Effects of BRL 38227, sodium nitroprusside and verapamil on collateral perfusion following acute arterial occlusion in the rabbit isolated ear

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1 We have used an isolated, buffer-perfused, rabbit ear model of acute arterial occlusion to investigate the effects of the nitrovasodilator sodium nitroprusside, the potassium channel activator BRL 38227 (the active (–)-enantiomer of cromakalim) and the calcium antagonist, verapamil, on collateral perfusion in the absence of pharmacological tone.

2 Verapamil was the most potent vasodilator ($EC_{50} = 72.6 \pm 32.0$ nM) of 5-hydroxytryptamine/histamine-induced tone in the rabbit isolated perfused ear. Sodium nitroprusside and BRL 38227 were less potent with respective EC_{50} values of 488 ± 75 nM and 296 ± 40 nM. Following inhibition of endothelium-derived relaxing factor (EDRF) synthesis, the potency of BRL 38227 was significantly ($P < 0.001$) increased with an EC_{50} of 55.6 ± 5.0 nM.

3 BRL 38227 at 500 nM and 3 μ M induced substantial increases in collateral perfusion following arterial ligation in the absence of pharmacological tone compared to control. Furthermore 3 μ M BRL 38227 completely reversed the attenuation of collateral perfusion which followed inhibition of EDRF synthesis with 100 μ M N^G-nitro-L-arginine methyl ester (L-NAME).

4 Sodium nitroprusside (500 nM and 3 μ M) induced modest improvements in collateral perfusion in the early stages after arterial occlusion.

5 Verapamil did not influence collateral perfusion at either of the concentrations used (50 nM and 3 μ M), even though it was a potent vasodilator.

6 The results of this study indicate that BRL 38227, and to a much lesser extent sodium nitroprusside, selectively improve collateral perfusion following arterial occlusion, even in the presence of effects of EDRF on acute collateralization, while verapamil has no effect. Furthermore, BRL 38227 also improves collateral perfusion following inhibition of EDRF synthesis. It remains to be established whether BRL 38227 has beneficial actions in acute arterial occlusion *in vivo*.

Keywords: Rabbit ear; microangiography; collateral flow; sodium nitroprusside; BRL 38227; verapamil; N^G-nitro-L-arginine methyl ester; endothelium-derived relaxing factor

Introduction

Pre-existing collateral vessels provide an alternative pathway to flow following arterial occlusion and thus have an important role in limiting tissue ischaemia and infarction. The function of these vessels is therefore critical to the maintenance of tissue perfusion following acute arterial occlusion. It is well established in both the canine and feline hindquarter circulations that acute arterial occlusion is accompanied by the rapid diversion of flow (within minutes) through pre-existing collateral vessels, and can thus maintain adequate tissue perfusion distal to the occlusion (Winblad *et al.*, 1959; John & Warren, 1961; Rosenthal & Guyton, 1968; Schaub *et al.*, 1976). Despite the importance of collateral flow, little is known of the precise mechanisms governing the recruitment and opening of collateral vessels, although it appears that the pressure gradient across the occlusion may act as a driving force for collateral perfusion (Holman, 1949; Holman & Taylor, 1952). This is supported by the observations that the development of such flow in the canine femoral artery varies directly with the pressure distal to the ligation (Winblad *et al.*, 1959) and regression of collateral perfusion is seen when distal pressure is elevated by shunting hypoxic blood from the contralateral femoral artery to the occluded area (Winblad *et al.*, 1959). Furthermore, the conductance of collateral circulation is reduced by lowering proximal arterial pressure (Rosenthal & Guyton, 1968).

Possible candidates for stimulation of collateral perfusion

include chemical mediators originating from the ischaemic region (Lewis, 1940). In a recent study with a newly-developed rabbit isolated ear model of arterial occlusion we have shown that endothelium-derived relaxing factor (EDRF; Furchgott & Zawadzki, 1980), which is thought to be nitric oxide or closely related chemical species (Palmer *et al.*, 1987), plays a central role in both the initiation and maintenance of collateral perfusion (Randall & Griffith, 1992a,b). Indeed, when EDRF synthesis is inhibited by N^G-nitro-L-arginine methyl ester (L-NAME) the time-dependent development of collateral perfusion is severely impaired, and this inhibitory effect may be reversed by an excess of L-arginine. We have now investigated the effects of three pharmacologically different classes of vasodilators on collateral perfusion in this model. We have also investigated whether BRL 38227 influences collateral perfusion in the absence of EDRF activity to determine whether it might be effective in promoting collateral perfusion in pathological states in which EDRF activity has been reported to be impaired (e.g. hypertension: Lüscher & Vanhoutte, 1986; Mayhan *et al.*, 1987, atherosclerosis: Harrison *et al.*, 1987 or hypercholesterolemia: Verbeuren *et al.*, 1986).

Methods

Preparation of the rabbit ear vascular bed

Male New Zealand White rabbits (2–2.5 kg) were killed by cervical dislocation. An ear was removed and the central

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artery cannulated and perfused with Holman's solution (composition, mM: NaCl 120, KCl 5, CaCl₂ 2.5, NaH₂PO₄ 1.3, NaHCO₃ 25, sucrose 10 and D-glucose 11) at a flow rate of 3.5 ml min⁻¹. The physiological buffer also contained 5% (w/v) dextran (mol. wt.: 80,000) to increase its viscosity (to ca. 2.3 mPaS) and 10 μ M indomethacin to eliminate prostanoic activity. The buffer was gassed with 95% O₂/5% CO₂ and maintained at 35°C.

Experimental protocol

The ear was positioned vertically in front of an X-ray microscope (4 μ m focal spot diameter) and loose sutures placed around the central artery at its midpoint and apex (Figure 1). Following an equilibration period (1 h), microangiograms were taken of the resting preparations. The exposure time was 15 s at 30 kV. Radiographic contrast was achieved by a 35 s pulse of iohexol diluted 3 fold in the perfusion fluid (final iodine concentration 100 mg ml⁻¹). Use of this agent is without significant influence on either vasomotor tone or vascular responses during the period of X-ray exposure (Griffith *et al.*, 1988). A segment of central ear artery (24.5 $\pm$ 0.5 mm; *n* = 52) was then isolated by tightening the sutures and microangiograms were then taken at 2, 5, 10, 15, 30, 60 and 90 min after ligation. Preliminary experiments, in which only the midpoint of the central ear artery was ligated, indicated that there was rapid and complete retrograde filling of the central artery distal to the ligation due to anastomotic supply arising from the daughter branches of its apical bifurcation. This phenomenon has also been reported in the rabbit ear *in vivo* by other workers (Bellman *et al.*, 1959).

The perfusion pressure of the intact preparation was continuously monitored by means of a pressure transducer placed close to the inflow cannula. The pressure drop across the cannula was determined at the end of each experiment and subtracted from the recorded pressure.

The effects of vasodilators were assessed by addition to the perfusion fluid 30 min prior to ligation and the level of collateral perfusion was then determined. The concentrations of the vasodilators were chosen in each case to approximate to the EC₅₀ for causing relaxation of established tone and also a concentration that gave maximal dilatation. In order to determine these concentrations the vasodilator properties of the agents were determined in different ear preparations in which submaximal tone (ca. 60%, Randall & Griffith, 1991) was established by combination of 1 μ M 5-hydroxytryptamine

and 1 μ M histamine. In the case of BRL 38227 the effects of 10 μ M glibenclamide were examined by addition of this agent in the presence of the maximum concentration of BRL 38227 used.

In the case of BRL 38227 the effects on collateral perfusion were also assessed following inhibition of EDRF synthesis with N^G-nitro-L-arginine methyl ester (Randall & Griffith, 1991). In these experiments 100 μ M N^G-nitro-L-arginine methyl ester (L-NAME) was added to the perfusion fluid 60 min before ligation; 30 min after this addition; BRL 38227 was added to the perfusion fluid and after 30 min of preincubation the preparation was ligated and collateral perfusion assessed. The vasorelaxant properties of BRL 38227 were also assessed against pharmacological tone in preparations pretreated with 100 μ M L-NAME 30 min before addition of the vasoconstrictor agents. The concentrations of 5-hydroxytryptamine and histamine were reduced to 100 nM to give a level of tone equivalent to that found in the absence of L-NAME (Randall & Griffith, 1991).

Quantitation

The microangiograms were analysed by a semi-interactive image analysis system as previously described (Griffith *et al.*, 1988). A gold calibration grid was placed on the surface of the ear to determine the magnification factor and the radiographs were further magnified by means of an electronic image analysis system. The diameter of each vessel studied was measured 4 times and the results averaged. Filling of the segment isolated between ligatures was assessed as the area of the segment on the angiogram which had filled with radiographic contrast, relative to its preocclusion value. Angiography carried out in the absence of radiographic contrast, but following previous pulses, showed that there was no stasis of contrast medium in the segment. Similarly, real-time video imaging indicated that clearance of contrast from the segment via the low resistance pathways provided by arteriovenous shunts and arterial branches was rapid (<30 s). The filling of the segment with contrast medium therefore reflects perfusion via collateral vessels and not a time-dependent accumulation of radiographic contrast.

As an estimate of collateral flow and also as a marker for the vascular effects of the vasodilators and L-NAME, the diameter of a first order branch artery (ca. 200 μ m) situated close to but proximal (<3 mm) to the midpoint ligation (Figure 1) was measured as a function of time. This vessel was termed pG1 (i.e. proximal first order generation). These vessels are close to the isolated segment and constitute the main feed vessels to the collateral circulation (Randall & Griffith, 1982b). We have previously shown that the diversion of flow through these vessels to supply a collateral circulation is accompanied by dilatation while the diameter of more proximal first order arteries remains unaltered. The diameter of the central ear artery proximal to the midpoint ligation (pG0) (Figure 1) was also measured. We have previously shown that the diameter of this artery can increase as a consequence of passive distension by an increase in intravascular pressure (Griffith *et al.*, 1987; Randall *et al.*, 1990).

Data and statistical analysis

All data are given as the mean $\pm$ s.e.mean and were compared by either paired or unpaired Student's *t* tests or analysis of variance as appropriate. EC₅₀ for vasodilator responses were obtained from individual concentration-response curves as the concentration at which half maximal reduction in established tone occurred.

Drugs

All solutions were prepared on the day of the experiment. N^G-nitro-L-arginine methyl ester, 5-hydroxytryptamine as creatinine sulphate complex, histamine dihydrochloride, and

Figure 1 An angiogram of the rabbit isolated perfused ear preparation before arterial occlusion: (1) indicates the position of the midpoint suture, (2) the apical suture, (3) the segment of the central artery, (4) a first order branch generation proximal (pG1) to the midpoint suture and (5) the central artery proximal (pG0) to the segment. The disc at the bottom of the angiogram is the gold calibration grid with a diameter of 3 mm.

sodium nitroprusside (all from Sigma Chemical Company, Poole), were dissolved in saline. Verapamil hydrochloride (Sigma) and glibenclamide (Hoechst, Hounslow) were dissolved in absolute ethanol and BRL 38227 (a generous gift from Smith Kline Beecham, Surrey) was dissolved in 70% (v/v) ethanol. Iohexol (Omnipaque, Nycomed, U.K.) was diluted in the perfusion fluid. Indomethacin (Sigma) was dissolved in 5% (w/v) NaHCO_3 . All drugs were then diluted to the required concentrations in the Holman's solution.

Results

Effects of isolating a segment of the central ear artery

Perfusion pressure in the resting ear preparation was 37.9 ± 4.6 mmHg ($n = 16$). Figure 2a shows that 2 min after ligation, filling was $18.2 \pm 3.7\%$ of its preocclusion value ($n = 18$). Filling of the segment increased progressively with time and was $26.1 \pm 3.6\%$ at 15 min ($n = 18$), $31.1 \pm 4.1\%$ at 30 min ($n = 16$), $39.8 \pm 4.6\%$ at 60 min ($n = 16$) and $42.7 \pm 5.9\%$ at 90 min ($n = 9$) (Figure 2a). Perfusion pressure increased modestly with time after ligation, becoming significantly ($P < 0.05$) greater than preocclusion levels after 5 min. This change persisted throughout the experiment (Figure 2c).

Figure 2b shows that the diameter of pG1 increased significantly ($P < 0.05$) from a control value of 201 ± 13 μm ($n = 17$) to a value of 241 ± 21 μm ($n = 16$) 2 min after ligation.

This increase in diameter persisted throughout the period of the experiment peaking at 274 ± 18 μm ($n = 11$) 60 min after ligation (Figure 2b). There was also a significant ($P < 0.001$) increase in the diameter of pG0 from 705 ± 26 μm to 817 ± 51 μm post ligation (both $n = 18$), followed by a slow decline to values not significantly different from the pre-occlusion value (Figure 2d). In all subsequent experiments investigating the effects of vasodilators almost identical changes in the diameter of pG0 were observed so, for brevity, these data have been omitted.

Vasodilator properties of sodium nitroprusside, BRL 38227 and verapamil

In 5 preparations resting perfusion pressure was 18.8 ± 2.6 mmHg and following addition of 1 μM 5-hydroxytryptamine and 1 μM histamine, perfusion pressure rose by 91.4 ± 15.4 mmHg to 110 ± 16 mmHg. In these preparations sodium nitroprusside (1 nM– 10 μM) brought about concentration-related reductions in established tone with an EC_{50} of 488 ± 75 nM and a maximum reduction in established tone of $81.9 \pm 5.6\%$ (Figure 3a).

In 5 different preparations perfusion pressure increased from 26.0 ± 3.7 mmHg to 113 ± 22 mmHg following addition of the combination of vasoconstrictors. In these preparations BRL 38227 (10 nM– 30 μM) similarly induced concentration-dependent relaxations of established tone with an EC_{50} of 296 ± 40 nM, and a maximum reduction in tone of $80.0 \pm 1.8\%$ (Figure 3a). These parameters do not differ significantly from those obtained for sodium nitroprusside. In

Figure 2 (a) The level of filling (%) of the isolated segment of the central artery normalized with respect to the preocclusion control value; (b) the time course of changes in diameter of pG1; (c) changes in perfusion pressure with respect to time and (d) time-course of changes in diameter of pG0 in the isolated ear preparations following arterial occlusion. Ligation was performed at 0 min. In (b), (c) and (d) statistical differences between baseline preligation values and those obtained after ligation are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. Data are given as the mean with bars representing $\pm$ s.e.mean.

3 experiments $10\ \mu\text{M}$ glibenclamide completely reversed the reductions in tone evoked by $30\ \mu\text{M}$ BRL 38227, returning the level of tone to $118 \pm 14\%$ of that before the additions of BRL 38227. When added alone, glibenclamide did not influence perfusion pressure in the constricted preparations ($n = 3$).

In order to examine whether the vasodilator properties of BRL 38227 were influenced by EDRF activity, 7 preparations were pretreated with $100\ \mu\text{M}$ L-NAME prior to precontraction and construction of the concentration-response curves. In these preparations resting perfusion pressure was $20.7 \pm 1.9\ \text{mmHg}$ and following addition of L-NAME was unchanged with a value of $26.4 \pm 5.2\ \text{mmHg}$. Addition of 5-hydroxytryptamine and histamine (both $100\ \text{nM}$) increased perfusion pressure to $140 \pm 10\ \text{mmHg}$, which was not significantly different from that obtained in preparations treated with $1\ \mu\text{M}$ of the constrictors in the absence of L-NAME. Lemakalim ($10\ \text{nM}$ – $10\ \mu\text{M}$), in the presence of L-NAME, caused relaxations of tone with an EC_{50} of $55.6 \pm 5.0\ \text{nM}$ which was significantly ($P < 0.001$) less than that obtained in the absence of L-NAME (Figure 3b). The maximum relaxation was $86.3 \pm 4.6\%$ and did not differ from that obtained in the absence of L-NAME reported above.

In a further 5 preparations, basal perfusion pressure was $36.7 \pm 12.1\ \text{mmHg}$ and was increased by $109 \pm 31\ \text{mmHg}$ to $146 \pm 35\ \text{mmHg}$ following the addition of 5-hydroxytryptamine and histamine (both $1\ \mu\text{M}$). In these preparations verapamil ($1\ \text{nM}$ – $1\ \mu\text{M}$) caused relaxations of established tone with an EC_{50} of $72.6 \pm 32.0\ \text{nM}$ and a maximum reduction in

tone of $63.8 \pm 11.4\%$ (Figure 3a). The EC_{50} was significantly less than that for sodium nitroprusside ($P < 0.001$) and BRL 38227 ($P < 0.01$) making verapamil the most potent of the vasodilators studied. The maximum vasodilator response to verapamil did not differ significantly from those found for either sodium nitroprusside or BRL 38227.

Effect of sodium nitroprusside on collateral perfusion following arterial occlusion

In 11 preparations, resting perfusion pressure was $50.9 \pm 8.4\ \text{mmHg}$ and following addition of $500\ \text{nM}$ sodium nitroprusside perfusion pressure was unaltered with a value of $44.1 \pm 8.5\ \text{mmHg}$; 2 min after ligation, filling of the isolated segment was $33.2 \pm 5.3\%$ of its preocclusion value and was significantly ($P < 0.05$) greater than that seen in the absence of the vasodilator (Figure 4). The degree of filling increased progressively with time being $42.7 \pm 3.2\%$ at 15 min, $49.2 \pm 4.0\%$ at 30 min reaching a maximum of $53.0 \pm 4.8\%$ at 90 min. For the first 15 min the level of filling was significantly greater than in the control preparations. Immediately following ligation, perfusion pressure was $57.7 \pm 9.7\ \text{mmHg}$ and not significantly different from the preocclusion value. Perfusion pressure increased significantly ($P < 0.05$) 5 min after ligation and reached $89.0 \pm 14.6\ \text{mmHg}$ 90 min after occlusion. Prior to ligation the diameter of pG1 was $253 \pm 16\ \mu\text{m}$ and this was unaffected by addition of sodium nitroprusside ($252 \pm 16\ \mu\text{m}$). After ligation the diameter of pG1 increased progressively and was significantly ($P < 0.05$) greater than resting 15 min after ligation ($284 \pm 20\ \mu\text{m}$); 90 min after ligation the diameter was $354 \pm 28\ \mu\text{m}$.

In 9 different preparations, perfusion pressure was $44.2 \pm 12.7\ \text{mmHg}$ and was not significantly affected by addition of $3\ \mu\text{M}$ sodium nitroprusside ($36.7 \pm 12.0\ \text{mmHg}$). Following isolation of the segment by ligation, filling was $25.0 \pm 6.5\%$ and increased to $41.9 \pm 4.9\%$ after 5 min of occlusion. This level of filling was maintained throughout the remainder of the experiment being $46.1 \pm 4.6\%$ (15 min), $44.8 \pm 9.5\%$ (60 min) and $40.9 \pm 10.0\%$ at 60 min (Figure 4). In the 5–30 min after ligation the level of filling was significantly ($P < 0.05$) greater than that in control preparations. In preparations receiving $3\ \mu\text{M}$ sodium nitroprusside,

Figure 3 (a) The vasodilator effects of sodium nitroprusside ($\square$), BRL 38227 ($\blacksquare$) and verapamil ($\blacktriangle$) in ear preparations precontracted with 5-hydroxytryptamine and histamine (both $1\ \mu\text{M}$). (b) The vasodilator properties of BRL 38227 in the absence ($\blacksquare$; data from a) and the presence of $100\ \mu\text{M}$ N^G -nitro-L-arginine methyl ester (L-NAME), ($\square$) in preparations precontracted with 5-hydroxytryptamine and histamine. Data are given as the mean with bars representing $\pm$ s.e.mean.

Figure 4 The effects of sodium nitroprusside (SNP) on collateral perfusion in the rabbit ear model of arterial occlusion. The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time is indicated by ($\blacksquare$) and ($\square$) for perfusion with $500\ \text{nM}$ and $3\ \mu\text{M}$ sodium nitroprusside respectively. Control data (taken from Figure 2a) are indicated by ($\blacktriangle$) and are included for comparison. Statistical differences between control and preparations perfused with sodium nitroprusside are indicated by: $\dagger P < 0.05$ and $\dagger\dagger P < 0.01$ for preparations receiving $3\ \mu\text{M}$ SNP and $* P < 0.05$ and $** P < 0.01$ for those perfused with $500\ \text{nM}$. Data are given as the mean with bars representing $\pm$ s.e.mean.

perfusion pressure remained at a constant level following addition of the agent (36.9 ± 12.0 mmHg) and also following ligation such that after 90 min it was 50.0 ± 12.8 mmHg. The preligation diameter of pG1 was similarly not significantly affected by sodium nitroprusside being 202 ± 21 μ m in its absence and 235 ± 21 μ m in its presence. However, immediately following ligation the diameter of these vessels increased to 274 ± 25 μ m and remained at this level throughout the experiment.

Effect of BRL 38227 on collateral perfusion following arterial occlusion

BRL 38227 (500 nM) did not affect perfusion pressure which was 44.3 ± 9.1 mmHg in its absence and 39.5 ± 7.6 mmHg in its presence (both $n = 6$); 2 min after ligation, filling of the isolated segment was $42.2 \pm 7.6\%$ and this increased to $71.6 \pm 9.0\%$ at 10 min after which time the level of filling remained at this level (Figure 5a). Throughout the experiment the level of filling was significantly ($P < 0.01$) greater than corresponding values obtained from the control preparations not treated with the vasodilator. In preparations receiving 500 nM BRL 38227, perfusion pressure remained similar to the preocclusion values for the entirety of the ligation period (Figure 5c). Figure 5b indicates that prior to ligation, BRL 38227 did not affect the diameter of pG1, while after ligation there was significant ($P < 0.05$) dilatation which peaked at 30 min with a value of 297 ± 36 μ m.

In the preparations which received 3 μ M BRL 38227, perfusion pressure was 46.9 ± 14.0 mmHg in its absence and 43.4 ± 14.0 mmHg in its presence (both $n = 6$). In these preparations ligation was followed by substantial improvements in segmental filling compared to preparations not receiving the vasodilator, being $46.4 \pm 9.8\%$ after 2 min and increasing progressively with time reaching a maximum of $77.2 \pm 7.4\%$ at 90 min (Figure 5a). Throughout the 90 min of occlusion, the level of filling was significantly ($P < 0.001$) greater than that found in control preparations not receiving the drug but not significantly different from those perfused with 500 nM of the agent. Following ligation, perfusion pressure remained at a level not significantly different from the preocclusion value (Figure 5c). The diameter of pG1 (178 ± 24 μ m) was also unaffected by BRL 38227 alone (178 ± 28 μ m) but increased significantly ($P < 0.05$) to 233 ± 29 μ m within 2 min of ligation and reached a plateau around 250 μ m after 10 min (Figure 5b).

Effects of BRL 38227 on collateral perfusion in the presence of L-NAME

In the 9 preparations treated with 100 μ M L-NAME perfusion pressure was 29.3 ± 1.8 mmHg before addition and 42.1 ± 7.0 mmHg afterwards and these values were not significantly different. Following subsequent addition of BRL 38227 to the physiological salt solution, perfusion pressure was 33.1 ± 3.3 mmHg. In these preparations, filling of the isolated segment was significantly ($P < 0.05$) greater than that seen in the presence of L-NAME alone (Randall & Griffith, 1992a,b) for 15–60 min after ligation (Figure 6a). There were no significant differences in segmental filling between preparations treated with 3 μ M BRL 38227 alone and those receiving both BRL 38227 and L-NAME. Addition of L-NAME to preparations prior to BRL 38227 did not significantly alter the diameter of pG1 (224 ± 41 μ m cf. 231 ± 38 μ m). However, after ligation there was dilatation of pG1 comparable to that seen in the presence of BRL 38227 alone (Figure 6b). This contrasts the lack of effect on pG1 seen in the presence of L-NAME alone (Randall & Griffith, 1992a,b). Throughout the ligation period in the presence of L-NAME and BRL 38227, perfusion pressure was unaltered and this contrasts the post-ligation pressor effect seen in the presence of L-NAME alone (Figure 6c).

Figure 5 The effects of BRL 38227 on collateral perfusion and perfusion pressure in the rabbit ear model of arterial occlusion. (a) The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time in preparations perfused with 500 nM (■) and 3 μ M (□) BRL 38227. The (▲) indicates control data taken from Figure 2a. Statistical differences between control and drug treated preparations are indicated by † $P < 0.001$ for perfusion with 3 μ M and * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ for 500 nM. (b) The diameter changes in pG1 during arterial ligation in preparations perfused with 500 nM (■) and 3 μ M (□) BRL 38227. Statistical differences between baseline diameters and those obtained after ligation are indicated by * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$. (c) Variation in perfusion pressure in preparations perfused with 500 nM (■) and 3 μ M BRL 38227. In (b) and (c) perfusion with the vasodilator was started 30 min before ligation and the preparations were ligated at 0 min. Data are given as the mean, with bars representing $\pm$ s.e.mean.

Figure 6 The effects of BRL 38227 on collateral perfusion and perfusion pressure in the rabbit ear model of arterial occlusion in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME). (a) The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time in preparations perfused with 3 μM (■) BRL 38227 alone (data taken from Figure 5a), in the presence of L-NAME (▲) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (□). Statistical differences between preparations treated with L-NAME and those treated with L-NAME plus BRL 38227 are indicated by * $P < 0.05$. (b) The variation of the diameter of pG1 with time in preparations perfused with 100 μM L-NAME (□) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (■). (c) The variation of perfusion pressure with time in preparations perfused in the presence of L-NAME (□) alone (data taken from Randall & Griffith, 1992b) and preparations receiving 3 μM BRL 38227 and 100 μM L-NAME (■). In (b) and (c) perfusion with L-NAME occurred 60 min before ligation, addition of the vasodilator was started 30 min before ligation and the preparations were ligated at 0 min. Data are given as the mean, with bars representing $\pm$ s.e.mean.

Effect of verapamil on collateral perfusion following arterial occlusion

Verapamil (50 nM) did not affect perfusion pressure which was 29.1 ± 8.8 mmHg before and 30.0 ± 9.1 mmHg after its addition (both $n = 5$); 2 min after ligation, filling of the isolated segment was $13.0 \pm 6.1\%$ and this increased to $20.0 \pm 8.7\%$ at 15 min reaching a maximum of $42.7 \pm 10.5\%$ at 90 min. The level of segmental filling was not significantly different from that in control preparations (Figure 7). In preparations perfused with verapamil, perfusion pressure remained similar to the preocclusion values following ligation. Verapamil did not affect the baseline diameter of pG1 (238 ± 15 μm before and 245 ± 14 μm after addition), while after ligation there was only significant ($P < 0.05$) dilatation between 60–90 min, with a maximum value of 311 ± 35 μm at 60 min.

In the preparations which received 3 μM verapamil, perfusion pressure was 32.2 ± 4.5 mmHg in its absence and 33.3 ± 4.6 mmHg in its presence (both $n = 6$). Two minutes after ligation filling was $30.7 \pm 3.8\%$ and reached a maximum of $39.4 \pm 8.6\%$ after 15 min (Figure 7). The level of filling was statistically similar to that found in the control preparations for the entirety of the ligation period. Perfusion pressure remained significantly ($P < 0.05$) greater than the resting value for the 90 min after ligation. The diameter of pG1 was also unaffected by verapamil (209 ± 19 μm cf. 227 ± 15 μm) but increased to 273 ± 329 μm within 2 min of ligation and remained elevated throughout the experiment.

Discussion

We have recently demonstrated that EDRF plays a central role in collateral perfusion after arterial occlusion (Randall & Griffith, 1992a,b). In the present study we have investigated the effects of exogenous vasodilators on collateral perfusion and show that BRL 38227 substantially enhances collateral perfusion over a period of 90 min, that sodium nitroprusside causes improvement in the early stages after occlusion (ca. 15 min) but that verapamil, which was the most potent vasodilator in pharmacologically-constricted preparations, was without significant effect.

The rabbit ear model of arterial occlusion is well suited for investigating the role of purely vascular responses in acute

Figure 7 The effects of verapamil on collateral perfusion in the rabbit ear model of arterial occlusion. The variation of filling (normalized with respect to preocclusion controls) of the isolated segment of central artery with time is indicated by (■) and (□) for perfusion with 50 nM and 3 μM verapamil respectively. The (▲) indicates control data from Figure 2a and has been included for comparison. Data are given as the mean, with bars representing $\pm$ s.e.mean.

collateralization since there is little complication from humoral, nervous or metabolic regulation. It was considered appropriate to carry out these occlusion experiments in the absence of pharmacological constriction, since acutely ischaemic organs are generally at minimal tone. Previous studies have indicated that filling of a segment of central ear artery isolated between ligatures is a reliable indicator of collateral perfusion (Randall & Griffith, 1992a,b). In addition to filling of the isolated segment we have also reported on the selective diameter changes of primary arterial branches (pG1) proximally close to the occlusion which feed the collateral circulation. Indeed in the present study there was a similar correlation between the dilatation of pG1 and segmental filling as noted previously (Randall & Griffith, 1992a,b). In our previous study we also noted that G1 vessels located proximally further away (10–30 mm) from the midpoint ligation did not undergo diameter changes after ligation indicating that only the pG1 vessels adjacent to the ligation feed the collateral circulation (Randall & Griffith, 1992b).

Arterial occlusion in control preparations was followed by time-dependent filling of the isolated segment, approaching a plateau after 60 min such that pre-existing collateral vessels restored filling of the isolated segment to nearly half of the preocclusion level. These observations are consistent with previous *in vivo* studies in which occlusion of the canine superficial femoral artery was followed by restoration of blood flow (between one third and one half control levels) within 5 to 8 min (John & Warren, 1961). Collateral flow then reaches a plateau at approximately 70% of the control level at 30 min (John & Warren, 1961; Rosenthal & Guyton, 1968). In the current study, segmented filling was accompanied by diameter changes of pG1 which increased by approximately 20% representing a ca. 80% increase in conductance and this change persisted throughout the time course of the experiment.

We have also considered the variations of perfusion pressure and diameter of pG0 with time which give insight into the effects of arterial occlusion on the distribution of flow. Ligation of the control preparations resulted in only a modest increase in perfusion pressure, despite the main arterial supply to the distal half of the ear having been occluded. These findings are analogous to those reported following acute occlusion of the canine superficial femoral artery *in vivo* (Winblad *et al.*, 1959) and are consistent with the diversion of flow limiting the rise in pressure proximal to the occlusion. In preparations receiving BRL 38227, which had the greatest influence on collateral perfusion, perfusion pressure was unaltered compared to preocclusion values. This is consistent with the vasodilator opening collateral pathways and facilitating the redistribution of flow. Conversely, in the presence of 3 μ M verapamil, which did not influence collateral function, perfusion pressure increased in a slow, time-dependent fashion similar to that observed in the control experiments. Occlusion of all preparations was accompanied by an immediate increase in the diameter of pG0 which probably reflects the previously described phenomenon of 'paradoxical dilatation' in response to increased distal resistance (Griffith *et al.*, 1987; Randall *et al.*, 1990).

The net effect of any vasodilator on collateral perfusion will be governed by the actions of the agent on collateral vessels and alternative arterial pathways not providing collateral perfusion. Selectivity may be influenced by alterations of tissue responsiveness occurring as a result of hypoxia/ischaemia following occlusion and these changes may be specific for a particular agent. For vasodilators which act on ion channels, the relative distribution of channels between collateral and non-collateral pathways may also govern selectivity. Furthermore changes in hypoxia may alter the activity of these channels and this may also influence activity. Selective vasodilatation or vascular steal of collateral or non-collateral pathways will therefore determine the particular effects of a vasodilator on collateral perfusion.

BRL 38227 significantly increased collateral perfusion com-

pared to control preparations in our model. Furthermore, in the pharmacologically-constricted preparations the vasodilator actions of BRL 38227 were fully reversed by glibenclamide, confirming that this vasodilator action occurs through activation of ATP-sensitive potassium channels. Improvement in collateral perfusion in the presence of BRL 38227 was indicated by the substantial increases in segmental filling coupled with increases in diameter of pG1. Thus 3 μ M BRL 38227 caused a 50% increase in the diameter of pG1 which is substantially greater than that seen in control preparations (ca. 20%). The actions of BRL 38227 would appear specific for the collateral vessels since there was dilatation of the feed vessels (pG1) and increased segmental filling. An action on the low resistance outflow pathways from the segment may be excluded since dilatation of these vessels would have increased exit of perfusate from the segment leading to reduced filling and this was not the case. Interestingly, BRL 38227 was equieffective at improving collateral perfusion at both the supramaximal concentration and the EC_{50} for relaxation of induced tone. This implies that the EC_{50} for relaxation of tone has maximal effects on collateral perfusion so that BRL 38227 is more potent as a collateral vasodilator than as a net relaxant of tone in the whole ear preparation. Heterogeneity in responses to cromakalim are known to occur in other species such as the rat where the agent is a potent vasodilator of small mesenteric arteries but is without action on small cerebral arteries (McCarron *et al.*, 1991). Similarly the *in vivo* vasodilator responses to BRL 38227 in the rat (Gardiner *et al.*, 1991) and cromakalim in the rat and rabbit (Cook & Hof, 1988) show marked regional differences.

That ATP-sensitive potassium channel activators may selectively dilate pre-existing collateral vessels has already been shown by Lamping & Gross (1984) in the canine coronary bed. In their study Lamping & Gross demonstrated that nicorandil improved collateral blood flow following acute coronary occlusion but only when aortic pressure was maintained artificially. The actions of nicorandil must be viewed with caution since this agent contains a nitroxy substituent group and may have nitrovasodilator activity (Edwards & Weston, 1990) dependent on guanosine 3':5'-cyclic monophosphate (cyclic GMP) elevation (Endoh & Taira, 1983). A selective action of potassium channel activators on pre-existing collateral vessels may therefore account for protection of the myocardium in ischaemia (Lampint *et al.*, 1984) and improved postreperfusion function (Grover *et al.*, 1989) which are known to occur in response to these agents. A physiological role for ATP-sensitive potassium channels in coronary vasodilatation has been demonstrated by Komaru *et al.* (1991) who showed that glibenclamide opposes arteriolar dilatations in response to coronary occlusion in the dog. Furthermore Angersbach & Nicholson (1988) have previously reported that cromakalim, nicorandil and pinacidil selectively increase blood flow to hypoxic regions of skeletal muscle in chronic tissue ischaemia in the rat hindlimb.

Sodium nitroprusside, which acts via the generation of nitric oxide, brought about improvements in collateral perfusion in the early stages after arterial occlusion. Both concentrations of the nitrovasodilator afforded an increase in filling above control levels for at least the first 15 min after occlusion. In common with BRL 38227, both concentrations of sodium nitroprusside had maximal effects on collateral perfusion which implies that this agent is also more potent on collateral vessels compared to its vasodilator effects of pharmacologically induced tone in the whole ear preparation. Nitroglycerin has been shown to increase collateral flow in the canine coronary circulation after occlusion (Capurro *et al.*, 1976) but this action is selective for newly developed collateral vessels, generated as a consequence of chronic arterial stenosis, as Lamping & Gross (1984) failed to detect any increase in true collateral flow following acute coronary occlusion in the dog.

The final agent investigated was the calcium antagonist,

verapamil. Although this agent acted as a potent vasodilator in pharmacologically constricted preparations it did not affect collateral perfusion and compared with the other vasodilators studied, had minimal effects on the diameter of pG1. Since there was no deterioration in collateral perfusion compared to control, this may suggest that the vasodilator is equipotent on collateral and non-collateral pathways since steal did not appear to occur or that the agent did not affect either pathway.

Our investigations have also considered whether BRL 38227 was effective following inhibition of EDRF activity, since this factor appears to be essential for normal collateral perfusion (Randall & Griffith, 1992a,b). In the vasodilator experiments, BRL 38227 was more potent (5 fold) in the presence of L-NAME compared to control. This was somewhat surprising since *in vivo*, the vasodilator properties of BRL 38227 in the rat are not, except in the case of the mesenteric circulation, influenced by L-NAME (Gardiner *et al.*, 1991). However, it is well established that responses to nitrovasodilators are augmented after inhibition of EDRF activity (Shirasaki & Su, 1985) and this is thought to be due to supersensitivity of the guanylyl cyclase (Moncada *et al.*, 1991). In the present context the increased potency of BRL 38227 cannot be explained through interactions at the level of guanylyl cyclase as the agent acts independently of cyclic GMP (Taylor *et al.*, 1988), and we have shown that the vasodilator responses were completely blocked by glibenclamide. Enhanced activity of vasodilators on loss of basal EDRF activity is not confined to nitrovasodilators, as vasodilator responses to an endothelium-independent agent isoprenaline are augmented after loss of the endothelium in rabbit aorta (White *et al.*, 1986). This interaction is explained by basal EDRF activity having a tonic vasodilator input which is abolished by endothelial removal, thus enabling other vasodilators to have greater impact. In the presence of L-NAME and BRL 38227, collateral perfusion was generally greater than that seen in the presence of L-NAME. Furthermore the level of perfusion was not significantly different from that seen in the presence of both EDRF activity and BRL 38227. Interestingly,

BRL 38227 abolished the post-ligation increase in perfusion pressure (ca. 100 mmHg) seen in the presence of L-NAME alone (Randall & Griffith, 1992a, b). The level of collateral perfusion seen in the present experiments indicates that BRL 38227 can act independently of EDRF activity and may therefore be effective at increasing collateral flow in states where EDRF activity has been reported to be impaired (e.g. hypertension, Lüscher & Vanhoutte, 1986; Mayhan *et al.*, 1987; atherosclerosis, Harrison *et al.*, 1987, or hypercholesterolemia, Verbeuren *et al.*, 1986). This observation may be of considerable therapeutic relevance since these states may predispose towards arterial occlusion.

The selective action of BRL 38227 on pre-existing collateral vessels requires explanation. Changes occurring during hypoxia or ischaemia may reduce the level of oxidative phosphorylation leading to a reduction in ATP production. This may result in membrane depolarization, suggesting that potassium channel activators would have a greater hyperpolarizing effect, and lead to selective dilatation of such vessels. A reduction in intracellular ATP concentrations would also potentiate the actions of drugs such as BRL 38227 which act on potassium channels sensitive to ATP. The impact of calcium channel blockers will be relatively unaffected by ATP levels and so verapamil will be without selective action on these vessels. An alternative explanation might be that the apparently selective action of potassium channel activators over other vasodilators reflects differences in ion channel populations between collateral and non-collateral vessels.

In conclusion, the present findings indicate a selective action for BRL 38227 on collateral perfusion and to a lesser extent for sodium nitroprusside. It remains to be established whether BRL 38227 may prove of therapeutic importance in acute occlusive arterial disease.

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