of verapamil and nifedipine on ST segment elevation elicited by i.v. administration of endothelin-1 (a) or the ET_B receptor-selective agonist, IRL-1620 (b) in anaesthetized rats. The animals were pretreated with vehicle (○), verapamil (●) (1 mg kg^{-1}, i.v.) or nifedipine (□) (200 μg kg^{-1}, i.v.) for 20 min before injection of ET-1 or IRL 1620 (1 nmol kg^{-1}, i.v.) at 0 min. Values are means with s.e.mean, $n=5$ for ET-1 and verapamil plus ET-1, $n=4$ for all other treatments. *$P<0.05$ (compared to ET-1 or IRL 1620 by Dunn's multiple contrast hypothesis test).

and ET_B (tentatively named ET_{B2} subtype, Warner *et al.*, 1993; Bax & Saxena, 1994) receptors involved in the generation of pressor action of ET-1 are, in part, coupled to calcium influx through dihydropyridine-sensitive calcium channels. These findings are consistent with previous studies demonstrating that activation of ET_A or ET_{B2} receptors located on the arterial smooth muscle leads to opening of voltage-gated L-type calcium channels (Sakata *et al.*, 1989; Gardner *et al.*, 1992; Sudjarwo *et al.*, 1995). On the other hand, the maximum depressor effect of ET-1 was greater in animals treated with verapamil or nifedipine than in control rats. The depressor action of ET-1 is thought to be mediated via activation of another subtype of ET_B receptors (tentatively named ET_{B1} subtype) located on endothelial cells through the release of nitric oxide and prostacyclin (De Nucci *et al.*, 1988; Hermán *et al.*, 1989). Therefore, an increased depressor response to ET-1 might be attributed to unmasking the pressor effect of the peptide. Significant potentiation of the depressor action of ET-1 by calcium antagonists occurred despite the fact that MABP was about 5 mmHg lower in verapamil or nifedipine-treated animals than in control rats immediately before injection of ET-1 and was observed only in conscious animals. Changes in basal MABP could affect the apparent vasodepressor/vasopressor responses to vasoactive agents. However, the small decreases in MABP observed after verapamil or nifedipine might have little, if any, effects on the actions of ET-1 and IRL 1620, as decreasing of MABP with hydralazine failed to modify the responses to these peptides. Anaesthesia and surgical stress are known to affect cardiovascular control mechanisms, which might be responsible for the differences in the magnitude of ET-1-induced changes observed in anaesthetized versus conscious animals.

The depressor and pressor actions of ET-1 and IRL 1620 were accompanied by a transient increase and prolonged decrease in heart rate. Attenuation of the ET-1 pressor response by calcium antagonists resulted in amelioration of the accompanying bradycardia in both anaesthetized and conscious rats. These findings would suggest that changes in heart rate were secondary to changes in MABP.

Previous studies have suggested that the pro-ischaemic action of ET-1 is possibly due to myocardial ischaemia related to coronary vasoconstriction, whereas a direct action of ET-1 on the myocardium might be responsible for the arrhythmogenic action of the peptide (Harada *et al.*, 1993). Therefore, the absence of arrhythmias in our experiments would indicate that ET-1 and IRL 1620 acted primarily on the coronary vascular smooth muscle rather than on the myocardium. Previous studies have shown that in addition to ET_A receptors, ET_B receptors also mediate the vasoconstrictor (Clozel *et al.*, 1992; Teerlink *et al.*, 1994) and pro-ischaemic effects (Filep *et al.*, 1995) of ET-1 in the rat coronary circulation. ET-1-induced contraction of coronary arteries and ST segment elevation can be attenuated by calcium antagonists (Kim *et al.*, 1989; Turner *et al.*, 1989; Suzuki *et al.*, 1992; Harada *et al.*, 1993). The present results showing that verapamil and nifedipine markedly, though not completely, protected the heart from ST segment elevation elicited by either ET-1 or IRL 1620, confirm and extend these observations. The pro-ischaemic action of IRL 1620 was unaffected by the ET_A receptor-selective antagonist, FR 139317, but was completely prevented by the

ET_A/ET_B antagonist bosentan (Filep *et al.*, 1995), indicating that under the present experimental conditions, IRL 1620 selectively activated ET_B receptors. Our data would also indicate that ET_A as well as the constrictor ET_B (ET_{B2}) receptor subtypes involved in mediating ST segment elevation in the rat heart are coupled to activation of voltage-gated calcium channels.

In addition to protecting the heart from ST segment elevation, verapamil and nifedipine prevented both ET-1 and IRL 1620-induced albumin extravasation in the coronary vascular bed of conscious rats. These findings lend further support to the notion that ET-1-induced albumin efflux via the ET_A receptor is related to calcium mobilization across postcapillary venules (Mayhan & Rubenstein, 1994). Although in the present study parallel changes were observed in MABP and albumin extravasation, previous results have demonstrated that an increase in albumin extravasation elicited by ET-1 is not merely a secondary effect to changes in MABP or perfusion pressure (Filep *et al.*, 1992; 1994; Lawrence *et al.*, 1995), but, as with other mediators, it can primarily be attributed to formation of interendothelial cell gaps exclusively in the venules (Grega *et al.*, 1986). Indeed, systemic vasodilatation cannot, *per se*, account for the observed effects of calcium antagonists, as hydralazine failed to affect albumin extravasation elicited by ET-1 or IRL 1620, despite the fact that it lowered basal MABP to comparable levels as seen after verapamil or nifedpine. One determinant of gap formation is a contractile mechanism within endothelial cells, which requires the influx of Ca^{2+} (Mayhan & Joyner, 1984; Paul *et al.*, 1990; Curry, 1992). However, electrophysiological studies indicate a lack of voltage-gated calcium channels in cultured endothelial cells (see Adams *et al.*, 1989; Curry, 1992). Although this conclusion may be compromised by the technical difficulty of measuring small calcium currents in whole cell patch-clamp experiments or by the loss of voltage-gated calcium channels during enzymatic digestion of endothelial cells, it seems unlikely that inhibition of ET-1-induced albumin extravasation by verapamil and nifedipine was related to inhibition of calcium influx into endothelial cells through voltage-gated calcium channels. ET-1 may induce gap formation directly and/or through release of secondary mediators, such as thromboxane A_2 or platelet-activating factor (Filep *et al.*, 1994). A direct gap forming effect of endothelin-1 is most likely to be mediated through activation of ET_B receptors (ET_{B1} subtypes), for ET_B receptor and ET_B receptor mRNA are predominantly expressed on endothelial cells derived from peripheral large vessels (Takayanagi *et al.*, 1991; Ogawa *et al.*, 1991). However, it should be noted that only a minor portion of the permeability enhancing effect of ET-1 is mediated via ET_B receptors in the rat coronary circulation (Filep *et al.*, 1995). Pharmacological studies indicated the presence of ET_A receptors on endothelial cells prepared from rat and human brain microvessels (Vigne *et al.*, 1990; Stanimirovic *et al.*, 1994) and the existence of ET-3-selective (tentatively named ET_C) receptors on bovine aortic endothelial cells (Emori *et al.*, 1990; Warner *et al.*, 1992). These observations raise the possibility that stimulation of these endothelin receptor subtypes might also lead directly to gap formation. However, it is not known at present whether coronary microvascular endothelial cells possess ET_A or ET_C receptors. The role of pericytes, a contractile cell in close apposition to capillary endothelial cells, in the regulation of gap formation is uncertain. It is not known at present whether or not these cells possess ET receptors. Alternatively, ET-1 may induce gap formation through release of thromboxane A_2 and platelet-activating factor predominantly via the activation of ET_A receptors (Filep *et al.*, 1994). Activation of ET_B receptors may also lead to thromboxane A_2 release, as demonstrated in the guinea-pig pulmonary circulation (D'Orleans-Juste *et al.*, 1994). Since ET_A and ET_{B2} receptors are coupled to voltage-gated calcium channels (see above) and the synthesis of platelet-activating factor and thromboxane A_2 requires the influx of calcium into cells (Ogletree, 1987; Prescott *et al.*, 1990), one may assume that verapamil and nifedipine inhibited the formation of these secondary mediators. An increase in capillary hydrostatic pressure would facilitate albumin extravasation only when gaps are formed (Grega *et al.*, 1986). Thus, attenuation of ET-1 or IRL 1620-induced coronary vasoconstriction by calcium channel blockers would decrease hydrostatic pressure in the coronary vascular bed, which, in turn, could contribute to the decrease in albumin extravasation.

An additional mechanism by which verapamil and nifedipine might have antagonized the cardiac actions of ET-1 is that they may have interfered with binding of ET-1 to its receptors. Indeed, another calcium antagonist, amlodipine has been reported to attenuate the density, but not the affinity, of ET-1 binding sites (presumably ET_A receptors) on cell membranes harvested from ischaemic-reperfused rat hearts (Nayler *et al.*, 1992). However, this effect may be secondary to protection against ischaemia-reperfusion-induced tissue injury. Nevertheless, it remains to be investigated whether or not verapamil and/or nifedipine could also interfere with ET-1 binding to ET_A and ET_B receptors in normal heart. A direct interaction between ET-1 and calcium antagonist appears to be unlikely as suggested by the failure of ET-1 to modify vasodepressor responses to nitrendipine in spontaneously hypertensive rats (Lawson *et al.*, 1992).

The peak plasma levels of ET-1 following i.v. administration of 1 nmol kg^{-1} peptide are at least two orders of magnitude higher (Filep *et al.*, 1994) than those detected in various myocardial ischaemia models (see Rubanyi & Polokoff, 1994). It is uncertain at present whether plasma ET-1 levels reflect local production and concentration of the peptide. However, if endogenous ET-1 plays an important role in the development of angina (Lüscher, 1991), the present findings that ET-1-induced ST segment elevation and albumin extravasation can be prevented by verapamil and nifedipine would be compatible with the known effects of calcium antagonists in this disease (Antman *et al.*, 1980; Follath, 1989).

In conclusion, the present study has demonstrated that verapamil and nifedipine protect the myocardium against the pro-ischaemic and microvascular permeability enhancing effects of ET-1 and IRL 1620 and suggest that both ET_A and vasoconstrictor ET_{B2} receptors mediating these actions of ET-1 are coupled to Ca^{2+} influx through dihydropyridine-sensitive calcium channels.

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