Effects of 4’-Chlorodiazepam on Cellular Excitation-Contraction Coupling and Ischemia-Reperfusion Injury in Rabbit Heart

David A. Brown, Miguel A. Aon, Fadi G. Akar, Ting Liu, Nicolas Sorarrain, and Brian O’Rourke
Institute of Molecular Cardiobiology, Division of Cardiology, Department of Medicine, The Johns Hopkins University School of Medicine, Baltimore MD 21205

Corresponding Author

Brian O’Rourke, Ph.D.
Professor of Medicine
Johns Hopkins University
School of Medicine
Institute of Molecular Cardiobiology
720 Rutland Ave.
1059 Ross Bldg.
Baltimore, MD 21205-2195
tel: 410-614-0034
fax: 410-955-7953
e-mail:bor@jhmi.edu

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Aims
Recent evidence indicates that the activity of energy-dissipating ion channels in the mitochondria can influence the susceptibility of the heart to ischemia-reperfusion injury. In this study, we describe the effects of 4′-chlorodiazepam (4-ClDzp), a well-known ligand of the mitochondrial benzodiazepine receptor, on the physiology of both isolated cardiomyocytes and intact hearts.

Methods
We used current- and voltage-clamp methods to determine the effects of 4-ClDzp on excitation-contraction coupling in isolated rabbit heart cells. At the level of the whole heart, we subjected rabbit hearts to ischemia/reperfusion in order to determine how 4-ClDzp influenced the susceptibility to arrhythmias and contractile dysfunction.

Results
In isolated rabbit cardiomyocytes, 4-ClDzp evoked a significant reduction in the cardiac action potential that was associated with a decrease in calcium currents and peak intracellular calcium transients. In intact perfused normoxic rabbit hearts, 4-ClDzp mediated a dose-dependent negative inotropic response, consistent with the observation that 4-ClDzp was reducing calcium influx. Hearts that underwent 30 min of global ischemia and 30 min of reperfusion were protected against reperfusion arrhythmias and post-ischemic contractile impairment when 4-ClDzp (24 μM) was administered throughout the protocol or as a single bolus dose given at the onset of reperfusion. In contrast, hearts treated with cyclosporin-A, a classical blocker of the mitochondrial permeability transition pore, were not protected against reperfusion arrhythmias.

Conclusions
The findings indicate that the effects on 4-ClDzp on both mitochondrial and sarcolemmal ion channels contribute to protection against post-ischemic cardiac dysfunction. Of clinical
relevance, the compound is effective when given upon reperfusion, unlike other preconditioning agents.

Introduction

Mitochondrial ion channels represent an important novel therapeutic target for the mitigation of ischemic injury in heart. Recently, a cardioprotective role for one specific channel complex, which is thought to include the mitochondrial benzodiazepine receptor (mBzr) (see 1 for review) and an inner membrane anion channel, has been elucidated 2, 3. The mBzr plays an important role in regulating mitochondrial membrane potential ($\Delta \Psi_m$) 3, 4 and apoptosis 5 in mammalian cardiac myocytes.

Ligands to the mBzr have been shown to inhibit at least two classes of ion channel in the mitochondrial inner membrane, the so-called mitochondrial megachannel (MMC), which has properties that resemble the macroscopic mitochondrial permeability transition inhibited by cyclosporin A (CsA), and the CsA-insensitive mitochondrial inner membrane anion channel (IMAC) 6 or centumpicosiemen anion channel (Mcts) 7. Furthermore, mBzR ligands have been shown to stabilize mitochondrial membrane potential ($\Delta \Psi_m$) in isolated cardiomyocytes subjected to oxidative stress 3. Given that oscillations in $\Delta \Psi_m$ were shown to directly influence action potential duration 3, the mBzR complex may be an important target for stabilizing cardiac excitability in the face of oxidative stress. The prototypical mBzR ligand, 4′chlorodiazepam (4-CIDzp), can reduce the lability in action potential duration in cells and prevent post-ischemic arrhythmias in guinea-pig hearts, leading to the proposal that regions of myocardium undergoing mitochondrial depolarization (“metabolic sinks”) contribute to the electrical inhomogeneity that results in arrhythmias. While numerous studies provide evidence that 4-CIDzp targets the mitochondrion, less is known about its possible influence on cellular excitation-contraction coupling, which could, theoretically, contribute to its protective effects.
In this study, we examined the effects of the most commonly used ligand to the mBzr, 4-ClDzp, on cellular action potentials, membrane currents and Ca\(^{2+}\) transients in isolated myocytes. At the level of the whole heart, we then investigated which actions of the compound might contribute to the cardioprotection provided by 4-ClDzp in a large animal global ischemia model.

**Methods**

**Animal Model.** Adult male New Zealand White rabbits (n = 36; body weight 2.1-3.3 kg) were housed in an animal facility with a 12:12 light/dark cycle and fed standard rabbit chow (Harlan High Fiber Rabbit Diet #2031). On the day of sacrifice, all rabbits were anesthetized by sequential administration of a ketamine and acepromazine cocktail (50 mg/kg and 5 mg/kg, respectively; i.m. injection) followed by 28 mg/kg of sodium pentobarbital delivered via a marginal ear vein. This study was conducted with prior approval from the Johns Hopkins University Animal Care and Use Committee and in accordance with guidelines established in the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

**Cell isolation.** Rabbits used for myocyte experiments (n=13) were anesthetized and hearts were placed on a modified Langendorff apparatus for the isolation of ventricular myocytes using protocols similar to those previously described \(^8\). Hearts were perfused with calcium-free Tyrodes solution containing (in mM): 140 NaCl, 1 MgCl\(_2\), 10 HEPES, 5 KCl, pH 7.5 (NaOH) for 5 minutes, followed by 8 minutes of perfusion with Tyrodes plus the addition of 1mg/mL collagenase, 0.17 mg/mL protease, and 10 \(\mu\)M CaCl\(_2\). After the digestion solution, the left
ventricle was cut away and minced in Tyrodes solution containing 10μM CaCl₂. The myocyte suspension was filtered and cells were gravity precipitated for 15 minutes. A series of 4 steps were used to gradually titrate calcium up to a final concentration of 2mM and cells were stored in DMEM (plus HEPES). Cardiomyocytes were placed in an incubator at 5% CO₂ (balance room air) at 37°C and were used 2-8 hours post-dispersion.

**Whole cell patch clamp.** Myocytes were perfused in a heated (37°C) flow-through chamber of an inverted microscope with an external solution of (in mM) 140 NaCl, 0.1 MgCl₂, 1 HEPES, 0.5 KCl, 10 glucose, and 2 CaCl₂. Borosilicate glass micropipettes (1-3 MΩ resistance) were used to establish the whole-cell patch clamp configuration using an Axopatch 1-D Amplifier (Axon Instruments). Cells were equilibrated with pipette solution containing (in mM) 130 K-glutamate, 9 KCl, 0.5 MgCl₂, 10 HEPES, 10 NaCl, 5 MgATP, and 80 μM Indo-1 pentapotassium salt (Molecular Probes) and dialyzed for 5 minutes prior to experimentation. Membrane currents were collected in voltage-clamp mode with a depolarizing step from the -80mV holding potential to a test potential of +10mV for 200 ms. For action potential waveforms, current-clamp mode was established, and action potentials were evoked by a 5 ms depolarizing step at a rate of either 0.2 or 1 Hz. Between 0.2 and 1Hz, we detected no statistically significant frequency-related differences in mean current amplitude or action potential duration, so the data were pooled and analyzed accordingly. Action potential and membrane current waveforms were transferred to a personal computer and analyzed with custom written software. (Ionview, B.O’R.). Indo-1 calcium transients were obtained during the step to +10mV and were analyzed using previously established methods ⁹, ¹⁰. All recordings were collected at a sampling rate of 1000Hz, and action potential waveforms were corrected to a glutamate offset potential of 13mV.
Calcium Current Experiments. A separate group of myocytes were used for calcium current experiments. Myocytes were equilibrated in potassium-free bath solution containing (in mM): 128 NaCl, 5 CsCl, 1 MgCl2, 10 NaHEPES, 2 CaCl2, and 10 glucose (pH 7.4). Cells were patch clamped as described above with pipettes containing (in mM): 110 CsCl, 0.4 MgCl2, 5 MgATP, 10 HEPES, 5 BAPTA, 20 TEA-Cl, and 5 glucose (pH 7.2). After equilibration at a holding potential of -80mV, cells were depolarized to -40mV for 10msec to inactivate Na currents, followed by a 150 msec test pulse ranging from -30mV to +50mV in 10mV increments (interpulse interval of 2 sec), after which cells returned to the holding potential of -80mV.

Hemodynamic Experiments. A separate group of rabbits (n=28) were used in ischemia-reperfusion experiments. Animals were anesthetized as described above. Upon the absence of reflexes, hearts were excised via midline thoracotomy and were placed on the cannula of a modified Langendorff apparatus attached to a Powerlab system (AD Instruments). Hearts were perfused with gassed (95/5 % O2/CO2) buffer containing (in mM): 118 NaCl, 24 NaHCO3, 1.2 KH2PO4, 4.75 KCl, 1.2 MgSO4, 2.0 CaCl2, and 10 glucose. A buffer-filled latex balloon (Harvard apparatus) was inserted through the mitral valve into the left ventricle and inflated to a pressure of 5 ± 1 mmHg. Hearts were suspended in a buffer-filled heating chamber maintained at 37°C and two electrodes were placed into the bath for volume-conducted electrocardiogram recordings. Hearts were perfused with constant pressure (73 ± 1 mmHg) and on-line coronary flow rate, heart rate, left ventricular developed pressure, maximal rates of contraction and relaxation, and electrocardiograms were monitored throughout the experiment and recorded on a personal computer. Hemodynamic parameters were analyzed using Chart software (Version 5, AD Instruments).
Drug administration and ischemia/reperfusion. After a 20 min equilibration period, hearts were subjected to the following protocols: 1) Control: 10 minutes normoxic perfusion, 30min/30min Ischemia/Reperfusion (n=7); 2) 4-ClDzp: 10 minutes perfusion with 4’chlorodiazepam at concentrations of either 12μM (n=4), 24μM (n=6), or 64μM (n=1), 30min/30min Ischemia/Reperfusion; 3) 4-ClDzp Bolus: 10 minutes normoxic perfusion, 30min/30min Ischemia/Reperfusion with a bolus of 300μM 4-ClDzp given at the onset of reperfusion (n=5); 4) CsA: 10 minutes normoxic perfusion with 0.2μmol/L Cyclosporin A, 30min/30min Ischemia/Reperfusion (n=5). For the delivery of 4-ClDzp, a stock solution of 4-ClDzp was prepared (using dimethyl sulfoxide as the solvent) and 4-ClDzp was added to fresh perfusion solutions at the beginning of each experiment (1 heart). Administration of dimethyl sulfoxide stock in the same concentrations alone had no effect on hemodynamic parameters or volume-conducted ECG (data not shown). With the exception of the bolus experiments, the compounds were present in the perfusate for the duration of the protocol (i.e., also during reperfusion). Preliminary results indicated that 24μM 4-ClDzp was the lowest concentration of drug that sufficiently provided protection against reperfusion arrhythmias, so this concentration was used in all subsequent cardiomyocyte experiments to characterize the cellular effects of the drug. In all intact heart experiments, global ischemia was established by stopping the perfusion to the heart, and reperfusion began when perfusion was restored.

Determination of arrhythmias. Arrhythmias were characterized in accordance with the Lambeth Conventions \cite{11} and scores were tabulated for the entire 30 minute reperfusion period using Score A as previously described by Curtis and Walker \cite{12}. Briefly, each heart was given a score based on the following criteria: 0 = < 50 ventricular premature beats; 1 = 50-499 ventricular premature beats; 2 = >500 ventricular premature beats and/or 1 episode of spontaneously
reverting ventricular tachycardia or ventricular fibrillation; 3 = >1 episode of spontaneously reverting ventricular tachycardia or fibrillation (<1 min total combined duration); 4 = 1-2 min of total combined ventricular tachycardia or fibrillation; 5 = > 2 min of ventricular tachycardia or fibrillation \(^{12}\).

Determination of Hemodynamic Parameters. Baseline measurements of left ventricular developed pressure, coronary flow, maximal rates of contraction and relaxation were made by taking the 30 second average during the last minute before the onset of ischemia.

Materials. All materials were obtained from Sigma-Aldrich unless otherwise noted.

Statistical analysis. Data are expressed as mean ± standard error, and statistics were calculated using Microsoft Excel for paired t-tests and SPSS software for ANOVA. Drug-induced changes in action potential duration, calcium current amplitude, as well as baseline effects of the drug on the whole heart were analyzed by using a paired t-test. Arrhythmia scores were compared with a one-way ANOVA, and planned between-group contrasts were made using Dunnett’s t-test based on the \(a \text{ priori}\) hypothesis that blocking the mBzr would decrease arrhythmias when compared to control \(^2\). Between-group differences in the development of ischemic contracture and in recovery of left ventricular developed pressure during reperfusion were analyzed using repeated-measures ANOVA and Dunnett’s post-hoc method of multiple comparisons.
Results

Action potential duration. Administration of 24μM 4-ClDzp reduced the duration of the action potential by 36% (Figure 1A). The times to 50 and 90% repolarization were decreased to a similar extent, indicative of a change in the net currents flowing during phase 2 of the action potential (Figure 1B, P < 0.05).

Effects on Excitation-Contraction Coupling. Consistent with the effects of 4-ClDzp to decrease the duration of the action potential plateau, voltage-clamp recordings of the total inward currents revealed a decrease in total inward current at 50 and 150 msec after the test pulse to +10mV (Figure 2 A and B). The late component of this current largely consists of overlapping L-type Ca\(^{2+}\) and K\(^{+}\) currents. When experimental conditions were optimized for measurement of \(I_{Ca}\), peak \(I_{Ca}\) was reduced significantly at test pulses from -10mV to +50mV (Figure 2 D and E). A decrease in Ca\(^{2+}\) current would also diminish the trigger for Ca\(^{2+}\) release from the sarcoplasmic reticulum and could explain the negative inotropic effects of the compound. Hence, we also measured intracellular Ca\(^{2+}\) transients. 4-ClDzp (24 μM) treatment led to a significant reduction in the amplitude of the cytosolic calcium transient, as reported using indo-1 (Figure 2 E and F). Peak calcium concentration was reduced by 28% in the presence of 24μM 4-ClDzp. (P < 0.05).

4-ClDzp effects on contractility. Administration of 4-ClDzp caused a dose-dependent decrease in the left ventricular developed pressure, ranging from little effect at 12μM to a 60% decrease in LVDP in 64μM 4-ClDzp (Figure 3). The effect on LVDP was entirely due to a decrease in the peak systolic pressure, as end diastolic pressure was not elevated in any of the groups after receiving 4-ClDzp (data not shown). Baseline effects of 24μM 4-ClDzp are presented in Table 1. While previous studies have shown no effect of 4-ClDzp on heart rate at concentrations
below $10\mu M$ \cite{13}, \cite{14}, our findings indicate that higher concentrations of 4-ClDzp led to a negative chronotropic effect that bordered on statistical significance. As a partial blocker of $I_{\text{Ca}}$ at a concentration of $24\mu M$, our results are consistent with previous studies showing that calcium channel blockers act as both negative inotropes and negative chronotropes in isolated heart preparations\cite{15}.

Left Ventricular Performance and Reperfusion Arrhythmias. Representative ECG traces and cumulative arrhythmia scores from whole-heart experiments are presented in Figures 4 and 5. Hearts that were perfused with 4-ClDzp, either when administered before ischemia or immediately upon reperfusion, had a significantly reduced arrhythmia score, tabulated for the entire 30 minute reperfusion period ($P < 0.05$ for both the $24\mu M$ pre-treatment group and the bolus group when compared with controls). In contrast, treatment with cyclosporin-A, a classical blocker of the permeability transition pore, was not effective at attenuating post-ischemic arrhythmias (Figure 4D and 5).

Development of ischemic contracture was prominent in hearts beginning at about 15 min into ischemia. Increased end diastolic pressure was observed in the control group, and this contracture was delayed in hearts that received $24\mu M$ 4-ClDzp prior to ischemia. End diastolic pressure was significantly lower for the last 4 minutes of ischemia in hearts that received $24\mu M$ 4-ClDzp prior to ischemia as compared to control hearts ($P < 0.05$, RM ANOVA), (Figure 6A). As expected, there was no difference in the development of ischemic contracture in the control versus 4-ClDzp bolus group, indicating that the mechanism of the post-ischemic protection was distinct from a change in the events occurring during the ischemic period.

Recovery of LVDP at reperfusion was significantly enhanced when hearts received 4-ClDzp when compared to control. Administration of $24\mu M$ 4-ClDzp led to greatly improved recovery of LVDP from 8.5 minutes of reperfusion throughout the duration of the protocol ($P<$
At the end of the 30 minute protocol, left ventricular developed pressure averaged 10 ± 4 mmHg in the control group, compared with 24 ± 2 mmHg in the 4-ClDzp group (Figure 6B). Recovery was biphasic, consisting of a transient increase and decrease in contractility immediately upon reperfusion, followed by a slow sustained recovery that was substantially greater in the 4-ClDzp treatment groups. The negative inotropic effect of 4-ClDzp (demonstrated above) abolished the initial transient increase in LVDP, however the steady state recoveries were similar between the 4-ClDzp treatment groups (LVDP was 26 ± 4 mmHg after 30 minutes of reperfusion in the bolus dose group; Figure 6B).

Hearts that received 4-ClDzp as a bolus at the onset of reperfusion also showed significant protection against reperfusion electrophysiological dysfunction. Arrhythmia scores were similar to those of the continuous 4-ClDzp treatment group (Figure 5). Again, while the protection against ischemic contracture observed by continuous 4-ClDzp administration could, in theory, contribute to the post ischemic protective effect against electrical and contractile dysfunction, the equivalent protection afforded by the compound given at reperfusion provides evidence that 4-ClDzp is acting primarily to prevent injury during the crucial minutes when flow is restored.
Discussion

In this study, we sought to characterize the cellular effects of 4’-chlorodiazepam (4-ClDzp), a ligand to the mitochondrial benzodiazepine receptor (mBzr), in order to understand the mechanism(s) behind its cardioprotective effect. Previous studies have shown that ligands to the mBzr can protect the heart against infarction \(^4\) and arrhythmias \(^2\), \(^16\) following ischemia. In the present study, we show that although 4-ClDzp has a partial inhibitory effect on excitation-contraction coupling, it not only reduces the incidence of reperfusion arrhythmias in the rabbit heart, but also markedly improves post-ischemic contractile performance when administered either prior to ischemia or as a bolus at reperfusion.

Cardioprotection provided by 4-ClDzp

Previous work by our group has shown that heart cells exposed to oxidative stress undergo periodic oscillations in action potential duration \(^17\), which were later found to be caused by oscillations in mitochondrial membrane potential (\(\Delta\Psi_m\)) and the consequent activation of ATP-sensitive K\(^+\) channels in the sarcolemma \(^3\). The lability in action potential profile elicited by oscillations in \(\Delta\Psi_m\) served as a substrate for arrhythmia in the intact heart \(^2\). A ligand to the mBzr, 4-ClDzp, stopped the oscillations in \(\Delta\Psi_m\) \(^3\) and was found to stabilize the action potential and significantly decrease the incidence of arrhythmias at the organ level \(^2\). In earlier studies, both the stability of \(\Delta\Psi_m\) and the protection from arrhythmias evoked by 4-ClDzp were not mimicked by cyclosporin-A, the classical blocker of the permeability transition pore \(^2\), \(^3\), supporting the view that mitochondrial ion channels open in a hierarchical fashion in response to oxidative stress \(^18\). This study corroborates our earlier work that 4-ClDzp is cardioprotective and re-emphasizes the importance of the mBzr complex as a therapeutic target for preventing arrhythmias that involve a mitochondrial ion channel target ‘upstream’ from the classical permeability transition pore. Previous patch-clamp studies of isolated mitoplasts support the
idea that two main mitochondrial ion channel types are inhibited by 4-CIDzp (Ro5-4864), a CsA-sensitive large conductance channel and a CsA-insensitive 100pS inner membrane anion channel (IMAC or Mcts) \(^6,7\). Our current working hypothesis is that IMAC is the principal target of 4-CIDzp and underlies the phenomenon of mitochondrial ROS-induced ROS release \(^18\).

In addition to the 4-CIDzp-mediated protection against arrhythmia, we also provide novel evidence that 4-CIDzp can protect against mechanical dysfunction. Administration of 4-CIDzp delayed the onset of ischemic contracture and also led to significantly improved recovery of LVDP at the onset of reperfusion. Importantly, application of a bolus of 4-CIDzp at the onset of reperfusion, a strategy with pertinent clinical relevance, also led to significantly greater contractile function. It is noteworthy that despite the negative inotropic effect of the drug, and that the extent of ischemic contracture was identical to the control group (Figure 6), exposure to the drug at reperfusion only still led to functional recovery during reperfusion.

It is plausible to suggest that as a partial inhibitor of \(I_{\text{Ca}}\) (discussed below), 4-CIDzp was able to delay the onset of ischemic contracture by reducing the calcium influx during the 30 minute ischemia (as hearts received the drug prior to ischemia). However, since this compound preserves electrical excitability and the action potential plateau for much longer into the ischemic period \(^2\), one would actually expect greater total \(\text{Ca}^{2+}\) influx during ischemia. This suggests that the main effect on ischemic contracture was mediated by more than just inhibition of \(I_{\text{Ca}}\). It is likely that the stabilizing effect of 4-CIDzp on mitochondrial function helps to maintain the energy supplies required for \(\text{Ca}^{2+}\) homeostasis both during and after ischemia. Moreover, the improved recovery upon reperfusion with a bolus dose of 4-CIDzp argues that delaying the ischemic contracture is not a major factor determining the outcome after ischemia (see Figure 6). At the onset of reperfusion, 4-CIDzp leads to improved developed pressure by a combination of at least two factors. Most importantly, the anti-arrhythmic properties that decreased the incidence of ventricular fibrillation and tachycardia allow the heart to successfully fulfill its role as a pump. Second, the compound may also help to reduce calcium influx (through
both direct Ca\textsuperscript{2+} channel inhibition and preservation of mitochondrial function) in early reperfusion, which could improve diastolic function and allow for better LVDP.

*Effects of 4-ClDzp on cellular electrophysiology*

While the protective properties of mBzr ligands are clear, very little is known about the effects of these drugs on cellular action potentials or contractility. In order to gain insight into the protective properties of mBzr ligands, we sought to characterize the cellular effects of 4-chlorodiazepam in isolated rabbit cardiomyocytes.

In this study, 4-ClDzp had significant effects on the duration of the cellular action potential, the peak calcium current, and the intracellular calcium transient. The shortening of the action potential, notably in the plateau phase, which we observed in isolated rabbit myocytes, is consistent with earlier experiments using guinea pig myocytes\textsuperscript{19-21}. Consistent with Phase 2 shortening of the action potential, cells that were exposed to 4-ClDzp displayed diminished calcium currents and transients. Notably, previous studies reported that some mBzr ligands decrease the inward calcium current (\(I_{Ca}\)) in cardiomyocytes\textsuperscript{20,22,23}, neuroblastoma cells\textsuperscript{24}, and adrenal glomerulosa cells\textsuperscript{25}. Our findings demonstrating reduced \(I_{Ca}\), shortening of the action potential (beginning in the plateau phase) and a reduction in the amplitude of intracellular calcium transients are consistent with these previous studies that showed a partial inhibition of \(I_{Ca}\) by 4-ClDzp.

At the level of the whole organ, we found a dose-dependent negative inotropic effect, which also supports the notion that 4-ClDzp may be decreasing the \(I_{Ca}\). This observation is in agreement with a previous study in isolated rat hearts\textsuperscript{13}, as well as in guinea pig papillary muscle preparations, where the 4-ClDzp-mediated decrease in tension was reversed by increasing the extracellular calcium concentration\textsuperscript{26}.
The protection from 4-ClDzp cannot be accounted for purely by reducing $I_{Ca}$.

Our results indicate that 4-ClDzp, at a concentration that provides protection against arrhythmias, is also reducing $I_{Ca}$. While a very large number of anti-arrhythmic agents act by reducing calcium influx (see 27, 28 for review), the cardioprotective effects of 4-ClDzp are distinct from traditional Class IV anti-arrhythmic agents for several reasons.

First, our previous work indicated that cardiomyocytes exposed to oxidative stress exhibit oscillations in membrane currents that occur independently from intracellular calcium concentrations 17. In subsequent work using quiescent cardiomyocytes, where L-type calcium channels are not active, oscillations in $\Delta \Psi_m$ evoked by oxidative stress were stabilized by the addition of 4-ClDzp 3.

Second, Akar et al. 2 showed that global ischemia induces gradual shortening of the cellular action potential (by activating $I_{KATP}$) that was delayed in hearts that received 4-ClDzp. Given that 4-ClDzp shortened the action potential in isolated myocytes in our study, but that 4-ClDzp prevented the shortening of the cellular action potential in the intact organ during ischemia 2, the properties of the compound that act to maintain $\Delta \Psi_m$ and prevent ATP depletion more likely underlie the effects of the compound during ischemia.

Third, administration of 4-ClDzp as a bolus at the onset of reperfusion successfully reduced the incidences of both arrhythmia and mechanical dysfunction to the same extent as hearts that were pre-treated with the drug. To the contrary, while a number of studies have noted cardioprotection elicited by pre-ischemic treatment of calcium channel blockers 29-32, the cardioprotective properties of calcium channel blockers are not evident when administered at the onset of reperfusion. For example, post-ischemic treatment with the $I_{Ca}$ blocker verapamil was ineffective at attenuating myocardial infarction 31 or stunning 30. Given the important clinical implications of drugs that are effective when administered at reperfusion, agents (such as 4-
CIDzp) that act by stabilizing $\Delta \Psi_m$ at reperfusion represent clinically relevant strategies for preventing electrical and mechanical dysfunction.

Finally, it should be noted that pleiotropic effects of 4-CIDzp we describe may actually strengthen the case for its clinical use. Given the propensity for the single compound to have desirable properties of reducing calcium overload and also stabilizing $\Delta \Psi_m$ in the face of metabolic stress, its use in mitigating reperfusion injury may be essentially ‘killing two birds with one stone’.

In conclusion, this study provides novel evidence, using a large animal model, that ligands to the mitochondrial benzodiazepine receptor can significantly reduce the incidence of arrhythmia and LV dysfunction, and that the drug may also have beneficial effects in reducing calcium overload in the face of ischemia-reperfusion challenge.

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**Acknowledgements**

None.

**Conflicts of Interest**

None declared
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Figure Legends

Figure 1: Effects of 4-ClDzp on rabbit ventricular action potential duration. (A) Representative action potential tracings approximating the mean action potential morphology in the study. (B) Mean time to 50 and 90% repolarization in myocytes used in the study (n=16 cells for each group); *, P < 0.05 versus control (paired t-test).

Figure 2: Transmembrane currents and calcium transients recorded from rabbit cardiomyocytes before and after the addition of 24μM 4-ClDzp. (A) Representative current tracing from the same myocytes before (black line) and after (blue line) exposure to 4-ClDzp. (B) Current density for rabbit myocytes (n=12) at 50 and 150 msec after a test pulse to +10 mV without (black bars) and with (blue bars) 4-ClDzp. (C) Representative calcium transients from the same cell in the absence (black line) and presence (blue line) of 4-ClDzp. (D) Current-voltage plot for calcium currents in the study. (E) Indo-1 mediated intracellular calcium transient recordings from the same cell under control (black line) and 4-ClDzp (blue line) conditions. (F) Peak calcium concentrations in rabbit cardiomyocytes with and without the addition of 4-ClDzp. *, P < 0.05 versus myocytes under control conditions.

Figure 3: Dose-response curve describing the negative inotropic effect of various concentrations of 4-ClDzp on left ventricular developed pressure (LVDP) in intact rabbit hearts. Animal numbers were n=7, 4, 6, and 1 for 0, 12, 24, and 64μM 4-ClDzp.
Figure 4: Representative left ventricular pressure tracings and ECG waveforms for the last 5 seconds of reperfusion in: (A) control hearts, (B) hearts receiving 24μM 4-ClDzp beginning 10 min prior to ischemia thru the duration of reperfusion, (C) hearts receiving a bolus of 4-ClDzp at the onset of reperfusion, and (D) hearts receiving 0.2μM cyclosporin-A 10 minutes prior to ischemia through the duration of reperfusion.

Figure 5: Arrhythmia scores tabulated for the entire 30 minute reperfusion period for hearts in the study. *, P < 0.05 versus control hearts (ANOVA with Dunnett’s t-test).

Figure 6: (A) Left ventricular end diastolic pressure for rabbit hearts during the 30 minute ischemia period. +, P < 0.05 for 24μM 4-ClDzp when compared to control (Repeated measures ANOVA). (B) Left ventricular developed pressure (LVDP) for the 30 minute reperfusion period for hearts receiving 4-ClDzp either prior to ischemia (24μM 4-ClDzp) or as a bolus at the onset of reperfusion. *, P < 0.05 for both 4-ClDzp groups when compared to control (Repeated measures ANOVA).
Table 1: Effects of 24μM 4-ClDzp on baseline myocardial hemodynamics

<table>
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<th>Baseline (n=7)</th>
<th>24μM 4-ClDzp (n=6)</th>
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<td>1446 ± 127</td>
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<td>179 ± 6</td>
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Figure 1

A.

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Figure 2

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Figure 4

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Figure 5:
Figure 6

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