British Journal of Pharmacology (2003) 139, 65–72
www.nature.com/bjp

Activation of ATP-dependent potassium channels is a trigger but not a mediator of ischaemic preconditioning in pigs

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1 Activation of ATP-dependent potassium channels (K_{ATP}) is involved in ischaemic preconditioning (IP). In isolated buffer-perfused rabbit hearts, activation of mitochondrial K_{ATP} – through a generation of free radicals – acted as a trigger rather than a mediator of IP; the isolated buffer-perfused heart preparation, however, favours free radical generation. In contrast, *in vivo* studies in rats and dogs suggested that activation of K_{ATP} acts as a mediator of IP's protection. A detailed analysis on the role of K_{ATP} in IP's protection *in vivo* by varying the time and dose of K_{ATP} blocker administration is, however, lacking.

2 In 54 enflurane-anaesthetized pigs, the left anterior descending coronary artery was perfused by an extracorporeal circuit. Infarct size (IS, %, TTC) following 90 min sustained low-flow ischaemia and 120 min reperfusion was 26.6 ± 3.5 (s.e.m.) ($n = 8$). IP with one cycle of 10 min ischaemia and 15 min reperfusion reduced IS to 6.5 ± 2.1 ($n = 7$, $P < 0.05$). Blockade of K_{ATP} with glibenclamide (0.5 mg kg^{-1} i.v., 50 $\mu g\,min^{-1}$ continuous infusion) starting 10 min before or immediately following the preconditioning ischaemia abolished IS reduction by IP (20.7 ± 2.7, $n = 7$ and 21.9 ± 6.6, $n = 6$, respectively) while having no effect on IS *per se* (22.2 ± 5.2, $n = 7$), supporting a trigger role of K_{ATP} in IP. In contrast, starting glibenclamide following the preconditioning ischaemia 10 min prior to the sustained ischaemia did not prevent IS reduction by IP (3.7 ± 2.3, $n = 6$), even when its bolus dose was increased to 1.5 mg kg^{-1} (26.6 ± 3.8 with IP *vs* 37.5 ± 2.9 without IP; $n = 7$ and 6 respectively, $P < 0.05$), thereby refuting a mediator role of K_{ATP} in IP.

3 In conclusion, activation of K_{ATP} in the immediate reperfusion following the preconditioning ischaemia is pivotal for triggering IP.
British Journal of Pharmacology (2003) **139,** 65 – 72. doi:10.1038/sj.bjp.0705225

Keywords: Ischaemia; reperfusion; ischaemic preconditioning; infarct size; ATP-dependent potassium channels; glibenclamide; pigs

Abbreviations: IP, ischaemic preconditioning; IS, infarct size; K_{ATP}, ATP-dependent potassium channels; LAD, left anterior descending

Introduction

Ischaemic preconditioning (IP) is a potent cardioprotective phenomenon, first described by Murry *et al.* (1986), in which brief episodes of ischaemia/reperfusion reduce infarct size resulting from a subsequent sustained ischaemia, and it has been confirmed in all species tested so far (Yellon *et al.*, 1998), possibly also including humans (Yellon & Dana, 2000; Heusch, 2001). The signal transduction cascade of IP involves endogenous triggers (such as adenosine, bradykinin, opioids and free radicals) and mediators (such as protein kinase C, protein tyrosine kinases and mitogen-activated protein kinases) (for a review, see Baxter, 1997; Schulz *et al.*, 2001). Conceptually, triggers are important during the preconditioning phase, while mediators are important during the sustained ischaemia. ATP-dependent potassium channels (K_{ATP}) are involved in the cardioprotection obtained by IP. Their blockade prior to the preconditioning ischaemia abolishes the infarct size reduction by IP in rats (Schultz *et al.*, 1997a, b), rabbits (Munch-Ellingsen *et al.*, 1996; Morita *et al.*, 1997; Munch-Ellingsen *et al.*, 2000), dogs (Gross & Auchampach, 1992; Sanada *et al.*, 2001) and pigs (Schulz *et al.*, 1994). Apart from the controversy on whether sarcolemmal or mitochondrial K_{ATP} are important for the observed cardioprotection (for a review, see Gross & Fryer, 1999; O'Rourke, 2000; Schulz, 2000; Oldenburg *et al.*, 2002a; Gross, 2002), it also remains controversial whether or not K_{ATP} act as triggers or mediators in IP's protection. *In vitro* studies in isolated buffer-perfused rabbit hearts support the notion that activation of K_{ATP} acts as a trigger rather than a mediator in preconditioning's protection (Pain *et al.*, 2000). In this study, 200 μM 5-HD (mitochondrial K_{ATP} antagonist) or glibenclamide (nonselective K_{ATP} antagonist) abolished the infarct size reduction by IP when administered to bracket the preconditioning ischaemic period, while having no effect on infarct size when administered to bracket the sustained ischaemic period.

In contrast, data from rats and dogs *in vivo* suggest that the activation of mitochondrial K_{ATP} is involved in both triggering and mediating IP's protection (Auchampach *et al.*, 1992; Gross & Auchampach, 1992; Fryer *et al.*, 2001). In these studies, 5-HD (Fryer *et al.*, 2001) or glibenclamide (Auchampach *et al.*, 1992; Gross & Auchampach, 1992) not only when given prior to (Auchampach *et al.*, 1992; Gross & Auchampach, 1992; Fryer *et al.*, 2001) but also when given immediately at the end of the preconditioning ischaemia (Gross &

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Auchampach, 1992; Fryer *et al*., 2001) or during the initial 10 min of the sustained ischaemic period (Auchampach *et al*., 1992) attenuated infarct size reduction by IP. From these results, the authors concluded that the activation of K_{ATP} acts as a mediator rather than a trigger of IP's. Opening of mitochondrial K_{ATP} leads to the generation of free radicals as demonstrated in vascular smooth muscle cells (Krenz *et al*., 2002; Oldenburg *et al*., 2002b) and isolated cardiomyocytes (Yao *et al*., 1999), and free radical generation is involved in triggering cardioprotection by IP (Tanaka *et al*., 1994; Patel *et al*., 2001; Skyschally *et al*., 2002). Therefore, the reperfusion phase following the preconditioning ischaemia might be pivotal for triggering preconditioning's protection, since the free radical production following short periods of ischaemia is increased only during the first few minutes of the subsequent reperfusion (Bolli *et al*., 1988, 1989; Skyschally *et al*., 2002).

Thus, part of the controversy on K_{ATP} acting as a trigger or mediator may relate to model (*in vitro vs in vivo*) or species differences (rabbit *vs* rat and dog). Importantly, the time of drug administration may also be decisive (treatment starting immediately following the end of the preconditioning ischaemia *vs* treatment starting just prior to the sustained ischaemia).

We have therefore now tested in an established pig model *in vivo* whether the activation of K_{ATP} acts as trigger or mediator in ischaemic preconditioning by varying the time and dose of drug administration. Pigs were used, since their coronary anatomy and infarct development closely resemble that observed in humans (Schaper *et al*., 1988). We used the K_{ATP} channel blocker glibenclamide to block both sarcolemmal and mitochondrial K_{ATP} channels.

Methods

The experimental protocols employed in this study were approved by the bioethical committee of the district of Düsseldorf, Germany, and they adhere to the guiding principles of the American Physiological Society.

Experimental preparation

A total of 54 Göttinger minipigs (20 – 40 kg) of either sex were initially sedated using ketamine hydrochloride (1 g i.m.) and then anaesthetized with thiopental (Trapanal, 500 mg i.v., Byk Gulden, Konstanz, FRG). Through a midline cervical incision, the trachea was intubated for connection to a respirator (Dräger, Lübeck, FRG). Anaesthesia was then maintained using enflurane (1 – 1.5%) with an oxygen/nitrous oxide mixture (40%:60%). Arterial blood gases were monitored frequently in the initial stages of the preparation until stable, and then, periodically throughout the study (Radiometer, Copenhagen, Denmark). Rectal temperature was monitored and kept between 37 and 38°C using heating pads. Pigs were instrumented for the measurement of left ventricular (LV) pressure and wall thickness (Heusch *et al*., 2000). After heparinization, the left anterior descending (LAD) coronary artery and adjacent vein were cannulated, and the artery was perfused from an extracorporeal circuit, including a roller pump. To induce ischaemia, blood flow into the cannulated LAD coronary artery was reduced (see below) by reducing the speed of the roller pump; a residual blood flow of approximately 15 – 20% of baseline was maintained.

Regional myocardial function and regional myocardial blood flow

Regional myocardial work was determined as the sum of the instantaneous LV pressure – wall thickness product over the time of the cardiac cycle. The maximal work value during systole is reported as WI (Heusch *et al*., 1996). Regional myocardial blood flow was measured with radioactive microspheres (Schulz *et al*., 1989), and in a subset of experiments ($n = 13$) regional myocardial oxygen consumption was measured by multiplying the arteriocoronary venous oxygen difference with the transmural myocardial blood flow at the crystal site. Myocardial efficiency was calculated as the ratio of WI to regional myocardial oxygen consumption during normoperfusion without and with glibenclamide either at a low dose (LD) or high dose (HD).

Morphology

Up to six transverse myocardial slices from each heart were incubated in triphenyl tetrazolium chloride solution to identify necrotic tissue (Schulz *et al*., 2002). The amount of infarcted tissue is expressed as a percentage of the LV area at risk, as determined by the microspheres technique (Post *et al*., 1998).

Experimental protocols (Figure 1)

Control (n = 8)

Following baseline measurements of systemic haemodynamics, WI and regional myocardial blood flow, coronary inflow was reduced to achieve a 90% reduction in WI. At 5 and 85 min ischaemia, measurements were repeated, and thereafter the myocardium was reperfused for 2 h.

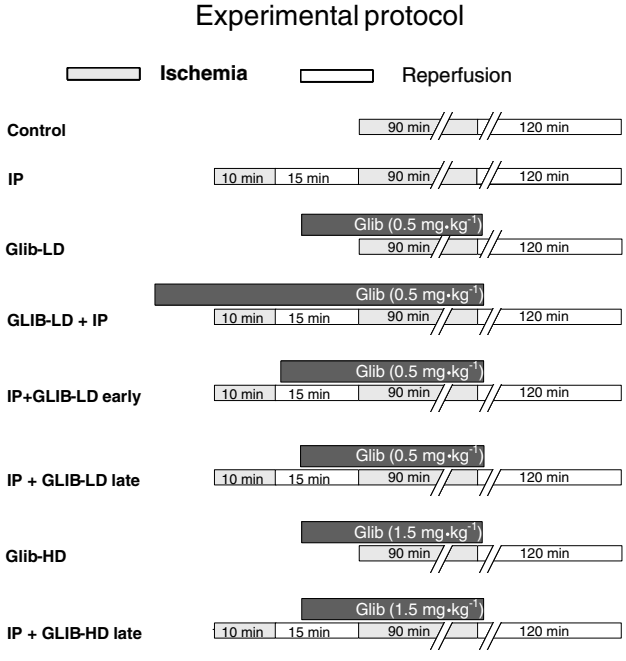

Figure 1 Schematic diagram of the experimental protocol.

Ischaemic preconditioning (IP, n = 7)

Following baseline measurements of systemic haemodynamics, WI and regional myocardial blood flow, the myocardium was subjected to one cycle of 10 min preconditioning ischaemia, with a 90% reduction in WI, and 15 min reperfusion. During reperfusion, coronary arterial pressure was maintained at the level measured prior to ischaemia by continuously adapting coronary inflow with the roller pump, thus permitting reactive hyperemia. Following reperfusion, coronary inflow was once again reduced to the same level as during the preconditioning ischaemia. Thereafter, the protocol was identical to that of the control group.

LD glibenclamide (Glib-LD, n = 7)

Following baseline measurements of systemic haemodynamics, WI, regional myocardial blood flow and oxygen consumption, glibenclamide dissolved in a mixture (ratio of 35:4:1) of NaCl (0.9%), ethanol (70%) and NaOH (80 mM), was given intravenously as a bolus dose of 0.5 mg kg^{-1} (LD) followed by a continuous infusion of 50 μg min^{-1} until the end of the sustained ischaemia. At 10 min after the bolus administration of glibenclamide, measurements of systemic haemodynamics, WI and regional myocardial blood flow and oxygen consumption were repeated before coronary inflow was reduced to achieve an approximately 90% reduction in WI. At 5 and 85 min ischaemia, measurements of systemic haemodynamics, WI and regional myocardial blood flow were repeated, and thereafter the myocardium was reperfused for 2 h.

Glib-LD + IP (n = 7)

The protocol of this group was identical to that of the IP group, except that starting 10 min before the preconditioning ischaemia, glibenclamide at a bolus dose of 0.5 mg kg^{-1} (LD) was administered followed by a continuous infusion of 50 μg min^{-1} until the end of the sustained ischaemia.

IP + Glib-LD early (n = 6) or late (n = 6)

The protocols of both groups were identical to that of the IP group, except that glibenclamide at a bolus dose of 0.5 mg kg^{-1} (LD) was administered immediately following the preconditioning ischaemia (early) or at 10 min prior to the sustained ischaemia (late), each followed by a continuous infusion of 50 μg min^{-1} until the end of the sustained ischaemia.

HD Glib (Glib-HD, n = 6)

Since a previous study in rabbits suggested that treatment with K_{ATP}-antagonists starting between the preconditioning and the sustained ischaemia required a higher drug concentration to be effective (Wang *et al*., 2001), glibenclamide was now administered at a bolus dose of 1.5 mg kg^{-1} (HD) followed by a continuous infusion of 150 μg min^{-1} until the end of the sustained ischaemia.

At 10 min after the bolus administration of glibenclamide, measurements of systemic haemodynamics, WI and regional myocardial blood flow and oxygen consumption were repeated before coronary inflow was reduced to achieve an approximately 90% reduction in WI. At 5 and 85 min ischaemia, measurements of systemic haemodynamics, WI and regional myocardial function and blood flow were repeated, and thereafter the myocardium was reperfused for 2 h.

IP + Glib-HD late (n = 6)

The protocol was identical to that of the IP group, except that glibenclamide was now administered at a bolus dose of 1.5 mg kg^{-1} (HD) 10 min prior to the sustained ischaemia followed by a continuous infusion of 150 μg min^{-1} until the end of the sustained ischaemia.

Data analysis and statistics

Data are reported as mean values ± s.e.m. Statistical analysis for all groups comprised two-way ANOVA for repeated measures and Fisher's LSD tests when significant overall effects were detected. Infarct size, area at risk and ischaemic subendocardial blood flow were analysed by one-way ANOVA and Fisher's LSD tests. Data on WI, regional myocardial oxygen consumption and myocardial efficiency during normoperfusion without and with glibenclamide at a LD or HD were compared by paired *t*-tests. A *P*-value less than 0.05 was accepted as indicating a significant difference in the mean values.

Results

Haemodynamics

Heart rate did not change significantly during the time course of the protocol within the individual groups and was also not different among the groups (Table 1). Glibenclamide given prior to ischaemia (Glib-LD, Glib-LD + IP, Glib-HD) increased LV peak pressure. However, except for an increased LV peak pressure at 5 min of the sustained ischaemia in the HD glibenclamide groups (Glib-HD, IP-Glib-HD late), systemic haemodynamics did not differ among groups during the sustained ischaemia (Table 1).

Regional myocardial function of the posterior control wall remained stable throughout the experimental protocol in each group. With glibenclamide at a low (Glib-LD) or high (Glib-HD) dose, WI of the anterior wall remained unchanged (Figure 2a). While regional myocardial oxygen consumption of the anterior myocardium remained unchanged following LD glibenclamide, it increased with the HD of glibenclamide (Figure 2b). In consequence, regional myocardial efficiency was reduced with the HD glibenclamide (Figure 2c).

During sustained ischaemia, WI did not differ among groups.

Infarct size

Area at risk (Figure 3a) and ischaemic myocardial blood flow (Figure 3b) were comparable between groups. Following 90 min ischaemia and 120 min reperfusion, infarct size was 26.6 ± 3.5% (Figure 3c). Infarct size was significantly reduced to 6.5 ± 2.1% by IP with one cycle of 10 min ischaemia and 15 min of reperfusion. LD glibenclamide did not change infarct size *per se* (22.2 ± 5.2%), but when starting 10 min before the preconditioning ischaema (20.7 ± 2.7%) or immediately

Table 1 Systemic haemodynamics

		Baseline	*Glibenclamide before ischaemia*	*IP*	*15 min reperfusion without or with glibenclamide*	*5 min ischaemia*	*85 min ischaemia*
HR	Control	99±2	ND	ND	ND	101±2	103±3
	IP	97±1	ND	97±1	96±1	96±1	98±1
	Glib-LD	96±1	96±1	ND	ND	96±1	97±2
	Glib-LD+IP	98±1	97±2	100±1	100±1	100±1	100±1
	IP+Glib-LD early	105±5	ND	107±5	108±5	108±5	108±5
	IP+Glib-LD late	102±3	ND	101±4	102±2	103±2	103±2
	Glib-HD	104±1	105±1	ND	ND	105±1	105±1
	IP+Glib-HD late	104±5	ND	103±6	105±6	103±6	103±6
LVPP	Control	95±3	ND	ND	ND	83±4*	88±2
	IP	97±4	ND	84±3*	97±4**	84±3*·**	83±3*
	Glib-LD	96±4	108±4*	ND	ND	87±3***	82±4*·***
	Glib-LD-IP	101±3	117±3*	100±7***	110±7**	92±4**·***	94±3***
	IP+Glib-LD early	96±3	ND	84±3*	101±3**	89±3**	87±3
	IP+Glib–LD late	93±2	ND	86±3	102±2**	92±4**	85±3
	Glib-HD	103±3	119±5*	ND	ND	100±4***.b	97±3***.c
	IP+Glib-HD late	104±4	ND	91±6*	116±11***	105±9[a]	97±6[c]
dP/dt	Control	1224±81	ND	ND	ND	1019±69	1378±57
	IP	1251±55	ND	1011±35	1118±75	994±55	1032±57
	Glib-LD	1415±164	1510±156	ND	ND	1172±95	1267±93
	Glib-LD+IP	1488±155	1573±175	1297±179	1416±275	1348±278	1114±196*·***
	IP+Glib-LD early	1330±109	ND	1066±60	1246±84	1120±69	1150±63
	IP+Glib-LD late	1203±130	ND	945±81	1014±68	966±86	995±66
	Glib-HD	1445±51	1497±74	ND	ND	1173±60***	1339±93
	IP+Glib-HD late	1439±113	ND	1166±133	1306±159	1247±130	1309±99
CAP	Control	116±3	ND	ND	ND	29±1*	30±2*
	IP	116±4	ND	30±2*	109±3**	29±2*·**	27±2*
	Glib-LD	118±2	129±6	ND	ND	33±3*·***	31±2*·***
	Glib-LD+IP	118±2	128±8	38±3*	107±12**·***	36±3**·***	38±3*·***
	IP+Glib-LD early	122±4	ND	28±4*	132±11**	31±1*·**	35±2*
	IP+Glib-LD late	115±1	ND	28±1*	127±3**	31±1*·**	29±1*
	Glib-HD	114±2	142±5*·[d]	ND	ND	32±2*·***	32±2*·***
	IP+Glib-HD late	117±5	ND	27±3*	141±19*·***	33±4*·**	33±5*
CBF	Control	40.2±3.7	ND		ND	6.0±0.8*	6.3±1.1*
	IP	32.5±5.2	ND	5.7±1.7*	38.9±4.2**	5.7±1.7*·**	6.0±1.5*
	Glib-LD	28.8±3.9	29.0±3.8	ND	ND	5.0±0.5*·***	4.6±0.5*·***
	Glib-LD+IP	25.5±2.0	27.6±2.6	4.4±0.4*·***	26.6±4.4**	4.4±0.4*·**·***	4.6±0.6*·***
	IP+Glib-LD early	39.7±4.1	ND	4.7±0.9*	59.7±5.6*·**	4.7±0.9*·**	4.7±0.9*
	IP+Glib-LD late	35.8±4.1	ND	5.4±0.8*	48.0±6.4*·**	5.3±0.8*·**	5.3±08*
	Glib-HD	42.6±5.1	42.8±5.1	ND	ND	6.0±1.1*·***	6.0±1.1*·***
	IP+Glib-HD late	34.9±4.7	ND	3.9±2.4*	41.8±11.1**	3.8±2.2*·**	3.8±2.2*
TMBF	Control	0.84±0.13	ND	ND	ND	0.11±0.01*	0.11±0.02*
	IP	0.70±0.05	ND	0.10±0.02*	ND	0.10±0.02*	0.10±0.02*
	Glib-LD	0.74±0.07	0.72±0.06	ND	ND	0.13±0.02*·***	0.13±0.02*·***
	Glib-LD+IP	0.72±0.08	0.71±0.05	0.09±0.01*	ND	0.10±0.01*·***	0.11±0.01*·***
	IP+Glib-LD early	0.75±0.07	ND	0.08±0.01*	1.30±0.14*·**	0.09±0.02*·**	0.11±0.02*
	IP+Glib-LD late	0.80±0.08	ND	0.09±0.02*	1.06±0.10**	0.09±0.02*·**	0.10±0.02*
	Glib-HD	0.76±0.07	0.79±0.07	ND	ND	0.10±0.02*·**	0.11±0.02*·***
	IP+Glib-HD late	0.79±0.09	ND	0.07±0.01*	0.93±0.13**	0.07±0.01*·**	0.09±0.01*

Control ($n=8$); IP, ischaemic preconditioning ($n=7$); Glib-LD, glibenclamide LD starting 10 min prior to the sustained ischaemia (0.5 mg kg^{-1} i.v. followed by 50 μg min^{-1}, $n=7$); Glib-LD+IP, glibenclamide LD starting 10 min prior to the preconditioning ischaemia+IP ($n=7$); IP+Glib-LD early, IP+glibenclamide LD starting immediately at the end of the preconditioning ischaemia ($n=6$); IP+Glib-LD late, IP+glibenclamide LD starting 10 min prior to the sustained ischaemia ($n=7$); Glib-HD, glibenclamide HD starting 10 min prior to the sustained ischaemia (1.5 mg kg^{-1} i.v. followed by 50 μg min^{-1}, $n=7$); IP+Glib-HD late, IP+glibenclamide HD starting 10 min prior to the sustained ischaemia; HR, heart rate (beats min^{-1}); LVPP, left ventricular peak pressure (mmHg); dP/dt, maximum of the first derivative of LV pressure (mmHg s^{-1}); CAP, coronary arterial pressure (mmHg); CBF, coronary blood flow (ml min^{-1}); TMBF, transmural myocardial blood flow (ml min^{-1} g^{-1}). *$P<0.05$ vs baseline. **$P<0.05$ vs preceding value, ***$P<0.05$ vs glibenclamide. [a]$P<0.05$ vs all groups; [b]$P<0.05$ vs control, IP, Glib-LD, IP+Glib-LD early; [c]$P<0.05$ vs IP, Glib-LD, IP+Glib-LD late; [d]$P<0.05$ vs Glib-LD, Glib-LD+IP. ND; not determined.

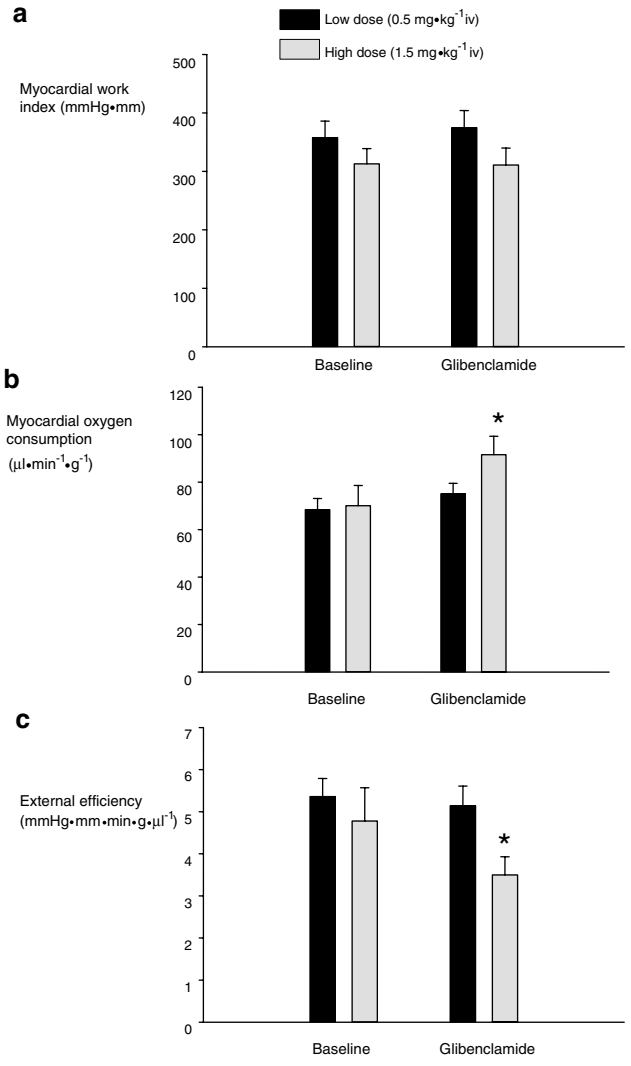

Figure 2 A low-dose or high-dose glibenclamide did not alter regional myocardial function—expressed as a work index—of the anterior wall (a). While regional myocardial oxygen consumption of the anterior myocardium remained unchanged following a low-dose glibenclamide, it significantly increased following pretreatment with high-dose glibenclamide (b). Thus, regional myocardial efficiency was reduced following the high-dose glibenclamide treatment (c). $^*P<0.05$ *vs* baseline and low dose.

following the preconditioning ischaemia ($21.9\pm16.6\%$) abolished the protection by IP. In contrast, LD glibenclamide starting 10 min prior to the sustained ischaemia did not prevent infarct size reduction by IP ($3.7\pm2.3\%$). Pretreatment of the myocardium with HD glibenclamide *per se* increased infarct size following 90 min ischaemia and 120 min reperfusion to 37.5 ± 2.9 ($P<0.05$ *vs* control and LD-Glib). However, the HD of glibenclamide starting 10 min prior to the sustained ischaemia did not prevent IP's protection, since infarct size was still significantly reduced to $26.6\pm3.8\%$ ($P<0.05$ *vs* HD-Glib).

Discussion

The major finding of the present study is that blockade of K_{ATP} with glibenclamide just before the sustained ischaemia

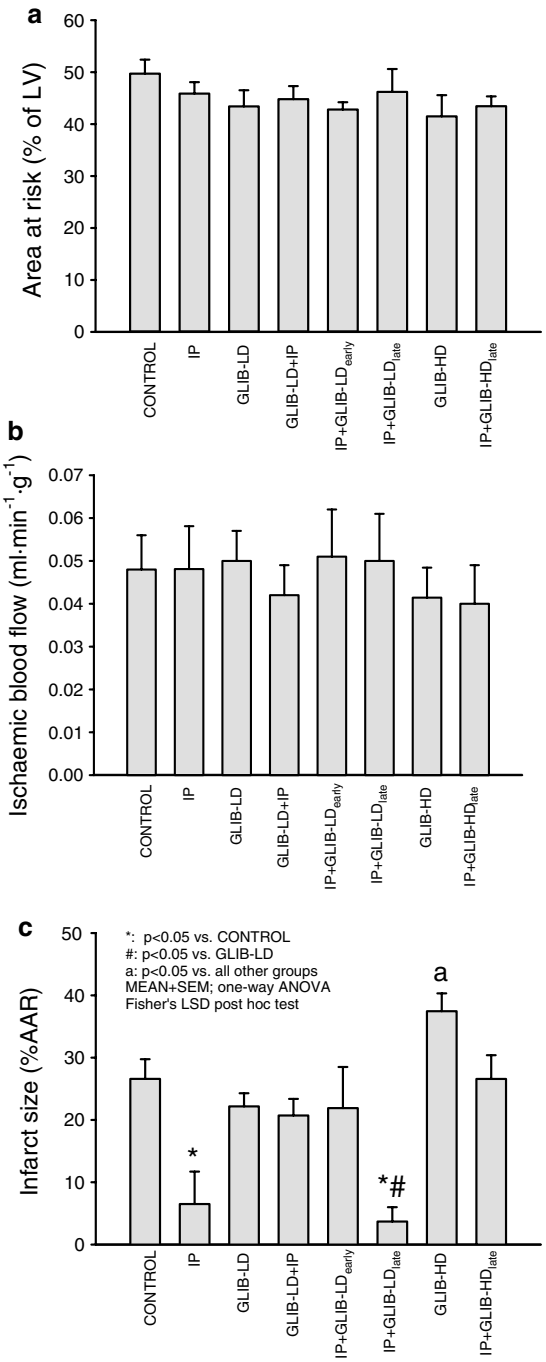

Figure 3 Area at risk (% of left ventricle, a), ischaemic blood flow ($ml\,min^{-1}\,g^{-1}$, b) and infarct size (% of the area at risk, c) in all the groups. Area at risk and ischaemic blood flow did not differ between the groups. Infarct size was significantly reduced in hearts preconditioned by one cycle of 10 min ischaemia and 15 min reperfusion (IP) compared to hearts undergoing 90 min ischaemia only (control). A low-dose glibenclamide (Glib-LD) did not alter infarct size *per se*, but completely abolished the infarct size reducing effect of IP when given before (Glib-LD + IP) or immediately following (IP + Glib-LD early) the preconditioning cycle of 10 min ischaemia and 15 min reperfusion. A low-dose glibenclamide did not affect the infarct size reducing effect of ischaemic preconditioning when given 10 min before the sustained ischaemia (IP + Glib-LD late). The high-dose glibenclamide (Glib-HD) increased infarct size *per se*, but when given 10 min prior to the sustained ischaemia did not abolish IP's protection (IP + Glib-HD late).

did not affect the infarct size reducing effect of IP, whereas blockade of K_{ATP} before the preconditioning ischaemia or during the immediate reperfusion following the preconditioning ischaemia abolished IP's protection, suggesting that activation of K_{ATP} is a trigger rather than a mediator of IP in pigs.

Critique of methods

The strengths and limitations of the experimental model have been discussed in detail elsewhere (Schulz *et al*., 1998).

HD glibenclamide increased infarct size following 90 min sustained ischaemia without or with IP over the respective infarct sizes obtained in the control or IP groups, but it did not abolish the cardioprotective action of IP as such. Also in a previous study in rabbits *in vivo*, glibenclamide increased infarct size above that in the respective control group (Thornton *et al*., 1993). In the present study, the HD glibenclamide increased LV pressure during early sustained ischaemia. However, such an increase in LV pressure is unlikely to account for the observed increase in infarct size, since a similar increase in LV pressure by aortic banding did not affect the extent of myocardial infarction in the same animal model (Heusch *et al*., 2000; Post *et al*., 2000). A more likely explanation for the increased infarct size with the HD glibenclamide is a decreased myocardial efficiency (Figure 2). Such decreased efficiency following blockade of K_{ATP} was recently demonstrated also in isolated rat hearts perfused at constant flow (Laclau *et al*., 2002), and a decreased efficiency of oxygen usage in the presence of a limited oxygen supply during ischaemia is indeed expected to favour the development of irreversible tissue damage. In the present study, anaesthesia was maintained by enflurane. Enflurane reduced infarct size in rabbits *in vivo*, although the infarct size reduction by enflurane was less than that obtained with halothane or isoflurane (Cope *et al*., 1997). In rabbits, the reduction of infarct size by isoflurane involved free radical production, since it was blocked by the free radical scavenger MPG (Tanaka *et al*., 2002). In contrast, in the same animal model as in the present study, scavenging free radicals by vitamin C had no effect on infarct development *per se*, while the infarct size reduction by IP was completely abolished (Skyschally *et al*., 2003), arguing against a significant enflurane/free radical-mediated cardioprotection in the present study. In isolated cardiomyocytes, isoflurane and servoflurane induced flavoprotein oxidation through opening of mitochondrial K_{ATP} channels (Kohro *et al*., 2001) and reduced the number of trypan-blue-positive cardiomyocytes following ischaemia/reperfusion (Zaugg *et al*., 2002), the latter effect being additive to that of diazoxide-induced cardiomyocyte protection. Again, LD glibenclamide – which effectively inhibited cardioprotection by IP – did not affect infarct size *per se*. The increase in infarct size observed with the higher dose of glibenclamide in the present study was also observed in rabbits *in vivo* anaesthetized with sodium pentobarbital (Thornton *et al*., 1993). Therefore, although we cannot definitely rule out any effect of enflurane on infarct size development *per se*, the above findings make a significant contribution of enflurane to the results of the present study unlikely.

In the present study, we were not able to distinguish between sarcolemmal and mitochondrial K_{ATP} in the cardioprotection by IP. In a recent study in pigs, 5-HD when given prior to the preconditioning ischaemia failed to attenuate infarct size reduction by IP, suggesting that mitochondrial K_{ATP} are less important in pigs (Schwartz *et al*., 2002). However, in this study a single dose of 5 or 10 mg kg^{-1} 5-HD (which equals approximately 40 – 80 μM) was administered 15 min prior to the preconditioning ischaemia.

This finding contrasts previous reports in dogs (Auchampach *et al*., 1992) and rats (Fryer *et al*., 2001) in which 5-HD abolished the infarct size reduction by IP, and a more recent report in which 5-HD also abolished the IP-induced reduction of arrhythmias in dogs (Vegh & Parratt, 2002). Potential explanations for the observed differences might again relate to species differences (pigs *vs* dogs and rats), the dose of 5-HD (dogs 300 (Vegh & Parratt, 2002) – 500 (Auchampach *et al*., 1992) μM) or the intensity of the preconditioning stimulus. Schwartz *et al*. used two cycles of preconditioning ischaemia/reperfusion, while the other two studies used only a single cycle of ischaemia/reperfusion. It is plausible that it is more difficult to block the protective effect of multiple preconditioning cycles as opposed to a single cycle of ischaemia/reperfusion (Gross, 2002). Indeed, a less intense preconditioning stimulus primarily involves bradykinin (Schulz *et al*., 1998) and prostaglandins (Gres *et al*., 2002), while a more intense preconditioning stimulus involves adenosine (Schulz *et al*., 1998) and opioids (Schulz *et al*., 2000). For adenosine, however, it has recently been shown in rabbit hearts that its cardioprotection cannot be blocked by 5-HD (Cohen *et al*., 2001).

K_{ATP} as trigger vs *mediator*

The signal transduction of IP can be classified into triggers, such as adenosine, bradykinin, opioids and free radicals, and mediators, such as protein kinase C, protein tyrosine kinase and mitogen-activated protein kinases. Free radicals can trigger the entrance into a preconditioned state by activating protein kinases (for review see Schulz *et al*., 2001).

Although undoubtedly activation of K_{ATP} is involved in IP's protection, their involvement as a trigger or a mediator is still controversial (Auchampach *et al*., 1992; Gross & Auchampach, 1992; Pain *et al*., 2000; Fryer *et al*., 2001). While part of the controversy might relate to the use of either *in vitro* (Pain *et al*., 2000) or *in vivo* (Gross & Auchampach, 1992; Fryer *et al*., 2001) models or species differences (rabbit *vs* rat *vs* dog), the present study highlights another important issue, that is, time of drug administration. In anaesthetized rats (Fryer *et al*., 2001) and dogs (Gross & Auchampach, 1992), blockade of K_{ATP} with 5-HD (Fryer *et al*., 2001) or glibenclamide (Gross & Auchampach, 1992) attenuated infarct size reduction by IP when administered either prior to or immediately following the preconditioning ischaemia. These results were confirmed also in the present study in pigs. However, when – in the present study – glibenclamide treatment was started several minutes after established reperfusion, it did not block preconditioning's protection, both at a LD and HD. This finding confirms the results obtained in the isolated rabbit hearts, in which treatment with 5-HD or glibenclamide just prior to the sustained ischaemia failed to block infarct size reduction by IP (Pain *et al*., 2000), but remains in contrast to a study in dogs in which glibenclamide given during the early phase of the sustained ischaemia still abolished infarct size reduction by IP (Auchampach *et al*., 1992).

Conclusion

The results of the present study reconcile some of the existing controversies on the role of K_{ATP} in the signal transduction cascade of IP, in that activation of K_{ATP} in the immediate reperfusion following the preconditioning ischaemia is pivotal for *triggering* IP. Full reperfusion is not necessarily mandatory for IP's protection, since in the same pig model, a brief episode of no-flow ischaemia increased the tolerance to subsequent sustained severe low-flow ischaemia without an intermittent period of full reperfusion (Schulz *et al*., 1995). This cardioprotective effect was again glibenclamide-sensitive and apparently mediated by activation of K_{ATP}.

The signal cascade downstream of the activation of K_{ATP} involves the generation of free radicals *in vitro* (Pain *et al*., 2000). Also *in vivo*, free radicals are generated upon immediate reperfusion following short periods of ischaemia/reperfusion (Bolli *et al*., 1988; 1989; Skyschally *et al*., 2003), and clearly those radicals are involved in IP's protection in pigs (Skyschally *et al*., 2003). However, further studies are required to elucidate the role of mitochondrial *vs* sarcolemmal K_{ATP} in pigs *in vivo*.

References

AUCHAMPACH, J.A., GROVER, G.J. & GROSS, G.J. (1992). Blockade of ischaemic preconditioning in dogs by the novel ATP dependent potassium channel antagonist sodium 5-hydroxydecanoate. *Cardiovasc. Res.,* **26,** 1054 – 1062.

BAXTER, G. (1997). Ischaemic preconditioning of myocardium. *Ann. Med.,* **29,** 345 – 352.

BOLLI, R., JEROUDI, M.O., PATEL, B.S., DuBOSE, C.M., LAI, E.K., ROBERTS, R. & McCAY, P.B. (1989). Direct evidence that oxygen-derived free radicals contribute to postischemic myocardial dysfunction in the intact dog. *Proc. Natl. Acad. Sci. U.S.A.,* **86,** 4695 – 4699.

BOLLI, R., PATEL, B.S., JEROUDI, M.O., LAI, E.K. & McCAY, P.B. (1988). Demonstration of free radical generation in ''stunned'' myocardium of intact dogs with the use of the spin trap α-phenyl *N*-tert-butyl nitrone. *J. Clin. Invest.,* **82,** 476 – 485.

COHEN, M.V., YANG, X.-M., LIU, G.S., HEUSCH, G. & DOWNEY, J.M. (2001). Acetylcholine, bradykinin, opioids, and phenylephrine, but not adenosine, trigger preconditioning by generating free radicals & opening mitochondrial K_{ATP} channels. *Circ. Res.,* **89,** 273 – 278.

COPE, D.K., IMPASTATO, W.K., COHEN, M.V. & DOWNEY, J.M. (1997). Volatile anesthetics protect the ischemic rabbit myocardium from infarction. *Anesthesiology,* **86,** 699 – 709.

FRYER, R.M., HSU, A.K. & GROSS, G.J. (2001) Mitochondrial K_{ATP} channel opening is important during index ischemia and following myocardial reperfusion in ischemic preconditioned rat hearts. *J. Mol. Cell Cardiol.,* **33,** 831 – 834.

GRES, P., SCHULZ, R., JANSEN, J., UMSCHLAG, C. & HEUSCH, G (2002). Involvement of endogenous prostaglandins in ischemic preconditioning in pigs. *Cardiovasc. Res.,* **55,** 626 – 632.

GROSS, G.J. (2002). The role of mitochondrial KATP channels in the antiarrhythmic effects of ischaemic preconditioning in dogs. *Br. J. Pharmacol.,* **137,** 939 – 940.

GROSS, G.J. & AUCHAMPACH, J.A. (1992). Blockade of ATP-sensitive potassium channels prevents myocardial preconditioning in dogs. *Circ. Res.,* **70,** 223 – 233.

GROSS, G.J. & FRYER, R.M. (1999). Sarcolemmal versus mitochondrial ATP-sensitive K^+ channels and myocardial preconditioning. *Circ. Res.,* **84,** 973 – 979.

HEUSCH, G. (2001). Nitroglycerin and delayed preconditioning in humans. Yet another new mechanism for an old drug? *Circulation,* **103,** 2876 – 2878.

HEUSCH, G., POST, H., MICHEL, M.C., KELM, M. & SCHULZ, R. (2000). Endogenous nitric oxide and myocardial adaptation to ischemia. *Circ. Res.,* **87,** 146 – 152.

HEUSCH, G., ROSE, J., SKYSCHALLY, A., POST, H. & SCHULZ, R. (1996). Calcium responsiveness in regional myocardial short-term hibernation and stunning in the *in situ* porcine heart – inotropic responses to postextrasystolic potentiation and intracoronary calcium. *Circulation,* **93,** 1556 – 1566.

KOHRO, S., HOGAN, Q.H., NAKAE, Y., YAMAKAGE, M. & BOSNJAK, Z.J. (2001). Anesthetic effects on mitochondrial ATP-sensitive K channel. *Anesthesiology,* **95,** 435 – 440.

KRENZ, M., OLDENBURG, O., WIMPEE, H., COHEN, M.V., GARLID, K.D., CRITZ, S.D., DOWNEY, J.M. & BENOIT, J.N. (2002). Opening of ATP-sensitive potassium channels causes generation of free radicals in vascular smooth muscle cells. *Basic Res. Cardiol.,* **97,** 365 – 373.

LACLAU, M.N., DOS SANTOS, P., BOUDINA, S., TARIOSSE, L., RAFFARD, G., DIOLEZ, P., BONORON-ADÈLE, S. & GARLID, K.D. (2002). The essential role of the mitochondrial ATP-sensitive potassium channel in cardiac inotropy. *Circulation,* **106** (Suppl)**,** 11 – 197.

MORITA, Y., MURAKAMI, T., IWASE, T., NAGAI, K., NAWADA, R., KOUCHI, I., AKAO, M. & SASAYAMA, S. (1997). KATP channels contribute to the cardioprotection of preconditioning independent of anaesthetics in rabbit hearts. *J. Mol. Cell Cardiol.,* **29,** 1267 – 1276.

MUNCH-ELLINGSEN, J., BUGGE, E. & YTREHUS, K. (1996). Blockade of the KATP-channel by glibenclamide aggravates ischemic injury, and counteracts ischemic preconditioning. *Basic Res. Cardiol.,* **91,** 382 – 388.

MUNCH-ELLINGSEN, J., LOKEBO, J.E., BUGGE, E., JONASSEN, A.K., RAVINGEROVA, T. & YTREHUS, K. (2000). 5-HD abolishes ischemic preconditioning independently of monophasic action potential duration in the heart. *Basic Res. Cardiol.,* **95,** 228 – 234.

MURRY, C.E., JENNINGS, R.B. & REIMER, K.A. (1986). Preconditioning with ischemia: a delay of lethal cell injury in ischemic myocardium. *Circulation,* **74,** 1124 – 1136.

O'ROURKE, B. (2000). Myocardial K_{ATP} channels in preconditioning. *Circ. Res.,* **87,** 845 – 855.

OLDENBURG, O., COHEN, M.V., YELLON, D. & DOWNEY, J.M. (2002a). Mitochondrial K_{ATP} channels: role in cardioprotection. *Cardiovasc. Res.,* **55,** 429 – 437.

OLDENBURG, O., QIN, Q., SHARMA, A.R., COHEN, M.V., DOWNEY, J.M. & BENOIT, J.N. (2002b). Acetylcholine leads to free radical production dependent on K_{ATP} channels, G_i proteins, phosphatidylinositol 3-kinase and tyrosine kinase. *Cardiovasc. Res.,* **55,** 544 – 552.

PAIN, T., YANG, X.-M., CRITZ, S.D., YUE, Y., NAKANO, A., LIU, G.S., HEUSCH, G., COHEN, M.V. & DOWNEY, J.M. (2000). Opening of mitochondrial K_{ATP} channels triggers the preconditioned state by generating free radicals. *Circ. Res.,* **87,** 460 – 466.

PATEL, H.H., HSU, A., MOORE, J. & GROSS, G.J. (2001). BW373U86, a δ opioid agonist, partially mediates delayed cardioprotection via a free radical mechanism that is independent of opioid receptor stimulation. *J. Mol. Cell Cardiol.,* **33,** 1455 – 1465.

POST, H., SCHULZ, R., BEHRENDS, M., GRES, P., UMSCHLAG, C. & HEUSCH, G. (2000). No involvement of endogenous nitric oxide in classical ischemic preconditioning in swine. *J. Mol. Cell Cardiol.,* **32,** 725 – 733.

POST, H., SCHULZ, R., VAHLHAUS, C., HÜSING, J., HIRCHE, H., GALLAGHER, K.P. & HEUSCH, G. (1998). Impact of resting and ischemic blood flow on infarct probability in ischemic preconditioning – a new approach to infarct size-blood flow data by logistic regression. *J. Mol. Cell Cardiol.,* **30,** 2719 – 2728.

SANADA, S., KITAKAZE, M., ASANUMA, H., HARADA, K., OGITA, H., NODE, K., TAKASHIMA, S., SAKATA, Y., ASAKURA, M., SHINOZAKI, Y., MORI, H., KUZUYA, T. & HORI, M. (2001). Role of mitochondrial and sarcolemmal K_{ATP} channels in ischemic preconditioning of the canine heart. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H256 – H263.

SCHAPER, W., GÖRGE, G., WINKLER, B. & SCHAPER, J. (1988). The collateral circulation of the heart. *Prog. Cardiovasc. Dis.,* **31,** 57 – 77.

SCHULTZ, J.E.J., QIAN, Y.Z., GROSS, G.J. & KUKREJA, R.C. (1997a). The ischemia-selective KATP channel antagonist, 5-hydroxydecanoate, blocks ischemic preconditioning in the rat heart. *J. Mol. Cell Cardiol.,* **29,** 1055 – 1060.

SCHULTZ, J.J., YAO, Z., CAVERO, I. & GROSS, G.J. (1997b). Glibenclamide-induced blockade of ischemic preconditioning is time-dependent in intact rat heart. *Am. J. Physiol.,* **41,** H2607 – H2615.

SCHULZ, R. (2000). Editorial: ischemic preconditioning. *Basic Res. Cardiol.,* **95,** 271.

SCHULZ, R., BELOSJOROW, S., GRES, P., JANSEN, J., MICHEL, M.C. & HEUSCH, G (2002). p38 MAP kinase is a mediator of ischemic preconditioning in pigs. *Cardiovasc. Res.,* **55,** 690 – 700.

SCHULZ, R., COHEN, M.V., BEHRENDS, M., DOWNEY, J.M. & HEUSCH, G. (2001). Signal transduction of ischemic preconditioning. *Cardiovasc. Res.,* **52,** 181 – 198.

SCHULZ, R., GRES, P. & HEUSCH, G. (2001). Involvement of endogenous opioids in ischemic preconditioning, but not in short-term hibernation in pigs. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H2175 – H2181.

SCHULZ, R., MIYAZAKI, S., MILLER, M., THAULOW, E., HEUSCH, G., ROSS JR J. & GUTH, B.D. (1989). Consequences of regional inotropic stimulation of ischemic myocardium on regional myocardial blood flow and function in anesthetized swine. *Circ. Res.,* **64,** 1116 – 1126.

SCHULZ, R., POST, H., SAKKA, S., WALLBRIDGE, D.R. & HEUSCH, G. (1995). Intraischemic preconditioning. Increased tolerance to sustained low-flow ischemia by a brief episode of no-flow ischemia without intermittent reperfusion. *Circ. Res.,* **76,** 942 – 950.

SCHULZ, R., POST, H., VAHLHAUS, C. & HEUSCH, G. (1998). Ischemic preconditioning in pigs: a graded phenomenon. Its relation to adenosine and bradykinin. *Circulation,* **98,** 1022 – 1029.

SCHULZ, R., ROSE, J. & HEUSCH, G. (1994). Involvement of activation of ATP-dependent potassium channels in ischemic preconditioning in swine. *Am. J. Physiol. Heart Circ. Physiol.,* **267,** H1341 – H1352.

SCHWARTZ, L.M., WELCH, T.S. & CRAGO, M.S. (2002). Cardioprotection by multiple preconditioning cycles does not require mitochondrial K_{ATP} channels in pigs. *Am. J. Physiol. Heart Circ. Physiol.,* **283,** H1538 – H1544.

SKYSCHALLY, A., SCHULZ, R., GRES, P., KORTH, H.-G. & HEUSCH G. (2003). Attenuation of ischemic preconditioning by scavenging of free oxyradicals with ascorbic acid in pigs. *Am. J. Physiol. Heart Circ. Physiol.,* **284,** H698–H703.

TANAKA, K., WEIHRAUCH, D., KEHL, F., LUDWIG, L.M., LADISA, J.F., KERSTEN, J.R., PAGEL, P.S. & WARLTIER, D.C. (2002). Mechanism of preconditioning by isoflurane in rabbits: a direct role for reactive oxygen species. *Anesthesiology,* **97,** 1485 – 1490.

TANAKA, M., FUJIWARA, H., YAMASAKI, K. & SASAYAMA, S. (1994). Superoxide dismutase and N-2-mercaptopropionyl glycine attenuate infarct size limitation effect of ischaemic preconditioning in the rabbit. *Cardiovasc. Res.,* **28,** 980 – 986.

THORNTON, J.D., THORNTON, C.S., STERLIN, D.L. & DOWNEY, J.M. (1993). Blockade of ATP-sensitive potassium channels increases infarct size but does not prevent preconditioning in rabbit hearts. *Circ. Res.,* **72,** 44 – 49.

VEGH, A. & PARRATT, J.R. (2002). The role of mitochondrial K_{ATP} channels in antiarrhythmic effects of ischaemic preconditioning in dogs. *Br. J. Pharmacol.,* **137,** 1107 – 1115.

WANG, S., CONE, J. & LIU, Y. (2001). Dual roles of mitochondrial K_{ATP} channels in diazoxide-mediated protection in isolated rabbit hearts. *Am. J. Physiol. Heart Circ. Physiol.,* **280,** H246 – H256

YAO, Z., TONG, J., TAN, X., LI, C., SHAO, Z., KIM, W.C., VANDEN HOEK, T.L., BECKER, L.B., HEAD, C.A. & SCHUMACKER, P.T. (1999). Role of reactive oxygen species in acetylcholine-induced preconditioning in cardiomyocytes. *Am. J. Physiol.,* **277,** H2504 – H2509.

YELLON, D.M., BAXTER, G.F., GARCIA-DORADO, D., HEUSCH, G. & SUMERAY, M.S. (1998). Ischaemic preconditioning: present position and future directions. *Cardiovasc. Res.,* **37,** 21 – 33.

YELLON, D.M. & DANA, A. (2000). The preconditioning phenomenon. A tool for the scientist or a clinical reality? *Circ. Res.,* **87,** 543 – 550.

ZAUGG, M., LUCCHINETTI, E., SPAHN, D.R., PASCH, T. & SCHAUB, M.C. (2002). Volatile anesthetics mimic cardiac preconditioning by priming the activation of mitochondrial K_{ATP} channels via multiple signaling pathways. *Anesthesiology,* **97,** 4 – 14.

(Received November 28, 2002
Revised January 14, 2003
Accepted February 03, 2003)