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The antiarrhythmic effects of the nucleoside transporter inhibitor, R75231, in anaesthetized pigs

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1 The effect of R75231, an inhibitor of purine nucleoside transport, were examined on ischaemic arrhythmias in anaesthetized pigs.

2 In closed chest pigs ($n = 4$), R75231 exerted a moderate dose-dependent decrease in mean arterial blood pressure (from 97 ± 4 mmHg to 95 ± 4, 90 ± 1 and 83 ± 2 mmHg at 25, 50 and 100 $\mu g\,kg^{-1}$ respectively) and produced a dose-related shift to the left of the blood pressure dose-response curve to intravenous bolus doses of adenosine. The degree of inhibition of adenosine uptake by R75231, assessed *ex vivo* in erythrocyte suspensions, was $43 \pm 5\%$, 64 ± 13 and $114 \pm 15\%$ at doses of 25, 50 and 100 $\mu g\,kg^{-1}$ respectively.

3 In open chest pigs, intravenous injection of R75231 (50 $\mu g\,kg^{-1}$; $n = 6$ and 100 $\mu g\,kg^{-1}$; $n = 10$) induced a dose-related decrease in both systolic and diastolic arterial blood pressure which was more marked than in closed-chest pigs (mean pressure 86 ± 4 to 70 ± 2 mmHg and 88 ± 6 to 60 ± 6 mmHg with 50 and 100 $\mu g\,kg^{-1}$ respectively), without affecting heart rate or myocardial contractility. Coronary artery occlusion in these pigs caused a secondary decrease in blood pressure. This was not observed in controls ($n = 10$). The lower dose of R75231 did not exert any antifibrillatory effects, whereas the higher dose significantly reduced the incidence of ventricular fibrillation, from 80% in control pigs to 30%. Neither dose modified the incidence of ventricular tachycardia (33% and 40% with 50 and 100 $\mu g\,kg^{-1}$ respectively, compared to 30% in controls) or had any effect on the total number of ventricular ectopic beats (85 ± 47 and 130 ± 31 vs 110 ± 19 in controls). R75231, at a dose of 100 $\mu g\,kg^{-1}$, also attenuated the ischaemia-induced shortening of QRS-interval, but neither dose modified the ST-segment depression seen following occlusion.

4 These results show that the nucleoside transport inhibitor, R75231, exerts an antifibrillatory effect in a model of severe myocardial ischaemia in a dose which completely inhibits adenosine uptake *ex vivo*. However, while this agent has minimal haemodynamic effects in closed chest animals, the reduction in blood pressure induced by R75231 in open-chest pigs cannot be excluded as a possible contributory mechanism of the antiarrhythmic effects of this drug.

Keywords: Myocardial ischaemia; coronary artery occlusion; pigs; nucleoside transport inhibition; adenosine; R75231; arrhythmias

Introduction

Since the demonstration in 1970 by Olsson that the accumulation and subsequent release of adenosine is an early consequence of myocardial ischaemia there has been increasing interest in its potential action as a cardioprotective substance. There is a substantial literature (for a recent excellent review see Forman *et al.*, 1993) on the cardioprotective effects of adenosine against myocardial injury induced by reperfusion, involving a variety of mechanisms such as modulation of neutrophil infiltration (Olafsson *et al.*, 1987), preservation of vascular function (Babbitt *et al.*, 1989) and reduction of neutrophil free radical generation (Cronstein *et al.*, 1990). In addition to injury resulting from myocardial ischaemia and reperfusion, adenosine has been shown to protect against coronary occlusion-induced ventricular arrhythmias in rats (Fagbemi & Parratt, 1984) and dogs (Wainwright & Parratt, 1988). Adenosine has long been known to be protective in treating supraventricular arrhythmias in man (Belhassen & Pelleg, 1984) and its clinical use is largely limited to treatment of these arrhythmias (Barber, 1992) because of the frequency of side effects (such as chest pain and dyspnoea; Rankin *et al.*, 1989) associated with its action at A_2-adenosine receptors which are universally distributed throughout the body. As a result, little is known about its ability to reduce ischaemia-induced ventricular arrhythmias in the clinical setting. This therefore restricts not only the use of adenosine but also of stable A_2-adenosine analogues as potential antiarrhythmic therapy. Since adenosine production occurs as an early response to ischaemia, an alternative approach to antiarrhythmic therapy could be to utilize the protective effects of endogenous adenosine by enhancing its concentration at the site(s) where it is required.

Adenosine exerts a role as a 'retaliatory metabolite' (Newby, 1984; Fredholm *et al.*, 1983); that is, its production is increased or 'switched on' by harmful stimuli (such as myocardial ischaemia) as a compensatory mechanism to try to override the underlying problem (e.g. by vasodilatation). This endogenous production of adenosine is, in general, temporary and very localized. The adenosine produced is generally prevented from spreading further than the site of injury by, firstly, rapid uptake by the endothelial cells and, secondly and more slowly, by the erythrocytes, both of which catabolise adenosine to inactive metabolites. If the uptake transport process into endothelial cells were to be inhibited then this would prolong the time adenosine is present in the local area. Thus nucleoside transport inhibition should effectively raise the endogenous levels of adenosine at the site where it is needed and, if the hypothesis that adenosine acts as an 'endogenous cardioprotective substance' is correct (Fagbemi & Parratt, 1984), this kind of intervention could be expected

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to modify the consequences of acute myocardial ischaemia.

Previous studies with the few existing nucleoside transport inhibitors such as dipyridamole (Wainwright & Parratt, 1988) and lidoflazine (Coker *et al.*, 1982) have indeed shown protection against ventricular arrhythmias during coronary occlusion in dogs. Furthermore, dipyridamole (Blumenthal *et al.*, 1981), lidoflazine (Flameng *et al.*, 1981), mioflazine (Wood *et al.*, 1985) and dilazep (Gupta *et al.*, 1989) have all been shown to reduce infarct size and/or improve functional recovery after myocardial ischaemia in canine hearts. Most of these agents, however, are not 'pure' nucleoside transport inhibitors. Dipyridamole is well documented as possessing the ability to inhibit vascular phosphodiesterase isoenzymes (Ahn *et al.*, 1989) and enhance prostacyclin synthesis (Blass *et al.*, 1980), whereas dilazep possesses calcium antagonist activity. These are effects which are seen at micromolar concentrations and could therefore contribute to their observed cardioprotective effects. R75231 (2-(aminocarbonyl)-*N*-(4-amino-2,6-dichlorophenyl)-4-[5,5-*bis*-(4-fluorphenyl)pentyl]-1-piperazine acetamide (free base); Ijzerman *et al.*, 1989) is a lidoflazine analogue which has been described as a specific inhibitor of nucleoside transport (Van Belle & Jansen, 1991). The term specific is applied in so far as it has been shown to be devoid of activity in a wide range of *in vitro* and *in vivo* systems designed to test activity at specified receptors and it is also ineffective against adenosine deaminase, nucleoside phosphorylase, adenosine kinase and phosphodiesterase (Van Belle *et al.*, 1992) in micromolar concentrations. In addition to this apparent specificity for nucleoside transport inhibition, R75231 has an added advantage over existing transport inhibitors in that it has a longer duration of action (hours compared to minutes in the cases of dilazep and dipyridamole; Van Belle & Janssen, 1991) and is orally active, compared to the variable oral bioavailability with dipyridamole (Stringer *et al.*, 1992). Its potency *in vitro* is comparable with other transport inhibitors (IC_{50} for inhibition of adenosine uptake by packed erythrocytes: 1.1×10^{-8} M for R75231 vs 1.9×10^{-8} M and 1.75×10^{-8} M for dipyridamole and myoflazine respectively; Van Belle & Janssen, 1991). Thus R75231 possesses properties which would improve its potential for therapeutic use over existing nucleoside transport inhibitors. The aim of the present study therefore was to assess the ability of R75231 to suppress ventricular arrhythmias in a model of severe ischaemia in open-chest anaesthetized pigs.

Methods

Animal preparation

Large White/Welsh Landrace cross breed pigs (25–40 kg) were used in the study. Sedation prior to anaesthesia was achieved by intramuscular injection of azaperone (Stresnil; Janssen), and anaesthesia induced by halothane (4% in oxygen). The animals were then intubated and maintained on halothane anaesthesia (1% in air) until an intravenous line had been introduced. The pigs were ventilated with a Palmer respiration pump (16 strokes min^{-1}) and the stroke volume and oxygen content of inspired air were adjusted to maintain arterial CO_2 and O_2 tensions of 45–50 mmHg and 100 mmHg respectively (giving a pH of approximately 7.4). Catheters were placed in the aorta and vena cava via the femoral vessels for measurement of arterial blood pressure and administration of drugs respectively. At this point the halothane was discontinued and chloralose (100 mg kg^{-1}, i.v.) was administered. Intracardiac catheters were placed under fluoroscopic control (Siemens image intensifier) in the pulmonary artery (via the left external jugular vein; Swan Ganz 7Fr) and the left ventricular cavity (via the left carotid artery; Angiomedics pigtail catheter 7Fr) for measurement of pulmonary artery pressure (PAP) and left ventricular pressure (LVP).

Studies in closed-chest pigs

A preliminary study to assess an adequate dosage of R75231 was performed in 4 pigs which were prepared for haemodynamic measurement as described above. In each pig dose-response curves to adenosine were obtained before and after administration of each of three cumulative doses of R75231 (25, 50 and 100 $\mu g\ kg^{-1}$). Adenosine was administered as a bolus intravenous injection (maximum injection volume 0.5 ml) over a 2–3 s period. Twenty minutes were allowed between completion of each dose-response curve and administration of R75231, and a further 20 min before starting the next dose-response curve, to allow assessment of the effects of R75231 on baseline haemodynamics.

Venous blood samples were withdrawn prior to administration of R75231 and 20 min after each dose of the drug for *ex vivo* assessment of the extent of adenosine uptake inhibition as described below.

Studies in open-chest pigs subjected to coronary artery occlusion

Following preparation as described above a thoracotomy was performed midsternally, from the xyphoid cartilage to the clavicle, and the pericardium opened to gain access to the coronary vessels. The left anterior descending coronary artery (LAD) was dissected free below the second major diagnonal branch, approximately two thirds of the distance from the origin to the apex of the heart, and a silk ligature placed around it. The ends of the ligature were threaded through a small piece of polythene tubing to produce a snare, such that when the tubing was clamped in place the coronary artery was occluded. All surgical procedures were carried out to conform with the guidelines of the UK Home Office (Project Licence PPL60/00307).

A total of 26 pigs were entered into this part of the study and were allocated to either control (solvent vehicle; 10 pigs), R75231 50 $\mu g\ kg^{-1}$, i.v. (6 pigs) or R75231 100 $\mu g\ kg^{-1}$, i.v. (10 pigs) groups prior to surgery. The pigs were allowed to stabilize for approximately 1 h after surgery before any intervention. Blood samples were taken for arterial blood gas and pH analysis immediately prior to and 15 min after drug administration. Twenty minutes after drugs were given the coronary artery was occluded by tightening the snare.

Arrhythmia analysis

Arrhythmia analysis was performed under the guidelines of the Lambeth Convention (Walker *et al.*, 1988). The arrhythmias following coronary artery occlusion were analysed over 1 min intervals for the 30 min occlusion time to obtain a profile of distribution and a total count of ventricular arrhythmias. The percentage incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) was noted. The time to VF was recorded in all animals which developed this arrhythmia and was calculated as a mean for those animals. When ventricular fibrillation did occur, the hearts were defibrillated (within 40–60 s) by direct cardioversion to allow the experiment to continue until 30 min post-occlusion.

Data acquisition

In all experiments blood pressures were recorded with Gould capacitance transducers and the signals passed through a Buxco haemodynamnics analyser (Buxco Electronics, U.S.A.) for calculation of systolic, diastolic and mean pressures, heart rate (from the electrocardiogram; ECG), left ventricular end-diastolic pressure (LVEDP), $LVdP/dt_{max}$ and $LVdP/dt/P$ (as indices of myocardial contractility). $LVdP/dt/P$ was taken as the preferable index of LV contractility, since it is independent of afterload, and was calculated by the analyser as $LVdP/dt$ divided by (LVP-LVEDP) at the point on the dP/dt wave where LVP-LVEDP equalled 30 mmHg. All parameters

were measured by the analyser on a beat to beat basis and the values for a 1 min period automatically meaned by a personal computer (Vanilla Computers, England). The 1 min recordings were then stored on the hard disk for subsequent analysis. Numerical values were constantly updated on the visual display unit and wave pressure traces were continuously displayed on a six channel chart recorder (Gould). Calculation of rapid and transient responses, e.g. after an intravenous bolus dose of adenosine, was confirmed by measurement of the peak of the response from the chart recording. The ECG was recorded from lead II of standard limb leads, and the signal processed by an ECG analyser (Buxco Electronics, U.S.A.) to measure R-wave amplitude, R–R, QT and QRS intervals and positive and negative fluctuations in ST-segment. As with the haemodynamic data, the numerical data were continuously displayed and stored on computer and the ECG trace constantly monitored on the chart paper.

Ex vivo *estimation of adenosine uptake inhibition by R75231*

Estimation of the extent of nucleoside transport inhibition by R75231 was made by determining adenosine uptake into erythrocytes isolated from blood samples taken before and after the cumulative administration of 25, 50 and 100 $\mu g\ kg^{-1}$ R75231 in closed chest pigs. Five 4 ml venous blood samples were withdrawn at different times (see below) from each pig ($n = 4$) into syringes containing 1 ml acid/citrate/dextrose (sodium citrate 62 mM, citric acid 25 mM, glucose 86 mM) and centrifuged for 5 min at 3000 *g*. An erythrocyte suspension was made by suspending 100 μl of the packed erythrocytes in 500 μl of MOPS-NaCl buffer (10 mM 4-morpholinopropanesulphonic acid in isotonic saline, pH = 7.4). The erythrocyte samples were then taken through the assay protocol as summarized in Figure 1. The final supernatant was stored frozen until analysis of adenosine, inosine and hypoxanthine content was performed by high performance liquid chromatography (h.p.l.c.) using the method described by Wynants & Van Belle (1985).

Calculation of the extent of inhibition of adenosine uptake into the erythrocyte and inosine and hypoxanthine release from the erythrocyte were calculated by the following formulae:

$$\%\ \text{inhibition adenosine uptake in } ex\ vivo \text{ sample} = \frac{(A_x - A_o) \times 100}{(A_c - A_o)}$$

$$\%\ \text{inhibition inosine/hypoxanthine efflux in } ex\ vivo \text{ sample} = \frac{(I_c - I_x) \times 100}{(I_o - I_c)}$$

Where: A_o, I_o = % of [total nucleoside] in time zero sample with no R75231 present (0% inhibition).

A_c, I_c = % of [total nucleoside] in time zero sample with 1 μM R75231 present (100% inhibition).

A_x, I_x = % of [total nucleoside] in sample taken after *in vivo* administration of 25, 50 or 100 $\mu g\ kg^{-1}$ R75231.

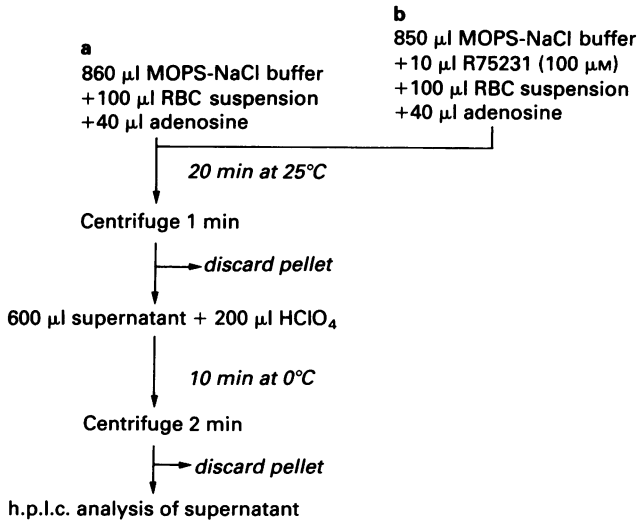

Figure 1 Flow diagram to illustrate the assay protocol for the determination of adenosine uptake by suspended erythrocytes.

Statistics

All values are expressed as mean ± s.e.mean of n experiments. Changes within each group were compared by a paired t test, whereas differences between groups were assessed by an independent t test or Mann Whitney U-test. Changes in the incidences of events were analysed by Fishers Irwin (Chi^2 with Yates correction) test. Results were considered to be statistically significant at $P < 0.05$.

Results

Studies in closed-chest pigs

Table 1 summarizes the changes in blood pressure, heart rate and LV$dP/dt/P$ following intravenous administration of cumulative doses of R75231. There was a moderate, dose-related fall in mean blood pressure (which was significant at the highest dose) within 15 min of drug administration. There were no significant effects on either heart rate or LV$dP/dt/P$. R75231 caused a dose-related shift to the left of the dose-response curve for the diastolic blood pressure response to intravenous bolus injections of adenosine (Figure 2). With the doses used there was no observed bradycardia or decrease in LV$dP/dt/P$ following adenosine injection. The blood pressure responses to adenosine prior to administration of R75231 persisted for 1.5 to 2 min. Following R75231 the responses were progressively prolonged to 4–5 min at the highest dose.

Haemodynamic effects of R75231 in open-chest pigs prior to coronary artery occlusion

The intravenous administration of R75231 produced a dose-related decrease in mean arterial pressure which started

Table 1 Haemodynamic changes in response to cumulative doses of R75231 in closed-chest pigs

		Dose R75231 ($\mu g\ kg^{-1}$)		
	Control	25	50	100
Mean arterial pressure (mmHg)	97 ± 4	95 ± 4	90 ± 1	83 ± 2*
Heart rate (beats min^{-1})	121 ± 10	123 ± 10	122 ± 17	115 ± 14
LV$dP/dt/P$ (s^{-1})	24.0 ± 0.9	21.7 ± 1.1	21.5 ± 1.0	20.9 ± 1.6
n	4	4	4	4

*$P < 0.05$ (paired t test) compared to control value prior to any drug administration. Post-drugs values were measured 10 min after drug administration.

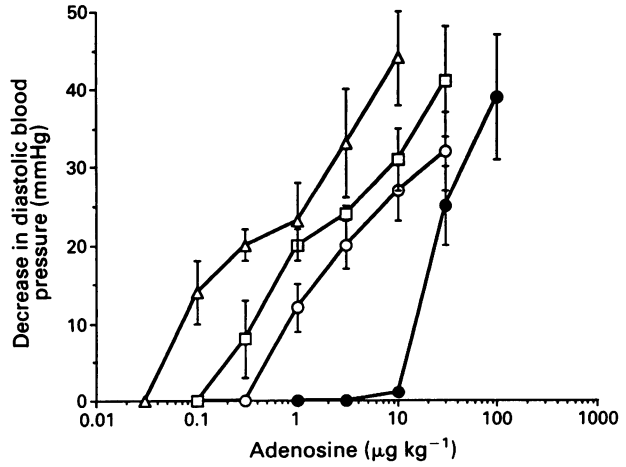

Figure 2 Decrease in diastolic pressure in response to intravenous bolus doses of adenosine prior to (●) and following cumulative administration of 25 (○), 50 (□) and 100 μg kg^{-1} (△) R75231 in closed-chest pigs ($n = 4$ pigs).

immediately following injection and reached a maximum within 5 min. Arterial pressure remained depressed for the duration of the experiment and thus by the time the coronary artery was occluded mean blood pressure was reduced by 16 ± 3 mmHg and 28 ± 6 mmHg by 50 μg kg^{-1} and 100 μg kg^{-1} R75231 respectively. This blood pressure response to R75231 was greater than that seen in closed-chest pigs. Neither dose of R75231 had any effect on any other haemodynamic parameters (Table 2).

Haemodynamic effects of coronary artery occlusion in controls and pigs treated with R75231

A 30 min period of coronary artery occlusion in control pigs resulted in no change in heart rate or arterial blood pressure compared to pre-occlusion values (Table 2). There was, however, a significant reduction in myocardial contractility assessed by both LV$dP/dt_{max}$ and LV$dP/dt/P$. In both groups of pigs given R75231, coronary artery occlusion resulted in decreases in LV$dP/dt_{max}$ and, in the case of the lower dose, LV$dP/dt/P$ which were similar to those seen in control pigs. Furthermore, in both groups given R75231, coronary artery occlusion resulted in a secondary fall in blood pressure, despite the fact that blood pressure had already decreased following drug administration.

The effects of R75231 on electrocardiographic changes seen during coronary artery occlusion

In control pigs, coronary artery occlusion had no effect on the QT interval (238.6 ± 23.7 ms preocclusion vs 214.9 ± 23.6 ms post-occlusion), but there was a significant depression of the ST-segment (from 32 ± 2 to −14 ± 6 μV: $P <$ 0.05), which became evident within 2 min post-occlusion and was maintained for the 30 min occlusion period. Coronary occlusion also resulted in a significant shortening of the QRS interval, from 27.1 ± 1.9 ms immediately prior to occlusion to 23.9 ± 1.2 ms 30 min post-occlusion ($P < 0.05$). Neither dose of R75231 exerted any effects on the ECG prior to occlusion, nor did it modify the ischaemia-induced ST-segment depression. However, the shortening of the QRS-interval was prevented by both doses of R75231 (24.2 ± 0.3 and 26.3 ± 2.0 ms 30 min post-occlusion vs 23.9 ± 0.3 and 24.1 ± 1.8 ms preocclusion with 50 and 100 μg kg^{-1} respectively).

The effects of R75231 on arrhythmias following coronary artery occlusion

Coronary artery occlusion in control pigs resulted, within 2 min, in the development of spontaneous ventricular arrhythmias which occurred over two phases. The first phase (phase 1a) appeared during the 2–10 min post-occlusion period and consisted mostly of single ventricular premature beats with some salvos (couplets and triplets). The second phase, commencing 18–20 min post-occlusion, was usually abrupt in onset and generally consisted of one or two 'warning beats' followed by ventricular fibrillation. The average time to the onset of VF in the 8 pigs which fibrillated was 25.5 ± 2.1 min post-occlusion. The incidence of ventricular tachycardia (identified as a run of 4 or more consecutive ectopic beats) was low in this model (30%), whereas the incidence of ventricular fibrillation was high (80%).

Ventricular ectopic activity following coronary artery occlusion in pigs given either dose of R75231 was similar to that seen in controls and showed a similar pattern of distribution. The total number of premature ventricular beats was also similar to controls in both groups (Figure 3). The higher (100 μg kg^{-1}) dose of R75231, however, significantly reduced the incidence of ventricular fibrillation, from 80% in controls to 30% ($P < 0.05$; Figure 4). Neither dose influenced the incidence of ventricular tachycardia and the time to onset of ventricular fibrillation was similar to that seen in controls (22.9 ± 3.9 min; $n = 5$ and 19.7 ± 2.8 min; $n = 3$ for 50 and 100 μg kg^{-1} R75231).

Table 2 The effects of R75231 (50 and 100 μg kg^{-1}; given at −20 min) and coronary occlusion (at 0 min) on haemodynamics in anaesthetized open-chest pigs

	Control			*R75231* (50 μg kg^{-1})			*R75231* (100 μg kg^{-1})		
	−21 min	−1 min	+30 min	−21 min	−1 min	+30 min	−21 min	−1 min	+30 min
n	10	10	10	6	6	6	10	10	10
Heart rate (beats min^{-1})	84 ± 5	89 ± 12	85 ± 7	108 ± 7	107 ± 7	96 ± 5	103 ± 8	96 ± 1	95 ± 8
Arterial pressure (mmHg)									
Systolic	109 ± 8	100 ± 13	97 ± 7	109 ± 3	91 ± 3*	84 ± 5[a]	112 ± 6	82 ± 8**	60 ± 8[a]
Diastolic	72 ± 7	64 ± 8	64 ± 3	75 ± 4	60 ± 2*	50 ± 2[a]	76 ± 6	48 ± 5**	35 ± 5[b]
Mean	84 ± 7	75 ± 10	75 ± 4	86 ± 4	70 ± 2*	61 ± 2[b]	88 ± 6	60 ± 6**	49 ± 4[a]
LVEDP (mmHg)	4.6 ± 0.4	3.9 ± 0.1	5.0 ± 0.8	3.9 ± 0.7	3.6 ± 0.4	4.9 ± 0.1	5.9 ± 0.6	4.2 ± 0.2	4.3 ± 0.2
LV$dP/dt_{max}$ (mmHg s^{-1})	1603 ± 139	1716 ± 258	1211 ± 171[b]	2176 ± 260	2446 ± 128	1113 ± 408[a]	2219 ± 249	2090 ± 376	1463 ± 294[a]
LV$dP/dt/P$ (s^{-1})	18.4 ± 1.0	20.1 ± 2.0	15.9 ± 0.7[a]	20.5 ± 1.8	22.1 ± 1.5	18.2 ± 0.9[b]	20.4 ± 1.1	21.2 ± 1.6	20.2 ± 1.4

*$P < 0.05$; **$P < 0.01$ compared to −21 min (pre-drug) value. [a]$P < 0.05$; [b]$P < 0.01$ compared to −1 min (preocclusion) value. (Paired *t* test).

Nucleoside uptake inhibition by R75231

Table 3 gives adenosine, inosine, hypoxanthine and total nucleoside concentrations in the assay samples generated from blood withdrawn before and after *in vivo* administration of R75231. The inhibition of adenosine uptake into the erythrocytes, calculated from the % contribution of adenosine to total nucleoside content, increased with increasing dose of R75231. The contributions of both inosine and hypoxanthine to total nucleoside count were decreased with increasing doses of R75231, demonstrating a reduced efflux of these nucleosides from the erythrocytes, since this is achieved by the same transport protein as adenosine uptake. This, in tandem with the reduced uptake of adenosine, reflects a slowed rate of conversion of adenosine to these two metabolites by the erythrocytes. The total nucleoside concentration in samples obtained from blood withdrawn after *in vivo* administration of R75231 tended to increase with the two lower doses. Although nucleoside concentrations were not measured in samples which had not had adenosine added, the same concentration of adenosine (40 μM) was added to all assay samples. This may reflect an increase in circulating nucleoside levels following administration of R75231.

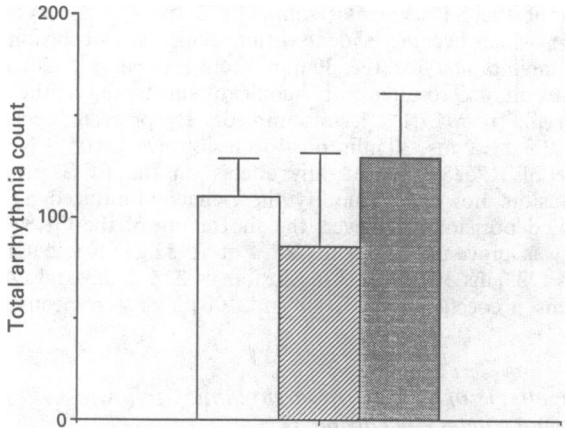

Figure 3 Total number of ventricular arrhythmias (seen as single ectopic beats, salvos and ventricular tachycardia) during 30 min of coronary occlusion in control pigs (open column) and pigs given either 50 (hatched column) or 100 μg kg^{-1} (shaded column) R75231.

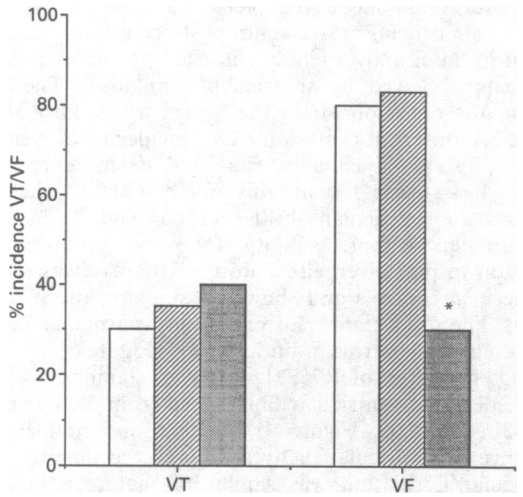

Figure 4 Percentage incidence of ventricular tachycardia (VT) and ventricular fibrillation (VF) following coronary artery occlusion in control pigs (open columns) and pigs given 50 (hatched columns) or 100 μg kg^{-1} (shaded columns) R75231. *$P<0.05$ compared to control group (Fischer Irwin test).

Discussion

The protective effects of adenosine against ventricular arrhythmias have been demonstrated previously in a number of different experimental models (reviewed in Boachie-Ansah *et al.*, 1993). In our own laboratory we have shown that exogenous administration of adenosine markedly reduces the incidence and severity of ischaemic arrhythmias in both rats (Fagbemi & Parratt, 1984) and greyhound dogs (Wainwright & Parratt, 1988). More recently, a study in beagle dogs demonstrated that intracoronary adenosine was able to terminate noradrenaline-induced ventricular tachycardia (Friedrichs & Merrill, 1991). Although adenosine is a useful agent in reducing arrhythmias (usually of supraventricular origin) in patients (Barber, 1992), the major problem with the clinical use of adenosine as an antiarrhythmic agent is that it is often poorly tolerated in patients due to intense chest pain (Rankin *et al.*, 1989) and occasionally dyspnoea and AV-block. Although these side effects are often transient and seldom require treatment there has been an increasing interest in exploiting the adenosine produced by the heart during a period of myocardial ischaemia by developing drugs which either enhance adenosine release, or prolong its biological lifetime, to act as a locally produced antiarrhythmic agent. A number of strategies for enhancing local adenosine concentration have already been employed. For example, inhibitors of adenosine deaminase, which converts adenosine to inosine, have been tested against both reperfusion injury and arrhythmias in rat isolated hearts (Zhu *et al.*, 1990), where treatment with erythro-9-(2-hydroxy-3-nonyl)adenine was found to reduce the incidence of reperfusion-induced

Table 3 Inhibition of uptake of adenosine into erythrocytes by R75231

	Time zero − *R75231*	*Time zero* + *R75231* (1 μM)	*R75231* in vivo 25 μg kg^{-1}	50 μg kg^{-1}	100 μg kg^{-1}
Adenosine					
[μM]	0.9 ± 0.2	3.1 ± 0.1	1.6 ± 0.2	2.4 ± 0.5	3.9 ± 0.8
% [total nucleoside]	0.5 ± 0.1	1.9 ± 0.3	1.1 ± 0.2	1.4 ± 0.4	2.1 ± 0.8
% inhibition	0	100	42 ± 5	64 ± 13	114 ± 14
Inosine					
[μM]	175 ± 11	166 ± 25	137 ± 35	184 ± 23	192 ± 24
% [total nucleoside]	96 ± 0.5	93.6 ± 0.6	95.2 ± 1.8	93.9 ± 2.5	93.1 ± 1.4
% inhibition	0	100	33 ± 12	87 ± 16	121 ± 22
Hypoxanthine					
[μM]	6.2 ± 1.8	4.4 ± 1.5	4.4 ± 0.7	5.8 ± 0.7	5.5 ± 1.4
% [total nucleoside]	3.4 ± 0.4	2.5 ± 0.7	3.1 ± 0.5	2.9 ± 0.6	2.7 ± 0.8
% inhibition	0	100	33 ± 5	55 ± 3	75 ± 9
[Total nucleoside]	182 ± 14	173 ± 21	144 ± 15	199 ± 15	202 ± 18

See text for details of calculations.

ventricular fibrillation, an effect which was attributed to both adenosine accumulation and inhibition of the production of superoxide radicals during the final step of adenosine metabolism. In another study an improvement in myocardial functional recovery following reperfusion was observed with 2-deoxyformycin, an effect which was attributed to an accelerated repletion of adenine nucleotide stores (Bolling *et al.*, 1990). Finally, acadesine (AICA riboside; 5-amino-1-β-D-ribofuranosyl-imidazole-4-carboaxmide) which increases intracellular adenosine concentrations only during periods of increased ATP metabolism (by an unclarified mechanism) has been shown to suppress arrhythmias and reduce ischaemic myocardial injury in dogs (Gruber *et al.*, 1989). Most of these interventions, however, do not raise adenosine concentration in the interstitium, unlike nucleoside transport inhibitors, which would allow adenosine better access to its receptors. A few studies with existing nucleoside transport inhibitors, such as lidoflazine and dipyridamole have demonstrated antiarrhythmic effects (Coker *et al.*, 1982; Wainwright & Parratt, 1988). In the present study we have shown a marked antifibrillatory effect of a new nucleoside transport inhibitor derived from lidoflazine, R75231, in a model of severe myocardial ischaemia in pigs.

R75231 has been described as a highly selective and potent inhibitor of the transport process which removes adenosine from the vicinity of its receptors (Ijzerman *et al.*, 1989). Under conditions of an increased oxygen demand in the myocardium the breakdown of purine nucleotides within the myocardial cell to adenosine monophosphate (AMP) is accelerated (Van Belle *et al.*, 1987). Until fairly recently the classical concept of adenosine release from cardiac myocytes was that AMP is converted to adenosine inside the myocyte by cytosolic 5'-nucleotidase, after which the adenosine was transported out of the cell by a transport protein. With such a mechanism a nucleoside transport inhibitor would be expected to retain adenosine within the cell, where clearly it would not reach its receptors. Recent evidence, however, has led to the growing belief that dephosphorylation to adenosine takes place outside the cell by an ecto 5'-nucleotidease on the endothelial cell membrane, since it is now known that a number of species possess low AMP conversion activity in the cytosol of the myocyte (Nees, 1989a; Borst & Schrader, 1991). Thus adenosine is most likely to be produced in the interstitium. Furthermore, even if AMP were to be converted to adenosine within the myocyte, it is now considered probable that simple diffusion accounts for the efflux of adenosine, rather than a transport process, which would therefore be unaffected by nucleoside transport inhibition (Van Belle, 1993). Once adenosine is in the interstitium it is transferred into the endothelial cell by a transporter protein, which has been localized on coronary microvessels (Parkinson & Clanachan, 1991) and cultured endothelial cells (Nees, 1989b). Degradation to inosine and hypoxanthine then takes place in the cytosol of the endothelial cell. The consequence of inhibition of this transport protein would therefore be an accumulation of adenosine in the interstitial spaces where it can gain better and more prolonged access to its receptors on the myocyte and vascular smooth muscle. In addition, efflux of inosine from the endothelial cells will be reduced since this is achieved by the same transport protein. Nucleoside transport inhibition, therefore, does not affect the production of adenosine but alters the ratio between adenosine and its metabolites in the interstitium. Leakage of adenosine through the clefts between endothelial cells may also occur, providing an access route to the lumen.

Although we did not measure tissue levels of adenosine in the present studies, indirect evidence of *in vivo* nucleoside transport inhibition by R75231 was obtained from the assays in packed erythrocytes which demonstrated an *ex vivo* dose-related inhibition of adenosine uptake, and of inosine efflux. Although this is not a direct measure of endothelial cell uptake *in vivo*, this method is regarded as an efficient tool to assess uptake inhibition *ex vivo* since it is representative for other cells with similar uptake mechanisms (Van Belle & Janssen, 1991). Other evidence to show that R75231 was inhibiting adenosine uptake *in vivo* in the doses used in the present study is the enhanced response to intravenous bolus doses of adenosine in closed-chest animals. The dose-response curves show a dose-related shift to the left of the depressor response to adenosine. Following administration of the higher dose of R75231, baseline blood pressure was approximately 10 mmHg lower than before administration of any dose of R75231 (Table 1). This could conceivably have reduced vasodilator reserve for observing maximal depressor responses. However injections of adenosine were not associated with any bradycardia or LV$dP/dt/P$ which could have contributed to the fall in arterial pressure, which suggests that the depressor responses seen in response to adenosine after the highest dose of R75231 were due to peripheral vasodilatation.

The main question pertaining to this study is what is the mechanism underlying the antifibrillatory effect of R75231? Is it due to elevation of adenosine levels within the heart or due to the reduction in afterload seen on administration of R75231? The hypotensive response to R75231 may play a part in the antiarrhythmic effects, since a reduction in afterload would decrease cardiac work and myocardial oxygen consumption, which would ultimately decrease the severity of ischaemia. While this is an appealing explanation, we have shown in this same model that other agents which decrease afterload to a similar degree, either by an action at A_2-adenosine receptors (e.g. CV1808; Wainwright & Parratt, 1991) or independent of adenosine receptors (e.g. pirsidomine; Wainwright & Martorana, 1993), display a different antiarrhythmic profile from that seen with R75231 in that they reduce total arrhythmia count, but have no effects on the incidence of ventricular fibrillation. Furthermore, R75231 did not modify the ST-segment changes which are indicative of the severity of ischaemia, suggesting a lack of anti-ischaemic effect. The role of the reduction in afterload in the antifibrillatory action of R75231 is therefore open to question but could be assessed more clearly by determining its antiarrythmic activity in closed-chest animals where a much smaller reduction in afterload by R75231 was seen.

The blood pressure response to R75231 is of interest however, since a primary aim in the development of these agents is to minimize systemic effects. In both closed-chest and open-chest pigs the response was probably due to an increase in circulating adenosine levels rather than a direct vasodilator effect of the drug since firstly, responses of a similar magnitude would be expected in both preparations (with the same starting pressure) on administration of a directly-acting vasodilator and, second, there is no evidence to suggest that R75231 possesses vasodilator properties, either direct or indirect (Van Belle *et al.*, 1992). The enhanced depressor response to R75231 in open-chest pigs may be related to a stimulation of adenosine production by the thoracotomy, since this was the only difference between the two preparations. Similarly, the secondary hypotensive response to coronary occlusion seen in treated pigs probably reflects the R75231-assisted leakage of adenosine, released by the heart in response to ischaemia, into the systemic circulation. This would not occur in control pigs since any adenosine produced by the heart would be rapidly taken up by the endothelial cells.

If R75231 exerts its antiarrhythmic effects by increasing local cardiac adenosine concentrations, rather than by the systemic responses described above, another question which arises relates to which of the actions of adenosine on the heart could be responsible. At the vascular level, A_2-receptors mediate a direct vasodilatation (King *et al.*, 1990), while a similar receptor type on the endothelium causes the release of endothelium-derived nitric oxide (Rose'Meyer & Hope, 1990). R75231 has been shown to reduce basal coronary resistance in rabbit isolated blood-perfused hearts (Galinanes *et al.*, 1993), but it would be difficult to envisage how a

coronary vasodilator effect could improve the situation within the ischaemic myocardium of a heart with the low collateral flow present in this model (<6%). Adenosine also acts via A_2-receptors to release prostacyclin from endothelial cells (Karwatowska-Prokopczuk *et al.*, 1988) and inhibit platelet function (Paul *et al.*, 1990). Prostacyclin has known antiarrhythmic properties and has been shown to be released into coronary venous blood in the early stages of ischaemia (Coker & Parratt, 1983). Similarly, substances released from platelets in response to ischaemia, such as thromboxane A_2, could contribute to the ischaemic arrhythmias (Coker *et al.*, 1981) and inhibition of platelet activation by adenosine could therefore be of importance. As mentioned above, however, the antiarrhythmic profile of R75231 is quite different from those seen with selective A_2-adenosine agonists in this model (Wainwright & Parratt, 1991; Wainwright *et al.*, 1992) which suggests an alternative mechanism.

The reduction in ventricular fibrillation by R75231 bears a closer similarity to the antiarrhythmic profile seen in this model with an A_1-adenosine agonist (**R**-PIA; Wainwright & Parratt, 1993). A_1-adenosine receptors are predominant on the sino-atrial node, where they mediate a bradycardia (Belardinelli *et al.*, 1983; Collis *et al.*, 1983), the atrioventricular node, where they slow conduction (Clemo & Belardinelli, 1986) and on the ventricular myocyte, where they modify the responses to sympathetic nerve stimulation (Schrader *et al.*, 1979). Any one of these effects could mediate an antiarrhythmic effect (reviewed by Boachie-Ansah *et al.*, 1993). While the antiarrhythmic effect of **R**-PIA was associated with a reduced heart rate, R75231 did not produce a bradycardia *per se*. However, there is evidence from this study that A_1-adenosine receptors were activated. The profound fall in blood pressure in response to R75231 would be expected to elicit a reflex tachycardia, which it did not. While it could be argued that anaesthesia depressed reflex activity (which is less likely in the chloralose anaesthetized animal than with barbiturate anaesthesia), we have observed a reflex tachycardia following administration of a selective A_2 agonist in this pig model, but not with an infusion of adenosine (which acts at both A_1- and A_2-receptors) producing the same degree of hypotension (Wainwright *et al.*, 1992). Since A_1-adenosine receptors on the sino-atrial node mediate a direct bradycardia and those at sympathetic nerve endings modulate catecholamine release it is reasonable to assume that the lack of reflex tachycardia seen with both adenosine and R75231 is due to A_1-receptor activation which overrides the reflex.

The cardioprotective effects of R75231 have also been demonstrated in other studies. In a model of rabbit isolated hearts subjected to global ischaemia, R75231 improved functional recovery and cardiac output on restoration of flow (Masuda *et al.*, 1991). R75231 has also been shown to decrease mortality and infarct size in a model of catecholamine-induced myocardial toxicity in rabbits (Van Belle *et al.*, 1992). In this respect it demonstrates a similar cardioprotective profile to other nucleoside transport inhibitors. However, as outlined in the introduction, R75231 has a number of advantages over the existing agents, such as an improved bioavailability and longer duration of action, which may make it more suitable for clinical use (Van Belle & Janssen, 1991). The speculation over whether or not the antiarrhythmic effect of R75231 is mediated by one or other (or both) of the subtypes of adenosine receptor can only be answered by concomitant administration of selective antagonists at the A_1- and A_2-receptors with R75231.

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