Enhanced role for the opening of potassium channels in relaxant responses to acetylcholine after myocardial ischaemia and reperfusion in dog coronary arteries

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1 Anaesthetized dogs were subjected to 1 h occlusion of the left circumflex coronary artery followed by 2 h of reperfusion. Relaxant responses were examined in coronary artery rings removed proximal (nonischaemic) or distal (ischaemic) to the site of occlusion.

2 Relaxant responses to acetylcholine (ACh) were similar in nonischaemic and ischaemic artery rings. In addition ACh-induced relaxation of nonischaemic and ischaemic artery rings was equally susceptible to inhibition of nitric oxide (NO) synthase using L-N^G-nitroarginine (L-NOARG, 10^{−4} M), or to inhibition of soluble guanylate cyclase using 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{−5} M).

3 In nonischaemic arteries, the relaxation to ACh was unaffected by high K⁺ (67 mM) but in ischaemic arteries, the maximum relaxation to ACh was significantly reduced from 113 ± 6 to 60 ± 2% (ANOVA, *P* < 0.05). Tetraethylammonium (TEA, 10^{−3} M), an inhibitor of large conductance calcium activated potassium (BK_{Ca}) channels did not inhibit the response to ACh in nonischaemic arteries but in ischaemic arteries TEA significantly shifted the concentration response curve to ACh to the right (pEC₅₀; nonischaemic, 7.07 ± 0.25; ischaemic, 6.54 ± 0.21, *P* < 0.01, ANOVA) without decreasing the maximum relaxation. TEA did not affect the responses to sodium nitroprusside in either nonischaemic or ischaemic arteries.

4 In conclusion, ischaemia/reperfusion did not change the sensitivity of endothelium-dependent relaxation to L-NOARG or ODQ indicating that ischaemia did not affect the contribution of NO or cyclic GMP to ACh-induced relaxation. However, in ischaemic arteries the opening of the BK_{Ca} channels contributed to relaxation caused by ACh whereas TEA had no effect in nonischaemic arteries. The factor responsible for the opening of this potassium channel was a factor other than NO and may be endothelium derived hyperpolarizing factor (EDHF).

Keywords: Acetylcholine; dog coronary arteries; ischaemia and reperfusion; nitric oxide; potassium channels; sodium nitroprusside

Abbreviations: BK_{Ca}, large conductance calcium activated potassium channel; DMSO, dimethyl sulphoxide; EDHF, endothelium-derived hyperpolarizing factor; K_{ATP}, ATP-sensitive potassium channel; L-NOARG, L-N^G-nitroarginine; NO, nitric oxide; ODQ, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one; SNP, sodium nitroprusside; TEA, tetraethylammonium; U46619, 9, 11-dideoxy-9 α , 11 α -epoxymethano-prostaglandin F_{2 α}

Introduction

The endothelium regulates vascular tone by the release of a number of factors including endothelium-derived relaxing factor (EDRF), characterized as nitric oxide (NO, Palmer *et al.*, 1987), endothelium-derived hyperpolarizing factor (EDHF, Taylor & Weston, 1988; Furchgott & Vanhoutte, 1989; Komori & Vanhoutte, 1990; Garland *et al.*, 1995; Mombouli & Vanhoutte, 1997) which remains to be identified and prostaglandins such as PGI₂ (Moncada *et al.*, 1976). EDHF opens potassium channels to hyperpolarize cells leading to closure of voltage-operated calcium channels to cause relaxation (Garland *et al.*, 1995). The type of potassium channels involved remains unclear but the calcium-sensitive potassium channels and ATP-sensitive potassium (K_{ATP}) channels are most commonly proposed to be involved (Garland *et al.*, 1995).

Even though EDHF can cause relaxation, its effect is transient (Komori & Vanhoutte, 1990) and in some studies it contributes to relaxation only when the NO system is blocked (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). This

suggests that EDHF may operate as a reserve system when the NO system is impaired which, for example, can occur in artery diseases (Garland *et al.*, 1995). Hence the contribution of EDHF to vasodilatation may be increased where there is endothelial dysfunction such as in the presence of hypercholesterolaemia and perhaps after myocardial ischaemia and reperfusion.

Myocardial ischaemia/reperfusion has been reported to cause selective attenuation of the endothelium-dependent relaxation of isolated coronary arteries (Ku, 1982; Sobey *et al.*, 1990; Dauber *et al.*, 1992; Sobey *et al.*, 1992) without impairing the function of the smooth muscle cells. However, Quillen *et al.*, (1990) and Winn & Ku (1992) both reported that ACh-induced relaxation of dog coronary arteries was unaffected by ischaemia and reperfusion. This then raises the question as to whether EDHF might contribute to endothelium-dependent relaxation after ischaemia and reperfusion to help to maintain responses to endothelium-dependent dilators, however the mechanism of endothelium-dependent relaxation has not previously been investigated in ischaemic arteries. The aim of this study was to compare the sensitivity of endothelium-dependent relaxation in nonischaemic and pre-

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viously ischaemic arteries to inhibition of NO synthase, guanylate cyclase and K⁺ channel opening to determine whether ischaemia and reperfusion altered the contribution of NO and EDHF to relaxation of the coronary arteries.

Methods

All experimental procedures were performed within the guidelines of the National Health & Medical Research Council of Australia and were approved by the Pharmacology and Physiology subcommittee of the University of Melbourne Animal Experimentation Ethics Committee.

Experimental preparation

Mongrel dogs ($n=23$) of either sex (14–47 kg) were sedated with an intramuscular injection of xylazine (0.2–0.6 mg kg⁻¹, Bayer, Australia) and anaesthetized by intravenous injection of sodium thiopentone (25–30 mg kg⁻¹, Abbott Australasia, Australia) followed by α -chloralose (70 mg kg⁻¹, i.v.) which was supplemented as necessary. The dogs were ventilated artificially with room air plus additional oxygen as necessary to maintain arterial blood gas and pH levels within the normal physiological range. Arterial pressure was measured by inserting a catheter into the left femoral artery and connecting it to a pressure transducer (Spectromed, U.S.A.) and mean and phasic arterial pressure were recorded using a Grass chart recorder (model # 7E, U.S.A.).

A left thoracotomy was performed and the heart was suspended in a pericardial cradle. Left ventricular pressure (LVP) was measured with a pressure tip transducer (Millar, U.S.A.) passed through the left atrium into the left ventricle. Peak positive LV dp/dt, an index of myocardial contractility, was obtained by a differential coupler. The heart rate was measured using a cardiometer coupler. A 1–2 cm segment of the left circumflex coronary artery was dissected clear of connective tissue for placement of an electromagnetic flow probe (internal diameter 2.0 or 2.5 mm) which was connected to a flow meter (AE Delft, Netherlands) for measurement of mean and phasic coronary blood flow. Commencing at the start of the surgery, normal saline (10 ml kg⁻¹ h⁻¹, i.v.) was infused to compensate for fluid loss throughout the experiments. A stabilization period of 15 min was allowed following the surgery.

Lignocaine hydrochloride (50 mg, i.v., Astra, Australia) was given 20 min prior to coronary artery occlusion. Total occlusion of the left circumflex artery, distal to both the probe and the first main branch, was performed with artery forceps. After 60 min of artery occlusion, the forceps were removed to start reperfusion for 2 h. The blood was allowed into the myocardium gradually to limit the ischaemia-induced hyperaemia which reduces the incidence of reperfusion arrhythmias. In sham ischaemic preparations, five dogs were instrumented and remained anaesthetized for the same period of time as dogs subjected to ischaemia/reperfusion.

Experimental protocol

At the end of the ischaemia/reperfusion (or sham) protocol, the dogs were killed with an anaesthetic (pentobarbitone, Boehringer, Australia) overdose. The heart was excised and the circumflex artery was isolated. Sections of the coronary artery proximal (nonischaemic) and distal (ischaemic/reperfused) to the point of occlusion were isolated and cleared of superficial connective tissue. Rings were stored in Krebs-bicarbonate

solution (composition (mM): NaCl 118.0, KCl 4.7, KH₂PO₄ 1.2, MgSO₄·7H₂O 1.2, (+)-glucose 5.0, NaHCO₃ 25.0, CaCl₂·2H₂O 2.5) overnight at 4°C.

Coronary artery rings, 5 mm in width, were mounted between two stainless steel wires with one end linked to an isometric force transducer (model # FT03, Grass Medical Instruments, U.S.A.) connected to a chart recorder (model # R-02, Rikadenki Kogyo Co., Japan). The other end was anchored to a glass rod submerged in a siliconized, jacketed, 10 ml organ bath filled with Krebs-bicarbonate solution containing indomethacin (3×10^{-5} M) maintained at 37°C with a pH of 7.4 and continuously aerated with 95% O₂, 5% CO₂. Rings were equilibrated to a resting tension of 4 g for 30 min. All artery rings were submaximally precontracted using 9, 11-dideoxy-9 α , 11 α -epoxymethano-prostaglandin F_{2 α} (U46619, $3-30 \times 10^{-9}$ M) and the endothelial integrity was confirmed with a single addition of acetylcholine (ACh, 10^{-5} M) and all rings showed relaxation of at least 65% of active tension. After washout and re-equilibration for 30 min, U46619 ($3-30 \times 10^{-9}$ M) was added to induce active tension which was matched between various treatment groups. Relaxant responses were examined in proximal and distal coronary artery rings removed from 18 dogs subjected to ischaemia/reperfusion and in five dogs subjected to sham ischaemia. Control relaxant responses to ACh were determined in artery rings from all dogs and control responses to sodium nitroprusside (SNP) in artery rings from four ischaemic/reperfused dogs. In artery segments isolated from ischaemic/reperfused dogs relaxant responses were also examined in the presence of the following treatments: (i) L-N^G-nitroarginine (L-NOARG, 10^{-4} M, ACh $n=7$, SNP $n=4$) to inhibit NO synthase; (ii) L-NOARG (10^{-4} M) plus haemoglobin (Hb, 2×10^{-5} M, ACh $n=4$) to bind NO; (iii) 1H-[1,2,4]oxadiazolo[4,3-a]quinoxaline-1-one (ODQ, 10^{-5} M, ACh $n=7$, SNP $n=4$) to inhibit soluble guanylate cyclase; (iv) High K⁺ (67 mM) solution plus nifedipine (3×10^{-7} M, ACh $n=4$). Concentrations of K⁺ higher than 25 mM inhibit the activity of K⁺ channels (Chen & Suzuki, 1989) and concentrations of 60–80 mM K⁺ have previously been used to investigate the role of EDHF in coronary artery relaxation (Kilpatrick & Cocks, 1994; Drummond & Cocks, 1996). The L-type Ca²⁺ channel blocker, nifedipine, was included to prevent contraction in response to the K⁺-induced hyperpolarization (Kilpatrick & Cocks, 1994); (v) Tetraethylammonium (TEA, 10^{-3} M, ACh $n=5$, SNP $n=4$) to inhibit large conductance calcium activated potassium (BK_{Ca}) channels; (vi) Glibenclamide (10^{-6} M, ACh $n=4$) to inhibit ATP-sensitive potassium (K_{ATP}) channels; or (vii) TEA (10^{-3} M) plus L-NOARG (10^{-4} M, ACh $n=5$).

In artery rings from sham ischaemic dogs relaxant responses to ACh ($n=5$) were examined in the presence of the following treatments: (i) L-NOARG (10^{-4} M); (ii) ODQ (10^{-5} M); or (iii) TEA (10^{-3} M).

The response to ACh was also examined in the presence of dimethyl sulphoxide (DMSO), the vehicle for ODQ and glibenclamide. DMSO had no effect on the ACh-induced relaxation and therefore this data was included in the control group.

Drugs and chemical used

Bovine haemoglobin, glibenclamide, indomethacin, nifedipine, L-N^G-nitroarginine, sodium nitroprusside, tetraethylammonium bromide and U46619 were obtained from Sigma Co. (U.S.A.). Acetylcholine perchlorate was obtained from BDH Chemicals (England) and ODQ was purchased from Sapphire

(Australia). All drugs were dissolved in normal saline (0.9% w v⁻¹ NaCl), except for acetylcholine perchlorate which was dissolved in distilled water, nifedipine and U46619 which were prepared in ethanol (100%) and U46619 further diluted in sodium bicarbonate (0.25 M). ODQ and glibenclamide were dissolved in 100% DMSO and glibenclamide was further diluted in 50% DMSO.

Data presentation and statistical analysis

The results are expressed as the mean $\pm$ s.e.mean and *n* indicates the number of experiments (dogs). Where possible, concentration-response curves from isolated coronary artery rings were computer fitted to a sigmoidal curve (REAP, Gamma Research Systems, Australia) to enable the calculation of the sensitivity (pEC₅₀) to agonists. However, where there is a substantial inhibition of the maximal relaxation that prevents the data being fitted to a sigmoid curve, pEC₅₀ cannot be determined. The calculated values for the pEC₅₀ and maximum relaxation were compared using one way analysis of variance (ANOVA) with *post hoc* multiple comparisons using the Bonferroni test (Instat, GraphPad Software, U.S.A.). The concentration-response curves generated before and after antagonist pretreatments were also compared with two-way ANOVA (CSS, StatSoft Inc., U.S.A.). In experiments where data was paired, that is where responses in nonischaemic (proximal) and ischaemic (distal) arteries from the same dog were compared, statistical analysis was carried out using a two tailed Student's *t*-test (Instat). In all cases, statistical differences were accepted when *P* $\leq$ 0.05.

Results

Effect of ischaemia on relaxant responses to ACh and SNP

The responses to ACh and SNP were similar in non-ischaemic (proximal) and ischaemic (distal) segments of coronary artery taken from dogs subjected to coronary artery occlusion and reperfusion (Figure 1). ACh-induced relaxation was similar in

proximal and distal segments of coronary artery from sham ischaemic dogs and responses were also similar to those observed in corresponding artery segments from ischaemic dogs (Table 1).

Figure 1 Concentration response curves to ACh (*n* = 18) or SNP (*n* = 4) in nonischaemic or ischaemic coronary artery rings. The bar graphs show that the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: ACh 12 $\pm$ 2; SNP 11 $\pm$ 2, ischaemic: ACh 14 $\pm$ 2; SNP 11 $\pm$ 2]. The responses to ACh or SNP were not affected by myocardial ischaemia/reperfusion. All values are means $\pm$ s.e.mean shown by vertical lines.

Table 1 A comparison of responses to ACh in proximal (to the occlusion site) and distal (to the occlusion site) artery segments obtained from dogs subjected to ischaemia/reperfusion (I/R) and sham ischaemia/reperfusion (Sham)

Treatment		n	pEC ₅₀	R _{max} (%)	pEC ₅₀	R _{max} (%)
			Nonischaemic (proximal)		Ischaemic (distal)	
I/R	Control	18	7.20 $\pm$ 0.10	94 $\pm$ 6	7.30 $\pm$ 0.10	99 $\pm$ 6
	L-NOARG (10 ⁻⁴ M)	8	6.70 $\pm$ 0.12	39 $\pm$ 6*	6.67 $\pm$ 0.26	30 $\pm$ 8*
	L-NOARG (10 ⁻⁴ M) + Hb (2 $\times$ 10 ⁻⁵ M)	4	6.61 $\pm$ 0.09	23 $\pm$ 10*	n.d.	15 $\pm$ 9*
	ODQ (10 ⁻⁵ M)	7	6.76 $\pm$ 0.21	50 $\pm$ 11*	7.13 $\pm$ 0.15	46 $\pm$ 8*
	High K ⁺ (67 mM)	4	7.36 $\pm$ 0.18	85 $\pm$ 6	7.50 $\pm$ 0.14	60 $\pm$ 2*#
	TEA (10 ⁻³ M)	5	7.07 $\pm$ 0.25	107 $\pm$ 9	6.54 $\pm$ 0.21*	94 $\pm$ 8
	Glibenclamide (10 ⁻⁶ M)	4	7.23 $\pm$ 0.06	90 $\pm$ 6	7.54 $\pm$ 0.08	112 $\pm$ 4
	TEA (10 ⁻³ M) + L-NOARG (10 ⁻⁴ M)	5	6.14 $\pm$ 0.22*	51 $\pm$ 5*	n.d.	20 $\pm$ 7*#
			Sham (proximal)		Sham (distal)	
	Control	5	7.36 $\pm$ 0.16	98 $\pm$ 4	7.18 $\pm$ 0.20	93 $\pm$ 6
Sham	L-NOARG (10 ⁻⁴ M)	5	7.00 $\pm$ 0.11	26 $\pm$ 16†	n.d.	15 $\pm$ 8†
	ODQ (10 ⁻⁵ M)	5	6.84 $\pm$ 0.21	39 $\pm$ 11†	7.00 $\pm$ 0.25	39 $\pm$ 19†
	TEA (10 ⁻³ M)	5	6.92 $\pm$ 0.15	101 $\pm$ 5	6.81 $\pm$ 0.15	97 $\pm$ 6

n = the total number of experiments, the number in parentheses indicates the number of experiments where a pEC₅₀ was calculated if not equal to *n*. n.d. = not determined due to inability to fit data to a sigmoid curve. * = significantly different from I/R control (*P* < 0.01, ANOVA, Bonferroni multiple comparisons tests). † = significantly different from sham control (*P* < 0.05, ANOVA, Bonferroni multiple comparison tests). # = significantly different from I/R nonischaemic (*P* < 0.05, Student's paired *t*-tests).

Effect of inhibition of NO on the responses to ACh and SNP in nonischaemic and ischaemic arteries

In the nonischaemic and ischaemic arteries, L-NOARG, an inhibitor of NO synthase, significantly reduced the maximum relaxation to a similar extent (Table 1), indicating non-competitive inhibition of the ACh-induced relaxation (Figure 2). In the presence of L-NOARG plus Hb, the maximum relaxation to ACh was reduced to $23 \pm 10\%$ in nonischaemic

Figure 2 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of ODQ ($n=7$, 10^{-5} M) or L-NOARG ($n=8$, 10^{-4} M). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to a similar level with U46619 [(nm) nonischaemic: control 12 ± 3 ; ODQ 6 ± 1 ; L-NOARG 4 ± 1 , ischaemic: control 12 ± 3 ; ODQ 5 ± 1 ; L-NOARG 3 ± 1]. The maximum relaxation to ACh was reduced by all treatments. Nonischaemic and ischaemic coronary arteries were equally sensitive to L-NOARG and ODQ. All values are means $\pm$ s.e.mean shown by vertical lines. *Significantly different from control (ANOVA, $P < 0.05$).

arteries and $15 \pm 9\%$ in ischaemic arteries although in neither case did this achieve statistical significance in comparison to responses in the presence of L-NOARG alone. The effect of L-NOARG on ACh-induced relaxation of proximal and distal arteries from sham ischaemic dogs was similar (Table 1). L-NOARG did not affect the sensitivity or maximum relaxation to SNP in nonischaemic or ischaemic arteries (Table 2).

Effect of inhibition of guanylate cyclase on the responses to ACh and SNP in nonischaemic and ischaemic arteries

Concentration-response curves were generated for ACh in the presence of ODQ, a selective inhibitor of soluble guanylate cyclase in nonischaemic and ischaemic arteries precontracted by U46619 (Figure 2). In the presence of ODQ, the maximum relaxation was significantly depressed in nonischaemic and ischaemic arteries (Table 1). The sensitivity to ACh was not affected by ODQ in either nonischaemic or ischaemic arteries (Table 1). In addition ACh-induced relaxation was equally susceptible to inhibition by ODQ in proximal and distal artery segments from sham ischaemic dogs (Table 1). ODQ significantly reduced the pEC_{50} of SNP in both nonischaemic and ischaemic arteries (Table 2).

Effect of non-selective inhibition of potassium channels on the responses to ACh in nonischaemic and ischaemic arteries

Figure 3 shows the effect of high K⁺ plus nifedipine on concentration-response curves to ACh in nonischaemic and ischaemic arteries. L-type Ca²⁺ channels were blocked with nifedipine to prevent contraction in response to the K⁺-induced depolarization. Thus the level of precontraction of the artery rings using U46619 was similar in the absence or presence of K⁺. High K⁺ did not affect the sensitivity or maximum relaxation to ACh in the nonischaemic coronary artery (Table 1). In contrast, there was a significant reduction in the maximum relaxation in the ischaemic arteries (Table 1). In addition the maximum relaxation to ACh in the presence of high K⁺ in ischaemic arteries was significantly less than that observed in nonischaemic arteries under the same conditions (Table 1).

Effect of selective inhibition of potassium channels on the responses to ACh and SNP in nonischaemic and ischaemic arteries

Glibenclamide was used to inactivate the K_{ATP} channel and TEA to inhibit the BK_{Ca} channel. In the nonischaemic artery, consistent with the high K⁺ data, neither TEA nor glibenclamide affected the sensitivity or maximum relaxation to ACh (Figure 4). By contrast, in the ischaemic arteries, TEA shifted the ACh concentration response curves to the right (Figure 4) with a significant reduction of the pEC_{50} without

Table 2 A comparison of responses to SNP in nonischaemic and ischaemic artery segments

Treatment	n	Nonischaemic		Ischaemic	
		pEC_{50}	R_{max} (%)	pEC_{50}	R_{max} (%)
Control	4	7.28 ± 0.13	130 ± 15	7.27 ± 0.21	115 ± 9
ODQ (10^{-5} M)	4	$5.66 \pm 0.51^*$	$94 \pm 8^*$	$5.26 \pm 0.40^*$	117 ± 8
L-NOARG (10^{-4} M)	4	7.24 ± 0.26	112 ± 3	7.43 ± 0.22	130 ± 7
TEA (10^{-3} M)	4	7.19 ± 0.14	113 ± 3	7.30 ± 0.08	109 ± 3

n = total number of experiments. * = significantly different from control ($P < 0.001$, ANOVA, Bonferroni multiple comparisons test).

Figure 3 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of high K⁺ (67 mM) plus nifedipine (3×10^{-7} M, $n=4$). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: control 10 ± 1 ; high K⁺ 14 ± 2 ; ischaemic: control 12 ± 3 ; high K⁺ 13 ± 2]. Responses to ACh were unaffected by high K⁺ in the nonischaemic arteries but significantly impaired in the ischaemic vessels. All the values are means $\pm$ s.e. mean shown by vertical line. *Significantly different from control (ANOVA, $P < 0.05$).

depressing the maximum response (Table 1). TEA had no effect on ACh-induced relaxation of proximal or distal artery segments from sham ischaemic dogs (Table 1). Unlike TEA, glibenclamide did not inhibit the ACh-induced response (Table 1, Figure 4).

To determine whether it was NO or another factor that opened the potassium channels, responses to SNP were examined in the absence or presence of TEA. In both nonischaemic and ischaemic coronary arteries, TEA did not affect the response to SNP (Table 2) suggesting that NO did not open the BK_{Ca} channel to cause relaxation.

Effect of combined inhibition of NO and potassium channels on the responses to ACh in nonischaemic and ischaemic arteries

The response to ACh was examined after blocking the contribution of NO and opening of BK_{Ca} channels to cause relaxation simultaneously using L-NOARG plus TEA. In nonischaemic coronary arteries, L-NOARG plus TEA inhibited the response to ACh with a significant depression in the maximum relaxation but the level of inhibition was similar to that achieved by L-NOARG alone (Table 1). In

Figure 4 Concentration response curves to ACh in nonischaemic and ischaemic coronary artery rings in the absence ($n=18$) or presence of TEA ($n=5$, 10^{-3} M) or glibenclamide ($n=4$, 10^{-6} M). The bar graphs show that in the different treatment groups the nonischaemic and ischaemic artery rings were precontracted to similar levels with U46619 [(nm) nonischaemic: control 10 ± 1 ; TEA 7 ± 2 ; glibenclamide 9 ± 1 ; ischaemic: control 10 ± 2 ; TEA 6 ± 1 ; glibenclamide 7 ± 1]. In the nonischaemic coronary arteries ACh-induced relaxation was unaffected by TEA or glibenclamide. By contrast in the ischaemic artery rings TEA inhibited the response to ACh. All values are means $\pm$ s.e. mean shown by vertical lines. *Significantly different from control (ANOVA, $P < 0.05$).

contrast, in the ischaemic arteries, TEA plus L-NOARG almost abolished the response to ACh such that the maximum relaxation in the ischaemic artery was significantly less in comparison to the nonischaemic and control ischaemic arteries (Table 1).

Discussion

We investigated the effect of 60 min of coronary artery occlusion and 120 min reperfusion *in vivo* on subsequent endothelium-dependent relaxation of dog coronary arteries *in vitro*. Ischaemia/reperfusion did not affect the responses to the endothelium-dependent vasodilator ACh. In addition the responses to ACh in nonischaemic and ischaemic coronary arteries were equally impaired by inhibition of NO or soluble guanylate cyclase. However, whereas responses to ACh in nonischaemic arteries were unaffected by depolarization with high K⁺, in ischaemic arteries the same treatment caused a significant reduction in the endothelium-dependent relaxation. TEA, but not glibenclamide, significantly reduced endothe-

lium-dependent relaxation in ischaemic arteries indicating that opening of BK_{Ca} channels was involved in the relaxation.

In the present study, the responses were compared between nonischaemic artery segments which were isolated proximal to the occlusion site and ischaemic/reperfused artery rings which were dissected distal to the occlusion site. Studies in which dogs were submitted to sham ischaemia confirmed that there were no differences in the responses to agonists generated in proximal and distal portions of the circumflex coronary arteries as reported earlier (Coughlan *et al.*, 1994). In addition, in the sham ischaemic tissue the sensitivity of proximal and distal segments to inhibition of ACh-induced relaxation by L-NOARG, ODQ and TEA was similar. Therefore if there is a difference in the responses generated from nonischaemic and ischaemic arteries, this is not caused by tissue variation across artery segments, but arises due to prior exposure to ischaemia and reperfusion.

There are several studies that have demonstrated that myocardial ischaemia and reperfusion selectively impairs endothelium-dependent relaxation of isolated coronary arteries (for reviews Hearse *et al.*, 1993; Sobey & Woodman 1993) although there are also reports that the coronary conductance arteries are unaffected by ischaemia (Quillen *et al.*, 1990; Winn & Ku, 1992). In the present study ischaemia/reperfusion did not affect the absolute response to ACh even though we employed a protocol similar to previous studies from this laboratory where endothelium-dependent relaxation was impaired (Sobey *et al.*, 1990; 1992). Ischaemia was confirmed by an absence of blood flow and an impairment of cardiac contractility measured *in vivo* (data not shown). Bauer *et al.*, (1993) have suggested that administration of vasodilators before ischaemia may prevent vascular dysfunction but in this study no vasodilators were administered *in vivo*. Thus the divergent effects of ischaemia on vascular function remain unexplained. Even though ischaemia/reperfusion did not alter the total response to ACh we hypothesized that an enhanced contribution of K⁺ channel opening could compensate for impaired NO release. As no previous studies have investigated the effect of ischaemia on the mechanism of endothelium-dependent dilatation we compared the contribution of NO and BK_{Ca} channel opening in non-ischaemic and ischaemic coronary arteries.

L-NOARG caused a non-competitive inhibition of the relaxant response to ACh with a 70% reduction in the maximum response in both nonischaemic and ischaemic arteries. The incomplete blocking effect observed with L-NOARG is not due to an inadequate concentration of L-NOARG (10^{-4} M) since a higher concentration (3×10^{-4} M) did not produce any greater inhibitory effect (data not shown) and additional treatment with Hb did not cause a further significant inhibition. Thus, these results suggest that the ACh-induced relaxation of the dog coronary artery is predominantly mediated by NO and that ischaemia/reperfusion does not affect the sensitivity of the relaxation to inhibition of NO synthesis or action. Whether ischaemia/reperfusion may affect the contribution of cyclic GMP, the second messenger for NO, to endothelium-dependent dilatation was investigated by determining the sensitivity of ACh-induced relaxation to ODQ, a selective inhibitor of soluble guanylate cyclase (Garthwaite *et al.*, 1995; Schrammel *et al.*, 1996). ACh-induced relaxation of nonischaemic and ischaemic/reperfused arteries were equally sensitive to inhibition by ODQ indicating that ischaemia/reperfusion does not affect the activity of guanylate cyclase.

There is now considerable evidence that NO may not be the only factor released by ACh to cause relaxation. A second

factor that could contribute to endothelium-dependent relaxation is EDHF (Garland *et al.*, 1995; Mombouli & Vanhoutte, 1997), an unidentified factor which causes relaxation by opening K⁺ channels to cause hyperpolarization. To determine whether the opening of K⁺ channels might be contributing to the endothelium-dependent relaxation we examined the responses to ACh in the presence of a depolarizing concentration of K⁺ (67 mM). To prevent K⁺-induced contraction we also added the L-type Ca²⁺ channel blocker nifedipine (Kilpatrick & Cocks, 1994). The rings were then precontracted with the thromboxane mimetic U46619 to the same level as in previous treatment groups so that the relaxant responses were not affected by differences in active tension. In the nonischaemic coronary arteries ACh-induced relaxation was unaffected by high K⁺ whereas in the ischaemic/reperfused arteries there was a significant inhibition of the endothelium-dependent relaxation. This suggests that the opening of K⁺ channels only contributes to ACh-induced relaxation after the coronary arteries have been exposed to a period of ischaemia/reperfusion.

The type of K⁺ channel that mediates vascular smooth muscle relaxation remains a matter of debate. The types most commonly proposed to mediate endothelium-dependent relaxation are the K_{ATP} channel (Garland *et al.*, 1995) and BK_{Ca} channel (Najibi *et al.*, 1994; Najibi & Cohen, 1995). Therefore we examined the effects of two K⁺ channel blockers TEA, which at the concentration used (10^{-3} M) is selective for the BK_{Ca} channel (Nelson, 1993) and glibenclamide (Kuramaru & Sakai, 1996), which is selective for the K_{ATP} channel. Neither inhibitor affected ACh-induced relaxation of the nonischaemic coronary arteries, as expected given that responses in nonischaemic tissue were insensitive to high K⁺. However TEA caused a significant decrease in the sensitivity to ACh in the ischaemic/reperfused artery rings whereas glibenclamide had no effect. Thus it appears that the BK_{Ca} and not the K_{ATP} channel, was involved in the ACh-induced relaxation of the ischaemic/reperfused artery.

Several previous studies have reported that endothelium-dependent relaxation of coronary arteries in normal circumstances does not involve K⁺ channel opening but if NO synthesis is inhibited, a K⁺ sensitive component of the relaxation is revealed (Kilpatrick & Cocks 1994; Drummond & Cocks, 1996). Thus it was proposed that an endothelium-derived K⁺ channel opener, possibly EDHF, acted as a reserve to NO to maintain vasodilatation. In this study however we did not observe any effect of K⁺ on ACh-induced relaxation in the absence or presence of NO synthase inhibition in nonischaemic arteries. The reason for the difference between the present observation and those of Cocks and colleagues (Kilpatrick & Cocks 1994; Drummond & Cocks 1996) is not known but may involve the use of different agonists (ACh versus bradykinin) or species (dog versus pig and cow).

There are no other reports of the effect of ischaemia on the mechanism of endothelium-dependent dilatation. However, the results of this study are similar to the observations of Cohen and his colleagues (Najibi *et al.*, 1994; Najibi & Cohen, 1995) who investigated the effect of hypercholesterolaemia on the mechanism of endothelium-dependent relaxation of rabbit carotid arteries. They reported that hypercholesterolaemia did not affect the relaxant responses to ACh nor was the sensitivity of that relaxation to inhibition of NOS different between normal and hypercholesterolaemic arteries. However elevated K⁺ impaired ACh-induced relaxation in hypercholesterolaemic, but not normal arteries (Najibi *et al.*, 1994). Further it was demonstrated that in hypercholesterolaemic arteries charybotoxin also inhibited the endothelium-dependent relaxation

indicating that it was the BK_{Ca} channel that was involved in the relaxation (Najibi & Cohen, 1995). Thus it appears that pathological states such as ischaemia/reperfusion and hypercholesterolaemia may alter the mechanism of endothelium-dependent relaxation even in the absence of any change in the absolute magnitude of the relaxation.

The question then arises as to what factor is responsible for the opening of the potassium channels. In the study of Tare *et al.*, (1990), hyperpolarization and relaxation caused by NO infusion in guinea-pig uterine artery were reduced by a NO synthase inhibitor, L-N^G-monomethylarginine or Hb. Bolotina *et al.*, (1994) also demonstrated that relaxation to NO was blocked by charybdotoxin, a specific inhibitor of calcium-dependent potassium channels, in rabbit aortic smooth muscle cells. By contrast other studies reported that endothelium-dependent relaxation but not endothelium-dependent hyperpolarization was inhibited by methylene blue or Hb in rat (Chen *et al.*, 1988) or rabbit arteries (Huang *et al.*, 1988) indicating a factor other than NO caused hyperpolarization. Thus, both NO and EDHF have been suggested to open the potassium channels leading to hyperpolarization in response to ACh. In the present study, the NO released by SNP did not cause the opening of BK_{Ca} channels as the responses were not affected by the presence of TEA in either nonischaemic or ischaemic arteries. It might be argued that the form of NO

released by SNP is not the same as that released endogenously by ACh, since studies showing that the endogenous form of EDRF may be a precursor of NO (Greenberg *et al.*, 1990). This would imply that the exogenous NO might not be able to interact with the potassium channels to cause hyperpolarization. However, there are other studies showing the capability of SNP to cause hyperpolarization (Huang *et al.*, 1988; Tare *et al.*, 1990). Therefore the present findings indicate that most likely a factor other than NO is released upon ACh stimulation in ischaemic arteries. This factor might be EDHF since the factor of interest here and EDHF share some common features in that both cause opening of the K_{Ca} channels and are released from endothelium upon agonist stimulation (Mombouli & Vanhoutte, 1997).

In conclusion, NO is the predominant factor mediating endothelium-dependent relaxation upon agonist-stimulation in nonischaemic dog isolated coronary arteries. By contrast, after ischaemia and reperfusion, in addition to the release of NO upon agonist stimulation, there is another factor released from the endothelium. This factor is possibly EDHF which then acts on the BK_{Ca} channels to cause hyperpolarization leading to relaxation. Thus this study provides evidence that ischaemia and reperfusion activates a reserve system, hyperpolarization to contribute to endothelium-dependent relaxation through the opening of BK_{Ca} channels.

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