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CARDIOVASCULAR SYSTEM

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# The cardioprotective effects of $Na^+/H^+$ exchange inhibition and mitochondrial $K_{ATP}$ channel activation are additive in the isolated rat heart

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**Abstract** The mechanisms of recovery of the isolated rat heart were studied after 30 min of global ischemia. Functional recovery was assessed by the percentage recovery of developed pressure after 30 min reperfusion and by the magnitude of the contracture on reperfusion. After a control ischemia, developed pressure recovered to only 12±2% of pre-ischemic control and the reperfusion contracture was very large (81±6 mmHg). Activation of the mitochondrial $K_{ATP}$ channel with 100 μM diazoxide present throughout ischemia and reperfusion improved recovery of developed pressure to 36±3% and reduced the reperfusion contracture (53±4 mmHg). Inhibition of the sodium/hydrogen exchanger with 10 μM cariporide caused a larger recovery of developed pressure to 72 ±4% and further reduced the reperfusion contracture (11 ±3 mmHg). The combination of both drugs increased recovery of developed pressure to 96±4% and the reperfusion contracture remained small (11±5 mmHg). The effectiveness of the timing of exposure to these drugs was explored. When both diazoxide and cariporide were applied 2 min before the end of ischaemia and remained present during reperfusion the recovery of developed pressure was 81±4% and the reperfusion contracture was small (12±3 mmHg); neither was significantly different to the recovery when both drugs were present throughout ischemia and reperfusion. We conclude that mitochondrial damage, blocked by diazoxide, and the coupled exchanger pathway, blocked by cariporide, are two of the principal damage pathways and functional recovery appears to be complete when both are blocked. The combination of these drugs is also highly effective when given 2 min before the end of ischemia.

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## Introduction

The degree of recovery of cardiac function on reperfusion after moderate periods of ischemia is of great importance clinically. Many patients who suffer acute cardiac ischemia will now be treated with either thrombolysins or primary angioplasty which facilitate reperfusion of the ischemic region in the hope of recovery. In addition, during both cardiac surgery and transplantation, hearts are kept ischemic for variable periods of time and then reperfused. Thus, there is great interest in identifying the damage pathways which operate during ischemia and/or reperfusion and finding therapeutic approaches that improve recovery [24].

The discovery by Murry et al. [32] that several short periods of ischemia reduced the infarct size produced by a subsequent long period of ischemia was an early indication that the damage mechanisms could be modified by endogenous pathways. This finding has generated great interest in the pathway(s) involved [38, 47]. Currently it is thought that the preconditioning ischemias release various triggers substances (e.g. adenosine, adrenergic transmitters, ATP, endothelin) which bind to G-protein-coupled receptors and cause the activation of protein kinase C (PKC). PKC phosphorylates one or more effector proteins that provide protection, presumably by inhibiting an existing damage pathway. One effector protein is the mitochondrial $K_{ATP}$ channel which believed to be phosphorylated by preconditioning resulting in increased opening of the channel [14, 36]. This is thought to provide protection at least in part by minimizing the $Ca^{2+}$ loading of mitochondria [19, 28]. Thus, mitochondrial $Ca^{2+}$ loading contributes to damage observed on reperfusion and the importance of this pathway can be examined by using agents which open the mitochondrial $K_{ATP}$-channel. Diazoxide is a potent mitochondrial channel

opener and, as expected, improves recovery when applied to the ischemic heart [11, 42]. However, diazoxide has other mechanisms of action which may contribute to its cardioprotective action [18, 33].

Another damage pathway is provided by the coupled exchanger mechanism. The heart becomes very acid during ischemia due to the accumulation of lactate and protons. At the end of ischemia the acidosis recovers rapidly and one important pathway for the extrusion of $H^+$ is the $Na^+/H^+$ exchanger whose cardiac isoform is known as NHE1 [44]. Each proton extruded by this mechanism causes the entry of a $Na^+$ and consequently a rapid rise of $[Na^+]_i$ can often be observed on reperfusion [45]. The elevation of $[Na^+]_i$ changes the equilibrium for the $Na^+/Ca^{2+}$ exchanger and results in $Ca^{2+}$ entry in exchange for $Na^+$ extrusion. This is generally thought to be the cause of the large increase in total $Ca^{2+}$ on reperfusion first described by Shen and Jennings [37]. The large rise in $[Ca^{2+}]_i$ is thought to cause damage by a range of mechanisms including activating proteases and causing $Ca^{2+}$ overload of mitochondria. The importance of this coupled exchanger mechanism is most clearly demonstrated by the improvement in recovery from ischemia caused by inhibitors of NHE1 [20, 31]. We have recently proposed that inhibition of NHE1 also has a role in the protection caused by preconditioning [45, 46] but this remains controversial with other groups reaching different conclusions [4, 16].

These two damage pathways are well established but numerous others have been proposed including free radical damage [5], membrane tearing due to hypercontracture [35], and apoptosis [12]. As outlined above the best evidence for the role of a particular pathway is provided by an appropriate inhibitor and is then evident from the degree of improvement in recovery provided by that inhibitor. Simplistically, if inhibitors to all the important pathways were available, then one might achieve complete recovery from ischemia. Thus, complete recovery in the presence of a cocktail of inhibitors would imply that the most important damage pathways had been identified and inhibited. An extension to this argument is that if two inhibitors alone each produce a certain degree of recovery, then if independent pathways are involved one might expect to observe an additive effect. Conversely, if the inhibitors act on the same pathway, though perhaps at different points, they may have little additive effect when applied together.

We have applied this approach to the recovery of developed pressure after 30 min of ischemia. For the two pathways described above there are reliable inhibitors and we have examined the recovery with each separately and then together. The combination gave a full recovery of developed pressure and the reperfusion contracture was greatly reduced. We have also used this approach to examine the key processes that contribute to the improved recovery observed in preconditioning. Finally, we have examined how effective these drugs and their combination are when used at various stages during ischemia and reperfusion. The results suggest that damage associated with the coupled exchanger pathway and with mitochondrial $K_{ATP}$ channels dominate in the recovery from ischemia and that this combination of drugs are effective when used in the final stage of ischemia and reperfusion.

## Materials and methods

The experiments were performed on Langendorff-perfused rat hearts from female Sprague-Dawley rats [34, 45, 46]. These experiments were approved by the Animal Ethical Committee of the University of Sydney. Rats (200–250 g) were anaesthetized with pentobarbitone, the hearts were excised, and perfused with a Tyrode solution at 10 ml/min (12–15 ml/min per g wet weight) at 37°C. The perfusate had the following composition (mM): NaCl 119, KCl 4, $NaH_2PO_4$ 1.2, $MgSO_4$ 1.2, $NaHCO_3$25, $CaCl_2$ 1, glucose 11. The solutions were equilibrated with 95% $O_2$/5% $CO_2$ to give a pH of 7.4. Hearts were continuously stimulated at 2 Hz after the sinoatrial node was excised and the atrio-ventricular node was crushed. The low rate of stimulation was chosen to minimize the consequences of the low $O_2$ content of the perfusate which lacks haemoglobin [1]. It has been shown that stimulation at 2 Hz compared to 5 Hz leads to improved metabolite levels in the isolated heart [8] and that the ischemic contracture occurs earlier in hearts stimulated at 5 Hz compared to 2 Hz [45]. Isovolumic left ventricular developed pressure (LVDP) was monitored with a balloon in the left ventricle.

### Experimental approach

Ischemia was produced by stopping perfusion inflow to the heart while the heart was maintained at 37°C. The standard period of ischemia was 30 min; preconditioning consisted of three periods of 5 min ischemia each followed by 5 min reperfusion and then followed by the standard 30 min of ischemia. Diazoxide was obtained from Sigma and a stock solution of 100 mM in dimethylsulphoxide was used; this resulted in 1 part in 1,000 dimethylsulphoxide in the final solution. The NHE1 inhibitor, cariporide (also known as HOE 642 or 4-isopropyl-3-methlsulphonylbenzoyl-guanidine methanesulphonate), was donated by Hoechst, 65926 Frankfurt/Main, Germany. A stock solution of 10 mM in $H_2O$ was used.

In some experiments we wished to ensure that the drug was in place in the first few seconds of reperfusion. In these experiments the heart was perfused for 15 s after 28 min of ischemia using solution containing the drug. This period is sufficient to clear the perfusion line and vascular volume of the heart with new solution containing the drug. Ischemia was then continued until 30 min and reperfusion started in the normal way using the drug-containing solution. Thus, in this procedure the drug was present for the final 2 min of ischemia and throughout reperfusion.

Recovery from ischemia was assessed by the recovery of LVDP and by the magnitude of the contracture on reperfusion. Recovery was quantified from the LVDP after 30 min of reperfusion and was expressed as a percentage of control LVDP. The reperfusion contracture was the additional increase in diastolic pressure observed from the end of ischemia to the peak during reperfusion. We also monitored a number of other parameters including the ischemic contracture (peak diastolic pressure during contracture), the time to onset of ischemic contracture and the increase in diastolic pressure after 30 min reperfusion. However, we do not report on this information in any detail for the following reasons. The ischemic contractures were highly variable and showed no significant changes in our experiments even in the presence of diazoxide and NHE1 inhbition where others have shown decreases [7, 10]. The time of onset of the ischemic contracture was in the range of 12–18 min and was significantly shortened by the presence of the $K_{ATP}$ channel blocker 5-hydroxydecanoate (5HD) to 6–8 min but unaffected by any other condition. The resting pressure after 30 min reperfusion

correlated very highly with the reperfusion contracture and had a similar magnitude and is therefore not presented.

### Intracellular sodium measurements

$[Na^+]_i$ was measured using the fluorescent indicator SBFI loaded in its membrane-permeable acetoxymethyl (AM) ester form. The resulting fluorescent signals were calibrated by standard methods; correction was made for the changes in autofluorescence which occur during ischemia [34]. We have previously established that this method measures ionic concentrations in the epicardium and myocardium to a depth of about 0.1–0.2 mm [43]. On reperfusion $[Na^+]_i$ rises rapidly reaching a peak at about 5 min, e.g. Fig. 1B. To quantify this peak we measured the peak $[Na^+]_i$ occurring within the first 5 min. When this peak was absent, e.g. Fig. 1F, the $[Na^+]_i$ after 5 min of reperfusion was measured.

### Statistics

All data are expressed as mean±SEM; *n* values for the main groups are shown in Fig. 2. Comparison between treatment groups was made by one-way analysis of variance (ANOVA) using the Student-Newman-Keuls correction for multiple comparisons. Statistical significance was taken as $P<0.05$. The statistical significance of selected comparisons are given in the text and on the figures.

## Results

### Diazoxide and cariporide present throughout ischemia and reperfusion

After 30 min of global ischemia at 37°C rat hearts showed very little recovery of developed pressure at 30 min (12 ±2%) and there was a very large reperfusion contracture (81±6 mmHg), e.g. Fig. 1A. Mean data and statistics are shown in Fig. 2. We first tested the effect of 100 μM diazoxide applied 5 min before the start of ischemia and present throughout ischemia and reperfusion. This concentration has been shown to produce a maximal protective effect [11]. As expected diazoxide significantly improved recovery of developed pressure to 36±3% and also significantly reduced the reperfusion contracture to 53 ±4 mmHg, e.g. Fig. 1C. This data supports earlier studies

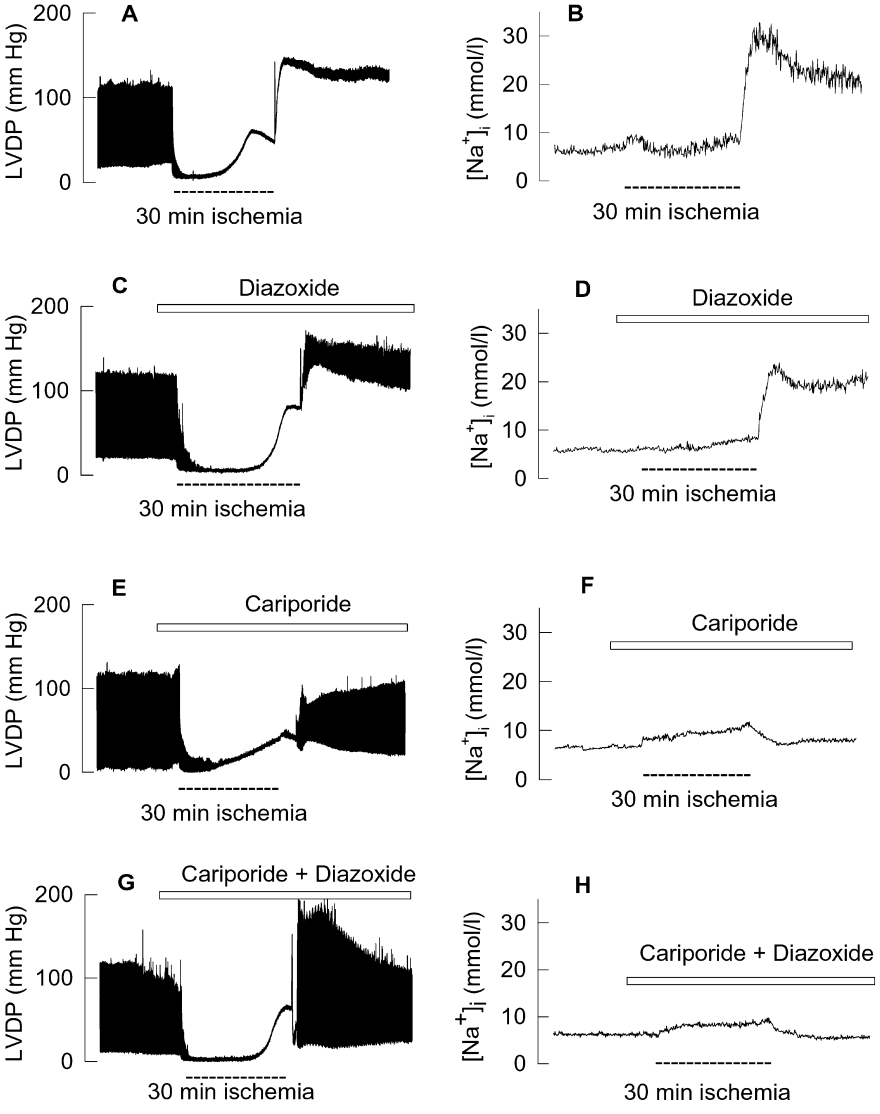


**Fig. 1A–H** Representative records of left ventricular developed pressure (LDVP) and intracellular sodium $[Na^+]_i$ in Langendorff-perfused rat hearts before, during and after a 30-min period of global ischemia. **A** LVDP in a control ischemia and reperfusion. Note very large contracture at the moment of reperfusion and failure of LVDP to recover on reperfusion. **C** Diazoxide (100 μM) present for 5 min preceding ischemia and present throughout ischemia and reperfusion. **E** Cariporide (10 μM) present throughout ischemia and reperfusion. **G** Diazoxide (100 μM) and cariporide (10 μM) present throughout ischemia and reperfusion. **B** $[Na^+]_i$ during control 30 min ischemia. Note minimal rise of $[Na^+]_i$ during ischemia but large and rapid rise of $[Na^+]_i$ on reperfusion. **D** Diazoxide (100 μM) present throughout ischemia and reperfusion. **F** Cariporide (10 μM) present throughout reperfusion and ischemia. Note absence of transient rise of $[Na^+]_i$ on reperfusion. **H** Diazoxide (100 μM) and cariporide (10 μM) present throughout ischemia and reperfusion

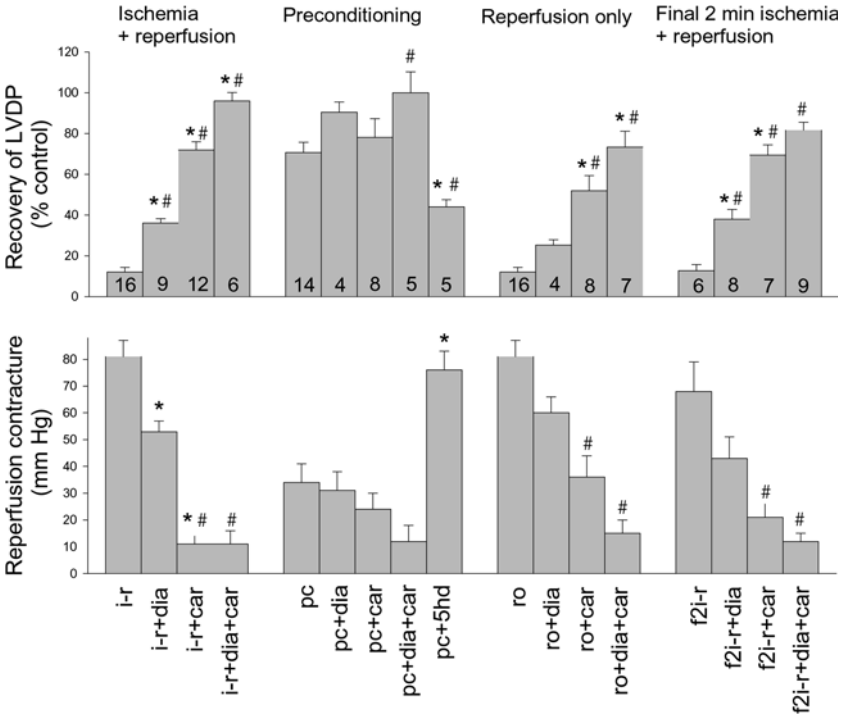


**Fig. 2** Graphical representation of recovery from LVDP (*upper panels*) and size of reperfusion contracture (*lower panels*). Number of hearts shown for each data set shown at the bottom of each column. Experimental paradigm shown above *upper panel* and in the labelling of individual columns below *lower panel*. *i-r* Drug present throughout ischemia and reperfusion, *pc* preconditioning, *ro* reperfusion only, *f2i-r* final 2 min of ischemia and reperfusion, *dia* diazoxide, *car* cariporide, *5hd* 5-hydroxydecanoate, *significantly different at $P<0.05$ from preceding bar, # significantly different at $P<0.05$ from first column in group

that closure of mitochondrial $K_{ATP}$ channels contributes to reperfusion damage and that an opener can partially reverse the damage [11, 42]. It is also well known that inhibiting NHE1 contributes to recovery after ischemia [20, 31]. We used cariporide (10 μM) which produces a near-maximal inhibition of NHE1 [46] and in these initial experiments we applied it throughout ischemia and reperfusion. This concentration of cariporide produced a large recovery of developed pressure (72±4%) and the reperfusion contracture was very small (11±3 mmHg) (Fig. 1E).

Given that diazoxide and cariporide are thought to improve recovery by different mechanisms it was of interest to combine the two drugs. In the presence of the two drugs there was complete recovery of developed force at 30 min (96±4%, which was not significantly different to the original control). Often, as in Fig. 1G, the initial developed pressure was substantially greater than control, suggesting that there might still be some degree of calcium overload at this time but that cellular regulatory mechanisms were subsequently able to remove the excess. The reperfusion contracture was also very small (11±5 mmHg).

## Intracellular sodium changes during reperfusion

We have previously shown that in the present model of ischemia, the rise in $[Na^+]_i$ during ischemia is small but there is a large and transient rise of $[Na^+]_i$ on reperfusion with a peak of 21.9±3.3 ($n$=6) at 5 min, e.g. Fig. 1B [34, 45]. We have also shown that cariporide eliminates the rise of $[Na^+]_i$ on reperfusion, e.g. Fig. 1F ($[Na^+]_i$ after 5 min reperfusion 7.9±0.5 mM, $n$=6). It is thought that the protective effect of cariporide arises in part from the reduced $Ca^{2+}$ entry occurring on reperfusion secondary to this greatly reduced $[Na^+]_i$ during reperfusion [21, 29, 46]. Since diazoxide is thought to improve recovery by a different mechanism, it was of interest to measure the rise of $[Na^+]_i$ on reperfusion in the presence of diazoxide. As expected the peak $[Na^+]_i$ on reperfusion in diazoxide (19.3 ±1.6 mM) was not significantly reduced compared to control reperfusion (Fig. 1D). However, when both diazoxide and cariporide were present the $[Na^+]_i$ on reperfusion was significantly reduced to 8.0±0.5 mM (Fig. 1H), a value which was not significantly different to that in cariporide.

## Ischemic preconditioning

To explore the extent of protection provided by these two mechanisms in the preconditioned heart, we added either diazoxide or cariporide to the perfusate 5 min before the 30-min ischemia. As shown in Fig. 2 neither the addition of diazoxide nor the addition of cariporide produced significant changes in the recovery from ischemic preconditioning judged by recovery of LVDP or reperfusion contracture. However, the combination of both cariporide and diazoxide did produce a significant increase in recovery which was 100±10% of the control. The reperfusion contracture was also decreased but the significance value was only $P<0.07$.

To gain additional insight into the contribution of $K_{ATP}$ channel opening in the protection of ischemic preconditioning we used 5HD, a blocker of mitochondrial $K_{ATP}$ channels (100 μM). 5HD significantly reduced the recovery of LVDP from 71±5% in preconditioned hearts

to 44±4%. The reperfusion contracture was significantly increased (56±7 mmHg).

### Timing of drug application

The timing of drug application which produces functional benefit can potentially help to identify the period when particular damage mechanisms are active and provides critical information for the use of a drug clinically. Here we compare the effectiveness of NHE1 inhibition and mitochondrial $K_{ATP}$ activation when applied during reperfusion-only and in the final 2 min of ischemia and reperfusion.

Figure 2 (third panel) shows the main results when the various drugs were applied during reperfusion only. Diazoxide in this period produced an apparent recovery of 25±3% but this was not significantly different to ischemia alone. Cariporide produced a substantial recovery of 52±7% and also caused a significant reduction in the reperfusion contraction as we have previously reported in this preparation [46]. The combination of diazoxide and cariporide produced a recovery that was significantly greater than either drug alone at 73±8% and further reduced the reperfusion contracture. This is an interesting result because although diazoxide alone had no significant effect, in combination with cariporide there was an additive effect.

Figure 2 shows the main results when the various drugs were applied in the final 2 min of ischemia and reperfusion. Figure 3 shows some representative pressure records. Figure 3A shows a record of this procedure with no added drugs and shows little recovery of LVDP (13 ±3%) and a large reperfusion contracture (68±12 mmHg). The 15-s perfusion after 28 min of ischemia had no significant effect on either of these parameters compared to the control ischemia. In Fig. 3B diazoxide was applied in the final 2 min of ischemia and reperfusion and produced a moderate recovery of LVDP (38±5%) which significantly greater than ischemia-alone. The reperfusion damage showed a trend to reduction that did not quite reach significance. Figure 3C shows the robust recovery produced by the cariporide in the final 2 min of ischemia and reperfusion (69±5%) and we confirm that this is not significantly different to the recovery caused by cariporide present throughout ischemia and reperfusion (72±4%) [46]. The reperfusion contraction was also significantly smaller than the control for this group. When both cariporide and diazoxide were applied in the final 2 min of ischemia and reperfusion (Fig. 3D) there was a pronounced early recovery which subsequently declines slightly so that at 30 min LDVP was 81±4%. This recovery was significantly greater than with diazoxide alone but just failed to meet statistical significance for an increase over cariporide alone ($P<0.07$). Nevertheless this result was not significantly different to the recovery seen when both drugs were present throughout ischemia and reperfusion (96±4%) or during preconditioning (100 ±10%). The reperfusion contracture (12±3 mmHg) was also greatly reduced by this combined treatment.

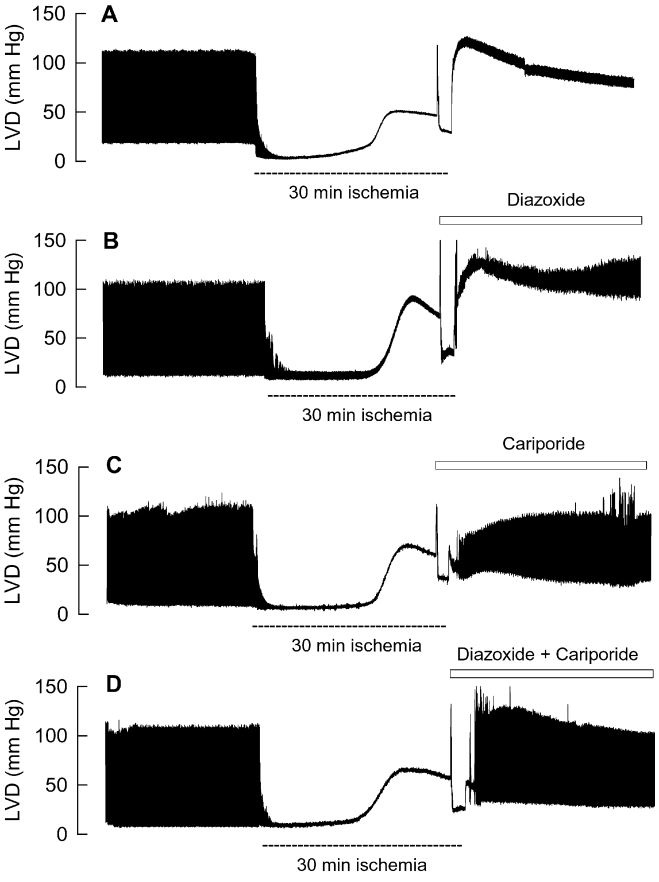


**Fig. 3A–D** Representative records of LVDP in hearts subjected to a 15-s period of perfusion after 28 min of ischemia. Ischemia was then continued until 30 min and reperfusion restarted in the normal manner. **A** Control with no drug application. Note failure of recovery and the large reperfusion contracture similar to the control in Fig. 1. **B** Diazoxide (100 μM) applied in the final 2 min of ischemia and throughout reperfusion. Note some recovery of LDVP apparent. **C** Cariporide (10 μM) applied in the final 2 min of ischemia and throughout reperfusion. Note very small reperfusion contracture and substantial recovery of LVDP. **D** Diazoxide (100 μM) and cariporide (10 μM) applied in the final 2 min of ischemia and throughout reperfusion. Note increased LVDP present from early in reperfusion and a slow decline over the 30 min period

## Discussion

### Limitations of the model of ischemia

The Langendorff-perfused rat heart has been used extensively for studies of the functional, metabolic and electrophysiological consequences of ischemia. The recovery of developed pressure and the magnitude of the reperfusion contracture have often been used to assess ischemic/reperfusion damage and have been shown to correlate with other markers of cell damage such as histological changes, protein release, frequency of arrhythmias [39]. In the saline-perfused heart the absence of red cells and haemoglobin causes a very large reduction in

$O_2$ carrying capacity which is partially offset by increasing the partial pressure of $O_2$ and by increasing the perfusion rate. Nevertheless there is still a reduced $O_2$ supply (for further discussion see [2]). We have chosen to minimize the consequences of this reduced $O_2$ supply by reducing the stimulation rate from the normal (endogenous) rate of 5 Hz to 2 Hz. Thus the consequences of ischemia will be somewhat slower in onset in our model than those studies which maintain the rate at 5 Hz. It is not clear which of these models most closely simulates the process of the damage in the human heart during clinical episodes of ischemia.

## Damage mechanisms on reperfusion after 30 min of ischemia

The improvement of recovery produced by diazoxide applied throughout ischemia and reperfusion is roughly similar to previous reports (for review see [36]). While the hypothesis that diazoxide acts by mitochondrial $K_{ATP}$ opening has wide support [11, 14, 19, 28, 36, 42] there is also evidence that diazoxide has other mechanisms of action which could contribute to cardioprotection [18, 33]. The fact that 5HD, an inhibitor of mitochondrial $K_{ATP}$ channels, reversed much of the effects of preconditioning supports the view that this is an important mechanism in the present experiments. The improvement in recovery caused by NHE1 inhibition is also comparable to many others in the literature and it is widely accepted that this protection arises through preventing calcium accumulation secondary to the coupled exchanger hypothesis (for review see [30]).

Our most striking finding is that when diazoxide and cariporide were combined the recovery of LVDP was complete (not significantly different to 100%) and the magnitude of the reperfusion contracture was reduced to a very low level. This suggests that these two pathways are the main ones contributing to ischemia/reperfusion damage. However, a study by Hale and Kloner [17] using a similar approach but utilizing infarct size in rabbits reached a different conclusion. In their study diazoxide had no significant effect on infarct size when used alone in contrast to the majority of studies reviewed above [36] so the significance of absence of effect when combined with NHE1 inhibition is limited. A very recent study on Langendorff-perfused hearts reported results very similar to ours on developed pressure and showed that this was accompanied by a reduction in the infarct area [6].

What do these studies tell us about the mechanisms of recovery and the way they interact? The coupled exchanger mechanism is thought to lead to $Na^+$ and $Ca^{2+}$ loading but there is an unresolved dispute about whether this occurs mainly during ischemia or mainly during the early part of reperfusion (for discussion see [2, 30, 41]). The elevated $[Ca^{2+}]_i$ is thought to cause damage (1) by activating proteases with many deleterious consequences including cleavage of troponin and loss of $Ca^{2+}$ sensitivity [5], and (2) by entering mitochondria causing loss of oxidative phosphorylation and opening of the mitochondrial transition pore with subsequent further damage [40]. NHE1 inhibitors reduce the $Ca^{2+}$ entry and would be expected to ameliorate both the above pathways. The role of the mitochondrial $K_{ATP}$ channel in damage is less clear and a very active topic of research. One theory is that diazoxide may minimize the $Ca^{2+}$ entry into mitochondria by depolarizing the mitochondrial membrane potential [19, 28]. If this is the case then the additive effect of both agents observed in our experiments may reflect the additional preservation of mitochondrial function when both the $Ca^{2+}$ entry into the cell is reduced and the $Ca^{2+}$ uptake by the mitochondria is reduced.

An interesting feature of our results when both cariporide and diazoxide are present is that the earliest contraction are often greater than 100% control and then gradually declines to a steady but somewhat lower level. This is in contrast to all other records where the LVDP is initially small on reperfusion and gradually recovers. When measurements of systolic rise of calcium ($Ca^{2+}$ transients) are made during recovery from ischemia or hypoxia they are normally very large initially and then gradually recover [23, 25, 26]. Thus, it seems that in the presence of both cariporide and diazoxide reduces the loss of $Ca^{2+}$ sensitivity of the contractile proteins which normally prevents the early contractions responding to the large $Ca^{2+}$ transients. There are various possible causes of this improved $Ca^{2+}$ sensitivity. First, during $Ca^{2+}$ overload spatially and temporally inhomogeneous $Ca^{2+}$ release reduces the force because regions with reduced $Ca^{2+}$ release act as a compliance in series with the activated regions [3]. As $Ca^{2+}$ entry is reduced this cause of apparent reduction in $Ca^{2+}$ sensitivity will decline. Second, another possible cause of the reduced sensitivity is damage to troponin I caused by $Ca^{2+}$ activated proteases [9]. Decreased $Ca^{2+}$ entry will also reduce this factor. Finally, during ischemia $Ca^{2+}$ sensitivity is reduced by metabolic factors including inorganic phosphate [22]. Improved mitochondrial function will allow resynthesis of inorganic phosphate into ATP and phosphocreatine and will accelerate the recovery of $Ca^{2+}$ sensitivity

## Damage mechanisms reversed by preconditioning

Many studies of ischemic preconditioning suggest that activation of the mitochondrial $K_{ATP}$ channel is a key pathway which contributes to the improved recovery observed in the preconditioned heart [11, 36]. However, in the present study diazoxide alone applied throughout the long ischaemia and reperfusion produced only 36±3% recovery while ischaemic preconditioning produced 71 ±5% recovery suggesting that other mechanisms must also contribute to the improved recovery following ischemic preconditioning. Furthermore, addition of 5-HD, which would be expected to reverse the benefits of $K_{ATP}$ channel activation, only partially reduced the recovery of the preconditioned heart from 71±5% to 44±4%. This again suggests that there are pathways activated by precondi-

tioning which are unrelated to $K_{ATP}$ activation. We have previously proposed that inhibition of NHE1 also contributes to preconditioning and the observation that addition of cariporide to the preconditioned heart brings no significant additional recovery supports this interpretation [45, 46]. Although diazoxide alone or cariporide alone produced small but non-significant benefits when applied to the preconditioned heart, when applied in combination the recovery of LVDP increased to 100±10%. This recovery is complete and comparable to the recovery when the two drugs are applied to the ischaemic-only heart. The simplest interpretation is that the activation of the mitochondrial $K_{ATP}$ channel and the inhibition of the NHE1 produced by ischemic preconditioning are not maximal and when maximally effective concentrations of both drugs are applied the full benefits are seen and recovery is complete.

### Timing of application of cardioprotective drugs

The issue of the optimal timing of drug application is controversial. Many studies of the benefits of diazoxide showed that the optimum effect was obtained when the drug was applied before ischemia or in the early part of ischemia [7, 11]. From these and other studies it has been suggested that activation of $K_{ATP}$ channels may both act as a trigger for preconditioning and also act as the late effector of protection [13]. Studies of the application of diazoxide during reperfusion only have generally found little effect which we confirm [15]. However, application of the $K_{ATP}$ channel openers late in ischemia have sometimes found no effect [42], whereas other studies have observed moderate benefit [27] with our data supporting the latter study. Studies of $Ca^{2+}$ uptake by mitochondria have shown slow uptake throughout ischemia which can be inhibited by diazoxide [28] so it would be expected that some benefit of diazoxide on protection would be seen throughout ischemia. The additional benefit of diazoxide and cariporide when applied in the final 2 min of ischemia and reperfusion may represent the fact that the first few minutes of ischemia are the time when both the rise of $[Ca^{2+}]_i$ and the uptake of $Ca^{2+}$ by mitochondria are at their greatest [26, 28]. Thus, the additive effect of the two drugs during this period fits well with this interpretation.

The issue of the optimal timing for application of NHE1 inhibitors has also generated very variable results. Many studies find the optimum effect of NHE1 inhibition to be during ischemia whereas other find that presence of the drug during reperfusion-only is the most effective period. Clearly our data support the latter contention and the issues have been extensively discussed in recent reviews but remain unresolved [2, 31].

Given that the combination of diazoxide and cariporide applied throughout ischemia and reperfusion produced a complete recovery of LVDP after 30 min reperfusion, it is of interest to determine whether the benefits arise mainly during ischemia or reperfusion. When diazoxide and cariporide were both present during reperfusion only, the recovery of LVDP was 73±8%, which was significantly less than the recovery of 96±4% when both drugs were present throughout ischemia and reperfusion. The application of the drugs 2 min before the end of ischemia is intended to allow time for the drugs to reach their receptor so that the pathways are already activated or inhibited when reperfusion starts. This is particularly critical for cariporide because the $Na^+/H^+$ exchanger is activated very rapidly on reperfusion and the rise in $[Na^+]_i$ reaches its peak in less than 5 min [45]. For the combined drugs applied in the final 2 min of ischemia the mean recovery of LVDP was 82±4% and this value is not significantly different to the recovery seen when both drugs are present throughout ischemia and reperfusion.

## Conclusions

The main novel conclusions from this study are as follows. Two processes dominate the recovery from ischemia in the rat heart. These are (1) $Na^+$ and $Ca^{2+}$ loading, which follow from the coupled activity of NHE1 and NCX and can be prevented by NHE1 inhibitors, and (2) the mitochondrial damage, which is in part a consequence of closure of $K_{ATP}$ channels and can be reversed by mitochondrial $K_{ATP}$ channel openers. These two processes are additive and when both pathways are inhibited apparently complete recovery of function can be obtained. Furthermore, the benefits of the combined drugs are nearly complete when they are applied just before the end of the ischemic period.

Clinically these results are potentially important as a cardiologist performing angioplasty for an acute ischemic infarct could in principle apply this combination of drugs into the ischemic region prior to full reperfusion and gain the benefits of this kind of drug timing.

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