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# Amrinone reduces ischaemia–reperfusion injury in rat heart

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**Abstract**

We investigated the effects of amrinone on ischaemia-induced changes in myocardial function in isolated rat hearts. Isolated hearts from male Sprague–Dawley rats (150–275 g) were perfused with physiological salt solution at a constant flow rate. The effects of amrinone (30 μM) on left ventricular end diastolic pressure, positive and negative $dP/dt$, heart rate and coronary perfusion pressure were observed following global ischaemia and reperfusion. In normal hearts, amrinone had no effect on myocardial contractility, heart rate, coronary perfusion pressure or left ventricular end diastolic pressure. Ischaemia–reperfusion caused an increase in coronary perfusion pressure, left ventricular end diastolic pressure and creatine kinase outflow and amrinone (present from before ischaemia) decreased the rise in all of these parameters. However, when amrinone was added only after the ischaemia, it had no effect on coronary perfusion pressure or left ventricular end diastolic pressure. Thus, the effect on coronary perfusion pressure must be due to actions during the ischaemia phase. We suggest that amrinone has pharmacological properties which may be useful in reducing ischaemia–reperfusion injury. We speculate that this involves altering ischaemia-induced changes in intracellular $Ca^{2+}$ in the myocytes. 

*Keywords:* Amrinone; Ischaemia; (Rat); Reperfusion injury

## 1. Introduction

Amrinone and milrinone are selective inhibitors of cyclic GMP inhibitable phosphodiesterase or phosphodiesterase 3 (Beavo and Reifsnyder, 1990). Drugs of this type enhance myocardial contractility by preventing the breakdown of cyclic AMP in cardiac myocytes (Silver et al., 1988) and cause vasorelaxation due to inhibiting the breakdown of both cyclic AMP and cyclic GMP in vascular smooth muscle (Murray, 1990; Walsh et al., 1995). It has been demonstrated that amrinone and milrinone decrease infarct size in rabbit hearts with left coronary artery branch occlusion (Rump et al., 1993a, 1994, 1995) which has been attributed to a vasodilator action resulting in increased myocardial perfusion in the tissue surrounding the ischaemic zone (Rump et al., 1994). The reduction in ischaemic damage by amrinone in isolated rabbit hearts was not mimicked by the β-adrenoceptor agonist isoprenaline, which also elevates cyclic AMP levels (Rump et al., 1993b). This finding suggests that increased tissue levels of cyclic AMP cannot explain the actions of amrinone and milrinone in ischaemia–reperfusion and that these agents may have additional pharmacological actions. For example, in rat heart and isolated atria, milrinone decreased β-adrenoceptor induced tachycardia (Minatoguchi and Majewski, 1991), opposite to what would be expected of phosphodiesterase inhibition as β-adrenoceptor induced tachycardia is mediated by cyclic AMP. Furthermore, milrinone and to a greater extent amrinone have a very different profile of action compared to other phosphodiesterase 3 inhibitors in that they produce a larger degree of vasodilatation relative to their inotropic effects (Taira, 1987). In addition, their vasodilator action is greater than expected from their phosphodiesterase 3 inhibitory effects alone (Hidaka et al., 1984; Pang, 1992). The question arises whether amrinone and milrinone may possess other pharmacological actions independent of phosphodiesterase inhibition.

In the present study, we chose to investigate the effects of amrinone on global ischaemia followed by reperfusion

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of rat isolated hearts under conditions of constant coronary flow. This model has been widely used and is characterized by marked changes in cardiac parameters during the reperfusion stage. These changes include coronary constriction, elevated left ventricular end diastolic pressure and diminished myocardial contractility (Buja, 1998). The rat was chosen for two reasons. Firstly, in this model, we observed the unexpected sympatholytic effects of milrinone (Minatoguchi and Majewski, 1991). Secondly, rat heart is resistant to the inotropic actions of amrinone in comparison to other species (Alousi et al., 1983), thus, it may be easier to demonstrate non-phosphodiesterase-mediated effects.

## 2. Methods

### 2.1. Rat isolated atria

Male Sprague–Dawley rats (150–275 g) were killed by decapitation and the hearts were rapidly excised. The atria were dissected free of surrounding tissue and transferred to a 3-ml organ bath containing physiological salt solution A of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.5, $MgSO_4$ 0.45, $NaHCO_3$ 25, $KH_2PO_4$ 1.03, D-(+) Glucose 11.1 ascorbic acid 0.14 and EDTA 0.067 bubbled with 95% $O_2$–5% $CO_2$. Atrial rate was recorded using a Maclab recording system. After 45 min of equilibration, amrinone (final concentration 30 μM) or vehicle (final concentration 500 μM HCl) was added into the organ bath and allowed to equilibrate for a further 20 min. A cumulative concentration response curve to Bay K 8644 was then performed.

### 2.2. Vasorelaxation studies

Male Sprague–Dawley (250–300 g) rats were killed by decapitation. The thoracic aorta was removed and mounted as ring preparations as described previously (Van der Zypp and Majewski, 1998). Following a 45 min equilibration period, the viability of the tissues was assessed. Tissues, which failed to produce a 0.5 g increase in tension to phenylephrine (0.1 μM), were rejected. Successful removal of endothelial cells was confirmed by the inability of acetylcholine (10 μM) to induce relaxation in the presence of phenylephrine (0.1 μM). The tissue bathing solution was then replaced repeatedly with fresh drug-free physiological salt solution until a stable baseline tension was obtained. The tension was then adjusted to 2 g. Tissues were allowed to equilibrate for a further 45 min after which they were constricted with phenylephrine (0.1 μM). After the phenylephrine response had reached a stable plateau, amrinone and 3-isobutyl-1-methylxanthine (IBMX) were added cumulatively with 10 min between subsequent additions.

### 2.3. Langendorff perfused rat heart

Male Sprague–Dawley rats weighing 150–275 g were injected with heparin (400 iU, i.p.) and 10 min later were anaesthetised with sodium methohexitone (25 mg $kg^{-1}$, i.p.) and sodium amylobarbitone (63 mg $kg^{-1}$, i.p.). The trachea was cannulated and while the rats were being mechanically ventilated, their hearts were perfused in situ via retrograde cannulation of the aorta. The hearts were then excised rapidly and transferred to a Langendorff apparatus. Hearts were perfused at 37°C at 7 ml $min^{-1}$ with physiological salt solution (PSSB) of the following composition (mM): NaCl 118, KCl 4.7, $CaCl_2$ 2.52, $MgSO_4$ 1.66, $NaHCO_3$ 24.88, $KH_2PO_4$ 1.18, D-(+) Glucose 5.55, Na-pyruvate 2, bubbled with 95% $O_2$–5% $CO_2$. The hearts were housed in a temperature-regulated chamber and maintained at 37°C throughout the experiment. A water–ethanol filled latex balloon (size 4) attached to a metal cannula was inserted into the left ventricle and connected to a pressure transducer for measurement of ventricular pressure. Balloon volume was adjusted to maintain maximal developed left ventricular pressure and the end diastolic pressure was always under 12 mm Hg. Once balloon volume was set, it remained constant during the remainder of the experiment. Left ventricular end diastolic pressure, positive and negative $dP/dt$ and heart rate were continuously recorded with a computer based analogue digital recording system. Coronary perfusion pressure was measured via a pressure transducer connected by a side arm to the aortic inflow.

After 20 min of equilibration, amrinone or vehicle was added into the perfusing solution and the hearts allowed to equilibrate for a further 30 min. Global ischaemia was achieved by stopping perfusion of the heart for 30 min. To prevent a temperature drop due to interruption of the aortic perfusion, the heart was immersed in warmed physiological salt solution which had been pre-oxygenated with 95% $O_2$–5% $CO_2$, containing the drug under investigation or its vehicle. Following the ischaemic period the bath was drained and the hearts reperfused for 30 min. During the first 5 min of reperfusion, the effluent was collected for measurement of creatine kinase and lactate dehydrogenase by routine biochemical assay using Du Pont Dimension Chemical Analyser with standard Du Pont chemistries. In a separate series of experiments, amrinone (30 μM) was present in the perfusate only during reperfusion. In addition, the effect of amrinone (30 μM) on coronary perfusion pressure following ischaemia or normoxic perfusion was studied in the presence of the thromboxane mimetic, 9,11-dideoxy-11α9α-epoxymethano-prostaglandin $F_{2\alpha}$ (U46619, 30 nM).

### 2.4. Drugs and materials

Acetylcholine perchlorate, amrinone, 11-dideoxy-11-α9α-epoxymethano-prostaglandin $F_{2\alpha}$, (U466199), 1,4-

dihydro-2,6-dimethyl-5-nitro-4-(-2-(trifluoromethyl)-phenyl)-3-pyrinecarboxylic acid methyl ester (Bay K 8644), IBMX; Sigma (St. Louis, USA). Amrinone was initially dissolved in 0.05 M HCl and U46619 was initially dissolved in ethanol to give 3 mM stock solutions. Bay K 8644 was initially dissolved in 50% ethanol and IBMX was initially dissolved in $H_2O$ to give 1 mM stock solutions. All further drug dilutions were made with PSS.

### 2.5. Statistical analysis

Haemodynamic variables were compared by repeated measures two-way analysis of variance or by Student's paired or unpaired *t*-test. A $P$ value $< 0.05$ was considered to be statistically significant. Data are presented as mean ± S.E.M.

### 2.6. Animal statement

The investigation conforms with the Australian code of practice for the care and use of animals for scientific purposes published by the National Health and Medical Research Council.

## 3. Results

### 3.1. Effect of amrinone on Bay K 8644-induced rises in atrial rate

Bay K 8644 (0.001–3 μM) a calcium channel opening drug, increased the rate of beating of isolated rat atria in a concentration-dependent manner. This action was significantly attenuated by 30 μM amrinone (Fig. 1).

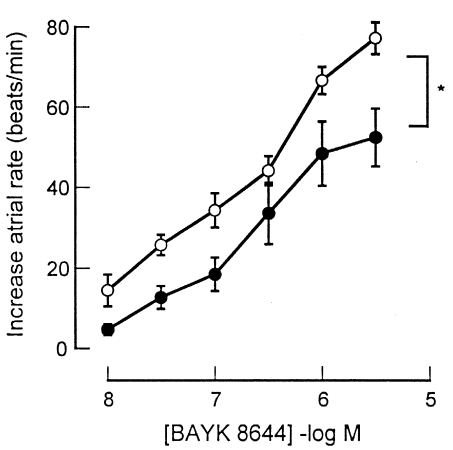


Fig. 1. The effect of amrinone on the tachycardic response of Bay K 8644 in rat isolated atria. Cumulative concentration response curves to Bay K 8644 (0.001–3 μM) were conducted in the presence of 30 μM amrinone (●) or its vehicle (○). Each point represents the mean ± S.E.M. ($n = 4$) and responses are expressed as the increase in atrial rate in beats/min. The rate of atrial beating was significantly reduced by amrinone ($^*P <$ 0.05, two-way analysis of variance).

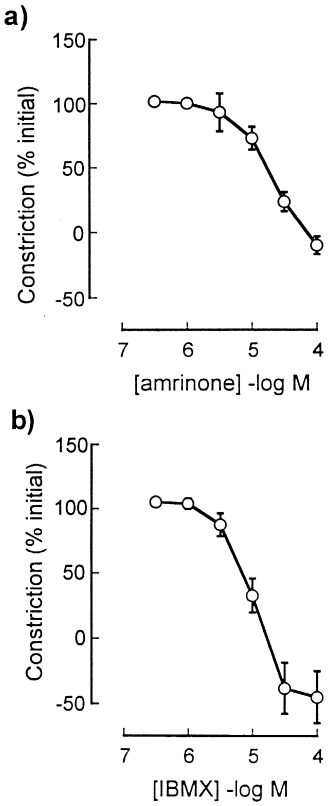


Fig. 2. The vasorelaxant effect of amrinone and IBMX in rat aortic rings. Cumulative concentration response curves to amrinone (0.3–100 μM) or IBMX (0.3–100 μM) were conducted in endothelium-denuded rat aortic rings constricted with phenylephrine (0.1 μM). Each point represents the mean ± S.E.M. ($n = 5$–30) and responses are expressed as a percentage of the initial phenylephrine-induced tone.

### 3.2. The vasorelaxant effect of amrinone and IBMX

The non-selective phosphodiesterase inhibitor IBMX (0.3–100 μM) and the phosphodiesterase 3 inhibitor amrinone (1–300 μM) both produced a concentration dependent relaxation of endothelium-denuded rat aortae constricted with 0.1 μM phenylephrine (Fig. 2). The potency of IBMX ($-\log EC_{50}$ 5.14 ± 0.10, $n = 5$) and amrinone ($-\log EC_{50}$ 4.83 ± 0.10, $n = 30$) in inducing relaxation was similar. For the subsequent cardiac studies, 10 μM IBMX and 30 μM amrinone was used.

### 3.3. Effect of global ischaemia and reperfusion on cardiac parameters

Ischaemia and reperfusion, produced by stopping the flow in Langendorff perfused rat hearts for 30 min and then reperfusing, produced several changes in the hearts (Fig. 3). There was a pronounced rise in left ventricular end diastolic pressure and concomitant reductions in myocardial contractility as measured by $dP/dt$. In addition, the coronary perfusion pressure was enhanced. There were minor effects on heart rate (see Fig. 3).

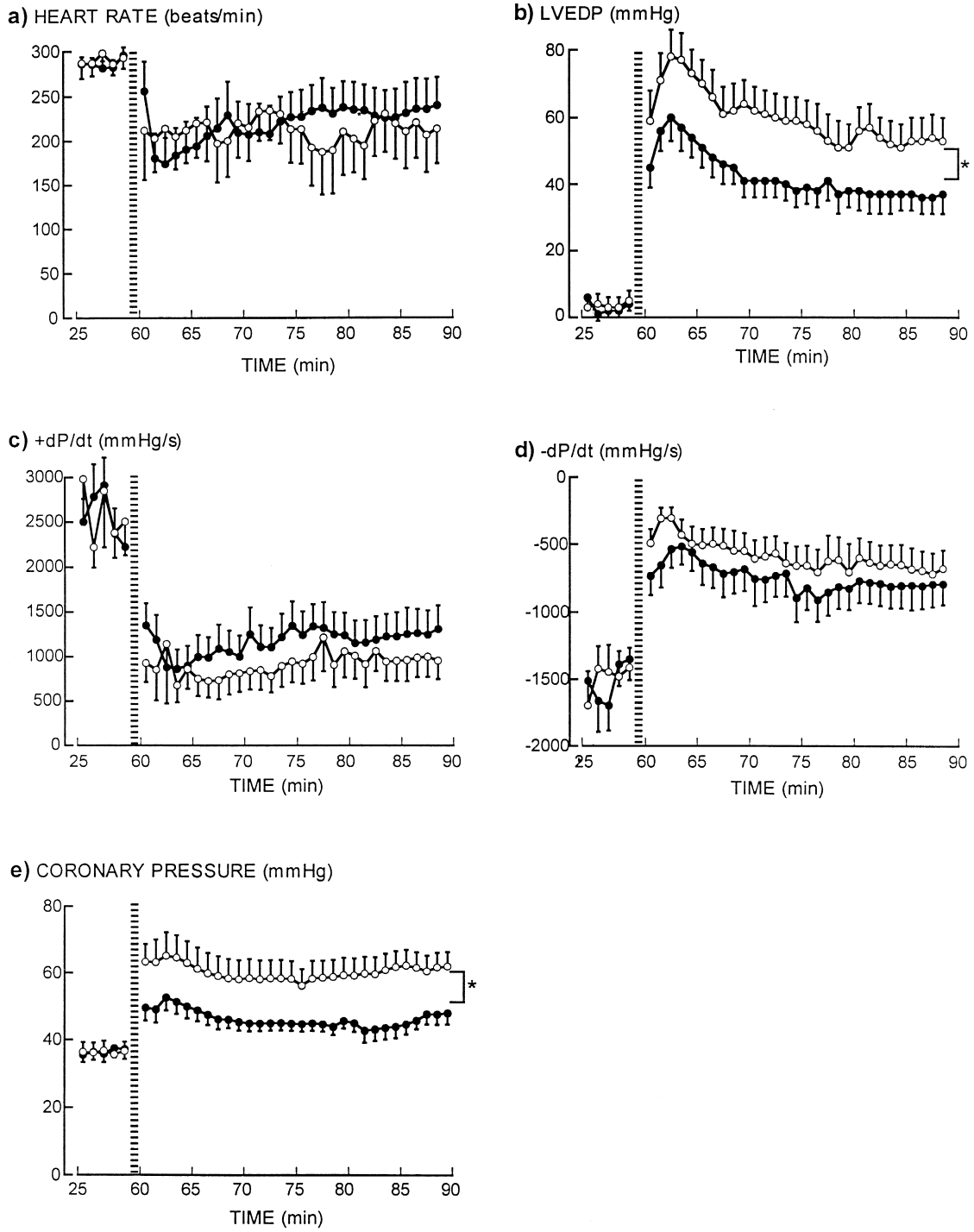


Fig. 3. The effect of amrinone on heart rate, left ventricular end diastolic pressure (LVEDP), $+dP/dt$, $-dP/dt$ and coronary perfusion pressure in rat isolated perfused hearts following 30 min of global ischaemia. These parameters were measured for 5 min prior to ischaemic insult and for 30 min after the initiation of reperfusion. Amrinone 30 μM (●, $n = 14$) or its vehicle (○, $n = 12$) were present in the perfusion solution prior to the 30 min period of global ischaemia and during the 30 min of reperfusion. Values are mean ± S.E.M. There was no significant difference in any of the cardiovascular parameters prior to ischaemia between the amrinone and vehicle treated hearts ($P > 0.05$, two-way analysis of variance). The post ischaemic left ventricular end diastolic pressure and coronary perfusion pressure were significantly reduced in the amrinone-treated hearts ($^{*}P < 0.05$, two-way analysis of variance).

### 3.4. Effect of amrinone on cardiac contractility

The effects of amrinone on cardiac contractility was assessed by examining the first 5 min of recordings in Fig. 3, prior to stopping coronary perfusion in Langendorff perfused rat hearts. Amrinone (30 μM) had no significant effects on heart rate, left ventricular end diastolic pressure, $dP/dt$ or coronary perfusion pressure (Fig. 3).

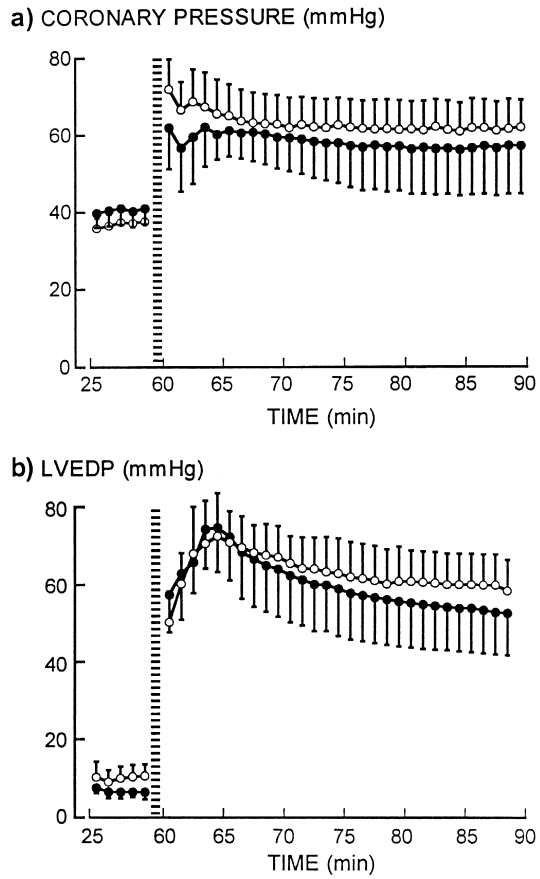


Fig. 4. The effect of amrinone on left ventricular end diastolic pressure (LVEDP) and coronary perfusion pressure in rat isolated perfused hearts following 30 min of global ischaemia. These parameters were measured for 5 min prior to ischaemic insult and for 30 min after the initiation of reperfusion. Amrinone 30 μM (●, $n = 4$) or its vehicle (○, $n = 4$) were present in the perfusion solution only during the 30 min of reperfusion. Values are mean ± S.E.M. There was no significant difference in any of the cardiovascular parameters prior to ischaemia between the amrinone and vehicle treated hearts ($P > 0.05$, two-way analysis of variance). Amrinone had no significant effect on either the post-ischaemic left ventricular end diastolic pressure or coronary perfusion pressure ($P > 0.05$, two-way analysis of variance).

### *3.5. Effect of pretreatment with amrinone on cardiac parameters during ischaemia and reperfusion*

When amrinone (30 μM) was present from 30 min before the cessation of coronary flow, the rise in left ventricular end diastolic pressure during reperfusion was attenuated (Fig. 3b), as was the rise in coronary perfusion pressure (Fig. 3e). Amrinone had no effect on the diminished heart rate (Fig. 3a) or myocardial contractility ($dP/dt$, Fig. 3c,d) during reperfusion.

### *3.6. Effect of time of addition of amrinone on coronary perfusion pressure and left ventricular end diastolic pressure*

When amrinone (30 μM) was present from 30 min before the cessation of coronary flow, the rise in coronary perfusion pressure and left ventricular end diastolic pressure during reperfusion were attenuated (Fig. 3b,e). However, when amrinone was added immediately after commencement of reperfusion, it had no effect on the coronary perfusion pressure or left ventricular end diastolic pressure during reperfusion (Fig. 4). However, if the coronary perfusion pressure was elevated using the thromboxane-mimetic U46619 (30 nM), subsequent addition of amrinone decreased the coronary perfusion pressure (Fig. 5). This effect of amrinone was seen in both normal and ischaemia-reperfused hearts which suggests that when there was an active tone in the coronary circulation, a vasodilator effect of amrinone could be revealed.

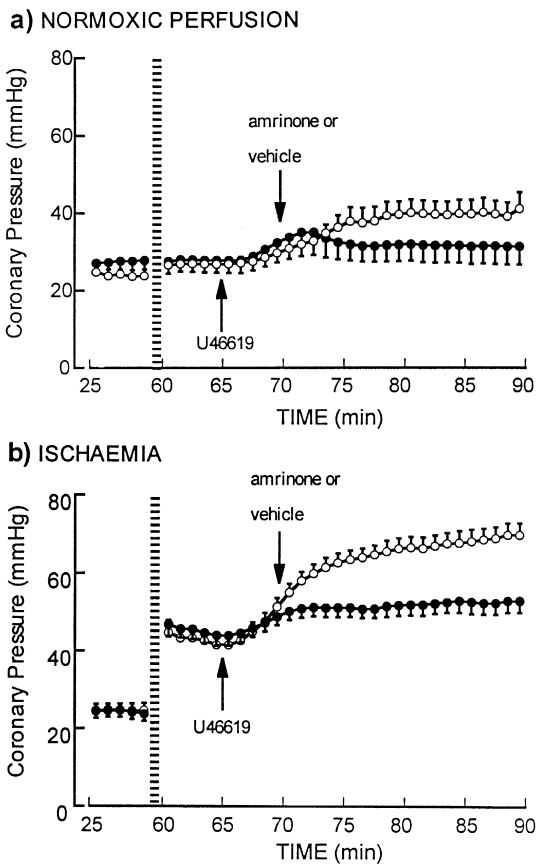


Fig. 5. The effect of amrinone on the increase in coronary perfusion pressure produced by U46619 in Langendorff perfused hearts following normoxic perfusion or ischaemia–reperfusion. These parameters were measured for 5 min prior to ischaemic insult and for 30 min after the initiation of reperfusion. U46619 (30 nM) was present in the perfusion solution 5 min after the initiation of reperfusion and amrinone 30 μM (●, $n = 5$) or its vehicle (○, $n = 4$) were present in the perfusion solution 5 min after the addition of U46619. Values are mean ± S.E.M. Following normoxic perfusion, U46619 (30 nM) increased coronary perfusion pressure to similar levels seen following ischaemia–reperfusion alone. Following ischaemia–reperfusion, U46619 increased coronary perfusion pressure above that produced by ischaemia alone. Amrinone inhibited the increase in coronary perfusion pressure produced by U46619 in both normoxic perfused and ischaemic hearts ($^{*}P < 0.05$ two-way analysis of variance).

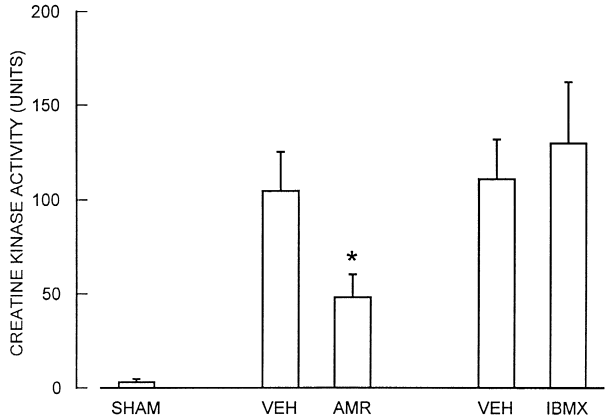


Fig. 6. The effect of amrinone and IBMX on creatine kinase levels in the coronary effluent following 30 min of global ischaemia. The coronary effluent was collected during the first 5 min of reperfusion. Creatine kinase was not detected in the effluent from hearts following a period of normoxic perfusion–reperfusion, however, its levels were markedly increased in hearts following ischaemia–reperfusion. Amrinone (30 μM, $n = 11$) present in the reperfusion solution prior to ischaemia significantly attenuated the rise in creatine kinase levels ($P < 0.05$, Student's *t*-test). In contrast, IBMX (10 μM, $n = 8$) had no significant effect on creatine kinase levels following ischaemia ($P > 0.05$, Student's *t*-test).

### *3.7. Creatine kinase and lactate dehydrogenase during reperfusion*

Creatine kinase and lactate dehydrogenase, indicators of myocardial injury, were determined in the coronary effluent collected during the first 5 min of reperfusion after 30 min of ischaemia. In preliminary experiments, it was found that the bulk of enzyme outflow occurred in the first 3 min and a slow decline thereafter to baseline at 30 min. Creatine kinase levels are almost undetectable in a 5-min sample of coronary effluent after normoxic perfusion but were elevated after ischaemia–reperfusion (Fig. 6). This increase was reduced when amrinone (30 μM) was present in the perfusion solution prior to the onset of ischaemia. In contrast, the non-selective phosphodiesterase inhibitor IBMX (10 μM) did not prevent the rise in creatine kinase activity in the perfusate (Fig. 6). Similarly, lactate dehydrogenase was almost undetectable in coronary effluent following sham ischaemia ($2.3 \pm 0.88$ units, $n = 3$) and this was elevated after ischaemia–reperfusion ($50.8 \pm 7.7$ units, $n = 11$). This increase was significantly reduced by amrinone ($27.9 \pm 4.6$ units, $n = 11$, $P < 0.05$, Student's *t*-test).

## 4. Discussion

Previously, we have reported that in rat isolated atria and in pithed rats, milrinone decreased responses to both noradrenaline and sympathetic nerve stimulation which is opposite to what would be expected of a phosphodiesterase 3 inhibitor which should potentiate this cyclic AMP dependent β-adrenoceptor response (Minatoguchi and Majewski, 1991). In the present study, we examined the structurally related phosphodiesterase 3 inhibitor amrinone (Alousi and Johnson, 1986) in rat isolated atria where tachycardia was produced using the $Ca^{2+}$ channel opener Bay K 8644 which produces tachycardia by increasing conductance through voltage-dependent $Ca^{2+}$ channels (Triggle and Rampe, 1989). In this case, amrinone significantly reduced the tachycardic response which leads us to conclude that amrinone possesses an anti-calcium effect. It should be noted, however, that amrinone (500 μM) does not block voltage-dependent $Ca^{2+}$ entry (Meisheri et al., 1980). This suggests a more subtle interaction with $Ca^{2+}$ signalling, which may involve receptor-operated $Ca^{2+}$ entry (Meisheri et al., 1980) or alterations in intracellular $Ca^{2+}$ movements (Tejerina et al., 1988).

Processes that seem to involve $Ca^{2+}$ are the changes in myocardial parameters during ischaemia and reperfusion. During ischaemia and reperfusion, metabolic changes have been suggested to be a result of $Ca^{2+}$ overload in the myocytes (Ferrari, 1995) leading to dysrhythms and other disturbances such as enhanced diastolic tension of the muscle (Piper et al., 1998). There is already some evidence that amrinone and milrinone decrease ischaemic damage in rabbit heart, although this has been attributed to a vasodilator effect (Rump et al., 1993b). In the present study, ischaemia followed by reperfusion, in rat perfused hearts resulted in a marked increase in coronary perfusion pressure and left ventricular end diastolic pressure accompanied by a reduction in myocardial contractility. Although contractility was not improved by pretreatment with amrinone, the rises in left ventricular end diastolic pressure and coronary perfusion pressure were reduced. The left ventricular end diastolic pressure is probably an indication of a tonic contracture of the muscle due to $Ca^{2+}$ overload (Silverman and Stern, 1994) and the ability of amrinone to reduce this parameter is consistent with an inhibitory effect on $Ca^{2+}$ overload. The ischaemia–reperfusion rise in coronary perfusion pressure was also reduced by amrinone. The rise in coronary pressure has been attributed to both endothelial changes (Nonami, 1997), changes in sensitivity to constrictor agents (Woodman, 1995) as well as a passive effect due to cell swelling and interstitial oedema leading to increased interstitial pressure (Garcia-Dorado and Oliveras, 1993; Miura, 1996) or pressure exerted on the intramural vessels due to the elevated resting tone of the cardiac muscle (Buja, 1998). Both left ventricular end diastolic pressure and coronary perfusion pressure remained elevated when amrinone was added only during the reperfusion stage suggesting that amrinone did not have a direct effect on the blood vessels but rather influenced some passive process either during ischaemia or immediately on reperfusion, possibly ischaemic damage due to oedema. It could be argued that amrinone was not an effective vasodilator, however, amrinone was able to reduce coronary perfusion pressure if the coronary circula-

tion was constricted using the thromboxane mimetic U46619 in both normal and ischaemia-reperfused hearts. This indicates that it did have vasodilator activity as has been reported previously (Rump et al., 1993a). We suggest that amrinone does not affect the ischaemia–reperfusion-induced rise in coronary perfusion when it is added after ischaemia because in this situation, the coronary circulation is not under active tone. Rather, following ischaemia–reperfusion, the increased coronary perfusion pressure is being maintained to some extent passively by high extramural or interstitial pressure exerted by the cardiac muscle. The concentration of amrinone that we used (30 μM) is not far different from the human therapeutic plasma concentration of 10–15 μM (Opie et al., 1991).

Amrinone reduced the ischaemia–reperfusion increase in creatine kinase and lactate dehydrogenase release, in addition to inhibitory effects on left ventricular end diastolic pressure and coronary perfusion pressure, which suggests a decrease in cellular damage in the rat hearts. IBMX the non-selective phosphodiesterase inhibitor had no effect on ischaemia-induced creatine kinase release. It should be noted that we measured enzyme release over the first 5 min only, which was the time where all the functional effects of amrinone on the reperfusion were also evident. It is possible that amrinone was delaying rather than preventing the ischaemic damage, but if this was so, it was not apparently functional over the 30 min after the onset of reperfusion. In another study, amrinone reduced the extent of myocardial ischaemia, as measured by NADH-fluorescence, during ischaemia in rabbit hearts (Rump et al., 1993a,b), although this was attributed to a vasodilator action. There are differences in the studies since Rump et al. (1993a) observed a vasodilator effect of amrinone when it was added subsequent to the ischaemia, whereas we did not. Furthermore, rabbits have a minimal native coronary collateral circulation (Cohen et al., 1994). This makes it unlikely that the cardioprotective actions of amrinone could be explained by a vasodilatory action producing an increase in collateral flow. We suggest that the reduced cellular damage by amrinone may be due to a decreased $Ca^{2+}$ overload in the myocytes, which would also explain the attenuation in the rise in left ventricular end diastolic pressure on reperfusion. The effect of amrinone cannot be explained by an action at phosphodiesterase 4 as the phosphodiesterase 4 inhibitor rolipram had no effect on infarct size following ischaemia–reperfusion in rat heart (McVey et al. 1999).

In summary, we suggest that amrinone reduces cellular damage during ischaemia–reperfusion by actions independent of phosphodiesterase inhibition. Since it also decreases left ventricular end diastolic pressure, we suggest that some mechanism that reduces the tonic contraction of the myocardium may be involved. We speculate that amrinone reduces $Ca^{2+}$ overload in the myocytes. This may represent a novel cardioprotective mechanism.

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