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HYPOXIC VASODILATATION IN ISOLATED, PERFUSED GUINEA-PIG HEART: AN ANALYSIS OF THE UNDERLYING MECHANISMS

BY NICOLAS VON BECKERATH, SABINE CYRYS, ANTON DISCHNER AND JÜRGEN DAUT*

From the Physiologisches Institut der Technischen Universität München, Biedersteiner Strasse 29, D-8000 München 40, Germany

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SUMMARY

1. The mechanisms underlying hypoxic dilatation of coronary arteries were studied in isolated guinea-pig hearts perfused with physiological salt solution at 37 °C. The hearts were perfused at a constant rate of 3–10 ml min^{-1}; coronary perfusion pressure (CPP) and isovolumetric left ventricular pressure (LVP) were measured with piezoresistive transducers.

2. Addition of the K^+ channel opener cromakalim (500 nM) to the perfusate caused a maximal vasodilatation in beating hearts, i.e. a decrease in CPP of about 50%. Switching from normal perfusate (partial pressure of O_2 (P_{O_2}), 650–700 mmHg) to hypoxic perfusate (P_{O_2}, 9–10 mmHg) caused a similar vasodilatation. Both of these effects were prevented by 2 μM-glibenclamide, a blocker of ATP-sensitive potassium channels. Hypoxic vasodilatation was accompanied by a marked decrease in LVP, which was reduced by 56 ± 22% (mean ± S.D.) in the presence of glibenclamide.

3. In hearts arrested by increasing the K^+ concentration of the perfusate to 15 mM, the addition of the adenosine-uptake inhibitor dipyridamole evoked a maximal vasodilatation and this was inhibited by 76 ± 7% in the presence of glibenclamide.

4. The adenosine antagonist 8-phenyltheophylline (8-PT; 5 μM) inhibited the vasodilatation induced by dipyridamole by 88 ± 10%. In contrast, hypoxic vasodilatation was unaffected by 5 μM 8-PT. This suggests that hypoxic dilatation of coronary arteries is not mediated by release of adenosine from cardiomyocytes.

5. In order to test whether release of endothelium-derived relaxing factor (EDRF) contributed to hypoxic vasodilatation we blocked EDRF synthesis with N^{ω}-nitro-L-arginine (NNA). When applied at a perfusion rate of 10 ml min^{-1} to arrested hearts, 10 μM-NNA increased CPP by 35% and prolonged the delay between application of hypoxic solution and half-maximal vasodilatation from 52 ± 9 to 129 ± 29 s.

6. Under control conditions the relation between perfusion rate and the CPP measured in the steady state was linear. In the presence of 10 μM-NNA coronary resistance was increased more than twofold at low perfusion rates; at perfusion rates between 4 and 10 ml min^{-1} coronary resistance decreased progressively. This change

* To whom correspondence should be addressed.

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in the pressure–flow relationship may be responsible for the alterations in the time course of hypoxic vasodilatation induced by NNA.

7. In order to test whether changes in energy metabolism in coronary smooth muscle cells were responsible for hypoxic vasodilatation we blocked glycolysis by replacing the glucose in the perfusate with deoxyglucose (DOG). DOG produced a near-maximal dilatation of coronary arteries that could be reversed by 2 μM-glibenclamide but was unaffected by 5 μM-8-PT.

8. Our results suggest that adenosine can induce the opening of ATP-sensitive potassium channels in smooth muscle cells of the coronary resistance vessels, but that this is not the principal mechanism underlying hypoxic vasodilatation. It is proposed that early hypoxic dilatation of coronary arteries is mediated by changes in the submembrane concentrations of adenine nucleotides in vascular smooth muscle cells.

INTRODUCTION

According to the classical adenosine hypothesis the dilatation of coronary arteries during hypoxia or after ischemia is related to the release of adenosine from hypoxic cardiomyocytes (Berne, 1963, 1980; Gerlach, Deuticke & Dreisbach, 1963). Recently it has been shown that hypoxic dilatation of coronary arteries in isolated perfused hearts can be prevented by application of glibenclamide, a blocker of ATP-sensitive potassium channels (K^+_{ATP} channels; Daut, Maier-Rudolph, von Beckerath, Mehrke, Günther & Goedel-Meinen, 1990). It was therefore suggested that hypoxic vasodilatation may be mediated by the following cascade of events: (i) opening of K^+_{ATP} channels in coronary smooth muscle cells, (ii) hyperpolarization of smooth muscle cells, (iii) reduction of the open-state probability of voltage-sensitive calcium channels, (iv) decrease in the free intracellular calcium concentration, and (v) relaxation of smooth muscle cells in coronary resistance vessels.

This hypothesis is not necessarily in conflict with the adenosine hypothesis, because adenosine might induce opening of K^+_{ATP} channels in coronary smooth muscle cells. In cardiomyocytes adenosine can indeed open K^+_{ATP} channels by a direct G protein-mediated mechanism, i.e. without soluble intracellular second messengers (Kirsch, Codina, Birnbaumer & Brown, 1990). Since the dilatation of coronary arteries induced by adenosine can be inhibited by glibenclamide (Daut *et al.* 1990) it appears possible that in vascular smooth muscle cells too, adenosine can open K^+_{ATP} channels. This would imply that hypoxic vasodilatation is related to the energy metabolism of cardiomyocytes because the release of adenosine appears to be linked to the cytosolic phosphorylation potential (Olsson & Bünger, 1987). Alternatively, the opening of the K^+_{ATP} channels may be mediated by the effects of hypoxia on the energy metabolism of the smooth muscle cells, for example via changes in the submembrane concentrations of ATP and/or ADP. Both alternative mechanisms have been found in other cell types (Ashcroft & Ashcroft, 1990; Kirsch *et al.* 1990), but neither of them has been shown to exist in coronary arteries.

Hypoxic dilatation of coronary blood vessels is most conspicuous in the small arterioles. The investigation of vasodilatory mechanisms in the microvasculature is difficult, because at least three different cell types are involved: coronary smooth muscle cells, cardiomyocytes, and coronary endothelial cells. In the terminal

arterioles these three cells types are closely apposed, and the restricted perivascular space between them allows for 'cross talk' by means of vasoactive substances released locally (Rovetto, Ford & Yassin, 1987; Bassingthwaighte, Wang & Chan, 1989). For elucidating the mechanisms of hypoxic vasodilatation quantitative information about the release of vasoactive compounds, about membrane transport processes and about the electrophysiology of the three cell types is required. These studies have to be done on isolated cells because the microvasculature is rather inaccessible for electrophysiological studies. On the other hand, it would be extremely difficult to mimic the cellular architecture and the chemical communication in the restricted interstitial space with isolated cells.

In view of these difficulties we decided to use isolated perfused guinea-pig hearts to reinvestigate the mechanism of hypoxic vasodilatation in the light of recent electrophysiological and biochemical findings. The principal question we asked was: is the opening of K^+_{ATP} channels during hypoxia caused by the release of adenosine or is it a consequence of changes in the energy metabolism of coronary smooth muscle cells? In trying to answer this question we studied the effects of the following interventions: (i) blockage of K^+_{ATP} channels with glibenclamide, (ii) blockage of adenosine uptake with dipyridamole, (iii) blockage of adenosine receptors with 8-phenyltheophylline, (iv) blockage of endothelium-derived relaxing factor (EDRF) synthesis with N^{ω}-nitro-L-arginine, and (v) blockage of glycolysis with deoxyglucose. Some of the results have been communicated in preliminary form (von Beckerath, Cyrys, Dischner & Daut, 1991).

METHODS

Guinea-pigs weighing 200–300 g were anaesthetized by intraperitoneal injection of 50 mg pentobarbitone per animal. The heart was quickly removed and the aorta was attached to a perfusion cannula made of polymethylmethacrylate. Throughout the experiment the coronary arteries were perfused at a constant rate through the aortic ostia, as shown in the schematic diagram of Fig. 1. The left ventricle was vented via a silicone tube inserted through the pulmonary vein. The perfusate could be changed with two inert electric valves (model LFYA, Lee, Frankfurt, Germany), which were coupled as indicated by the dotted lines. Constant flow was produced with two high-performance liquid chromatography (HPLC) pumps (model 2248, Pharmacia, Freiburg, Germany). At perfusion rates of 5–10 ml min^{-1} the pumps produced a pressure drop of 5–10 bar across a capillary tubing made of poly-ether-ether-ketone (PEEK, lumen 0·1 mm), which was shielded with stainless-steel tubing to minimize diffusion of gas across the wall. Since the resistance of the PEEK capillary was more than hundredfold larger than the resistance of the heart the perfusion rate changed less than 1 % when the resistance of the coronary arteries decreased during hypoxia or other interventions. The two PEEK capillaries were connected to a glass heat exchanger for reheating the solution to 37 °C.

With the electric valves positioned immediately in front of the heat exchanger the perfusate reaching the heart could be changed in less than 1 s. Ischemia could be introduced by opening another electric valve (see Fig. 1). The heart was submerged in a small temperature-controlled water bath at 37 °C. This increased the viability of the hearts considerably; usually the recordings lasted 4–6 h. Coronary perfusion pressure (CPP) in the aorta and isovolumetric left ventricular pressure (LVP) were measured with piezoresistive transducers (SensoNor 880). CPP was measured at a T-junction of the polymethylmethacrylate cannula to which the heart was attached. The transducer measuring LVP was connected to a water-filled latex balloon (Hugo Sachs Elektronik, Freiburg, Germany) inserted into the left ventricle through the pulmonary vein. When adjusting the volume of the balloon (pre-load 0–5 mmHg) care was taken to ensure that the balloon was still

slack so that the diastolic pressure of the left ventricle and not the compliance of the balloon was measured.

Immediately after excision the heart was perfused at a rate of 7 ml min^{-1} with a solution containing (mM): 105 NaCl; 15 KCl; 1 $CaCl_2$; 0·8 $MgCl_2$; 1 NaH_2PO_4; 24 $NaHCO_3$, 10 glucose and 5 sodium pyruvate. All solutions were pre-heated to 37 °C and equilibrated with 95% O_2–5% CO_2

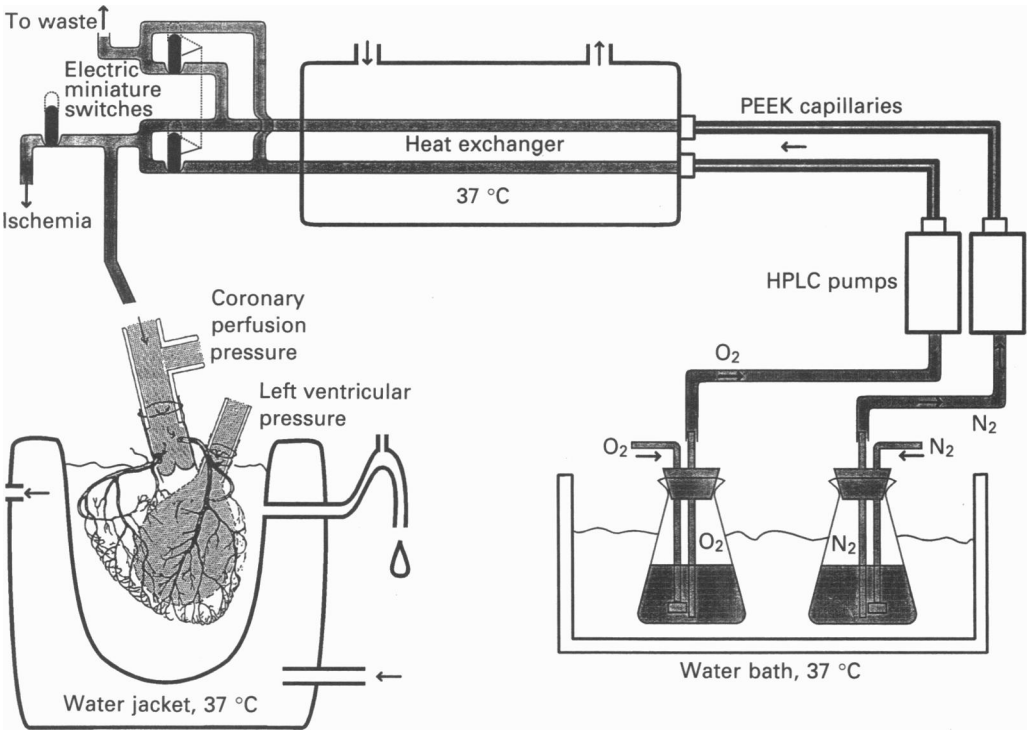

Fig. 1. Schematic diagram of the experimental set-up (see text).

in a water bath; the pH was 7·4. The elevated K^+ concentration caused diastolic arrest of the heart. Within 15 min the CPP usually rose to 50–80 mmHg. Subsequently the heart was perfused with pyruvate-free solution which otherwise had the same composition. For experiments on beating hearts we switched to a solution containing (mM): 116 NaCl; 4 KCl; 1 $CaCl_2$; 0·8 $MgCl_2$; 1 NaH_2PO_4; 24 $NaHCO_3$ and 10 glucose. The spontaneous heart rate was 3·5–4 Hz. In order to eliminate any effects of frequency changes on LVP, the hearts were electrically paced at a rate slightly above their spontaneous frequency. Hypoxia was induced by switching to an identical solution equilibrated with 95% N_2–5% CO_2 for at least 15 min. All connections were made of glass or stainless steel to minimize loss or uptake of oxygen. The P_{O_2} of the hypoxic perfusate was 9–10 mmHg in the aorta, and thus close to zero in the microvasculature.

When the heart had recovered from the dissection the perfusion rate was adjusted to give a CPP of about 60 mmHg. The range of perfusion rates was 3–10 ml min^{-1}, depending on the composition of the perfusate (see Results). Arrested hearts that did not develop a CPP of at least 60 mmHg at a perfusion rate of 10 ml min^{-1}, and beating hearts that did not produce an LVP of at least 50 mmHg were rejected. The pressure signals were stored on a digital audio tape-recorder (BioLogic, F-38130 Echirolles, France) at a sampling rate of 44 kHz. After the experiment the data were transferred to a microcomputer for analysis. The bit-mapped figures were then plotted on a Laser printer (Hewlett-Packard LaserJet II). The results reported below are given as means ± standard deviation (S.D.).

Drugs

Glibenclamide (from Hoechst, Frankfurt), cromakalim (BRL 34915) and lemakalim (BRL 38277, both from Beecham, Betchworth, Surrey), indomethacin and dipyridamole (both from Sigma, Deisenhofen, Germany) were dissolved in dimethyl sulphoxide to give stock solutions of 2 or 5 mM. The final concentration of dimethyl sulphoxide in the perfusate was 0·1 % or less and had no effect on CPP or LVP. 8-Phenyltheophylline (8-PT, from Sigma or ICN) was dissolved in 0·1 M-NaOH (2 mM); after addition of 8-PT the pH of the perfusate was readjusted to 7·4 with HCl. N^{ω}-Nitro-L-arginine (N^{5}[nitroamidino]-L-2,5-diaminopentanoic acid; from Sigma), abbreviated NNA, was dissolved in water; the pH of the stock solution (2 mM) was adjusted to 7·4 with NaOH.

RESULTS

Blockage of ATP-sensitive K^+ channels with glibenclamide

The coronary arteries of isolated guinea-pig hearts were perfused with physiological salt solution at a constant rate. The CPP and the isovolumetric LVP were measured with piezoresistive transducers. Changes in CPP were used to monitor changes in vascular resistance, i.e. dilation or constriction of coronary resistance arteries. In beating hearts the rate of perfusion was usually 10 ml min^{-1}. The calcium concentration in the perfusate was lowered to 1 mM in order to reduce the LVP and myocardial oxygen consumption. The importance of ATP-sensitive potassium channels (K^+_{ATP} channels) determining the tone of coronary resistance vessels is illustrated in Fig. 2. Application of the K^+ channel opener cromakalim (500 nM), which opens K^+_{ATP} channels, reduced CPP by $51 \pm 5\%$ ($n = 23$; Fig. 2*A*). The decrease in CPP was accompanied by a small diphasic change in LVP; in the steady state 500 nM-cromakalim decreased LVP by $12 \pm 10\%$. The effects of cromakalim were reversed within 10 min after wash-out of the drug.

Hypoxic vasodilatation was induced by switching to a solution equilibrated with 95 % N_2–5 % CO_2 (see Methods). Hypoxia caused a decrease in CPP similar to that induced by cromakalim. This was accompanied by a rapid decrease of systolic LVP and an increase in diastolic LVP ($n = 30$). Hypoxic vasodilatation was reversed within 2 min after readdition of oxygen. Subsequent addition of 1 μM-adenosine to the perfusate caused a decrease in CPP similar to that caused by hypoxia. However, there was almost no concomitant change in LVP ($< 2\%$; $n = 5$). Figure 2*B* shows the effects of glibenclamide, a blocker of K^+_{ATP} channels, on the changes in CPP and LVP caused by cromakalim and hypoxia. Application of 2 μM-glibenclamide increased CPP in beating hearts only slightly ($3{\cdot}7 \pm 2{\cdot}7$ mmHg; $n = 15$). The vasodilation induced by cromakalim and hypoxia could be prevented almost completely by 2 μM-glibenclamide ($n = 15$). This was probably due to blockage of K^+_{ATP} channels in coronary smooth muscle cells. The decrease in LVP during hypoxia was inhibited by 30–90 % in the presence of glibenclamide, depending on the duration of the hypoxia. On average, glibenclamide inhibited the decrease in LVP during a 30 s hypoxic period by $56 \pm 22\%$ ($n = 15$). This may be explained by blockage of K^+_{ATP} channels in cardiomyocytes because 'early contractile failure' is partly due to a shortening of the ventricular action potential (Allen & Orchard, 1987), which is mediated by the opening of K^+_{ATP} channels (Sanguinetti, Scott, Zingaro & Siegl, 1988).

The vasodilatory effect of adenosine was reduced to $29 \pm 4\%$ in the presence of

glibenclamide ($n = 4$), as illustrated in Fig. 2. This may be explained by the hypothesis that the glibenclamide-sensitive part of the effects of adenosine is mediated by the opening of K^+_{ATP} channels in coronary smooth muscle cells (see Discussion). In order to demonstrate that the coronary arteries could still dilate

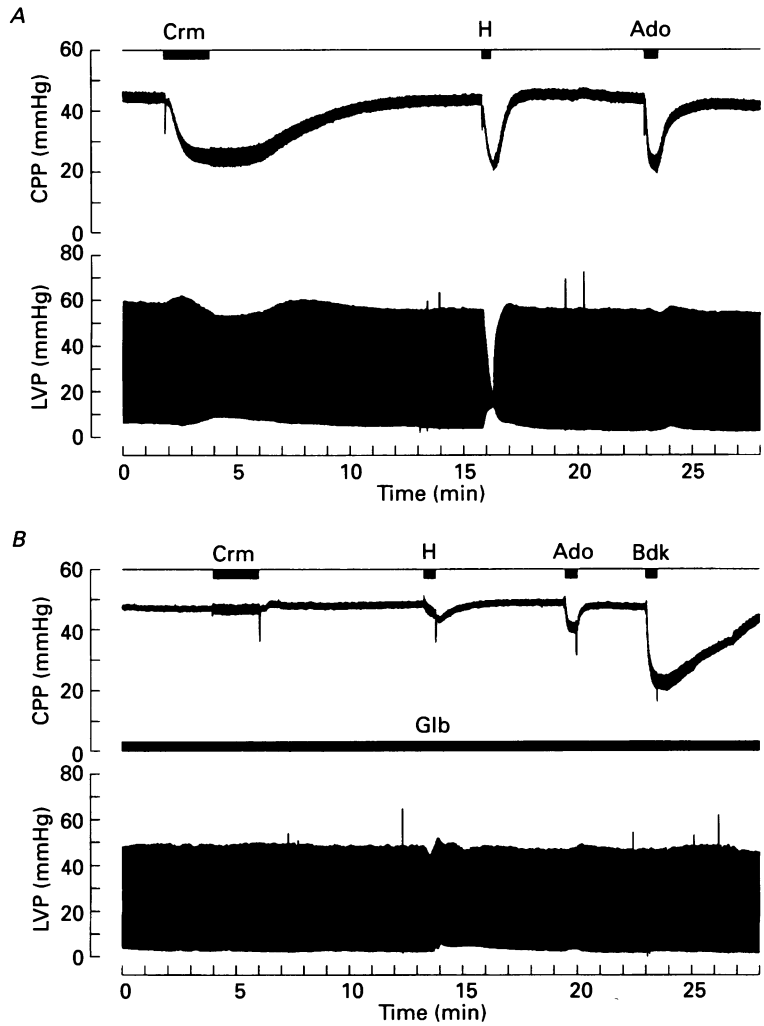

Fig. 2. The effects of cromakalim (Crm), hypoxia (H) and adenosine (Ado) on coronary perfusion pressure (CPP) and isovolumetric left ventricular pressure (LVP) in a beating heart. The perfusion rate was 10 ml min^{-1}. *A*, application of 500 nM-cromakalim, hypoxia, and 1 μM-adenosine under control conditions. *B*, application of 500 nM-cromakalim, hypoxia, 1 μM-adenosine, and 1 nM-bradykinin (Bdk) in the presence of 2 μM-glibenclamide (Glb). The solution changes are indicated by filled bars.

maximally in the presence of glibenclamide, we applied 1 nM-bradykinin, which is known to cause vasodilatation by inducing release of EDRF (Newby & Henderson, 1990). The vasodilatory effect of 1 nM-bradykinin was virtually unaffected by

glibenclamide. Similar results have been reported previously in arrested (Daut *et al.* 1990), but not in beating guinea-pig hearts.

At a perfusion rate of 10 ml min^{-1} beating guinea-pig hearts developed a CPP of 50–80 mmHg. Hypoxia, 1 μM-adenosine or 1 nM-bradykinin usually caused a reduction of CPP to 20–40 mmHg. The perfusion pressure could not be lowered further by any combination of vasodilatory stimuli, which suggests that under these conditions the resistance arteries were maximally dilated. The remaining pressure drop probably represents the lumped resistance of the capillaries, the large coronary arteries and the coronary veins.

Blockage of adenosine uptake with dipyridamole

In trying to elucidate the mechanism of action of adenosine it is important to discriminate between intravascular (luminal) and perivascular (abluminal) application of adenosine. It is well known that there can be considerable gradients of adenosine across the endothelium (Wangler, Gorman, Wang, DeWitt, Chan, Bassingthwaighte & Sparks, 1989; Mohrmann & Heller, 1990). Cardiomyocytes continuously release adenosine, and there is an active re-uptake of adenosine into myocytes and into coronary endothelial cells through a specific transporter (Rovetto *et al.* 1987). Thus the interstitial adenosine concentration is in a steady state determined by the rates of uptake and release of adenosine. The perivascular adenosine concentration can be increased by application of dipyridamole, which blocks adenosine uptake (Wangler *et al.* 1989). Figure 3*A* shows that addition of 500 nM-dipyridamole to the perfusate induced a vasodilatation similar to that caused by hypoxia. This vasodilatation was reversed within 20 min during wash-out of the drug. The right-hand part of Fig. 3*A* shows that the application of dipyridamole had no effect when it was repeated (about 50 min later) in the presence of 5 μM-8-PT (see below).

The main disadvantage of working with saline-perfused hearts is that the oxygen content of physiological salt solution equilibrated with 95% O_2–5% CO_2 at 37 °C is only about 10% of the oxygen content of arterial blood and that, in order to ensure adequate oxygenation, the rate of perfusion has to be correspondingly increased. In beating saline-perfused hearts it cannot be excluded that parts of the myocardium are hypoxic and that this alters coronary resistance. In most of our experiments we therefore arrested the heart by increasing the potassium concentration to 15 mM. This simplifies the experimental situation because oxygen consumption is greatly reduced and, as a result of this, the tone of the coronary arteries rises. Even so, the hearts had to be perfused at a rate of 4–10 ml min^{-1} to keep the CPP in the physiological range. The mean perfusion rate required to produce a CPP of 60 mmHg was $9{\cdot}1 \pm 1{\cdot}2$ ml min^{-1} ($n = 50$). This flow rate still leads to an increased synthesis of EDRF, mainly as a result of the increased shear stress at the vessel wall (Griffith & Edwards, 1990).

The elevated release of EDRF can interfere with the analysis of the cellular mechanisms of hypoxic vasodilatation. In some of our experiments we therefore added N^{ω}-nitro-L-arginine (NNA) to the perfusate, which prevents the synthesis of EDRF from L-arginine (see below). Figure 3*B* shows a repetition of the experiment of Fig. 3*A* with 15 mM-potassium and 10 μM-NNA in the perfusate. It can be seen

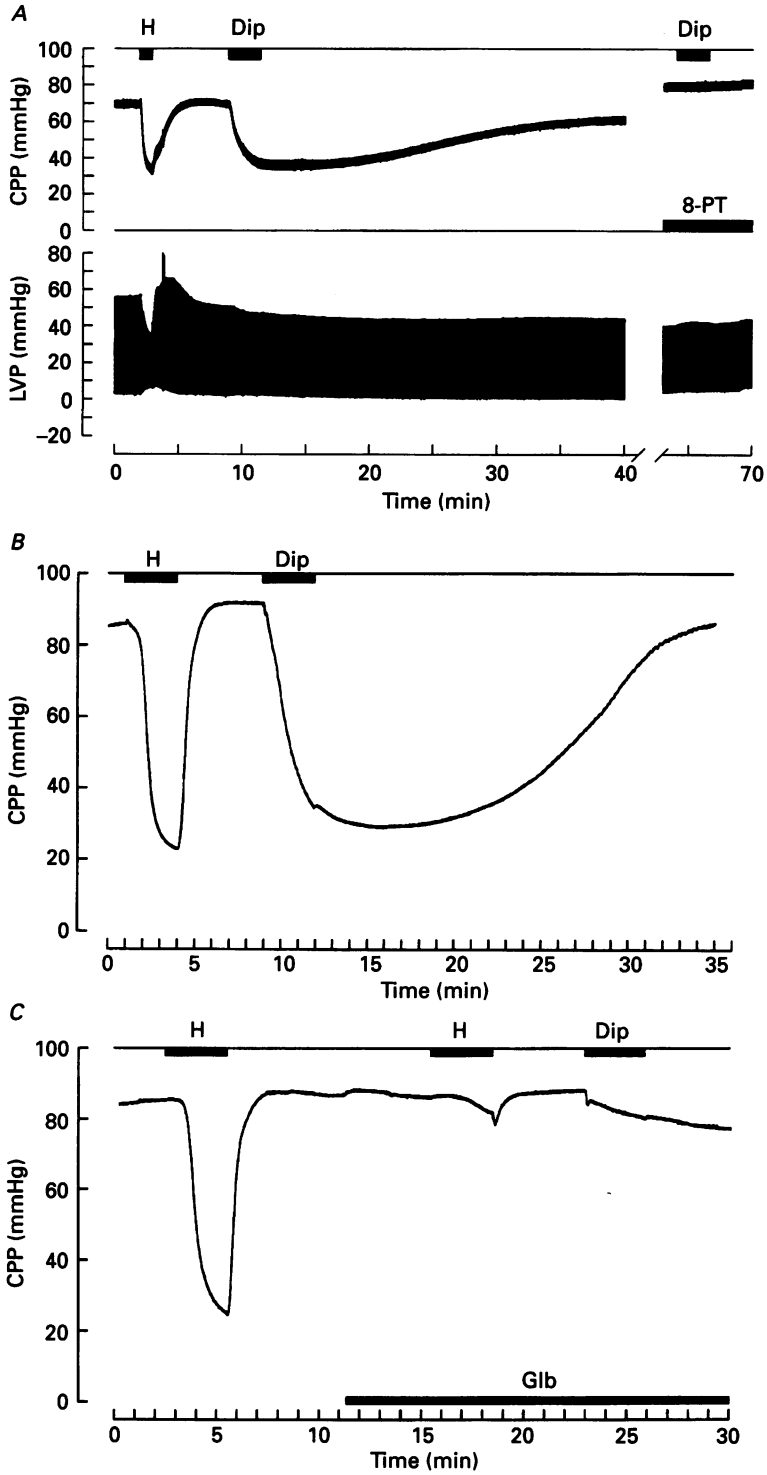

Fig. 3. For legend see facing page.

that the changes of CPP during hypoxia and during application of 500 nM-dipyridamole were qualitatively similar, but much larger than in the beating heart. The similarity between beating and arrested hearts illustrated in Fig. 3*A* and *B* suggests that the mechanisms of hypoxic and adenosine-induced vasodilatation are the same in beating and arrested NNA-perfused hearts. Thus the arrested heart appears to be a convenient model for studying the mechanisms of hypoxic vasodilatation because some of the uncontrolled variables are eliminated and the signal-to-noise ratio is increased.

The effects of glibenclamide on hypoxic vasodilatation and dipyridamole-dependent vasodilatation are illustrated in Fig. 3*C*. In the arrested heart 2 μM-glibenclamide increased CPP only by $1{\cdot}5 \pm 1{\cdot}4$ mmHg ($n = 25$). The vasodilatation caused by 500 nM-dipyridamole was reduced by $76 \pm 7\%$ ($n = 6$) in the presence of 2 μM-glibenclamide. This observation is consistent with the idea that the vasodilatory effect of perivascular adenosine is mainly due to the opening of K^+_{ATP} channels in coronary smooth muscle cells (see Discussion). It should be noted that the time course of the effects of dipyridamole varied considerably. Figure 3*B* and 4*A* show the two extremes in the delay between application of dipyridamole and its maximal effect. This variation may be due to differences between hearts in the permeability of the endothelial barrier which limits the diffusion of dipyridamole and the accumulation of adenosine in the interstitial space. The average delay between application of 500 nM-dipyridamole and half-maximal vasodilatation was $3{\cdot}5 \pm 2{\cdot}9$ min ($n = 14$). The magnitude of the inhibitory effects of glibenclamide (and 8-PT, see below) was not correlated with the time course of the effects of dipyridamole.

Blockage of adenosine receptors with 8-phenyltheophylline

The observation that both the effects of hypoxia and the effects of a presumed increase in perivascular adenosine concentration could be inhibited by addition of glibenclamide to the perfusate raises the possibility that hypoxic vasodilatation may be entirely attributable to release of adenosine from hypoxic cardiomyocytes. In order to test this hypothesis we looked at the effects of the xanthine derivative 8-PT, a blocker of adenosine receptors (Griffith, Meghji, Moody & Burnstock, 1981), on hypoxic and adenosine-induced vasodilatation. The vasodilatation evoked by 1 μM-adenosine in beating and arrested hearts was prevented completely by 5 μM-8-PT ($n = 8$, not shown). The vasodilatation evoked by 500 nM-dipyridamole was strongly inhibited by 5 μM-8-PT, as illustrated on the right-hand side of Fig. 3*A*.

Figure 4*A* shows a similar result obtained in an arrested heart. In this experiment

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Fig. 3. *A*, the effects of hypoxia (H) and 500 nM-dipyridamole (Dip) on CPP and LVP in a beating heart. The perfusion rate was 10 ml min^{-1}. Dipyridamole was washed out for about 50 min, then it was applied again (right), this time in the presence of 5 μM-8-phenyltheophylline (8-PT). *B*, the effects of hypoxia and dipyridamole (500 nM) on CPP in an arrested heart. The synthesis of EDRF was blocked with 10 μM-NNA. The perfusion rate was 8 ml min^{-1}. *C*, the effects of 2 μM-glibenclamide (Glb) on the vasodilatation induced by hypoxia and by 500 nM-dipyridamole. The records shown in *B* and *C* were from the same heart. *C*, was obtained before *B*; there was a 1 h interval to allow for complete wash-out of glibenclamide and dipyridamole.

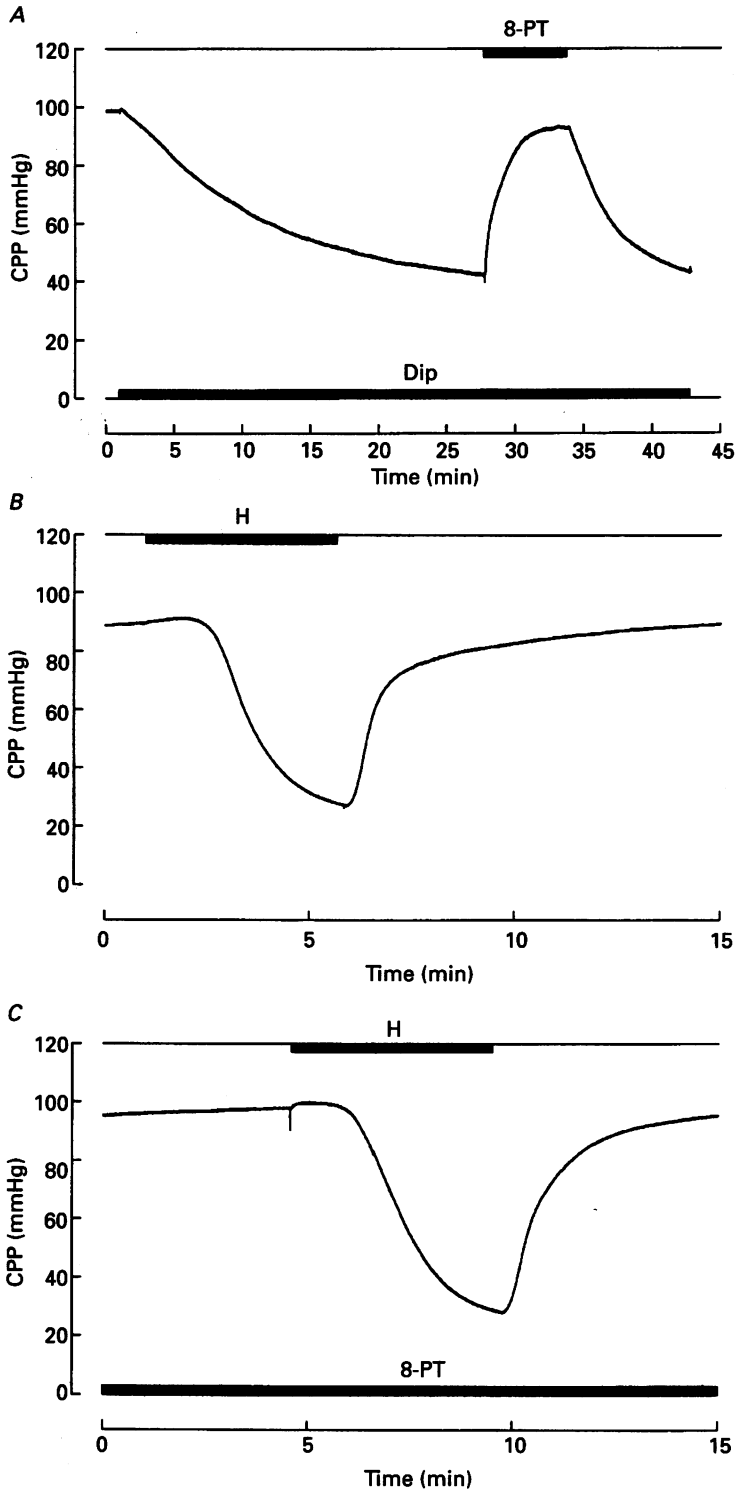

Fig. 4. For legend see facing page.

a near-maximal vasodilatation was induced by application of 500 nM-dipyridamole. Then 5 μM-8-PT was added in the continued presence of dipyridamole. The adenosine antagonist reversed the vasodilatation evoked by dipyridamole almost completely. The time course of the change in CPP during application of 8-PT presumably reflects the diffusion into the perivascular space and the binding of the drug. The blocking effect was complete within 5 min. Similar results were obtained in twelve further experiments. The average reduction of the dipyridamole-induced vasodilatation, measured 5 min after application of 5 μM-8-PT, was $88 \pm 10\%$. In dog hearts *in situ* 8-PT has also been found to inhibit the effects of both exogeneous adenosine (Wei, Kang & Merrill, 1989) and dipyridamole (Kanatsuka, Lamping, Eastham, Dellsperger & Marcus, 1989).

Figures 4*B* and *C* show the change in CPP induced by hypoxia in the same heart under control conditions, and after pre-incubation with 5 μM-PT for 5 min. It can be seen that the same concentration of 8-PT that abolished the vasodilatation evoked by dipyridamole had no effect on hypoxic vasodilatation in the same heart ($n = 10$). In beating hearts we found a similar clear-cut difference between the effects of 5 μM-8-PT on hypoxic and adenosine-induced vasodilatation. The amplitude of hypoxic vasodilatation was changed by less than 2% in the presence of 5 μM-8-PT, whereas the vasodilatation induced by 1 μM intravascular adenosine was blocked completely ($> 99\%$; $n = 5$). These findings argue against a causal link between an increased adenosine concentration in the interstitial space and early hypoxic vasodilatation.

With higher concentrations of 8-PT, however, the results were different. When the hearts were perfused with 20 μM-8-PT for 10 min (not shown) the decrease in CPP induced by 1 μM-adenosine ($n = 4$) or by hypoxia ($n = 4$) was greatly reduced or abolished (see also von Beckerath, Mehrke & Daut, 1990). This may be due to the fact that phenylxanthines can also produce effects other than blockage of adenosine receptors, for example inhibition of phosphodiesterase (see Discussion).

Blockage of EDRF synthesis with N^{ω}-nitro-L-arginine

It is now well established that release of nitric oxide (NO) from the endothelium can play an important role in the regulation of coronary tone, especially in saline-perfused hearts (Kelm & Schrader, 1990). Therefore we tried to block the release of EDRF with N^{ω}-nitro-L-arginine (NNA) and studied the resulting changes in hypoxic vasodilatation in the arrested guinea-pig heart. NNA is 70 times more potent as a specific inhibitor of EDRF synthesis than the commonly used N^{G}-monomethyl-L-arginine (Ishii, Chang, Kerwin, Huang & Murad, 1990). As can be seen from Fig. 5, addition of 10 μM-NNA increased CPP considerably. The measurements were usually started 15–30 min after application of the drug. At a perfusion rate of 10 ml min^{-1}, 10 μM-NNA increased CPP by $35 \pm 13\%$ ($n = 21$) in the first 15 min. After that, CPP continued to rise slowly, usually about 2–3 mmHg h^{-1}. The effects of NNA were not reversible after removal of the drug.

Fig. 4. *A*, inhibition of the vasodilatory effect of 500 nM-dipyridamole (Dip) by 5 μM-8-PT. *B*, hypoxic vasodilatation (H) in the same heart under control conditions. *C*, hypoxic vasodilatation in the same heart after pre-incubation with 5 μM-8-PT for 5 min. The perfusing solution contained 10 μM-NNA throughout the experiment. The perfusion rate was 4 ml min^{-1}.

The amplitude of hypoxic vasodilatation was much larger with NNA (compare Fig. 5*A* and *B*) because the CPP measured during hypoxia in the steady state was virtually the same as under control conditions. Surprisingly, the time course of hypoxic vasodilatation was changed considerably by NNA. Figure 5 shows that the

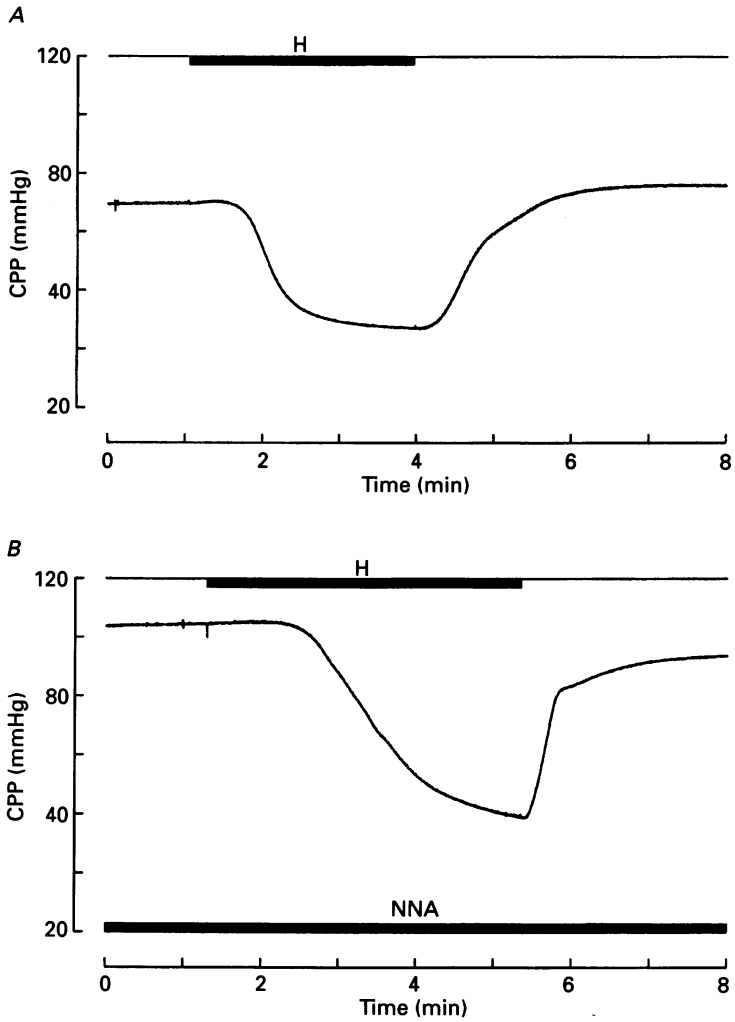

Fig. 5. The effects of NNA on hypoxic vasodilatation. *A*, control. *B*, 30 min after addition of 10 μM-NNA to the perfusate. The perfusion rate was 7 ml min^{-1} in *A* and *B*.

delay between application of hypoxic solution and the onset of the change in CPP became longer. At a constant flow rate of 10 ml min^{-1} the time interval between the application of hypoxic solution and half-maximal vasodilatation increased from 52 ± 9 s (control; $n = 47$) to 129 ± 29 s ($n = 11$) in the presence of NNA. At a first glance, this might be taken to indicate that the earliest phase of hypoxic vasodilatation was mediated by release of EDRF. However, it will be shown below that this is probably not so.

Another unexpected finding was that the relative increase in CPP produced by NNA at low rates of perfusion was much larger than that produced at high rates. When NNA was applied in hearts perfused at a rate of 5 ml min^{-1} it increased CPP by more than 100% ($n = 4$; not shown). In trying to understand this we looked at

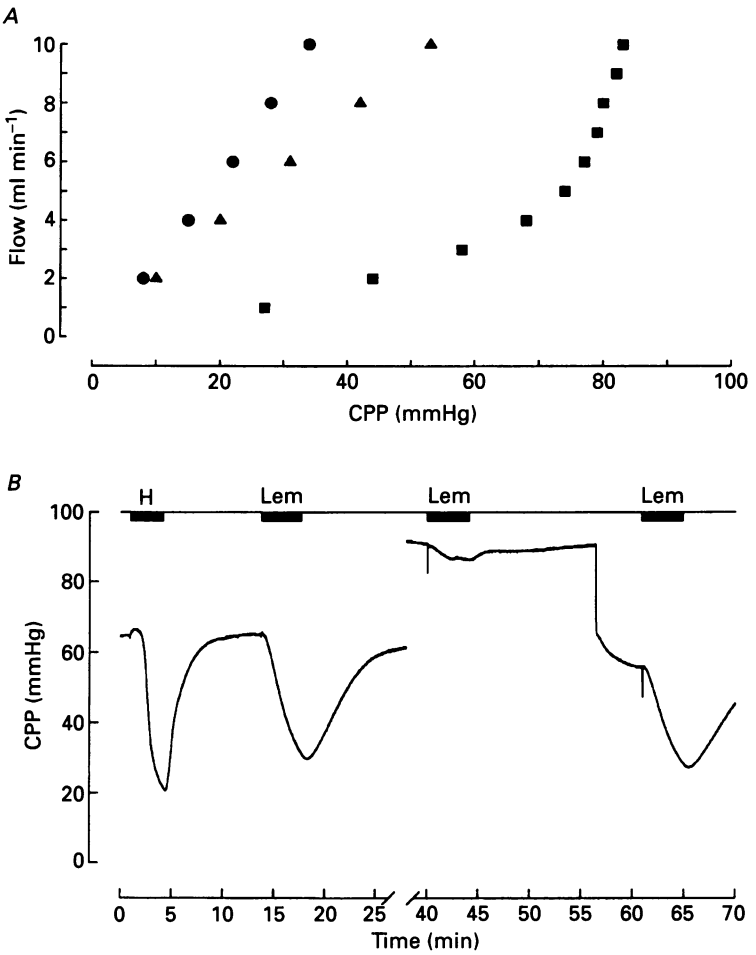

Fig. 6. *A*, the relation between flow rate and CPP in an arrested heart in the steady state; ▲, control; ■, 30 min after addition of NNA to the perfusate; ●, during hypoxia in the presence of NNA. *B*, the effects of the K^+ channel opener lemakalim (Lem) on CPP in an arrested heart treated with NNA. The flow rate was 4 ml min^{-1} (left, control), 10 ml min^{-1} (middle), and again 4 ml min^{-1} (right, re-control). Note the time interval between the two parts of the record.

the relationship between coronary flow and CPP with and without NNA. The results of a typical experiment are shown in Fig. 6*A*. Under control conditions the relation between the applied flow rate and the CPP measured in the steady state was linear in the range 2–10 ml min^{-1} ($n = 18$) (▲). During hypoxia, when the coronary arteries

were maximally dilated, the relationship between flow and pressure was linear both in the presence of NNA ($n = 3$; Fig. 6*A*, ●) and under control conditions ($n = 3$; not shown). In oxygenated hearts NNA increased the vascular resistance more than twofold at perfusion rates below 4 ml min^{-1} (■). At higher perfusion rates the

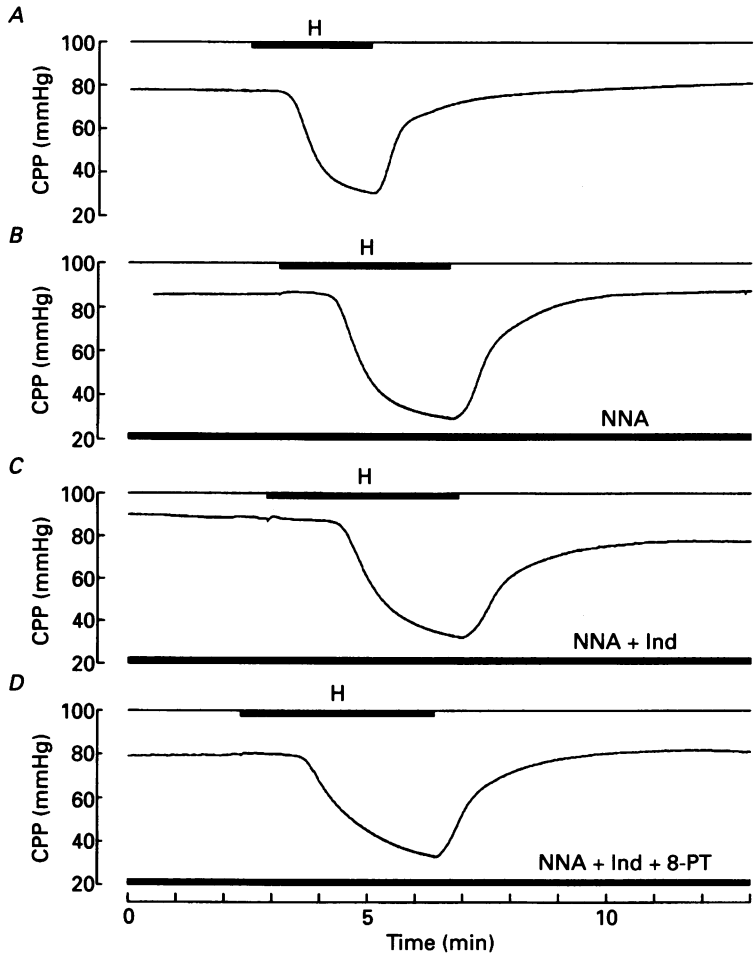

Fig. 7. The cumulative effect of various antagonists on the vasodilatation induced by hypoxia (H). *A*, control; perfusion rate 4 ml min^{-1}. *B*, with 10 μM-NNA. *C*, with 10 μM-NNA + 3 μM-indomethacin (Ind). *D*, with 10 μM-NNA + 3 μM-indomethacin + 5 μM-8-PT. In *B*, *C* and *D* the perfusion rate was 3 ml min^{-1}.

pressure–flow relationship was concave upwards, i.e. large changes in flow produced only small changes in pressure. Hence it might be that in this range a much larger vasodilatory stimulus was required to produce a given decrease in CPP. This could be the reason why in the presence of NNA it takes longer until the vasodilatation becomes obvious, i.e. more K^+ channels need to be opened to cause relaxation of the coronary smooth muscle cells (see Discussion).

The results presented in Fig. 6*B* are consistent with this interpretation. In this experiment a low concentration of the K^+ channel opener lemakalim (BRL 38227, the active enantiomer of cromakalim) was applied in the presence of 10 μM-NNA. At a perfusion rate of 4 ml min^{-1}, 100 nM-lemakalim caused a marked vasodilatation. When the same experiment was repeated at a perfusion rate of 10 ml min^{-1} the effect of lemakalim was drastically reduced. The right-hand side of Figure 6*B* shows the re-control after switching back to the lower perfusion rate. Since the K^+ channel openers are supposed to act directly on coronary smooth muscle cells, and not on the endothelium (Daut *et al.* 1990. Mehrke, Pohl & Daut, 1991*a*), it is unlikely that blockage of EDRF synthesis by NNA affected the mechanism of action of lemakalim. We therefore assume that the dose–response relationship of vasodilatory stimuli at high flow rates is distorted by application of NNA. In most experiments we reduced flow rate in the presence of NNA in order to remain in the linear part of the flow–pressure relationship. An example is shown in Fig. 7 (*A* and *B*). After the hypoxia under control conditions (perfusion rate, 4 ml min^{-1}) we applied 10 μM-NNA for 30 min, reduced the perfusion rate to 3 ml min^{-1} and repeated the hypoxia. Under these conditions ($n = 15$) the time course of hypoxic vasodilatation was changed much less than in the experiments in which the flow rate was kept constant (see Fig. 5).

Apart from EDRF, the endothelium releases several other autacoids, for example prostaglandins (Newby & Henderson, 1990). In coronary arteries the most important prostaglandin is prostacyclin, which can cause a marked vasodilatation. Thus increased secretion of prostacyclin by coronary endothelial cells might contribute to the decrease in CPP observed during hypoxia. This was tested by blocking cyclo-oxygenase activity with indomethacin, which prevents prostaglandin synthesis. After application of NNA the heart was perfused for 30 min with a solution containing 10 μM-NNA and 3 μM-indomethacin. It can be seen in Fig. 7*A* that indomethacin did not have any major effect on hypoxic vasodilatation ($n = 3$). Subsequently, we switched to a solution containing NNA, indomethacin, and 5 μM-8-PI. This solution also had no major effect on amplitude and time course of hypoxic vasodilatation (Fig. 7*D*). In contrast to this cocktail of ineffective inhibitors, 2 μM-glibenclamide alone prevented hypoxic vasodilatation (see Fig. 3*C*).

Blockage of glycolysis with deoxyglucose

The results presented so far show that our attempts to inhibit hypoxic vasodilatation by blocking the action of the known local regulators of vascular tone were unsuccessful. It is therefore necessary to consider the alternative hypothesis for the mechanism of hypoxic vasodilatation, namely that the opening of K^+_{ATP} channels may be related to changes in the energy metabolism of coronary smooth muscle cells. As a tool for altering cellular energy metabolism we used 2-deoxyglucose (DOG), which is known to block glycolysis. Figure 8*A* shows a typical experiment ($n = 9$) in which we studied the effect of deoxyglucose on the recovery from hypoxic vasodilatation. First, hypoxic vasodilatation was induced in the presence of 10 mM-glucose. After reoxygenation, hypoxia was repeated with a solution in which glucose was replaced by 10 mM-DOG. It is obvious that in the presence of DOG the coronary arteries could not constrict during re-oxygenation. After replacement of DOG by

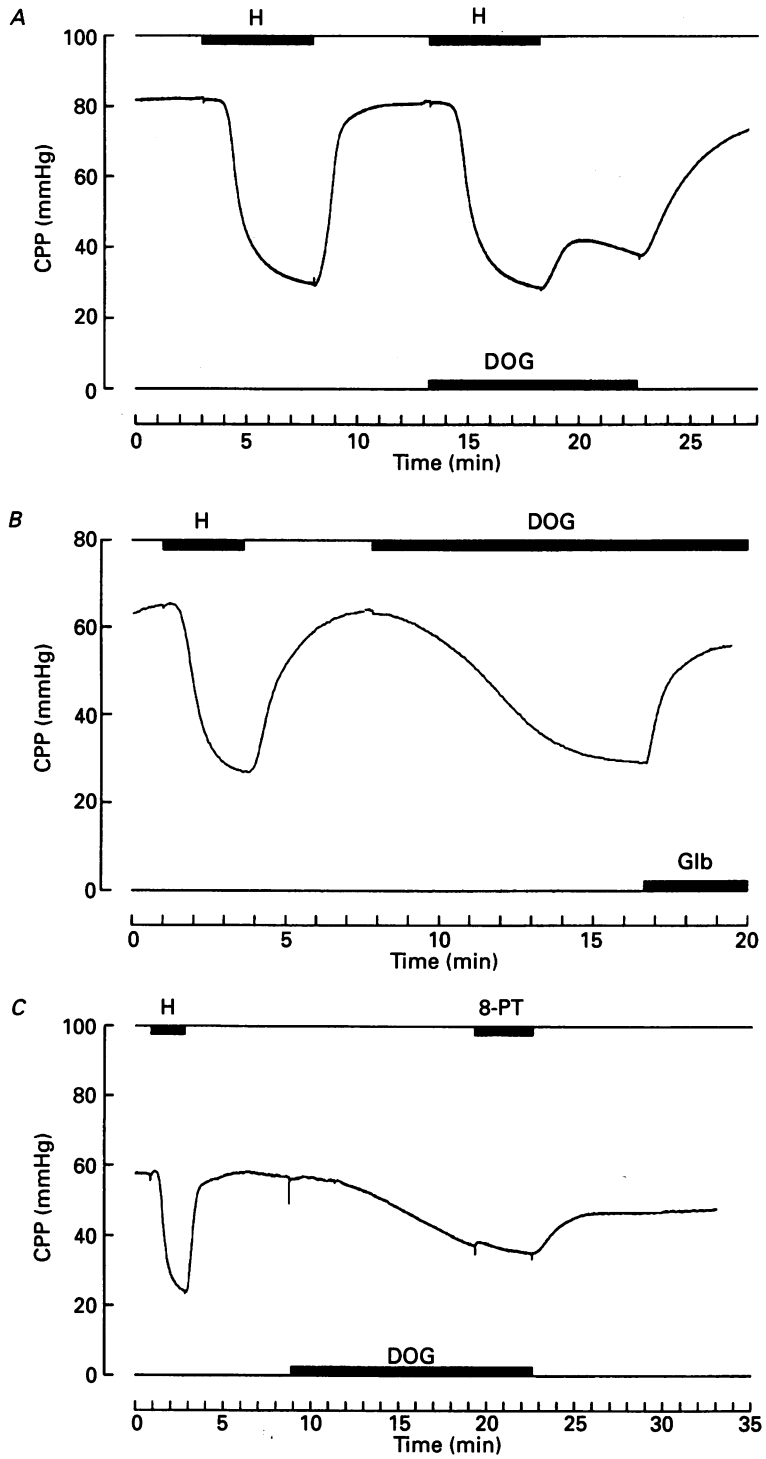

Fig. 8. For legend see facing page.

glucose the hypoxic vasodilatation was reversed completely within 10 min. The observation that glucose was necessary for the reversal of hypoxic vasodilatation is consistent with the idea that changes in cellular energy metabolism may be involved in the generation of hypoxic vasodilatation.

Even in the presence of oxygen, replacement of glucose by DOG caused a vasodilatation after a few minutes, as can be seen from Fig. 8*B*. The amplitude of this dilatation was $92 \pm 7\%$ of the amplitude of hypoxic vasodilatation ($n = 9$; no NNA added). The delay between application of DOG and the resulting vasodilatation showed considerable variation from one heart to the next, and in the course of experiments lasting several hours. The interval between application of DOG and the time when the amplitude of the DOG-induced vasodilatation reached 50% of its steady-state value ranged between 3·5 and 12 min; the mean was $7{\cdot}5 \pm 3$ min ($n = 9$). Figure 8*B* also shows the vasodilatation evoked by DOG could be inhibited by 2 μM-glibenclamide ($n = 5$). This suggests that the vasodilatation produced by DOG was also related to the opening K^+_{ATP} channels.

Now it could still be argued that the effects of DOG may be attributable to impairment of the energy metabolism of cardiac muscle cells, and not of coronary smooth muscle cells. The alteration of myocardial energy metabolism might then lead to release of adenosine and thus to agonist-induced opening of K^+_{ATP} channels. The experiment illustrated in Fig. 8*C* shows that this is probably not the case. The vasodilatation induced by DOG was not reversed by the adenosine antagonist 8-PT. The same concentration of 8-PT (5 μM) that reversed the effects of dipyridamole (Fig. 4*A*) produced hardly any change in CPP when added during the vasodilatation elicited by DOG ($n = 3$).

DISCUSSION

The role of the endothelium in hypoxic dilatation of coronary arteries

We have studied the mechanisms of hypoxic vasodilatation in isolated guinea-pig heart. In order to obtain interpretable measurements we have simplified the experimental situation as much as possible. HPLC pumps were used to produce a constant, pulsation-free perfusion of the coronary arteries, and electric miniature valves were used to allow for rapid switching between different solutions. In order to avoid changes in cardiac work the hearts were arrested by elevating the K^+ concentration of the perfusate to 15 mM. Under these conditions hypoxia produced a large and reproducible dilatation of coronary arteries. This approach appears suitable for correlating molecular mechanisms in isolated cells with the function of these cells in the microvasculature of the intact heart.

In vivo the coronary arterial and capillary endothelium forms a relatively tight barrier for solutes which are not actively transported (Bassingthwaighte *et al.* 1989). In the terminal arterioles this barrier delimits a perivascular space where substances

Fig. 8. The effects of replacing 10 mM-glucose in the perfusate with 10 mM-deoxyglucose (DOG). *A*, control hypoxia (left) in a heart treated with 10 μM-NNA, followed by a second period of hypoxia (right). During and after the second hypoxia glucose was replaced by DOG. *B*, application of DOG in a different heart, not treated with NNA. When a steady state was reached 2 μM-glibenclamide (Glb) was added. *C*, lack of effect of 8-PT on the vasodilatation induced by DOG in a different heart, not treated with NNA.

released locally can establish chemical communication between cardiac muscle cells, vascular smooth muscle cells, perivascular nerves and endothelial cells. Endothelial cells and cardiomyocytes release adenosine into the perivascular space, and both cell types take up adenosine through a specific nucleoside transporter (Nees, Herzog, Becker, Böck, Des Rosiers & Gerlach, 1985; Jarvis, 1988). The endothelial nucleoside transporter appears to have a much lower Michaelis–Menten constant (K_m) for adenosine than the nucleoside transporter present in the membrane of cardiomyocytes (Rovetto *et al.* 1987). The perivascular adenosine concentration rises when the nucleoside transporter is inhibited by dipyridamole (Kanatsuka *et al.*, 1989; Wangler *et al.* 1989). Recent electrophysiological experiments on cultured coronary endothelial cells suggest that coronary endothelial cells do not possess K^+_{ATP} channels (Mehrke *et al.* 1991*a*). However, microvascular coronary endothelial cells have adenosine receptors which can induce a rise in intracellular Ca^{2+}, and thus stimulate formation of EDRF and prostaglandins (Mehrke & Daut, 1990). A sustained hyperpolarization of coronary endothelium induced by adenosine has also been reported recently (Mehrke, Seiss-Geuder & Daut, 1991*b*). The endothelial adenosine receptors may be responsible for the glibenclamide-insensitive component of the effects of adenosine (Fig. 2).

We have found that the delay between application of hypoxic solution and half-maximal vasodilatation was increased more than twofold when EDRF formation was blocked with NNA (Fig. 5). This seems to be in line with the proposal that the release of EDRF contributes to hypoxic dilatation in various vascular beds (Hopwood, Lincoln, Kirkpatrick & Burnstock, 1989; Pohl & Busse, 1989). However, we consider it more likely that the increased delay was due to the effects of NNA on the pressure–flow relationship. In the presence of NNA the vascular slope resistance decreased drastically at high flow rates (Fig. 6). This is quite unlike the situation *in vivo*, where autoregulation of blood flow, i.e. the increase in arterial tone with increasing transmural pressure, tends to keep blood flow relatively constant (Berne & Rubio, 1979; Johnson, 1986). It has been suggested that in saline-perfused organs the flow-dependent vasodilatation, which is partly due to release of EDRF induced by shear stress at the arterial wall, opposes autoregulation (Griffith, Edwards, Davies & Henderson, 1989). Furthermore, with constant-flow perfusion autoregulation appears to be less pronounced than with constant-pressure perfusion (Borgdorff, Sipkema & Westerhof, 1988; Griffith & Edwards, 1990). Thus the linear relation between pressure and flow (Fig. 6) may be the combined result of autoregulation and flow-dependent vasodilatation. The higher resistance of coronary arteries in the presence of NNA can then be explained by the removal of the effects of EDRF. At flow rates higher than 5 ml min^{-1} another mechanism of flow-dependent vasodilatation may come into play, which is independent of the endothelium (Bevan, Joyce & Wellman, 1988).

The curvature in the relation between flow and pressure (Fig. 6*A*) may be the reason why at high flow rates the effects of small vasodilatory stimuli on CPP were substantially decreased in the presence of NNA (Fig. 6*B*), whereas the maximal vasodilatation was unaffected. Thus the relation between a vasodilatory stimulus (for example, a given increase in the open-state probability of K^+_{ATP} channels) and the measured decrease in CPP was altered by NNA, and this may account for the

increased delay in hypoxic vasodilatation. Furthermore, we found that hypoxic vasodilatation was blocked by glibenclamide (Fig. 3*C*) whereas the effects of the endothelium-dependent vasodilator bradykinin were unaffected by glibenclamide (Fig. 2*B*; see also Daut *et al.* 1990). Finally, inhibition of prostaglandin release by indomethacin had virtually no effect on hypoxic vasodilatation (Fig. 7). Taken together, our experiments suggest that release of vasoactive compounds by the endothelium does not play a major role in hypoxic dilatation of coronary arteries.

The role of adenosine in hypoxic dilatation of coronary arteries

The vasodilatory effect of adenosine in mammalian hearts *in situ* is restricted to arterial microvessels with a diameter smaller than 150 μm. Metabolic dilatation of coronary arteries also resides mainly in small arterioles, although it is distributed over a somewhat wider range of vessel diameters (Kanatsuka *et al.* 1989). In isolated, saline-perfused guinea-pig hearts the vascular resistance change induced by hypoxia or adenosine probably also reflects mainly the diameter changes of small arterioles. Our working hypothesis was that the changes in coronary resistance are largely determined by changes in the membrane potential of microvascular smooth muscle cells (Daut *et al.* 1990; Nelson, Patlak, Worley & Standen, 1990). The membrane potential of vascular smooth muscle cells is very sensitive to the open-state probability of K^+_{ATP} channels (Nelson *et al.* 1990). Glibenclamide has been reported to be a specific blocker of K^+_{ATP} channels (see Ashcroft & Ashcroft, 1990) which binds directly to the channel protein (Bernardi, Fosset & Lazdunski, 1988; Boyd, Aguilar-Bryan & Nelson, 1990). We tried to induce an accumulation of adenosine in the perivascular space of coronary microvessels by inhibiting adenosine uptake with dipyridamole. The vasodilatation induced by 500 nM-dipyridamole could be inhibited both by glibenclamide and by the adenosine antagonist 8-PT (Figs 3*C* and 4*A*). These results suggest that adenosine may hyperpolarize coronary smooth muscle cells by increasing the open-state probability of K^+_{ATP} channels. In recent patch-clamp experiments K^+_{ATP} channels that could be blocked by glibenclamide have been found in smooth muscle cells isolated from porcine (Inoué, Nakaya & Nakayama, 1990) and rabbit (J. Quayle and M. T. Nelson, unpublished observations) coronary arteries. Direct recordings of single channels opened by adenosine in coronary smooth muscle cells have not yet been reported.

Microelectrode measurements on isolated bovine coronary arteries (diameter, 3 mm) suggest that adenosine can hyperpolarize coronary smooth muscle cells by about 17 mV (Sabouni, Hargittai, Lieberman & Mustafa, 1989). In a recent study on rabbit coronary arteries (diameter, 300 μm) it was found that both adenosine (0·3–3 μM) and the K^+ channel opener cromakalim (1 μM) induce a marked hyperpolarization and relaxation of vascular smooth muscle cells (J. Garcia-Roldan, J. Brayden, J. Daut & M. T. Nelson, unpublished observations). Both of these effects could be blocked by glibenclamide. These findings support the hypothesis that the membrane potential of smooth muscle cells is an important determinant of coronary blood flow. The mechanism by which adenosine may open the K^+_{ATP} channels is still unknown. Kirsch *et al.* (1990) have presented evidence that in cardiomyocytes the open-state probability of K^+_{ATP} channels can be enhanced by direct interaction with the α-subunit of a G protein (G_i), and that this G protein may be coupled to the

adenosine receptor. This may also be the case in coronary smooth muscle cells, although it cannot be excluded that adenosine receptors can activate or modulate K^+_{ATP} channels via intracellular second messengers.

Experiments with adenosine antagonists like 8-PT and adenosine uptake inhibitors like dipyridamole have often been used as tests for the adenosine hypothesis (reviewed by Berne, 1980). The results of these studies have been controversial and inconclusive, and the absence of reproducible inhibition of hypoxic vasodilatation by adenosine antagonists has been regarded as one of the main arguments against the adenosine hypothesis. Our simplified Langendorff model allows better isolation of different vasodilatory mechanisms than previous approaches, because there are relatively few uncontrolled variables, and because the ratio between oxygen supply and demand is more favourable in the arrested heart. We found that the same concentration of 8-PT that inhibited the effects of dipyridamole and adenosine (5 μM) had no effect on hypoxic vasodilatation in the same hearts (Fig. 4). This makes it rather unlikely that the early hypoxic dilatation of coronary arteries described here was caused by perivascular accumulation of adenosine. However, it cannot be excluded that hypoxic cardiomyocytes release vasoactive agents other than adenosine that might play a role in hypoxic dilatation of coronary arteries.

The effects of higher concentrations of 8-PT (20 μM) are more difficult to interpret. These high-dose effects were often only partly reversible. In beating hearts they were associated with an increase in diastolic pressure. They may be related to a different site of action of 8-PT, perhaps phosphodiesterase inhibition, a well-known side effect of methylxanthines. These side effects of higher concentrations of adenosine antagonists may be one of the reasons for the variability of the results reported in the literature.

The role of the energy metabolism in vascular smooth muscle cells

Our attempts to influence hypoxic dilatation of coronary arteries with blocking substances like 8-PT, NNA and indomethacin all produced negative results (Fig. 7). We therefore reconsidered the hypothesis that hypoxia acts directly on coronary smooth muscle cells and that the opening of the K^+_{ATP} channels may be mediated by changes in intracellular metabolites. The open-state probability of the K^+_{ATP} channels might be increased by a fall in the submembrane ATP concentration, by a rise in submembrane ADP, by intracellular second messengers, or by some other consequence of impaired oxidative phosphorylation (Ashcroft & Ashcroft, 1990). Our observation that replacement of glucose by deoxyglucose prevented the reversal of hypoxic vasodilatation (Fig. 8*A*) supports the hypothesis that the opening of the K^+_{ATP} channels is related to changes in cellular energy metabolism.

DOG is taken up into the cells like glucose and accumulates mainly as deoxyglucose-6-phosphate (DOG-6-P) and DOG-1-P. DOG-6-P cannot be isomerized to deoxy-fructose-6-P and thus blocks glycolysis and prevents oxidation of glucose-1-P derived from glycogenolysis. We have found that application of DOG elicited a near-maximal vasodilatation in the arrested, oxygenated guinea-pig heart (Fig. 8*B*), and that this effect could be inhibited by glibenclamide. It is tempting to speculate that this vasodilatation was due to increased K^+_{ATP} channel activity mediated by changes in the submembrane ATP/ADP ratio of coronary smooth muscle cells. This would imply that oxidation of endogeneous lipids does not occur at a rate sufficient to

prevent changes in submembrane ATP and ADP. Another possible explanation for the vasodilatation produced by DOG is that glycolytic metabolism may be more effective than oxidative metabolism in suppressing K^+_{ATP} channel activity in the membrane vascular smooth muscle cells.

The effects of DOG in the oxygenated heart could not be inhibited by 5 μM-8-PT (Fig. 8*C*). Thus it is unlikely that vasodilatation evoked by DOG was an indirect consequence of an alteration in the energy metabolism of cardiac muscle cells, which could lead to an enhanced release of adenosine (Olsson & Bünger, 1987). Other interventions known to lower the cytosolic phosphorylation potential, for example uncoupling of mitochondria with 2,4-dinitrophenol or blockage of oxidative phosphorylation with cyanide, also caused a vasodilatation that could be inhibited by glibenclamide (Daut *et al.* 1990). These findings support the idea that in coronary smooth muscle cells, like in pancreatic B cells, the opening of K^+_{ATP} channels may be linked to cellular energy metabolism.

In conclusion, our results suggest that adenosine can open K^+_{ATP} channels, but that this is not what happens during the early phase of hypoxic vasodilatation. Nevertheless, it would be premature to assume that adenosine is not involved at all in hypoxic dilatation of coronary arteries. It cannot be excluded that release of adenosine and ATP from cardiomyocytes and endothelial cells may play a role in the vasodilatation observed after prolonged myocardial hypoxia or ischemia (Burnstock, 1989).

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