Br. J. Pharmacol. (1993), **109**, 386–393

Modulation of vasodilatation to levcromakalim by hypoxia and EDRF in the rabbit isolated ear: a comparison with pinacidil, sodium nitroprusside and verapamil

[1]Michael D. Randall & Tudor M. Griffith

Department of Diagnostic Radiology, University of Wales College of Medicine, Heath Park, Cardiff CF4 4XN

1 We have used an isolated buffer-perfused preparation of the rabbit ear to investigate the effects of hypoxia and inhibition of endothelium-derived relaxing factor (EDRF) synthesis on the vasodilator responses to the potassium channel opener, levcromakalim (the active (−)-enantiomer of cromakalim). The results obtained with levcromakalim have been compared with those for pinacidil, sodium nitroprusside and verapamil.

2 Levcromakalim relaxed preconstricted preparations with an $EC_{50} = 343 \pm 41$ nM and $R_{max} = 80.3 \pm 6.4\%$. Under hypoxic conditions the concentration-response curve was significantly ($P < 0.01$) shifted to the left with an $EC_{50} = 118 \pm 16$ nM and $R_{max} = 89.9 \pm 2.7\%$. Hypoxia did not influence relaxation to either pinacidil, sodium nitroprusside or verapamil.

3 Inhibition of EDRF synthesis with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) also significantly ($P < 0.001$) increased the vasodilator potency of levcromakalim ($EC_{50} = 56 \pm 5$ nM), and caused a similar shift in the concentration-response curve to sodium nitroprusside. It did not influence vasodilatation to either verapamil or pinacidil. The potentiation of vasodilator responses to levcromakalim by L-NAME was reversed by an excess of L-arginine.

4 Impairment of oxidative phosphorylation with 400 nM carbonyl cyanide *m*-chlorophenylhydrazone significantly ($P < 0.05$) increased the potency of levcromakalim ($EC_{50} = 120 \pm 20$ nM) but did not influence vasodilatation to pinacidil or endothelium-dependent relaxations to acetylcholine.

5 Vasodilatation to levcromakalim was augmented both by hypoxia and by inhibition of EDRF activity. Since impairment of oxidative phosphorylation increased the potency of levcromakalim but did not alter EDRF activity then the mechanism responsible for hypoxic facilitation of responses to levcromakalim is likely to be due to reduced ATP levels in hypoxic smooth muscle cells rather than a change in EDRF activity. These results suggest that levcromakalim may selectively dilate both hypoxic vessels and vessels with impaired EDRF activity. The results also point to important differences in the pharmacology of levcromakalim and pinacidil.

Keywords: Levcromakalim; sodium nitroprusside; verapamil; pinacidil; N^G-nitro-L-arginine methyl ester; L-arginine; hypoxia; endothelium-derived relaxant factor; potassium channel opener-sensitive potassium channels (KCO-channels); rabbit ear

Introduction

Levcromakalim (formerly BRL 38227), the active enantiomer of cromakalim, is a member of the novel class of vasodilators (Edwards & Weston, 1990) and hypotensive agents (Buckingham *et al.*, 1986) which are thought to act via the activation of potassium channels (Standen *et al.*, 1989) leading to hyperpolarization (Hamilton *et al.*, 1986; see Edwards & Weston, 1990). The potassium channel subtypes involved are thought to be regulated by both intracellular ATP and ADP concentrations (Noma, 1983; Misler *et al.*, 1986). In this respect, intracellular ATP closes these channels and high ADP favours opening, while potassium channel openers reduce channel sensitivity towards ATP thereby promoting channel opening (see Nichols & Lederer, 1991). These channels are selectively blocked by the hypoglycaemic sulphonylureas (Sturgess *et al.*, 1985).

In a recently developed model of acute collateral perfusion in the rabbit ear, we have previously shown that levcromakalim substantially improves collateral flow after acute arterial occlusion (Randall & Griffith, 1992). In this respect, levcromakalim had appreciably greater effects than sodium nitroprusside, while verapamil was without effect. Accordingly, we hypothesized that the selectivity shown by levcromakalim may relate to differences in the distribution of potassium channel opener-sensitive channels (KCO-channels) between pre-existing collateral vessels and non-collateral vessels, or alternatively that the collateral vessels may potentially be hypoxic and this could influence the action of vasodilators. Impairment of oxidative metabolism in hypoxia may reduce intracellular ATP levels, and could theoretically promote the opening of KCO channels. Indeed, this mechanism is thought to be responsible for hypoxic vasodilatation in the guinea-pig coronary vasculature, which is blocked by glibenclamide and mimicked by cromakalim and inhibition of oxidative phosphorylation with dinitrophenol (Daut *et al.*, 1990). That the activity of potassium channel activators may be influenced by hypoxia has also been proposed by Cook & Quast (1990). To test this hypothesis we have now examined the influence of hypoxia on vasodilatation to levcromakalim in the intact perfused ear of the rabbit and compared the results with those obtained with three other vasodilators, namely pinacidil which also acts via the opening of potassium channels, sodium nitroprusside (which acts via the generation of nitric oxide) and verapamil (which acts through blockage of voltage-sensitive calcium channels).

Hypoxia also influences vasorelaxation to endothelium-dependent vasodilators through either a reduction in EDRF synthesis or vascular smooth muscle sensitivity (Furchgott & Zawadzki, 1980; DeMey & Vanhoutte, 1983; Johns *et al.*, 1989; Warren *et al.*, 1989; Randall *et al.*, 1990). We have accordingly investigated the effects of inhibition of EDRF synthesis with N^G-nitro-L-arginine methyl ester (L-NAME)

[1] Author for correspondence.

on vasodilatation to levcromakalim, pinacidil, sodium nitroprusside and verapamil. Additionally, we have also used carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), an uncoupler of oxidative phosphorylation, to examine whether responses to levcromakalim and pinacidil are influenced by impaired ATP generation.

Part of this work was communicated to the January 1993 meeting of the British Pharmacological Society (Randall & Griffith, 1993).

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 artery cannulated and perfused with Holman's solution (composition mM: NaCl 120, KCl 5, $CaCl_2$ 2.5, NaH_2PO_4 1.3, $NaHCO_3$ 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 μM indomethacin to eliminate prostanoid activity. The buffer was gassed with either 95% O_2/5% CO_2 (normoxia, Po_2 = 500–600 mmHg) or 95% N_2/CO_2 (hypoxia, Po_2 = 20–30 mmHg) and maintained at 35°C from the start of the experiments.

Experimental protocols

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 in order to determine the actual perfusion pressures across the vascular bed.

To characterize the vasodilator responses, preparations were equilibrated for 1 h. Perfusion pressure was raised pharmacologically with the combination of 5-hydroxytryptamine and histamine in equimolar concentrations to achieve submaximal tone (ca. 60% of maximal tone, Randall & Griffith, 1991). Cumulative concentration-response curves were obtained in different preparations for levcromakalim, pinacidil, sodium nitroprusside and verapamil by addition of each agent to the reservoir containing the perfusion fluid in volumes less than 100 μl and perfusion pressure was monitored continuously. The results obtained under hypoxic perfusion were compared with control responses obtained from different preparations perfused under normoxic conditions.

In order to investigate the influence of EDRF on relaxation to these agents, different preparations were perfused with 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) which was included in the perfusion fluid 30 min before the construction of the concentration-response curves. We have previously shown that perfusion with L-NAME selectively abolishes endothelium-dependent relaxations to acetylcholine and inhibits basal EDRF activity (Randall & Griffith, 1991). The ability of excess L-arginine (10 mM) to reverse the effects of L-NAME on the relaxations to levcromakalim was investigated by its inclusion in the perfusion fluid 30 min after addition of L-NAME. Concentration-response curves to levcromakalim were then constructed after a further 30 min. The effects of L-arginine alone on vasodilatation to levcromakalim were also investigated by perfusion with 10 mM L-arginine prior to (30 min) and during preconstriction with 5-hydroxytryptamine and histamine. In the case of levcromakalim, experiments were performed to investigate the effects of L-NAME in combination with hypoxic perfusion which was carried out as described above.

The effects of impaired oxidative phosphorylation on vasodilatation to levcromakalim and pinacidil were also investigated. In these experiments, normoxic preparations were equilibrated and then preconstricted with supramaximal concentrations of 5-hydroxytryptamine and histamine (both 3 μM) and once maximal pressor effects had been achieved, carbonyl cyanide *m*-chlorophenylhydrazone (CCCP) was added to the perfusion fluid to achieve a concentration of 400 nM which resulted in a reduction in established tone. Once the level of established tone had stabilized at a constant value (after ca. 30 min) concentration-response curves were constructed for levcromakalim and pinacidil in different preparations. Since vasodilator responses may be influenced by the level of preconstriction (White *et al.*, 1986) the control concentration-response curves for levcromakalim and pinacidil were constructed against an equivalent level of induced tone. To achieve a level of tone comparable to that observed in the presence of 400 nM CCCP, it was found necessary to reduce the equimolar concentrations of 5-hydroxytryptamine and histamine to 300 nM. Subsequent analysis of the potencies of levcromakalim and pinacidil indicated that this reduction in the concentrations of vasoconstrictor agents did not influence vasodilatation to either agent.

To determine whether the chosen concentration of CCCP influenced EDRF activity, concentration-response curves were constructed for the endothelium-dependent vasodilator, acetylcholine, in the presence of CCCP and in control preparations preconstricted to a level of tone comparable to that in preparations receiving the metabolic inhibitor.

Data and statistical analysis

All data are given as the mean ± s.e.mean and were compared by either paired or unpaired Student's *t* tests or analysis of variance, as appropriate. EC_{50} values for vasodilator responses were obtained from individual concentration-response curves as the concentration at which half-maximal reduction in established tone occurred. These values were converted to the logarithmic values (pD_2) for statistical analysis.

Drugs

All solutions were prepared on the day of the experiment. N^G-nitro-L-arginine methyl ester, L-arginine hydrochloride, 5-hydroxytryptamine as creatinine sulphate complex, histamine dihydrochloride, acetylcholine chloride and sodium nitroprusside (all from Sigma Chemical Company, Poole, Dorset), were dissolved in saline. Verapamil hydrochloride (Sigma) glibenclamide (Hoescht, Hounslow, Middlesex) and carbonyl cyanide *m*-chlorophenylhydrazone (Sigma) were dissolved in abolute ethanol. Levcromakalim (a generous gift from Smith Kline Beecham, Surrey) and pinacidil (a generous gift from Leo, Bucks.) were dissolved in 70% (v/v) ethanol. All drugs were then diluted to the required concentrations in the Holman's solution.

Results

Vasodilator responses to levcromakalim, pinacidil, sodium nitroprusside and verapamil

The concentration-response curve for the vasoconstrictor effects of 5-hydroxytryptamine and histamine under control conditions is shown in Figure 1 and is described by an EC_{50} of 895 ± 190 nM and R_{max} value of 174 ± 8 mmHg.

In different preparations all 4 vasodilator agents used caused concentration-related reductions in submaximal (ca. 60%) tone induced by 1 μM 5-hydroxytryptamine and 1 μM histamine. Table 1 and Figures 2a, 3, 4 and 5 indicate that verapamil was the most potent vasodilator, levcromakalim and sodium nitroprusside were equipotent and pinacidil was considerably less potent. The vasodilator effects of both maximum concentrations of levcromakalim and pinacidil were reversed by addition of 10 μM glibenclamide (Table 1).

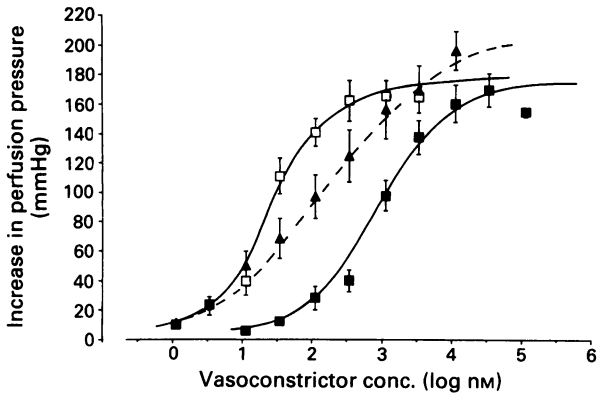

Figure 1 Concentration-response curves for the increase in perfusion pressure induced by the equimolar combination of 5-hydroxytryptamine and histamine during normoxia (■, $n = 8$), hypoxia (▲, $n = 5-7$), and in the presence of 100 μM N^G-nitro-L-arginine methyl ester (□, $n = 4-5$) in isolated perfused ear preparations of rabbit.

Effects of hypoxia on vascular responses

The concentration-response curve for 5-hydroxytryptamine plus histamine constructed during hypoxic perfusion is shown in Figure 1. In these 7 preparations, basal perfusion pressure was 40.1 ± 4.8 mmHg. Addition of equimolar amounts of vasoconstrictors (1 nM–10 μM) resulted in concentration-related increases in perfusion pressure, with an R_{max} of 191 ± 11 mmHg and the EC_{50} value of 407 ± 210 nM (Figure 1). Therefore, under hypoxic conditions the combination of vasoconstrictors was significantly ($P < 0.05$) more potent. To achieve a comparable level of tone in the vasodilator experiments to that used under normoxic conditions (ca. 60% of maximum), the equimolar concentration used to preconstrict the rabbit ear preparations under hypoxic conditions was 100 nM.

In the preconstricted preparations perfused with hypoxic buffer, all four vasodilators induced concentration-related reductions in established tone. It can be seen from Table 2 and Figure 2a that the EC_{50} value for the relaxation of tone by levcromakalim was significantly ($P < 0.001$) less than that under normoxic conditions. The maximum reduction in tone was 89.9 ± 2.7% and not significantly different from that obtained in normoxic control preparations. However, the EC_{50} values and the R_{max} values obtained for pinacidil, sodium nitroprusside and verapamil did not differ from their respective values obtained under normoxic perfusion (Table 2, Figures 3, 4 and 5). Once again, addition of 10 μM glibenclamide reversed the relaxation of tone induced by levcromakalim and pinacidil (Table 2).

Effects of inhibition of EDRF activity on vasodilator responses

Concentration-response curves were constructed for the combination of 5-hydroxytryptamine and histamine in 5 preparations pretreated with 100 μM L-NAME (Figure 1). Prior to the addition of L-NAME, basal perfusion pressure was 30.9 ± 4.1 mmHg and afterwards was 41.8 ± 7.6 mmHg. The EC_{50} value for the vasoconstrictor effects was 29.0 ± 8.6 nM and maximum increase in perfusion pressure was 166 ± 12 mmHg. The EC_{50} value was significantly ($P < 0.01$) lower than under normoxic conditions. To achieve a comparable level of tone to that used under normoxic control conditions (ca 60% of maximum), the equimolar concentration used to preconstrict the rabbit ear preparations in the presence of L-NAME was reduced to 100 nM.

In the presence of 100 μM L-NAME, all of the vasorelaxants caused concentration-related decreases in tone (Figures 2a, 3 and 4, Table 3). In the case of both verapamil and pinacidil, their potencies were not significantly different from those obtained under control conditions in the absence of the inhibitor. In the presence of L-NAME there were significant increases in the potency for both levcromakalim ($P < 0.001$) and sodium nitroprusside ($P < 0.001$). Furthermore, the EC_{50} value obtained for levcromakalim in the presence of L-NAME was also significantly ($P < 0.05$) less than that obtained under hypoxic conditions.

In order to characterize further the effects of L-NAME on vasorelaxation to levcromakalim, excess L-arginine (10 mM) was added to a further 7 preparations 30 min after the addition of 100 μM L-NAME. In the preparations treated with both L-NAME and L-arginine the concentration-response curve was shifted back to a similar position to that obtained under normoxic conditions (Table 3; Figure 2b). In the absence of L-NAME, treatment with L-arginine alone did not influence vasodilatation by levcromakalim, when the concentration-response curve was described by an EC_{50} value of 300 ± 84 nM and R_{max} of 76.2 ± 0.6% ($n = 5$). These parameters were not significantly different from those obtained in control preparations.

Table 1 Vasodilator properties of levcromakalim, pinacidil, sodium nitroprusside and verapamil under control conditions

	Levcromakalim	*Pinacidil*	*Sodium nitroprusside*	*Verapamil*
n	8	8	5	5
Basal perfusion pressure (mmHg)	40.3 ± 8.5	34.1 ± 5.9	18.8 ± 2.6	36.6 ± 12.6
Increase in tone (mmHg)	112 ± 19	101 ± 11	91.4 ± 15.4	109 ± 31
EC_{50} (nM)	343 ± 41	3,360 ± 850	488 ± 75	72.6 ± 32.0
Maximum relaxation (%)	80.3 ± 6.4	95.3 ± 3.5	81.9 ± 5.6	63.8 ± 11.4
% reversal of maximum relaxation by 10 μM glibenclamide	118 ± 14	78.2 ± 14.6		

Table 2 Vasodilator properties of levcromakalim, pinacidil, sodium nitroprusside and verapamil under hypoxic conditions

	Levcromakalim	*Pinacidil*	*Sodium nitroprusside*	*Verapamil*
n	12	8	6	4
Basal perfusion pressure (mmHg)	27.8 ± 6.2	38.1 ± 3.1	34.4 ± 8.1	24.8 ± 11.8
Increase in tone (mmHg)	111 ± 4	102 ± 12	94.0 ± 14.0	84.0 ± 19.0
EC_{50} (nM)	118 ± 16	2,950 ± 580	518 ± 130	95 ± 19
Maximum relaxation (%)	89.9 ± 2.7	94.9 ± 2.7	87.6 ± 3.2	64.8 ± 6.8
% reversal of maximum relaxation by 10 μM glibenclamide	96.2 ± 4.7	72.9 ± 11.0		

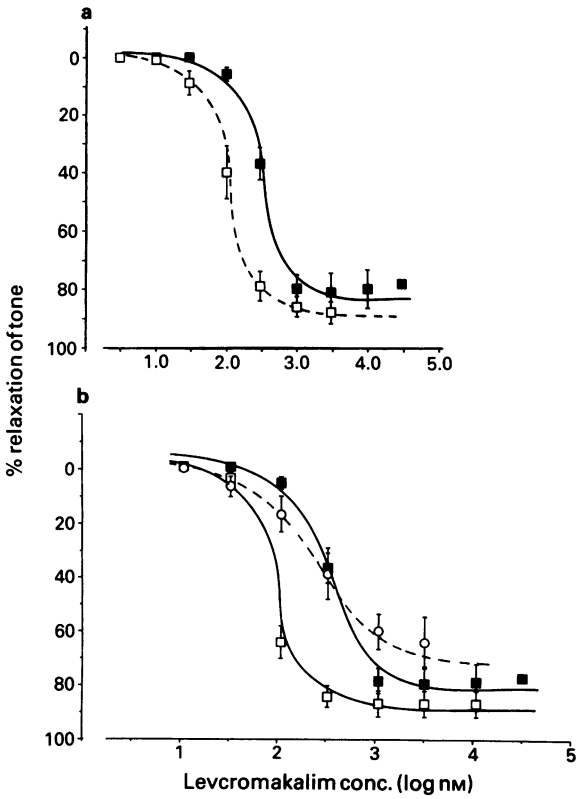

Figure 2 Concentration-response curves for the relaxation of established tone by levcromakalim in isolated perfused ear preparations of rabbit. (a) Under normoxic (■, $n = 8$) and hypoxic (□, $n = 12$) conditions. (b) In the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME) under normoxic conditions (□, $n = 7$), in the presence of L-NAME and 10 mM L-arginine (○, $n = 7$) and in the absence of L-NAME under normoxic conditions (■) (taken from a).

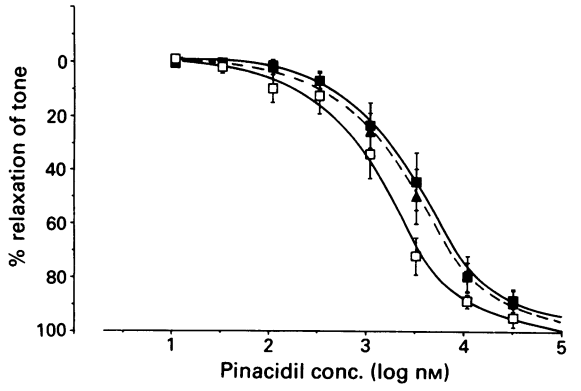

Figure 3 Vasodilatation to pinacidil in the preconstricted isolated perfused ear of preparations of rabbit under control conditions (■, $n = 8$), during hypoxic perfusion (▲, $n = 8$) and in the presence of 100 μM N^G-nitro-L-arginine methyl ester (□, $n = 8$).

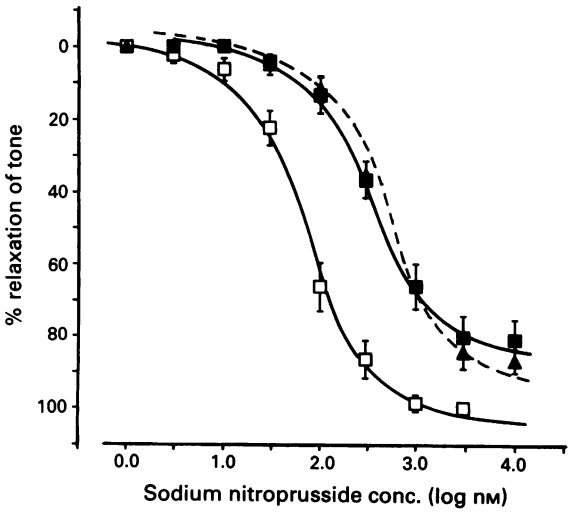

Figure 4 Concentration-response curves for the relaxation of established tone by sodium nitroprusside under normoxic conditions (■, $n = 5$), hypoxic conditions (▲, $n = 6$) and preparations perfused with 100 μM N^G-nitro-L-arginine methyl ester (□, $n = 6$).

Effects of combined inhibition of EDRF synthesis and hypoxia on vasodilatation to levcromakalim

In 8 preparations perfused with hypoxic buffer, resting perfusion pressure was 37.1 ± 7.1 mmHg and this was not significantly affected by addition of 100 μM L-NAME (43.5 ± 11.1 mmHg). Following addition of 5-hydroxytryptamine and histamine (both 100 nM) perfusion pressure rose by 151 ± 11 mmHg to 195 ± 15 mmHg. In these preparations the vasodilator responses to levcromakalim were described by an EC_{50} of 71 ± 13 nM and $R_{max} = 95.3 \pm 8.2\%$. Under these conditions levcromakalim was therefore significantly more potent than under hypoxic conditions alone ($P < 0.05$) or normoxic conditions ($P < 0.001$) alone, but not different from that in the presence of L-NAME under normoxia. The maximum reactivity was not significantly different under any of the above conditions.

Effects of impairment of oxidative phosphorylation with CCCP on vasodilatation to levcromakalim and pinacidil

In 7 preparations, perfusion pressure was 30.9 ± 10.6 mmHg and this was increased by 118 ± 13 mmHg to 149 ± 14 mmHg after addition of the combination of vasoconstrictors (both 3 μM). Following addition of 400 nM CCCP, the level of induced tone fell by 66.2 ± 13.1 mmHg so that total perfusion pressure was 82.7 ± 14.7 mmHg. Levcromakalim (10 nM–3 μM) in the presence of CCCP brought about concentration-related reductions in induced tone (EC_{50} = 120 ± 20 nM, R_{max} was 91.4 ± 2.9%) (Figure 6). In 3 preparations 10 μM glibenclamide was added in the presence of 3 μM levcromakalim and returned the level of tone to 96.1 ± 14.4% of the preconstricted level in the presence of CCCP.

Table 3 Vasodilator properties of levcromakalim, pinacidil, sodium nitroprusside and verapamil in the presence of 100 μM N^G-nitro-L-arginine methyl ester (L-NAME)

	Levcromakalim	*Levcromakalim + L-arginine*	*Pinacidil*	*Sodium nitroprusside*	*Verapamil*
n	7	7	8	6	5
Basal perfusion pressure (mmHg)	20.7 ± 1.9	27.1 ± 14.0	55.0 ± 8.9	17.2 ± 2.5	22.2 ± 2.8
Change in tone (+L-NAME, mmHg)	5.5 ± 5.7	23.4 ± 9.3	17.5 ± 10.0	13.3 ± 9.5	13.0 ± 9.4
Increase in tone (mmHg)	119 ± 9	130 ± 6	133 ± 18	127 ± 12	99.0 ± 16.1
EC_{50} (nM)	56 ± 5	273 ± 47	1,740 ± 370	71 ± 12	91 ± 19
Maximum relaxation (%)	86.3 ± 4.6	70.9 ± 10.0	95.9 ± 3.0	100 ± 2	62.7 ± 4.7

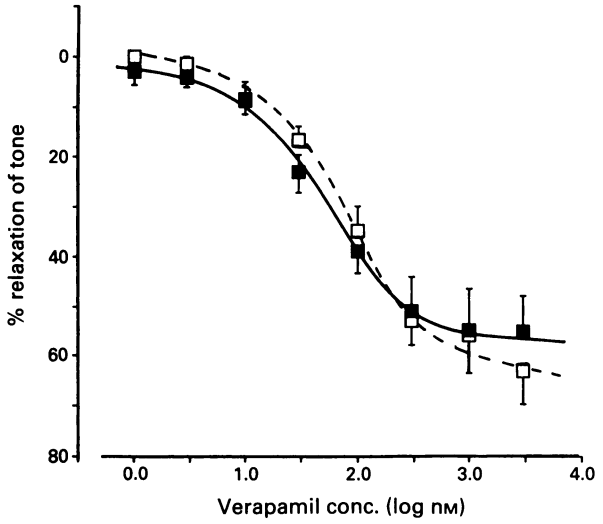

Figure 5 Concentration-response curves for vasodilatation to verapamil in the preconstricted isolated perfused ear preparations of rabbit under control conditions (■, $n = 5$) and during hypoxic perfusion (□, $n = 4$).

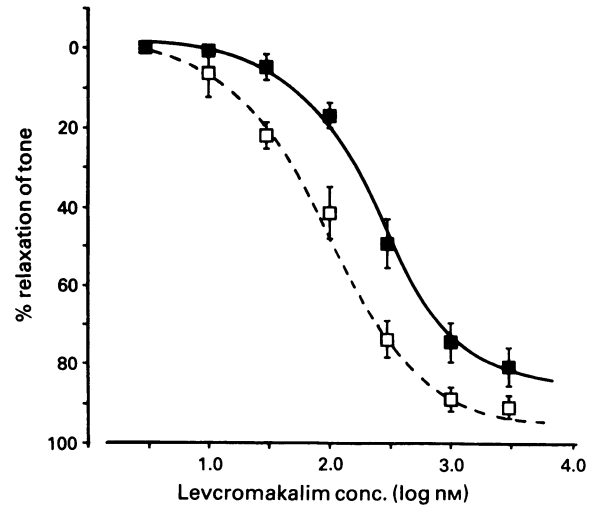

Figure 6 Concentration-response curves for the relaxation of established tone by levcromakalim in the absence (■, $n = 8$) and presence (□, $n = 7$) of 400 nM carbonyl cyanide *m*-chlorophenylhydrazone in isolated perfused ear preparations of rabbit.

As a control to these experiments, the vasodilator responses to levcromakalim were determined against a level of established tone comparable to that observed in the presence of CCCP. In these 8 control preparations, resting perfusion pressure was 23.4 ± 5.6 mmHg and following addition of 5-hydroxytryptamine and histamine (both 300 nM) rose by 50.9 ± 6.6 mmHg to 74.3 ± 7.3 mmHg. This increase in tone was not significantly different from that in the preparations receiving CCCP (51.8 ± 5.4 v 50.9 ± 6.6 mmHg). In these control preparations, where induced tone was ca. 30% of maximum, levcromakalim (10 nM–3 μM) brought about concentration-related reductions in established tone with an EC_{50} = 242 ± 42 nM, which is significantly greater ($P < 0.05$) than that observed in the presence of CCCP and with an R_{max} = 78.0 ± 5.4% which is significantly ($P < 0.05$) less than that in the presence of CCCP (Figure 6). In the 4 preparations which received 10 μM glibenclamide in the presence of 3 μM levcromakalim established tone returned to 126 ± 25% of control values.

In another group of control preparations basal perfusion pressure was 62.2 ± 8.4 mmHg and addition of 300 nM 5-hydroxytryptamine and histamine increased tone by a further 58.6 ± 8.1 mmHg to 121 ± 14 mmHg. In these preparations, pinacidil (10 nM–30 μM) relaxed tone with an EC_{50} value of 2.89 ± 0.87 μM and the R_{max} value was 87.4 ± 6.5% ($n = 8$). Subsequent addition of 10 μM glibenclamide partially reversed the vasodilator effects of 30 μM pinacidil such that induced tone was returned to 74.1 ± 6.1% of control. In the 11 preparations treated with CCCP, basal perfusion pressure was 38.6 ± 5.4 mmHg and was increased by 160 ± 10 mmHg to 198 ± 10 mmHg. After addition of CCCP the level of established tone was reduced to 55.0 ± 6.9 mmHg. In these preparations the vasodilator responses of pinacidil (10 nM–300 μM) were described by an EC_{50} value of 5.25 ± 1.26 μM and the R_{max} was 102 ± 6%. These values did not differ significantly from those obtained in the controls.

Effects of CCCP on endothelium-dependent vasodilatation to acetylcholine

In 6 different control preparations, perfusion pressure was 37.0 ± 9.4 mmHg and 5-hydroxytryptamine and histamine (both 300 nM) increased this by 71.7 ± 11.8 mmHg to 109 ± 12 mmHg. In these preparations, acetylcholine (3 nM–10 μM) induced concentration-related relaxations of established tone which were described by an EC_{50} = 91 ± 33 nM and a maximum inhibition of 90.2 ± 5.0%. In the experimental preparations, basal perfusion pressure was 35.0 ± 9.7 mmHg and was increased by 166 ± 18 mmHg to 201 ± 19 mmHg following addition of 3 μM 5-hydroxytryptamine and 3 μM histamine. Subsequent addition of CCCP (400 nM) caused perfusion pressure to fall by 75.7 ± 6.4 mmHg to 125 ± 15 mmHg after 30 min. In these preparations, acetylcholine (3 nM–10 μM) caused concentration-related relaxations of tone with an EC_{50} of 87 ± 17 nM and a maximum reduction in tone of 81.7 ± 8.8%. These values were not significantly different from those obtained in the absence of CCCP.

Discussion

The present investigation demonstrates that the vasodilator potency of levcromakalim is increased 3 fold under hypoxic conditions, compared to normoxia, while responses to pinacidil, sodium nitroprusside and verapamil are unaffected. To our knowledge this is the first demonstration that the vasodilator properties of levcromakalim are influenced by acute hypoxia. Furthermore, the differential effects of hypoxia on vasodilatation to levcromakalim and pinacidil are interesting in view of the common action of these agents on potassium channels.

That the potency of levcromakalim is augmented by hypoxia is not entirely unexpected, as levcromakalim is thought to act on potassium channels sensitive to intracellular ATP as glibenclamide, which has been shown to block ATP-sensitive potassium channels in other cell types, blocked the action of cromakalim (Standen *et al.*, 1989). It should be noted, however, that there is recent evidence that potassium channel openers may also act on other potassium channels including the calcium-activated subtypes (see Kajioka *et al.*, 1991). Furthermore, high intracellular ATP levels have previously been reported to depress the channel opening activity of pinacidil in guinea-pig isolated myocytes (Fan *et al.*, 1990). It is therefore conceivable that a reduction in metabolic activity leading to reduced ATP promotes the actions of potassium channel openers.

In addition to hypoxia, impairment of oxidative phosphorylation with CCCP, an uncoupler of oxidative metabolism, selectively increased the potency of levcromakalim without influencing vasodilator responses to either acetylcholine or pinacidil. In the present study, perfusion with CCCP resulted in reductions of established tone, an effect previously reported by Griffith *et al.* (1986) in both endothelium-intact and endothelium-denuded rabbit aorta. This is

consistent with a reduced supply of ATP for smooth muscle contraction or alternatively a non-specific vasodilator action. The present results indicate that under conditions associated with a reduction in ATP generation, the vasodilator properties of levcromakalim are augmented. This accords with findings in pancreatic β cells where metabolic inhibitors promote the opening of ATP-sensitive potassium channels (Misler *et al.*, 1986). We have therefore established a link between impaired oxidative metabolism and increased vasodilator potency for levcromakalim which may account for the hypoxic augmentation of vasodilatation.

That the responses to levcromakalim but not those to pinacidil were affected by hypoxia requires explanation. Despite their widely different chemical structures these agents are generally assumed to have a common mechanism of action through the activation of KCO-sensitive potassium channels. However, there is some evidence in the literature pointing to differences in the pharmacology of these agents (see Cook & Quast, 1990). Early studies indicated subtle haemodynamic differences between cromakalim and pinacidil. In the cat, for example, the hypotensive actions of cromakalim but not those of pinacidil are accompanied by reductions in renal vascular resistance (Longman *et al.*, 1988). McPherson & Angus (1990) also identified differences in the pharmacology of cromakalim and pinacidil, in that glibenclamide, phentolamine and alinidine non-competively inhibited the actions of cromakalim on the canine coronary artery while having competitive actions against pinacidil. More recently Lawson *et al.* (1992) have demonstrated that endothelin-1 discriminates between the actions of levcromakalim and that of pinacidil. In their study, subcontractile concentrations of endothelin-1 prevented the vasodilator actions of low concentrations of levcromakalim but not those of pinacidil, observations which led to them proposing that levcromakalim and pinacidil interact with different sites on the potassium channel. In the present context, hypoxia may be associated with increased endothelin-1 release (Rakugi *et al.*, 1990), but this would not account for the specific increase in the potency of levcromakalim, since an increase in endothelin-1 would be expected to reduce its dilator activity. The differences between levcromakalim and pinacidil identified in the current study may therefore be related to differences in the way these agents interact with potassium channels as suggested by Lawson *et al.* (1992).

Hypoxia may influence vascular responses in other ways, for example we have previously shown that it reduces the potency of acetylcholine as an endothelium-dependent vasodilator in the rabbit ear preparation (Randall *et al.*, 1990). This effect can be ascribed to either a reduction in either EDRF production or attenuation of its relaxant effects on vascular smooth muscle, or both (DeMey & Vanhoutte, 1983; Johns *et al.*, 1989; Warren *et al.*, 1989). The present results indicate that in hypoxia, vasodilatation to sodium nitroprusside is unaltered. Since this agent acts via stimulation of soluble guanylyl cyclase, and may be regarded as an analogue of EDRF, altered vascular smooth muscle responsiveness to EDRF does not appear to occur under the present experimental conditions. The impairment of endothelium-dependent relaxations in hypoxia that we have previously reported in the rabbit ear (Randall *et al.*, 1990) is therefore likely to be due to reduced EDRF synthesis.

Loss of basally released EDRF augments vasodilatation to endothelium-independent vasodilators (Shirasaki & Su, 1985; White *et al.*, 1986; Moncada *et al.*, 1991). To investigate whether hypoxia-induced changes in EDRF activity could account for the increase in potency for levcromakalim, experiments were carried out under normoxic conditions in which both agonist-stimulated and basal EDRF activity were abolished by perfusion with L-NAME (Randall & Griffith, 1991). Under these conditions the vasodilator potency of levcromakalim was increased 6 fold, a change greater than that in hypoxia (3 fold). In the absence of EDRF activity, sodium nitroprusside underwent a similar 7 fold increase in potency, while vasodilatation to verapamil and pinacidil were unaffected. That vasodilatation to nitrovasodilators is enhanced in the absence of EDRF has been known for sometime (Shirasaki & Su, 1985) and has been ascribed to increased sensitivity of guanylyl cyclase on loss of the basal EDRF input (Moncada *et al.*, 1991). Alternatively, basal EDRF activity has a tonic vasodilator input which is abolished, thus enabling other vasodilators to have greater impact. This idea is supported by augmented responses to the endothelium-independent agent isoprenaline in the rabbit aorta after endothelial loss (White *et al.*, 1986). In the present study loss of EDRF activity was accompanied by increased potency for both levcromakalim and sodium nitroprusside but not verapamil or pinacidil. The observation that the responses to verapamil and pinacidil were unaffected, suggests that non-specific augmentation through loss of tonic vasodilator input is not a contributory factor. Therefore, the increased potency of sodium nitroprusside observed in the present study is probably due to increased sensitivity of guanylyl cyclase on loss of the basal input of EDRF (Moncada *et al.*, 1991).

The increased potency of levcromakalim after inhibition of EDRF activity requires explanation. Since cromakalim acts independently of guanylyl cyclase (Taylor *et al.*, 1988) and the vasodilatation to levcromakalim in the present study was fully reversed by glibenclamide, then increased sensitivity of guanylyl cyclase presumably cannot account for this change. Non-specific effects of L-NAME are unlikely since destruction of the endothelium also results in increased potency of levcromakalim in the isolated rat mesenteric arterial bed (C.R. Hiley, personal communication). The possibility that L-NAME acts to uncouple oxidative phosphorylation in a manner akin to CCCP can be excluded because vasoconstrictor responses are augmented rather than impaired in the presence of L-NAME (Randall & Griffith, 1991). Nitric oxide, in some (Tare *et al.*, 1990; Garland & McPherson, 1992) but not all (Komori *et al.*, 1988) vascular preparations may exert a hyperpolarizing effect. If nitric oxide exerts a hyperpolarizing effect in the rabbit ear then inhibition of this input may lead to vascular smooth muscle depolarization, which might enable a potassium channel opener to have a greater hyperpolarizing effect leading to augmented vasodilatation. However, since only relaxations to levcromakalim and not those to pinacidil were augmented after L-NAME, such a mechanism would appear unlikely.

Associated with the hyperpolarizing effects of nitric oxide on vascular smooth muscle is the cyclic GMP-dependent activation of calcium-sensitive potassium channels leading to hyperpolarization and relaxation (Fujino *et al.*, 1991). This action of nitric oxide may account for its synergism with cromakalim reported by others (Rae & Corrêa, 1992). In the present experiments, loss of EDRF activity after inhibition with L-NAME may potentially enable levcromakalim to have greater impact through the above mechanism but why the responses to pinacidil are not similarly influenced is at present unclear.

Although hypoxia results in impaired endothelium-dependent relaxations (Randall *et al.*, 1990), it did not, in the present study, augment vasodilatation to sodium nitroprusside. This suggests that the level of hypoxia used did not alter basal EDRF activity for reductions in basal EDRF activity are known to augment relaxations to nitrovasodilators through supersensitivity of guanylate cyclase (Shirasaki & Su, 1985; Moncada *et al.*, 1991). That hypoxia does not substantially alter the basal release of EDRF accords with previous findings in bovine aorta endothelial cells (Richards *et al.*, 1991). Furthermore, previous studies have indicated that basal, but not stimulated, EDRF release is resistant to the effects of full inhibition of oxidative metabolism (Griffith *et al.*, 1986; 1987). CCCP, at the concentration used, did not impair endothelium-dependent vasodilatation to acetylcholine while previous work has shown that higher concentrations of CCCP do reduce agonist-stimulated but not basal EDRF activity (Griffith *et al.*, 1986). Therefore the experiments with

CCCP have effectively dissociated a reduction in oxidative metabolism and altered basal EDRF synthesis, indicating that a reduction in oxidative metabolism may augment relaxations to levcromakalim without influencing EDRF activity. This enables the conclusion to be drawn in that hypoxia, the augmentation of vasodilatation to levcromakalim is likely to be due to reduced oxidative metabolism leading to reduced ATP content in the vascular smooth muscle favouring the action of potassium channel activators.

The results of the present study indicate that the vasodilator potency of levcromakalim, but not that of pinacidil, is increased both in hypoxia and the absence of EDRF activity. That hypoxia may promote the action of levcromakalim is a novel and important finding and may indicate that this compound may be effective at increasing blood flow to acutely ischaemic organs. This complements previous work showing that cromakalim can increase blood flow to chronically ischaemia tissues (Angersbach & Nicholson, 1988). Our findings may also explain the selectivity shown by levcromakalim in substantially improving collateral flow after acute arterial occlusion (Randall & Griffith, 1992). The increase in potency found in hypoxia would appear independent of EDRF activity, and is probably related to a reduction in intracellular ATP levels in vascular smooth muscle. In addition to these findings is the observation that the potency of levcromakalim is also enhanced in the absence of basal EDRF, which may enable levcromakalim to dilate selectively vessels where endothelial activity is impaired, for example through various disease states eg. hypertension (Lüscher & Vanhoutte, 1986; Mayhan *et al.*, 1987), atherosclerosis (Harrison *et al.*, 1987) or hypercholesterolemia (Verbeuren *et al.*, 1986). The results also indicate differences in the pharmacology of levcromakalim and pinacidil, the significance of which remains to be determined.

This work was funded by a grant from the British Heart Foundation. The authors thank Professor G.M. Roberts of the University of Wales College of Medicine for his support and interest.

References

ANGERSBACH, D. & NICHOLSON, C.D. (1988). Enhancement of muscle blood cell flux and pO_2 by cromakalim (BRL 34915) and other compounds enhancing K^+ conductance, but not Ca^{2+} antagonists or hydralazine, in an animal model of occlusive arterial disease. *Naunyn-Schmiedebergs Arch. Pharmacol.*, **337**, 341–346.

BUCKINGHAM, R.E., CLAPHAM, J.C., HAMILTON, T.C., LONGMAN, S.D., NORTON, J. & POYSNER, R.H. (1986). BRL 34915, a novel antihypertensive agent: comparison of effects on blood pressure and other haemodynamic parameters with those of nifedipine in animal models. *J. Cardiovasc. Pharmacol.*, **8**, 798–804.

COOK, N.S. & QUAST, U. (1990). Potassium channel pharmacology. In *Potassium Channels: Structure, Classification, Function and Therapeutic Potential.* pp. 181–255, ed. Cook, N.S. Chichester, West Sussex: Halstead Press.

DAUT, J., MAIER-RULDOLPH, W., VON BECKERATH, N., MEHRKE, G., GÜNTHER, K. & GOEDEL-MEINEN, L. (1990). Hypoxic dilation of coronary arteries is mediated by ATP-sensitivie potassium channels. *Science*, **247**, 1341–1344.

DEMEY, J.G. & VANHOUTTE, P.M. (1983). Anoxia and endothelium-dependent reactivity in the canine femoral artery. *J. Physiol.*, **335**, 65–74.

EDWARDS, G. & WESTON, A.H. (1990). Potassium channel openers and vascular smooth muscle relaxation. *Pharmacol. Ther.*, **48**, 237–258.

FAN, Z., NAKAYAMA, K. & HIRAOKA, M. (1990). Multiple actions of pinacidil on adenosine triphosphate-sensitive potassium channels in guinea-pig ventricular myocytes. *J. Physiol.*, **430**, 273–295.

FUJINO, K., NAKAYA, S., NAKATSUKI, T., MIYOSHI, Y., NAKAYA, Y., MORI, H. & INOUE, I. (1991). Effects of nitroglycerin on ATP-induced Ca^{++}-mobilization, Ca^{++}-activated K channels and contraction of cultured smooth muscle cells of porcine coronary artery. *J. Pharmacol. Exp. Ther.*, **256**, 371–377.

FURCHGOTT, R.F. & ZAWADZKI, J.V. (1980). The obligatory role of endothelial cells in the relaxation of vascular smooth muscle by acetylcholine. *Nature*, **288**, 373–376.

GARLAND, C.J. & MCPHERSON, G.A. (1992). Evidence that nitric oxide does not mediate the hyperpolarization and relaxation to acetylcholine in the rat small mesenteric artery. *Br. J. Pharmacol.*, **105**, 429–435.

GRIFFITH, T.M., EDWARDS, D.H. & HENDERSON, A.H. (1987). Unstimulated release of endothelium-derived relaxing factor is independent of mitochondrial ATP generation. *Cardiovasc. Res.*, **21**, 565–568.

GRIFFITH, T.M., EDWARDS, D.H., NEWBY, A.C., LEWIS, M.J. & HENDERSON, A.H. (1986). Production of endothelium-derived relaxant factor is dependent on oxidative phosphorylation and extracellular calcium. *Cardiovasc. Res.*, **20**, 7–12.

HAMILTON, T.C., WEIR, S.W. & WESTON, A.H. (1986). Comparison of the effects of BRL 34915 and verapamil in the electrical and mechanical activity in rat portal vein. *Br. J. Pharmacol.*, **88**, 103–111.

HARRISON, D.G., FREIMAN, P.C., ARMSTRONG, M.L., MARCUS, M.L. & HEISTAD, D.D. (1987). Alterations of vascular reactivity in atherosclerosis. *Cir. Res.*, **61**, II-74–II-80.

JOHNS, R.A., LINDEN, J.M. & PEACH, M.J. (1989). Endothelium-dependent relaxation and cyclic GMP accumulation in rabbit pulmonary artery are selectively impaired by moderate hypoxia. *Circ. Res.*, **65**, 1508–1515.

KAJIOKA, S., NAKASHIMA, M., KITAMURA, K. & KURIYAMA, H. (1991). Mechanisms of vasodilatation induced by potassium channel activators. *Clin. Sci.*, **81**, 129–139.

KOMORI, K., LORENZ, R.R. & VANHOUTTE, P.M. (1988). Nitric oxide, Ach, and electrical and mechanical properties of canine arterial smooth muscle. *Am. J. Physiol.*, **255**, H207–H212.

LAWSON, K., BARRAS, M., ZAZZI-SUDRIEZ, E., MARTIN, D.J., ARMSTRONG, M. & HICKS, P.E. (1992). Differential effects of endothelin-1 on the vasorelaxant properties of benzopyran and non-benzopyran potassium channel openers. *Br. J. Pharmacol.*, **107**, 58–65.

LONGMAN, S.D., CLAPHAM, J.C., WILSON, C. & HAMILTON, T.C. (1988). Cromakalim, a potassium channel activator: a comparison of its cardiovascular haemodynamic profile and tissue specificity with those of pinacidil and nicorandil. *J. Cardiovasc. Pharmacol.*, **12**, 535–542.

LÜSCHER, T.F. & VANHOUTTE, P.M. (1986). Endothelium-dependent contractions to acetylcholine in the aorta of the spontaneously hypertensive rat. *Hypertension*, **8**, 344–348.

MAYHAN, W.G., FARACI, F.M. & HEISTAD, D.D. (1987). Impairment of endothelium-dependent responses of cerebral arterioles in chronic hypertension. *Am. J. Physiol.*, **253**, H1453–1440.

MCPHERSON, G.A. & ANGUS, J.A. (1990). Characterization of responses to cromakalim and pinacidil in smooth and cardiac muscle by use of selective antagonists. *Br. J. Pharmacol.*, **100**, 201–206.

MISLER, D.S., FALKE, L.C., GILLIS, K. & MCDANIEL, M.L. (1986). A metabolite regulated potassium channel in rat pancreatic B cells. *Proc. Natl. Acad. Sci. U.S.A.*, **83**, 7119–7123.

MONCADA, S., REES, D.D., SHULZ, R. & PALMER, R.M.J. (1991). Development and mechanisms of a specific supersensitivity to nitrovasodilators following inhibition of vascular nitric oxide synthesis in vivo. *Proc. Natl. Acad. Sci. U.S.A.*, **88**, 2166–2170.

NICHOLS, C.G. & LEDERER, W.J. (1991). Adenosine triphosphate-sensitive potassium channels in the cardiovascular system. *Am. J. Physiol.*, **261**, H1675–1686.

NOMA, A. (1983). ATP regulated K^+ channels in cardiac muscle. *Nature*, **305**, 147–148.

RAE, G.A. & CORRÊA, D.S. (1992). Effect of cromakalim on endothelium-dependent vasodilation in rat aortic rings. *J. Vasc. Res.*, **29**, 185.

RAKUGI, H., TABUCHI, Y., NAKAMARU, M., NAGANO, M., HIGASHIMORI, H., MIKAMI, H., OGIHARA, T. & SUZUKI, N. (1990). Evidence for endothelin-1 release from resistance vessels of rats in response to hypoxia. *Biochem. Biophys. Res. Commun.*, **169**, 973–977.

RANDALL, M.D., EDWARDS, D.H. & GRIFFITH, T.M. (1990). Activities of endothelin-1 in the vascular network of the rabbit ear: a microangiographic study. *Br. J. Pharmacol.*, **101**, 781–788.

RANDALL, M.D. & GRIFFITH, T.M. (1991). Differential effects of L-arginine on the inhibition of N^G-nitro-L-arginine methyl ester of basal and agonist-stimulated EDRF activity. *Br. J. Pharmacol.*, **104**, 743–749.

RANDALL, M.D. & GRIFFITH, T.M. (1992). Effects of 38227, sodium nitroprusside and verapamil on collateral perfusion following acute arterial occlusion in the isolated rabbit ear. *Br. J. Pharmacol.*, **106**, 315–323.

RANDALL, M.D. & GRIFFITH, T.M. (1993). The effects of hypoxia on vasodilatation to BRL 38227 in the isolated perfused rabbit ear. *Br. J. Pharmacol.* (in press).

RICHARDS, J.M., GIBSON, I.F. & MARTIN, W. (1991). Effects of hypoxia and metabolic inhibitors on production of prostacyclin and endothelium-derived relaxing factor by pig aortic endothelial cells. *Br. J. Pharmacol.*, **102**, 203–209.

SHIRASAKI, Y. & SU, C. (1985). Endothelium removal augments vasodilatation by sodium nitroprusside and sodium nitrite. *Eur. J. Pharmacol.*, **114**, 93–96.

STANDEN, N.B., QUAYLE, J.M., DAVIES, N.W., BRAYDEN, J.E., HUANG, Y. & NELSON, M.T. (1989). Hyperpolarizing vasodilators activate ATP-sensitive K^+ channels in arterial smooth muscle. *Science*, **245**, 177–180.

STURGESS, N.C., ASHFORD, M.J.L., COOK, D.L. & HALES, C.N. (1985). The sulphonylurea receptor may be an ATP-sensitive potassium channel. *Lancet*, **ii**, 474–475.

TARE, M., PARKINGTON, H.C., COLEMAN, H.A., NEILD, T.O. & DUSTING, G.J. (1990). Hyperpolarization and relaxation of arterial smooth muscle caused by nitric oxide derived from endothelium. *Nature*, **346**, 69–71.

TAYLOR, S.G., SOUTHERTON, J.S., WESTON, A.H. & BAKER, J.R.L. (1988). Endothelium-dependent effects of acetylcholine in rat aorta. A comparison with sodium nitroprusside and cromakalim. *Br. J. Pharmacol.*, **94**, 853–863.

VERBEUREN, T.J., JORDAENS, F.H., ZONNEKEYN, L.L., VAN HOVE, C.E., COENE, M.C. & HERMAN, A.G. (1986). Effect of hypercholesterolemia on vascular reactivity in the rabbit. *Circ. Res.*, **58**, 552–554.

WARREN, J.B., MALTBY, N.H., MACCORMACK, D. & BARNES, P.J. (1989). Pulmonary endothelium-derived relaxing factor is impaired in hypoxia. *Clin. Sci.*, **77**, 671–676.

WHITE, D.G., LEWIS, M.J., GRIFFITH, T.M., EDWARDS, D.H. & HENDERSON, A.H. (1986). Influence of endothelium on drug-induced relaxation of rabbit aorta. *Eur. J. Pharmacol.*, **121**, 19–23.

(Received November 16, 1992
Revised January 15, 1993
Accepted January 29, 1993)