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Investigation of mechanisms that mediate reactive hyperaemia in guinea-pig hearts: role of K_{ATP} channels, adenosine, nitric oxide and prostaglandins

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1 Reactive hyperaemia is a transient vasodilatation following a brief ischaemic period. ATP-dependent K^+ (K_{ATP}) channels may be important in mediating this response, however it is unclear whether mitochondrial K_{ATP} channels contribute to this in the heart.

2 We examined the involvement of K_{ATP} channels and the relative role of mitochondrial channels as mediators of coronary reactive hyperaemia and compared them to mechanisms involving NO, prostaglandins and adenosine in the guinea-pig isolated heart.

3 Reactive hyperaemic vasodilatation (peak vasodilator response and flow debt repayment) were assessed after global zero-flow ischaemia (5–120 s) in the presence of nitro-L-arginine methyl ester (L-NAME, 10^{-5} M, $n=9$), 8-phenyltheophylline (8-PT, 10^{-6} M, $n=12$) and indomethacin (10^{-5} M, $n=12$). Glibenclamide (10^{-6} M, $n=12$) a non-selective K_{ATP} channel inhibitor and 5-hydroxy-decanoic acid (5-HD, 10^{-4} M, $n=10$) a selective mitochondrial K_{ATP} channel inhibitor were also used. The specificity of the effects of glibenclamide and 5-HD ($n=6$ each) were confirmed using pinacidil (38 nmol–10 μmol) and diazoxide (42 nmol–2 μmol).

4 Glibenclamide was most effective in blocking the hyperaemic response (by 87%, $P<0.001$) although 5-HD and 8-PT also had a marked effect (40% inhibition, $P<0.001$ and 32%, $P<0.001$, respectively). L-NAME and indomethacin had little effect.

5 Perfusion with L-NAME and glibenclamide significantly reduced baseline coronary flow (22%, $P<0.01$ and 33%, $P<0.01$) while 8-PT, indomethacin and 5-HD had no effect.

6 K_{ATP} channels are the major mediators of the coronary reactive hyperaemic response in the guinea-pig. Although mitochondrial K_{ATP} channels contribute, they appear less important than sarcolemmal channels.

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Abbreviations: DMSO, dimethyl sulphoxide; 5-HD, 5-hydroxy-decanoic acid; K_{ATP}, ATP-dependent K^+; K_{max}, maximum flow; $K_{1/2}$, ischaemic duration required to produce half-maximal flow; L-NAME, nitro-L-arginine methyl ester; NO, nitric oxide; 8-PT, 8-phenyltheophylline

Introduction

Reactive hyperaemia is a transient local vasodilatation that occurs in response to an interruption of blood supply. This may be seen as a mechanism to repay the metabolic debt incurred during brief periods of ischaemia. Reactive hyperaemic responses occur in the coronary circulation and are impaired in left ventricular hypertrophy (Kingsbury *et al*., 2000). The exact mechanisms mediating coronary reactive hyperaemia are unclear although there are a number of possible mediators that may be involved. Adenosine, NO and prostaglandins have all been reported to contribute to the coronary hyperaemic response (Lee *et al*., 1992; Viau *et al*., 1997; Kingsbury *et al*., 2000), while recent work suggests that mechanisms involving ATP-dependent K^+ (K_{ATP}) channels may be important (Daut *et al*., 1990; Aversano *et al*., 1991; Kingsbury *et al*., 2000). As K_{ATP} channels have been shown to be involved in the coronary vasodilator responses to prostacyclin (Jackson *et al*., 1993) and adenosine (Daut *et al*., 1990; Orito *et al*., 1993; Duncker *et al*., 1995), and to have links with NO production in the coronary circulation (Ming *et al*., 1997; Schnitzler *et al*., 2000), it is conceivable that they are involved in the response to many of the putative mediators of coronary reactive hyperaemia.

We have recently described how in the guinea-pig heart, reactive hyperaemia is mediated by a combination of actions with relative contributions from K_{ATP} channels > adenosine > NO (Kingsbury *et al*., 2000). K_{ATP} channels are present on both sarcolemmal and mitochondrial membranes (Garlid *et al*., 1996; Liu *et al*., 1998; Szewczyk & Marbán, 1999) and glibenclamide, a widely used K_{ATP} channel antagonist, is active at both sites (Szewczyk & Marbán, 1999). Recent evidence suggests that both types of channels are involved in

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the cardioprotective effects associated with heat stress and preconditioning and that there may be 'cross-talk' between them (Hoag *et al*., 1997; Joyeux *et al*., 1998; Gross & Fryer, 1999). At present it is unclear whether mitochondrial K_{ATP} channels are involved in mediating reactive hyperaemic responses in the heart. To investigate this, we examined the role of mitochondrial K_{ATP} channels in reactive hyperaemia in the guinea-pig heart and compared the relative importance of their involvement to that of sarcolemmal K_{ATP} channels and mechanisms involving NO, prostaglandins and adenosine.

Methods

Langendorff heart preparation

Adult male guinea-pigs (935 ± 90 g) were sacrificed by cervical dislocation and the hearts rapidly removed and mounted vertically for perfusion according to a modified Langendorff technique as described previously (Kingsbury *et al*., 2000). Briefly, hearts were perfused retrogradely at a pressure of 50 mmHg with a modified buffered Krebs-Henseleit solution (pH 7.4) containing (mM) NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.1, $NaHCO_3$ 24, $CaCl_2$ 2.5, glucose 9 and pyruvate 2, equilibrated with a 95% O_2/5% CO_2 mixture and maintained at 37°C. The right atrium was opened to ensure free drainage from the coronary sinus. Hearts were paced at 250 beats min^{-1} using bipolar electrodes and a stimulator (Harvard Apparatus), mean coronary flow was measured using an ultrasonic flow probe (Transonic, T108) inserted in the aortic line, and left ventricular pressure measured isovolumetrically using a latex balloon (7 mm diameter, Linton Instrumentation) inserted into the ventricle *via* the left atrium. The balloon was connected to a pressure transducer (SensoNor 840) and was filled to set the end diastolic pressure at 8 mmHg. All measurements were displayed and processed by computer using Po-Ne-Mah Acquire Plus data acquisition software (Gould, 12 bit resolution, sampling rate 250 Hz).

Reactive hyperaemia

Reactive hyperaemic vasodilatation was assessed after global zero-flow ischaemia lasting 5, 10, 20, 40, 60 and 120 s. Flow was allowed to return to basal levels between ischaemic challenges and there was no evidence of any significant shift in basal flow following the repeated ischaemic insults. The peak vasodilator response and flow debt repayment were determined as described previously (Kingsbury *et al*., 2000). Flow debt incurred during occlusion was measured as the area between basal and zero flow during the occlusion, and debt repayment as the area under the reperfusion flow curve above basal flow; debt repayment was expressed as a percentage of the debt incurred.

The reactive hyperaemic response was assessed in the presence of the NOS inhibitor L-N^G nitroarginine methyl ester (L-NAME, 10^{-5} M, $n=9$), the adenosine A_1 receptor antagonist 8-phenyltheophylline (8-PT, 10^{-6} M, $n=10$) and the cyclo-oxygenase inhibitor indomethacin (10^{-5} M, $n=12$). Glibenclamide (10^{-6} M, $n=12$) a non-selective K_{ATP} channel inhibitor and 5-hydroxy-decanoic acid (5-HD, 10^{-4} M, $n=10$) a selective mitochondrial K_{ATP} inhibitor (Liu *et al*., 1998) were also used. L-NAME and 5-HD were dissolved directly in Krebs-Henseleit buffer to give a final concentration of 10^{-5} M and 10^{-4} M respectively and normal Krebs-Henseleit buffer was used in the control experiments. 8-PT was dissolved in 1 M NaOH and 100% ethanol (1 : 3) to give a stock solution which was then diluted with Krebs-Henseleit buffer (0.04 ml l^{-1}) to give a final concentration of 10^{-6} M and Krebs-Henseleit buffer with NaOH and ethanol (1 : 3, 0.04 ml l^{-1}) was used in control experiments. Similarly indomethacin was dissolved in 1 M NaOH to give a stock solution which was then diluted with Krebs-Henseleit buffer (100 μl l^{-1}) to give a final concentration of 10^{-5} M and Krebs-Henseleit buffer with NaOH (100 μl l^{-1}) was used in control experiments. Glibenclamide was dissolved in dimethyl sulphoxide (DMSO) which was then diluted with Krebs-Henseleit buffer (0.01 ml l^{-1}) to give a final concentration of 10^{-6} M and in these experiments Krebs-Henseleit buffer with DMSO (0.01 ml l^{-1}) was used in controls. After control responses were obtained from preparations perfused with Krebs-Henseleit buffer plus vehicle as described above, perfusion was changed to Krebs-Henseleit buffer plus the antagonist at the appropriate concentration. The preparations were allowed to stabilize for 20 min to allow flow to equilibrate to a new basal level. Preliminary time control experiments confirmed that the second cycle of ischaemic challenges were not significantly different from the first following 20 min control Krebs-Henseleit buffer perfusion. The responses to exogenous agonists and ischaemic challenges were repeated in the presence of the antagonist.

Antagonist specificity

The specificity of glibenclamide ($n=6$) and 5-HD ($n=6$) at the doses used in this protocol was confirmed by constructing dose-response curves to the non-selective K_{ATP} channel agonist pinacidil (38 nmol – 10 μmol) and the mitochondrial selective K_{ATP} channel agonist diazoxide (42 nmol – 2 μmol). Drugs were injected as bolus doses into the perfusion line below the flow probe over 3 s in a volume of 2 – 100 μl. Stock solutions of pinacidil and diazoxide were prepared in DMSO, with 1 : 1000 and 1 : 100 dilutions in Krebs-Henseleit buffer. DMSO at the concentration used had no effect on coronary flow.

Statistical analysis

Values are expressed as mean ± standard error of the mean. Dose response curves were analysed by fitting sigmoidal curves using non-linear regression analysis. ED_{50} and maximum values were obtained for each experiment and used for comparison of dose response curves. Peak hyperaemic flow response curves were analysed by fitting rectangular hyperbolic curves described by the equation $Y = K_{max} \cdot X / (K_{1/2} + X)$ where K_{max} is maximum flow and $K_{1/2}$ is the ischaemic duration required to produce half-maximal flow. K_{max} and $K_{1/2}$ values were used to compare curves. Statistical analysis of data using Student's unpaired *t*-test enabled the comparison of groups (an F value calculation was also performed to test for unequal variance between the groups. If significant variance was found, *t*-tests with Welch's correction for unequal variance were used). All statistical

analysis was performed using Prism analysis software (v3.00, GraphPad Software Inc), $P<0.05$ indicating statistical significance.

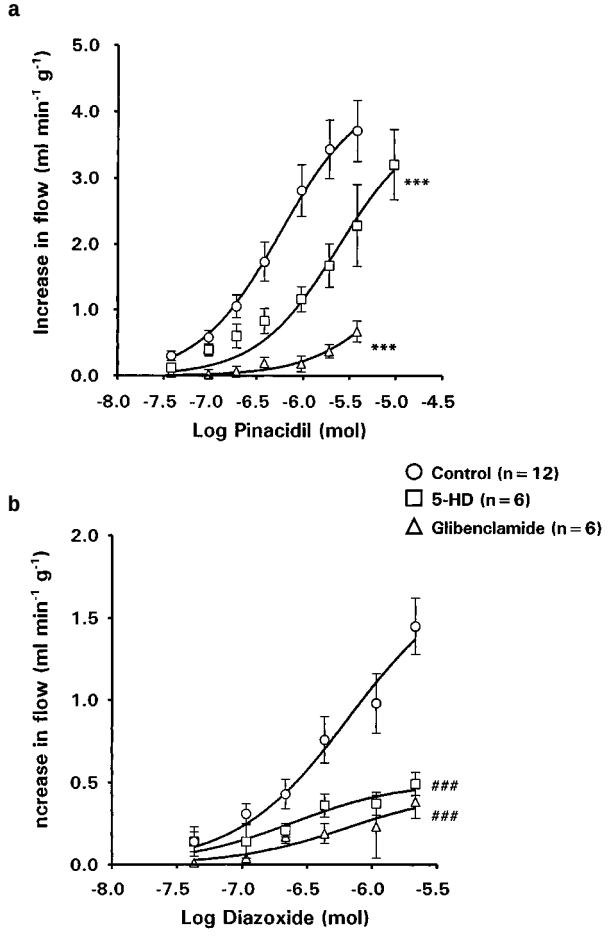

Figure 1 Concentration-response curves to illustrate pinacidil (a) and diazoxide (b) responses in the presence and absence of 5-hydroxy-decanoic acid (5-HD) and glibenclamide. In the presence of 5-HD the pinacidil dose response curve was shifted to the right. Glibenclamide shifted the dose response curve further to the right. The vasodilator response to diazoxide was inhibited equally by both 5-HD and glibenclamide with a decrease in maximum vasodilator response. *** $P<0.001$ *vs* control for ED_{50} and ### $P<0.001$ *vs* control for K_{max}.

Results

The baseline flow in control isolated hearts was 5.2 ± 0.1 ml min^{-1} g^{-1} with a left ventricular systolic pressure of 83 ± 4 mmHg and end diastolic pressure of 8 ± 0.5 mmHg. Bolus doses of the non-selective K_{ATP} channel agonist pinacidil resulted in a dose-dependent increase in coronary flow in all hearts. The characteristic sigmoidal dose response curves obtained are shown in Figure 1a. In the presence of the mitochondrial K_{ATP} inhibitor 5-HD (10^{-4} M) the pinacidil dose response curve was shifted to the right (Figure 1a) with a significant ($P<0.001$) increase in the ED_{50} value (Table 1). The non-selective K_{ATP} inhibitor glibenclamide (10^{-6} M) shifted the dose response curve further to the right (Figure 1a) with a significant increase in ED_{50} value (Table 1). Similarly bolus doses of the mitochondrial selective K_{ATP} channel agonist diazoxide also produced a dose-dependent increase in coronary flow in all hearts, although the vasodilation achieved was less than that observed with pinacidil (Figure 1b, Table 1). The vasodilator response to diazoxide was inhibited equally by both the mitochondrial K_{ATP} inhibitor 5-HD and the non-selective K_{ATP} inhibitor glibenclamide with a decrease in maximum vasodilator response ($P<0.001$, in both cases, Figure 1b) but no change in ED_{50} value (Table 1).

All hearts showed a reactive hyperaemic vasodilation in response to ischaemic challenges. The peak hyperaemic increase in flow increased with the duration of the ischaemic challenge to a maximum, which occurred in response to ischaemic challenges of between 60 and 120 s, Figures 2a–6a. This characteristic response produced rectangular hyperbolic curves. The overall control reactive hyperaemic flow response curve had a maximum vasodilator response (K_{max}) of 8.8 ± 0.1 ml min^{-1}g^{-1} and the ischaemic duration required to produce half-maximal flow ($K_{1/2}$) of 19.4 ± 0.6 s. The control percentage flow debt repayment calculated from the reactive hyperaemic response was $52\pm1\%$ for ischaemic challenges of between 5 and 40 s but this was reduced with longer ischaemic challenges with only a $42\pm2\%$ debt repayment of a 120 s ischaemic challenge (see, for example, Figures 2b–6b).

The NO synthesis inhibitor L-NAME (10^{-5} M) reduced basal coronary flow by 22% ($P<0.01$) but had relatively little effect on the peak reactive hyperaemic flow response curves (Figure 2a). While K_{max} was not significantly altered by L-NAME (Table 2) there was a significant ($P<0.01$) increase in $K_{1/2}$ (Table 2). The change in $K_{1/2}$ is due to a decreased

Table 1 Pinacidil and diazoxide dose response curve maxima and ED_{50} data during control perfusion and in the presence of 5-HD (10^{-4} M) and glibenclamide (10^{-6} M)

	Pinacidil		*Diazoxide*	
	Maximum vasodilator response (ml min^{-1} g^{-1})	*ED_{50}* (mol)	*Maximum vasodilator response* (ml min^{-1} g^{-1})	*ED_{50}* (mol)
Control ($n=12$)	4.3 ± 0.08	5.54×10^{-7} (4.69×10^{-7}–6.54×10^{-7})	1.8 ± 0.19	6.74×10^{-7} (3.12×10^{-7}–1.46×10^{-6})
5-HD ($n=6$)	3.9 ± 0.29	2.36×10^{-6}*** (1.33×10^{-6}–4.20×10^{-6})	0.51 ± 0.06***	2.40×10^{-7} (8.69×10^{-8}–6.64×10^{-7})
Glibenclamide ($n=6$)	2.1 ± 1.27	8.39×10^{-6}** (1.04×10^{-6}–6.76×10^{-5})	0.45 ± 0.09***	6.37×10^{-7} (1.37×10^{-7}–2.96×10^{-6})

**$P<0.01$, ***$P<0.001$ *vs* control. ED_{50} values are presented as the mean and 95% confidence limits.

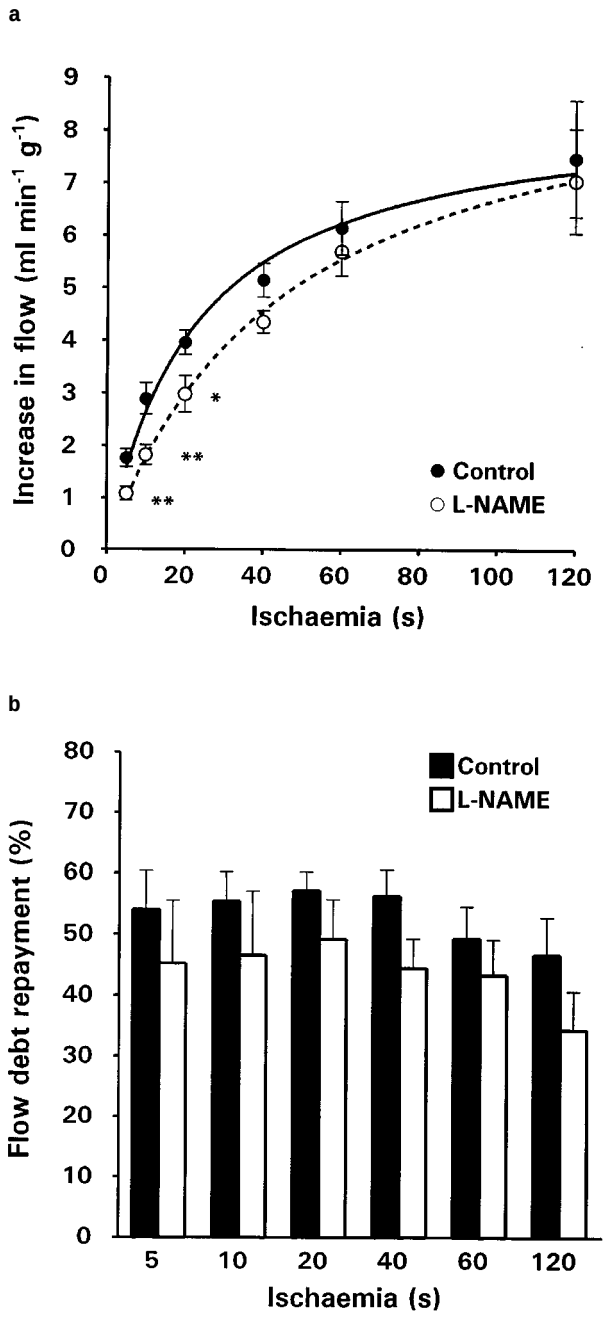

Figure 2 Peak reactive hyperaemic flow response (a) and flow debt repayment (b) in the presence and absence of L-NAME. L-NAME had relatively little effect on the peak reactive hyperaemic flow response curves but decreased vasodilator response to short ischaemic challenges. The percentage flow debt repayment tended to be reduced in the presence of L-NAME for all ischaemic challenges, although this did not reach statistical significance. $*P<0.05$, $**P<0.01$ *vs* control.

vasodilator response to short (5–20 s) ischaemic challenges in the presence of L-NAME ($P<0.05-P<0.01$ by one-way ANOVA followed by Bonferroni's Multiple Comparison test as a post-test, Figure 2a). The percentage flow debt repayment tended to be reduced in the presence of L-NAME for all ischaemic challenges, although this did not reach statistical significance (Figure 2b).

Table 2 K_{max} and $K_{½}$ values calculated from fitted curves in the presence of various antagonists. Due to the marked effect of glibenclamide, it was not possible to calculate these parameters

		K_{max} (ml min^{-1} g^{-1})	$K_{½}$ (s)
L-NAME	Control	8.59±0.43	22.31±3.18
($n=9$)	+Antagonist	9.69±0.39	44.99±4.03**
Indomethacin	Control	8.40±0.18	17.56±1.16
($n=7$)	+Antagonist	6.71±0.08	17.80±0.53
8-PT	Control	9.95±0.27	18.20±1.53
($n=12$)	+Antagonist	6.77±0.16***	16.89±1.25
Glibenclamide	Control	8.39±0.11	20.60±0.82
($n=12$)	+Antagonist	–	–
5-HD	Control	9.89±0.19	24.40±1.33
($n=10$)	+Antagonist	5.94±0.22***	25.58±2.58

$**P<0.01$, $***P<0.001$ *vs* the control response in the absence of antagonist.

The cyclo-oxygenase inhibitor indomethacin (10^{-5} M) had no effect on basal coronary flow and very little effect on the reactive hyperaemic response in these experiments. The peak flow response (Figure 3a) shows that the response to shorter ischaemic challenges is almost superimposed with a tendency for an attenuated response to longer ischaemic challenges. Although there is a slight reduction in K_{max} (Table 2), there were no significant differences in $K_{1/2}$ (Table 2) or flow debt repayment in the presence of indomethacin (Figure 3b).

The adenosine A_1 receptor antagonist 8-PT (10^{-6} M) had no effect on basal coronary flow but reduced both the peak flow response and the flow debt repayment independently of the duration of the ischaemic challenge (Figure 4). There was a parallel 32% ($P<0.001$) decrease in K_{max} (Table 2) which was reflected in a decrease in flow debt repayment of approximately 30% for all ischaemic challenges (Figure 4b).

The non-selective K_{ATP} inhibitor glibenclamide (10^{-6} M) reduced basal coronary flow by 33% ($P<0.01$) and had the largest effect of any of the compounds tested on the peak hyperaemic flow response (Figure 5a). Responses to ischaemic challenges of up to 40 s were reduced by 87% ($P<0.01-P<0.001$), with some residual vasodilator capacity to longer ischaemic challenges as demonstrated by the 49% reduction in response to 120 s ischaemia (Figure 5b). This pattern of antagonism differed from the other antagonists studied and fundamentally altered the relationship between peak hyperaemic flow response and duration of ischaemia changing the response curve from the characteristic rectangular hyperbolic curve to a straight line (Figure 5a). Due to this marked effect of glibenclamide, it was not possible to calculate K_{max} and $K_{1/2}$ (Table 2) Flow debt repayment was also significantly impaired by the presence of glibenclamide (Figure 5b).

The mitochondrial selective K_{ATP} channel inhibitor 5-HD (10^{-4} M) also inhibited both the peak hyperaemic flow response and flow debt repayment (Figure 6), although to a lesser extent than glibenclamide, but had no effect on basal coronary flow. 5-HD resulted in a parallel downward shift of the peak flow response curve (Figure 6a) with a 40% ($P<0.001$) decrease in K_{max} with no significant

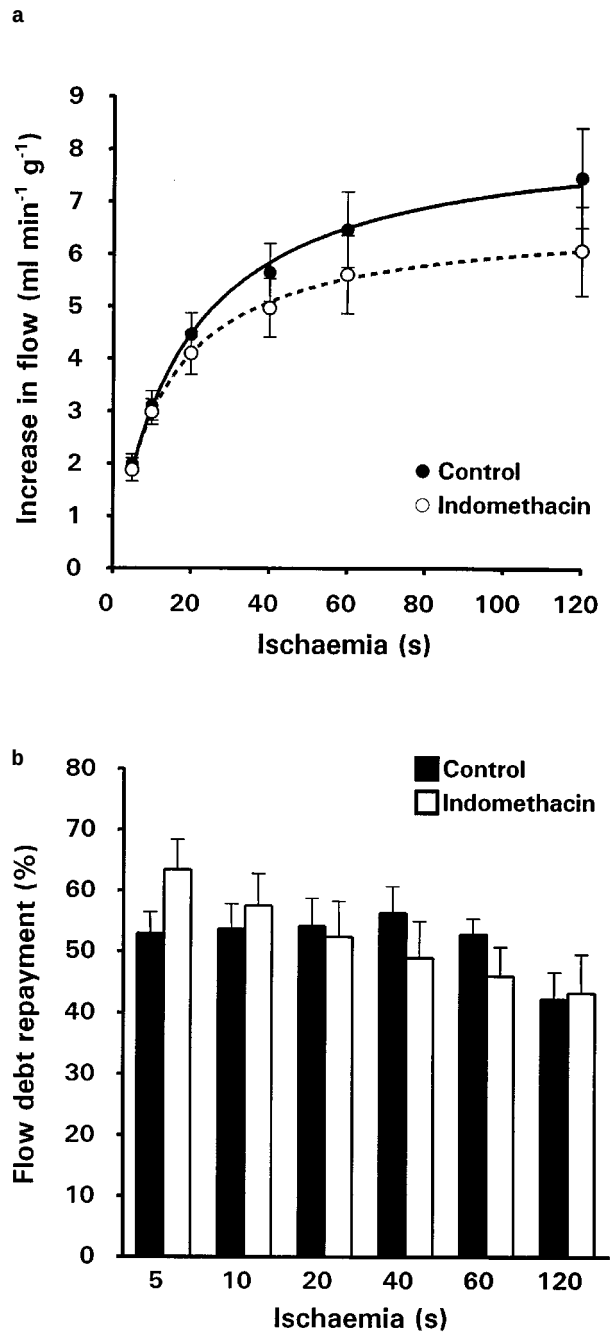

Figure 3 Peak reactive hyperaemic flow response (a) and flow debt repayment (b) in the presence and absence of indomethacin. Indomethacin had very little effect on the reactive hyperaemic response such that the response to shorter ischaemic challenges is almost superimposed with a tendency for an attenuated response to longer ischaemic challenges. There were no significant differences in flow debt repayment in the presence of indomethacin.

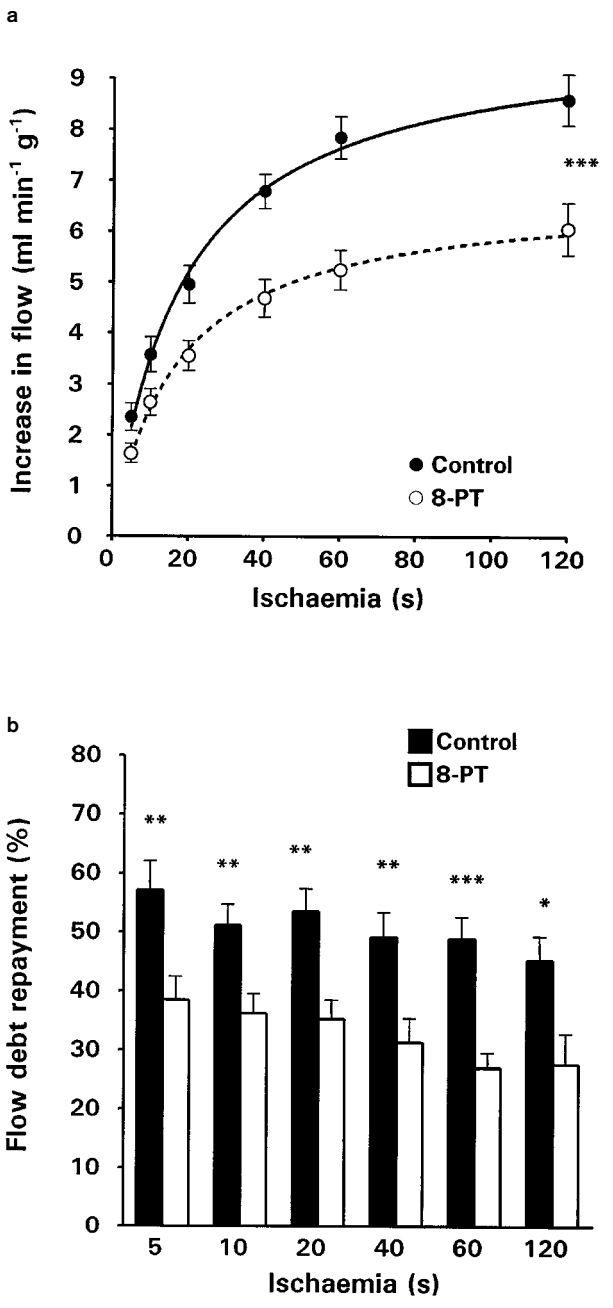

Figure 4 Peak reactive hyperaemic flow response (a) and flow debt repayment (b) in the presence and absence of 8-PT reduced both the peak flow response and the flow debt repayment independently of the duration of the ischaemic challenge. $*P<0.05$, $**P<0.01$, $***P<0.001$ *vs* control.

change in $K_{1/2}$ (Table 2). 5-HD also reduced the flow debt repayment (Figure 6b) and in contrast to glibenclamide had a greater effect with longer ischaemic challenges. While responses to 5 s ischaemia were decreased by 43% this effect steadily increased so that the flow debt repayment following 120 s ischaemia was reduced by 70%, Figure 6b.

Discussion

The key findings from these experiments are that: (a) non-selective K_{ATP} channel antagonism using glibenclamide was most effective in blocking the hyperaemic response although 5-HD and 8-PT also had a marked effect indicating that mitochondrial K_{ATP} channels and adenosine have a less important role than the sarcolemmal K_{ATP} channels; (b) L-NAME had little effect, although it would appear that NO is relatively more important in the response to shorter ischaemic

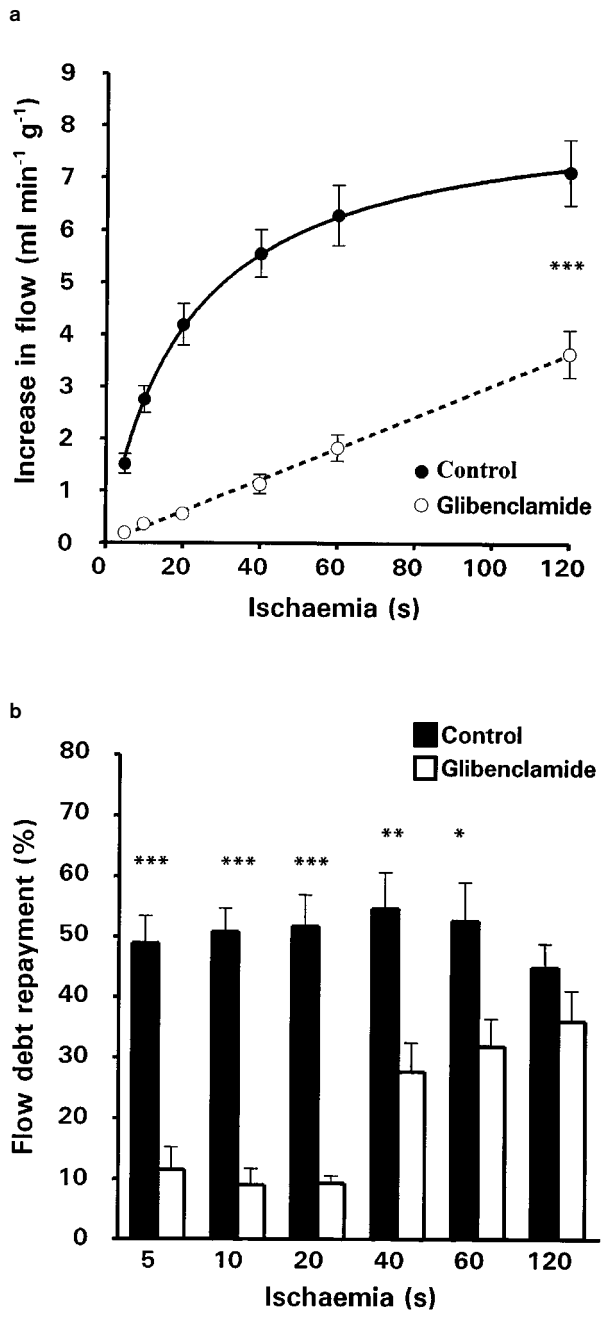

Figure 5 Peak reactive hyperaemic flow response (a) and flow debt repayment (b) in the presence and absence of glibenclamide. Glibenclamide had a large effect on the peak hyperaemic flow response (a). Responses to ischaemic challenges of up to 40 s were reduced, with some residual vasodilator capacity to longer ischaemic challenges. Flow debt repayment was also significantly impaired by the presence of glibenclamide. $*P<0.05$, $**P<0.01$, $***P<0.001$ *vs* control.

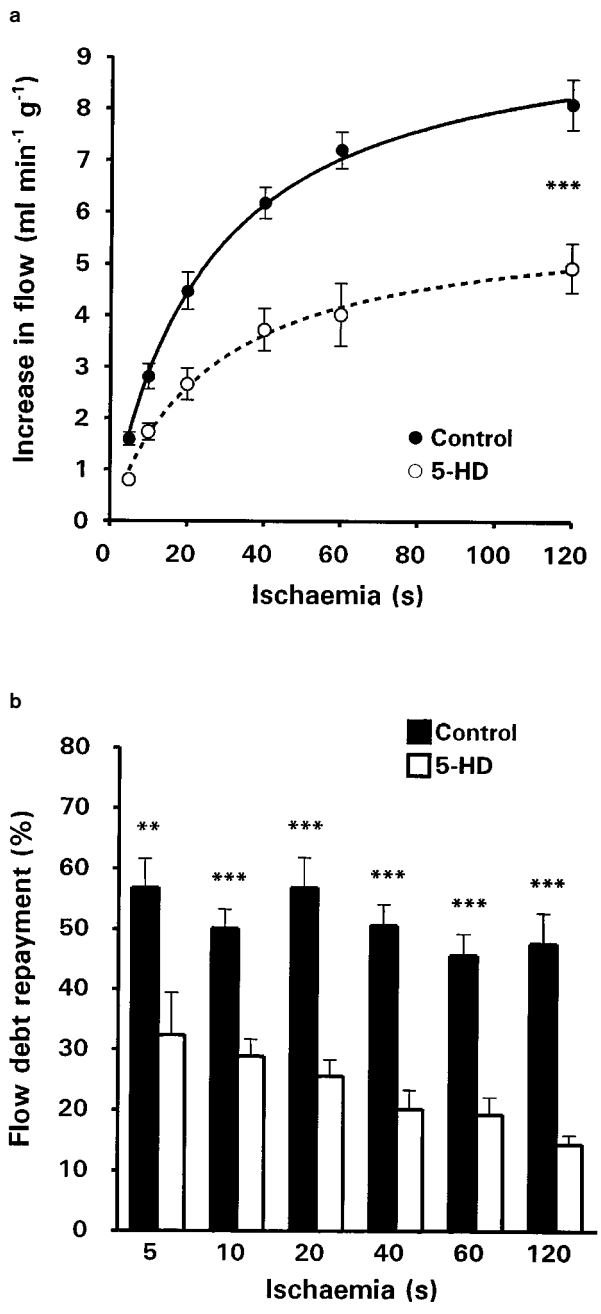

Figure 6 Peak reactive hyperaemic flow response (a) and flow debt repayment (b) in the presence and absence of 5-hydroxy-decanoic acid (5-HD). 5-HD inhibited both the peak hyperaemic flow response and flow debt repayment, but to a lesser extent than glibenclamide. Despite this, 5-HD had a greater effect with longer ischaemic challenges. $**P<0.01$, $***P<0.001$ *vs* control.

challenges, and (c) indomethacin appeared least effective indicating that cyclo-oxygenase products appear to have little if any role in reactive hyperaemia in the guinea-pig.

Perfusion with L-NAME and glibenclamide significantly reduced baseline coronary flow suggesting its dependence on both NO release and K_{ATP} channel activation, findings which are consistent with other studies (Kostic & Schrader, 1992; Aversano *et al*., 1993; Richmond *et al*., 1999). 5-HD had no effect on baseline flow, suggesting that it is the sarcolemmal rather than the mitochondrial channels that are involved.

The mechanisms underlying reactive hyperaemia have been investigated in recent years, with evidence of a role for NO (Pohl *et al*., 1994; Gryglewski *et al*., 1996; Gattullo *et al*., 1999; Andrieu *et al*., 1999), prostaglandins (Messina *et al*., 1977; Carlsson *et al*., 1987; Woditsch & Shror, 1992; Engelke *et al*., 1996), adenosine (Saito *et al*., 1981; Wei *et al*., 1988; 1989), and K_{ATP} channels (Daut *et al*., 1990; Aversano *et al*., 1991;

Lee *et al*., 1992) mediated vasodilatation. Other studies have shown that K^+_{ATP} channels together with prostaglandins (Viau *et al*., 1997), adenosine (Duncker *et al*., 1995) and NO (Shinoda *et al*., 1997) are involved and all act together in mediating the response (Shinoda *et al*., 1997; Ishibashi *et al*., 1998; Kingsbury *et al*., 2000). We have recently described how in the guinea-pig heart, reactive hyperaemia is mediated by a combination of actions with relative contributions from K_{ATP} channels > adenosine > NO (Kingsbury *et al*., 2000) and how the hyperaemic response is impaired in hypertrophic myocardium. Data from these studies support the contention that the reactive hyperaemic response is multifactorial with roles for NO, adenosine and most importantly K_{ATP} channels.

Perfusion with L-NAME had relatively little effect on the reactive hyperaemic response with no change in K_{max} and only a tendency to decrease the ischaemic flow debt repayment. However L-NAME did significantly reduce the peak hyperaemic flow response to short (5–20 s) ischaemic challenges which is consistent with previous work that has shown NO is more important as a mediator of the flow response to short ischaemic challenges (Hansen & Haunso, 1995; Kingsbury *et al*., 2000). This may reflect shear stress-induced NO release augmenting the reactive hyperaemic response rather than NO acting as a primary mediator (Gattullo *et al*., 1995).

Perfusion with indomethacin had very little effect on the coronary hyperaemic response: flow debt repayment was unchanged but there was a tendency for a reduction in the maximum flow response to ischaemic challenges >60 s. This suggests that cyclo-oxygenase products have little, if any, role as mediators of the reactive hyperaemic response in this preparation, and confirms the work of others (Gryglewski *et al*., 1996). The possibility remains that the cyclo-oxygenase pathway participates in myocardial reactive hyperaemia only when other mediators, such as NO, are inhibited (Puybasset *et al*., 1996).

Perfusion with 8-PT resulted in a significant reduction in the coronary reactive hyperaemic response irrespective of the duration of the preceding ischaemic challenge. This is consistent with previous work that has shown that adenosine is an important mediator, accounting for about 30% of the total hyperaemic response (Saito *et al*., 1981; Macho *et al*., 1995; Gryglewski *et al*., 1996; Kingsbury *et al*., 2000). Studies showing that adenosine deaminase was capable of reducing the hyperaemic response by around 90% (Wei *et al*., 1988) may be explained by considering that this would reduce the tissue adenosine levels while 8-PT selectively inhibits adenosine interaction with the adenosine A_1 receptor. Adenosine is also known to act *via* interaction with K_{ATP} channels (Orito *et al*., 1993) and adenosine deaminase would reduce both this and the A_1 adenosine receptor-mediated effects, and this may at least partially account for its greater efficacy.

Perfusion with glibenclamide had the greatest effect on the reactive hyperaemic response almost eliminating the response to ischaemic challenges of less than 40 s, although there was some residual vasodilator capacity to longer ischaemic challenges which was lacking with 5-HD. This is consistent with K_{ATP} channels having a direct role as a major mediator of reactive hyperaemia (Daut *et al*., 1990; Aversano *et al*., 1991; Lee *et al*., 1992; Viau *et al*., 1997; Kingsbury *et al*., 2000) and with their involvement in the vasodilator actions of other mediators (Jackson *et al*., 1993; Orito *et al*., 1993; Ming *et al*., 1997). As 5-HD also significantly antagonized the hyperaemic response it appears that the mitochondrial K_{ATP} channels are involved in the hyperaemic response just as they are with ischaemic preconditioning (Gross & Fryer, 1999), but since 5-HD reduced the reactive hyperaemic response to a lesser extent than glibenclamide, it appears that it is the sarcolemmal K_{ATP} channels that are of primary importance in coronary hyperaemia. However, as 5-HD reduced the hyperaemic flow debt repayment response to longer ischaemic challenges to a greater extent, it is possible that mitochondrial K_{ATP} channels may be increasingly important in the response to longer-lasting ischaemia.

The ability to distinguish pharmacologically mitochondrial K_{ATP} channels depends on the specificity of 5-HD. Evidence for this is supplied by comparing the actions of 5-HD against pinacidil, a well characterized non-specific K^+_{ATP} channel opener (Szewczyk & Marbán, 1999), and diazoxide, a mitochondrial-specific K_{ATP} channel agonist (Garlid *et al*., 1997; Liu *et al*., 1998). The concentration of diazoxide required to induce half maximal activation in sarcolemmal channels is 2000 times greater than that required for mitochondrial channels (0.40 *vs* 855 μM, Garlid *et al*., 1996). Thus although diazoxide is known to open sarcolemmal K_{ATP} channels in the millimolar range (Garlid *et al*., 1997), the micromolar levels used in these experiments specifically targeted mitochondrial channels. In contrast to the non-specific K_{ATP} channel antagonist glibenclamide which antagonized the effects of both pinacidil and diazoxide equally, 5-HD antagonized the response to diazoxide to a similar extent as glibenclamide but was much less potent in antagonizing the response to pinacidil as we demonstrate. This is consistent with 5-HD selectively inhibiting mitochondrial K_{ATP} channels in this preparation. This conclusion is supported by work in isolated hearts that showed that 5-HD at concentrations in the range 0.1 to 1 mM had little or no effect on sarcolemmal K_{ATP} channels (McCullough *et al*., 1991) while at a concentration of 100 μM completely abolished the effects of diazoxide (Garlid *et al*., 1997). 5-HD also significantly inhibited the ability of diazoxide to open reconstituted mitochondrial K_{ATP} channels (Garlid *et al*., 1997) and was found to virtually abolish pinacidil induced mitochondrial flavoprotein oxidation without inhibiting plasma-membrane potassium currents (Sato *et al*., 1998). Although this evidence strongly suggests that 5-HD at the concentration used in these experiments specifically interacts with mitochondrial K_{ATP} channels it is not clear how opening these channels could contribute to the hyperaemic response. It remains possible that the 5-HD inhibition of reactive hyperaemia is partially due to some non-selective effect.

In conclusion, K_{ATP} channels are the major mediators of the coronary reactive hyperaemic response. Although mitochondrial K_{ATP} channels play a part they appear to be less important than the sarcolemmal channels. Adenosine also has a role in mediating the response *via* adenosine A_I receptors. NO is also a factor, particularly in response to short periods of ischaemia while cyclo-oxygenase products appear to have little if any role.

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