Original Paper

The Blockade of Transmembrane Cl⁻ Flux Mitigates I/R-Induced Heart Injury via the Inhibition of Calpain Activity

Jian-Ying Zhang^a Feng Wu^b Xiao-Ming Gu^a Zhen-Xiao Jin^c Ling-Heng Kong^d
Yuan Zhang^a Jing-Jun Zhou^a Feng Gao^a

^aDepartment of Physiology, The Fourth Military Medical University, ^bDepartment of Cardiology, ^cDepartment of Cardiovascular Surgery, Xijing Hospital, Xi'an, ^dInstitute of Basic Medical Science, Xi'an Medical College, Xi'an, China

Key Words

Chloride • Calpain • Heart • Calcium • Ischemia/Reperfusion

Abstract

Aims: The aim of this study was to determine whether calpain is involved in Cl⁻-induced myocardial ischemia/reperfusion (I/R) injury. **Methods:** Isolated rat hearts were subjected to either 45 min of global no-flow ischemia followed by reperfusion or successive perfusion with Ca²⁺-free KH solution for 3 min and normal KH solution for 30 min, also known as Ca²⁺ paradox. **Results:** The hearts in the I/R group exhibited increases in myocardial injury area, LDH release, caspase 3 activity and apoptotic indices and a marked decline in cardiac performance. As was the case regarding the effects of MDL 28170, an inhibitor of calpain, treatment with 5 μM NPPB, 5 μM DIDS and low Cl⁻ significantly attenuated cardiac injury. Moreover, each of the treatments significantly protected against Ca²⁺ overload-induced injury in the setting of Ca²⁺ paradox. The Western blot and immunofluorescence data revealed that there was an increase in the percentages of calpain membrane-positive cells and the numbers of fragments resulting from the calpain-mediated proteolysis of α-fodrin in both the I/R and the Ca²⁺ paradox, indicating that the activation of calpain occurred. More importantly, these effects were mitigated by the blockade of transmembrane Cl⁻ flux, as was accomplished via MDL 28170. **Conclusion:** Our results provide evidence that the blockade of transmembrane Cl⁻ flux mitigates I/R-induced cardiac injury via the inhibition of calpain activity. They also indicate that intracellular Ca²⁺ overload regulates calpain activation in the setting of Cl⁻-induced injury.

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J.-Y. Zhang and F. Wu contributed equally to this project.

Dr. Jing-Jun Zhou,
or Dr. Feng Wu,

Dept. of Physiology, and Dept. of Cardiology, Xijing Hospital, The Fourth Military Medical University, No. 169, Changle West Road, Xi'an 710032 (P. R. China)
Tel. +86-29-84774521, E-Mail jizhou@fmmu.edu.cn, E-Mail wufeng@fmmu.edu.cn

Introduction

Since the discovery of Cl⁻ conductance in the Purkinje fibers by Hutter et al. [1], researchers have been exploring the role of Cl⁻ in the heart. Patch clamp studies have revealed that at least eight types of Cl⁻ channel currents have been recorded in cardiac cells, currents regulated by protein kinase A and protein kinase C, as well as other enzymes [2]. On the other hand, six Cl⁻ channel gene families, including the cystic fibrosis transmembrane conductance (CFTR) channels and the voltage-gated Cl⁻ channels, have been identified via molecular biology techniques [3]. Additionally, the Cl⁻/HCO₃⁻ exchangers, another family of sarcolemmal Cl⁻ transporters, are also found in the heart [4, 5]. Until now, functional studies in this field have demonstrated that transmembrane Cl⁻ flux is involved in the regulation of action potentials [6], Ca²⁺ transient [7], pH [5], and cell volume [8] and that abnormalities in this process are associated with the genesis of cardiac arrhythmias and cell death [6, 9].

Ischemia/reperfusion (I/R) injury often occurs during treatment for acute myocardial infarction and heart surgery, and is a focal point of research in the field of cardiology. Clinical studies have revealed that patients with postoperative atrial fibrillation exhibit high serum chloride concentrations following coronary artery bypass grafting surgery [10]; laboratory studies have demonstrated that interfering with transmembrane Cl⁻ flux with a Cl⁻ channel blocker such as SITS or NPPB protects cardiac cells against both I/R injury and anoxia/reoxygenation injury [11-13]. Therefore, it is worthwhile to investigate the mechanism underlying this phenomenon. A previous study revealed that Cl⁻ is involved in regulating intracellular Ca²⁺ levels in hypoxic/reoxygenated cardiac cells [5]. As intracellular Ca²⁺ overload is an important pathogenetic culprit of myocardial I/R injury, it is necessary to determine which Ca²⁺ binding protein mediates the cellular injury induced by Cl⁻.

Calpain belongs to a family of Ca²⁺-activated neutral proteases [14, 15]. In the setting of I/R, calpain is activated due to intracellular Ca²⁺ overload. Activated calpain cleaves many scaffold or functional proteins, such as fodrin [16], Na⁺/K⁺ ATPase [17], sarco- (endo)plasmic reticulum Ca²⁺-ATPase [18], and bid [19], causing membrane rupture and mitochondrial dysfunction, which leads to cell death. The blockade of calpain translocation and activation is an effective strategy for limiting I/R-induced heart injury [20]. Therefore, we hypothesized that calpain mediates Cl⁻-induced myocardial injury in the setting of I/R.

In this study, we firstly studied the role of Cl⁻ in myocardial I/R by studying the inhibition of Cl⁻ transport with non-selective blockers such as NPPB and DIDS, and the replacement of Cl⁻ with gluconate. Then we prepared Ca²⁺ paradox in hearts to induce intracellular Ca²⁺ overload [21, 22] and subsequently determined whether Cl⁻ mitigated Ca²⁺ overload-induced heart injury. We also evaluated calpain inhibitor MDL 28170, in both the hearts subjected to ischemia and reperfusion and the hearts inflicted by Ca²⁺ paradox. Finally, we evaluated the effects of these drugs on calpain activity. Our results demonstrated that the blockade of Cl⁻ transmembrane flux reduced calpain activity and protected against myocardial I/R injury.

Materials and Methods

Ethical information

The experimental procedures were approved by the Research Commission on Ethics of The Fourth Military Medical University and conformed to the Guidelines for the Care and Use of Laboratory Animals (NIH publication no. 80-23, revised in 1996).

The Langendorff perfusion and functional study

As described previously [22], adult male Sprague-Dawley rats weighing between 250 and 300 g were decapitated; their hearts were quickly removed, and the aortas were cannulated to a Langendorff apparatus using a three-way stopcock (Radnoti Glass Technology Inc., Monrovia, CA, USA). Retrograde perfusion in a nonrecirculating mode was established at a constant perfusion pressure of 80 mm Hg and 37°C. The Krebs-

Henseleit (KH) solution equilibrated with 95% O₂/5% CO₂ (pH 7.4) containing (in mM) 118 NaCl, 4.7 KCl, 1.2 MgSO₄, 1.2 KH₂PO₄, 1.25 CaCl₂, 25 NaHCO₃, and 11 glucose was used for perfusion. To measure left ventricular pressure, a latex balloon connected to a pressure transducer was inserted into left ventricle via the atria, and the volume in the balloon was adjusted to obtain a left ventricular end-diastolic pressure (LVEDP) of 5–10 mm Hg. The analog signal was acquired using Labchart 7 software (ADInstruments) and stored on a computer. The basal values of the functional parameters were recorded following a 15-min equilibration period and recording of the data was performed until the end of an experiment. Left ventricular-developed pressure (LVDP) was calculated as the difference between the systolic and the diastolic pressures. As the basal values of LVDP were slightly different from each other, LVDP recovery, which was expressed as a percentage of the basal value, was used to reflect cardiac function.

The study groups and the experimental protocol

As described in Fig. 1, the first series of experiments (A) were undertaken to determine the effects of several drugs on the hearts subjected to ischemia and reperfusion. For the control group, the hearts were perfused with a normal KH solution. In the drug control group, the drugs were given for 8 min, followed by a washout. Global no-flow ischemia was produced via the interruption of the three-way stopcock for a period of 45 min, during which coronary flow was reduced to zero. Coronary perfusion was then restored for 30 or 120 min. All drugs were administered during the 3-min perfusion period prior to the induction of ischemia and were administered again for the first 5 minutes of reperfusion. In this series of experiments, 30 min of reperfusion was allowed to evaluate cardiac function, whereas 120 min of reperfusion was performed to measure the size of the myocardial injury area.

The second series of experiments (B) were undertaken to evaluate the drugs' effects in Ca²⁺ paradox. The hearts was subjected to successive perfusion with Ca²⁺-free KH solution for 3 min and normal KH solution for 30 min, either with or without the drugs. The drugs were administered during a 1-min perfusion period prior to treatment with the Ca²⁺-free KH solution, during the entire period of treatment with the Ca²⁺-free KH solution, and for the first 2 minutes of treatment with the normal, Ca²⁺ containing KH solution. For the Ca²⁺-free KH solution, Ca²⁺ was omitted, and 0.1 mM EGTA was added to ensure the removal of any contaminating Ca²⁺.

For the low-Cl⁻ treatment, we chose gluconate as a substitute for Cl⁻ as reported previously [23], and the concentration of Cl⁻ in the modified KH solution was kept at 90 mM. To administer NPPB, DIDS, and MDL 28170, a stock solution of 0.1 M NPPB and MDL 28170 was prepared in DMSO, and another stock solution of 0.1 M DIDS was prepared with 0.1 M KHCO₃. The final concentration of DMSO was ≤ 0.01%, and the contents of K⁺ and HCO₃⁻ in KH solution were almost unchanged. Additionally, 0.01% DMSO was also included as a vehicle control (Fig. 1).

Myocardial injury area determination

The hearts were frozen, cut into slices, and incubated in 1.0% TTC for 20 min at 37 °C. The slices were subsequently placed in 10% formalin overnight. Viable myocardium was stained brick red, and the absence of TTC staining represented the areas of irreversibly injured myocardium. Myocardial injury area was determined using a computerized planimetry technique (OPTIMAS v. 5.2, BioScan Inc., Edmonds, WA, USA) and expressed as a percentage of the total area as described previously [22].

The assessment of lactate dehydrogenase (LDH) release and caspase 3 activity

Coronary effluent was collected during either the first 10 minutes of reperfusion or during the 2 minutes of returning the samples to Ca²⁺ containing perfusates (Fig. 1), and LDH, an indicator of cell death, was measured via a colorimetric LDH quantification assay kit. Specific activity was expressed in mU per ml of coronary effluent [22].

Caspase 3 activity was measured following the colorimetric assay kit protocols provided by Merck Millipore [24]. Briefly, after the left ventricular tissue was homogenized using a Bio-Gen PRO200 homogenizer, the cytosolic extract was collected via 10,000×g centrifugation and aliquoted at -70 °C. Approximately 60 µg proteins were incubated with the labeled substrate, DEVD-pNA, at 37 °C. The activity of caspase 3 was quantified using a spectrophotometer at 405 nm, and the data were normalized to the control group, which was given a value of 1.

TdT-mediated dUTP nick end labeling (TUNEL)

At the end of experiment (Fig. 1), 3- μ m-thick left ventricular sections were prepared and incubated with a permeabilization solution containing 20 μ g/ml proteinase K for 30 min at 37 °C. The sections were subsequently incubated with TUNEL reaction mixture at 37 °C for 1 hour. The nuclei were labeled with DAPI. Finally, the sections were analyzed via a laser-scanning confocal microscope equipped with a FV10-ASW system (Olympus FV1000, Japan). The percentage of the apoptotic index was calculated as the ratio between the TUNEL positive cell nuclei (green fluorescence) and the total number of nuclei (blue fluorescence), multiplied by one hundred as described previously [25].

The determination of calpain translocation levels

Ten minutes following reperfusion, or one minute after the return to a Ca²⁺ containing perfusate (Fig. 1), the left ventricular tissue was excised, and paraffin-embedded tissue sections were subsequently prepared. Following dewaxing and rehydration, the sections were stained with anti- μ -calpain at a dilution of 1:200 and incubated with a TRITC-labeled goat anti-rabbit IgG secondary antibody. DAPI was used to label the nuclei. Finally, the sections were scrutinized using a laser-scanning confocal microscope, and the images were analyzed using Image-Pro Plus 5.0 software (Media Cybernetics Inc, Rockville, MD, USA). The percentage of calpain membrane-positive cells was calculated as described previously [22].

Western blot

The left ventricular tissue was homogenized in lysis buffer containing 50 mM Tris-HCl (pH 7.3), 150 mM NaCl, 5 mM EDTA, and 1 mM dithiothreitol, with 1% Triton X-100 and a 1% protease inhibitor cocktail; the lysates were centrifuged for 15 min at 12,000 $\times$ g, and protein quantification was completed using a BCA protein assay kit to ensure equal loading amounts in each lane, or 40 μ g of protein. GAPDH was chosen as a loading control to ensure that the same amount was used for all samples. The proteins were resolved using an 8% SDS gel and transferred onto nitrocellulose membranes. The membranes were immunoblotted with a primary antibody against α -fodrin at a dilution of 1:1000 and subsequently incubated with a secondary antibody conjugated with peroxidase. Finally, the protein bands were probed via chemiluminescence, and calpain activity, which was quantified using the densitometric ratio between the calpain-cleaved fragment (approximately 150 kDa) and the full length of the protein (240 kDa), was analyzed using the Image Lab software package (Bio-Rad Laboratories, Herts, UK) as described previously [17, 22].

Fig. 1. Experimental protocols. During the first series of experiments (A), the hearts were exposed to 45 min of global no-flow ischemia followed by reperfusion, either with or without the drugs; a control group, a drug control group and a vehicle control group (Vehicle) were also included in the study. During the second series of experiments (B), the hearts were subjected to successive perfusion with Ca²⁺-free KH solution for 3 min and normal KH solution for 30 min, either with or without the drugs. The time at reperfusion with normal KH solution was set to 0. The arrows are the time points for the data measurements. MIA, myocardial injury area; 90 Cl, Cl⁻ in the modified KH solution was kept at 90 mM; MDL, MDL 28170; n, the number of hearts.

Drugs and chemicals

5-Nitro-2-(3-phenylpropylamino) benzoic acid (NPPB) and MDL 28170 were each purchased from Tocris Bioscience (Bristol, UK). 4,4'-Diisothiocyanatostilbene-2,2'-disulfonic acid disodium (DIDS) and gluconate were each purchased from Sigma (Shanghai, China). The rabbit anti-calpain and mouse anti-GAPDH antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). The mouse anti- α -fodrin antibodies were purchased from Enzo Life Sciences (Plymouth Meeting, PA, USA). The tetramethylrhodamine goat anti-rabbit IgG was purchased from Molecular Probes (Eugene, OR, USA). The colorimetric LDH quantification assay kit was purchased from Abnova (Walnut, CA, USA). The TUNEL kit was purchased from Roche (Mannheim, Germany). The caspase 3 colorimetric activity assay kit was obtained from Merck Millipore (Billerica, MA, USA). Triphenyltetrazolium chloride (TTC), 4',6-Diamidino-2-phenylindole dihydrochloride (DAPI), and additional chemicals were obtained from Sigma (Shanghai, China).

Statistical analysis

The data are expressed as the means $\pm$ SEMs. To compare the effects of the drugs on cardiac performance in the control hearts, a paired t-test was used. For the remaining data, the differences among the groups were assessed using ANOVA, followed by Tukey's test. Statistical significance was set at a probability value of 0.05.

Results

The effects of NPPB, DIDS and 90 mM Cl⁻ on myocardial injury area and cardiac performance in the control hearts

Compared with the control group, 10 μ M NPPB slightly but significantly increased the size of the myocardial injury area, whereas 5 μ M NPPB, 5 or 10 μ M DIDS, and 90 mM Cl⁻ did not have an effect on the size of the area (Table 1). Both 5 and 10 μ M NPPB transiently increased the LVDP and decreased the heart rate; however, these two parameters returned to baseline following the washout of the drug (Table 2). By contrast, DIDS also increased LVEDP; more importantly, this effect was reversible only when 5 μ M DIDS was used (Table 2). Ninety millimolar Cl⁻ transiently increased both the LVDP and the LVEDP, increases that did not reach 5% significance; both the LVDP and the LVEDP returned to normal following perfusion with normal KH solution (Table 2). Therefore, we chose 5 μ M NPPB, 5 μ M DIDS, and 90 mM Cl⁻ to block transmembrane Cl⁻ flux. Additionally, DMSO at 0.01% did not exert any effects on either myocardial injury area or cardiac function (Tables 1 & 2).

The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 attenuated I/R-induced cell death and improved cardiac functional recovery

Compared with the control subjects, the hearts in the I/R group had enlarged myocardial injury areas and exhibited increased LDH release, an indication of cell necrosis (Fig. 2). By contrast, NPPB, DIDS, 90 mM Cl⁻, and MDL 28170, an inhibitor of calpain, significantly reduced both the size of myocardial injury area and the amount of LDH release secondary

Table 1. The effects of the blockade of transmembrane Cl⁻ flux on myocardial injury area in the control hearts. The data are expressed as the means $\pm$ SEMs, n=6. *P<0.05 vs. Control. MIA, myocardial injury area, which was expressed as a percentage of the total area; Vehicle, 0.01% DMSO; NPPB, 5-Nitro-2-(3-phenylpropylamino) benzoic acid; DIDS, 4,4'-Diisothiocyanatostilbene -2,2'-disulfonic acid disodium; 10 and 5 represent the concentrations of NPPB and DIDS at 10 or 5 μ M; 90 Cl, Cl⁻ in the KH solution was partially substituted with gluconate, and its final concentration was kept at 90 mM

	Control	Vehicle	NPPB (10)	NPPB (5)	DIDS (10)	DIDS (5)	90 Cl
MIA (%)	1.2 $\pm$ 0.1	1.1 $\pm$ 0.2	7.8 $\pm$ 1.2*	1.3 $\pm$ 0.2	1.6 $\pm$ 0.3	1.1 $\pm$ 0.2	1.5 $\pm$ 0.3

Table 2. The effects of the blockade of transmembrane Cl⁻ flux on heart rate (HR), LVEDP, and LVDP in the control hearts. The data are expressed as the means $\pm$ SEMs, n=6. **P<0.01 vs. B. B, before; D_{max} represents the maximal value of LVDP/LVEDP during drug treatment at the corresponding heart rate; W, washout. Please refer to Table 1 for additional abbreviations

	time	HR (bpm)	LVEDP (mm Hg)	LVDP (mm Hg)
Vehicle	B	268 $\pm$ 13	7.6 $\pm$ 0.9	89 $\pm$ 3.7
	D _{max}	268 $\pm$ 11	7.5 $\pm$ 1.0	88 $\pm$ 3.9
	W	269 $\pm$ 12	7.7 $\pm$ 1.1	89 $\pm$ 3.5
NPPB (10 μ M)	B	265 $\pm$ 11	7.2 $\pm$ 0.9	91 $\pm$ 3.5
	D _{max}	221 $\pm$ 14**	7.5 $\pm$ 1.1	108 $\pm$ 5.5**
	W	261 $\pm$ 12	7.3 $\pm$ 0.8	93 $\pm$ 3.7
NPPB (5 μ M)	B	263 $\pm$ 13	8.1 $\pm$ 1.2	86 $\pm$ 4.2
	D _{max}	231 $\pm$ 15**	8.3 $\pm$ 1.1	101 $\pm$ 5.1**
	W	258 $\pm$ 16	8.2 $\pm$ 1.0	89 $\pm$ 3.8
DIDS (10 μ M)	B	270 $\pm$ 12	7.8 $\pm$ 1.1	87 $\pm$ 4.5
	D _{max}	246 $\pm$ 12**	17 $\pm$ 1.6**	100 $\pm$ 4.7**
	W	271 $\pm$ 16	15 $\pm$ 1.4**	72 $\pm$ 4.7**
DIDS (5 μ M)	B	266 $\pm$ 13	7.3 $\pm$ 1.1	90 $\pm$ 4.3
	D _{max}	208 $\pm$ 15**	11 $\pm$ 1.2**	102 $\pm$ 3.8**
	W	257 $\pm$ 15	7.5 $\pm$ 0.9	98 $\pm$ 4.5
90 Cl	B	271 $\pm$ 11	7.5 $\pm$ 0.8	87 $\pm$ 3.9
	D _{max}	256 $\pm$ 18	8.3 $\pm$ 1.3	93 $\pm$ 3.2
	W	268 $\pm$ 17	7.8 $\pm$ 1.1	85 $\pm$ 4.1

Fig. 2. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced necrosis in the hearts subjected to I/R. A. Representative TTC staining of a heart slice. (a) Control, (b) I/R, (c) NPPB+I/R, (d) DIDS+I/R, (e) 90 Cl+I/R. B. The group results regarding myocardial injury area. C. The group results regarding LDH release. Each bar represents the mean $\pm$ SEM, n=6. **P<0.01 vs. Control, ##P<0.01 vs. I/R. 90 Cl+I/R, ischemia/reperfusion with 90 mM Cl⁻. For a more detailed version of the protocol, please refer to the Methods section.

to I/R injury (Fig. 2). Furthermore, the hearts in the I/R group exhibited higher caspase 3 activity and apoptotic indices than those in the control group, and these effects were also attenuated by NPPB, DIDS, 90 mM Cl⁻, and MDL 28170 (Fig. 3).

Reperfusion following ischemia resulted in a marked decline in cardiac performance, as determined based on both the significant decrease in LVDP and the increase in LVEDP

Fig. 3. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced apoptosis in the hearts subjected to I/R. A. Representative TUNEL staining. The nuclei were counter-stained with DAPI (blue). (a) Control, (b) I/R, (c) NPPB+I/R, (d) DIDS+I/R, (e) 90 Cl+I/R. Scale bar, 20 μ m. B. The group results regarding the apoptotic indices. C. The group results regarding caspase 3 activity. The values were normalized to the control group, which was given a value of 1. Each bar represents the mean $\pm$ SEM, n=6. ** P <0.01 vs. Control, # P <0.05, ## P <0.01 vs. I/R. Please refer to Figure 2 for the abbreviations.

Fig. 4. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) promoted cardiac functional recovery following I/R. A. Representative left ventricular pressure (LVP) recordings. B. The group results regarding LVDP recovery and LVEDP at the end of the experiments. Each bar represents the mean $\pm$ SEM, n=10. ** P <0.01 vs. Control, # P <0.05, ## P <0.01 vs. I/R. Please refer to Figure 2 for the abbreviations.

(Fig. 4). Treatment with NPPB, DIDS, 90 mM Cl⁻, and MDL 28170 significantly improved the recovery of LVDP (Fig. 4). Furthermore, these agents also decreased the level of LVEDP,

Fig. 5. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced the myocardial necrosis induced by Ca²⁺ paradox (CaP). A. Representative TTC staining. (a) CaP, (b) NPPB+CaP, (c) DIDS+CaP, (d) 90 Cl+CaP. B. The group results regarding myocardial injury area. C. The group results regarding LDH release. Each bar represents the mean $\pm$ SEM, n=6. $^{##}P<0.01$ vs. CaP. 90 Cl+CaP, Ca²⁺ paradox with 90 mM Cl⁻. For a more detailed version of the protocol, please refer to Fig. 1.

Fig. 6. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced the amount of myocardial apoptosis induced by Ca²⁺ paradox (CaP). A. Representative TUNEL staining. The nuclei were counterstained with DAPI (blue). (a) CaP, (b) NPPB+CaP, (c) DIDS+CaP, (d) 90 Cl+CaP. Scale bar, 20 μ m. B. The group results regarding the apoptotic index. C. The group results regarding caspase 3 activity. The values were normalized to the control group, which was given a value of 1. Each bar represents the mean $\pm$ SEM, n=6. $^{#}P<0.05$, $^{##}P<0.01$ vs. CaP. 90 Cl+CaP, Ca²⁺ paradox with 90 mM Cl⁻.

although the difference between the NPPB group and the I/R group did not reach statistical significance (Fig. 4).

The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 attenuated Ca²⁺ paradox-induced cell death

As previously demonstrated, MDL 28170, an inhibitor of calpain, significantly rescued the hearts inflicted by Ca²⁺ paradox [25], an occurrence characterized by decreases in myocardial injury area, LDH release, apoptotic indices and caspase 3 activity (Fig. 5 & 6). More importantly, treatment with NPPB or DIDS or 90 mM Cl⁻ also significantly decreased myocardial injury area, LDH release and cellular apoptosis (Fig. 5 & 6).

Fig. 7. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) promoted cardiac functional recovery following Ca²⁺ paradox (CaP). A. Representative left ventricular pressure (LVP) recordings. B. The group results regarding LVDP recovery and LVEDP at the end of the experiments. Each bar represents the mean $\pm$ SEM, n=10. ^{##}P < 0.01 vs. CaP. 90 Cl+CaP, Ca²⁺ paradox with 90 mM Cl⁻.

The Ca²⁺ paradox induced a decrease in LVDP and an increase in LVEDP (Fig. 7). Treatment with NPPB, DIDS, 90 mM Cl⁻, and MDL 28170 significantly improved LVDP recovery and decreased the level of LVEDP following Ca²⁺ paradox (Fig. 7).

The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 attenuated calpain activation in the hearts subjected to either I/R or Ca²⁺ paradox

The immunofluorescence data revealed that compared with the control group, the hearts subjected to ischemia and reperfusion exhibited higher percentages of μ -calpain (Fig. 8) and larger amounts of cleaved 150-kDa fragments resulting from the specific calpain-mediated proteolysis of α -fodrin (Fig. 9). These effects were blunted by MDL 28170 (Fig. 8 & 9). Our data were consistent with those of previous studies [17, 20], suggesting that the activity of calpain was increased. Similar to the findings observed following MDL 28170 treatment, treatment with NPPB, DIDS and 90 mM Cl⁻ significantly reduced the percentage of μ -calpain that translocated to the sarcolemma and the extent of the proteolysis of fodrin, findings indicative of the inhibition of calpain (Fig. 8 & 9).

To determine whether the blockade of transmembrane Cl⁻ flux inhibited calpain by decreasing Ca²⁺ overload, we measured calpain activity in the setting of Ca²⁺ paradox, a well-known experimental model for studying Ca²⁺ overload. As was the case following MDL 28170 treatment, the blockade of transmembrane Cl⁻ flux with NPPB, DIDS, and 90 mM Cl⁻ not only rescued the damaged hearts but also decreased both the percentages of membrane-translocated calpain and the numbers of cleaved 150-kDa fragments as a result of the Ca²⁺ paradox (Fig. 8 & 9). These results suggest that intracellular Ca²⁺ overload regulates calpain activation in the setting of Cl⁻-induced heart injury.

Fig. 8. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced the sarcolemmal translocation of μ -calpain in both the I/R hearts and the hearts inflicted by Ca²⁺ paradox (CaP). A. representative confocal images. The nuclei were counterstained with DAPI (blue). (a) Control, (b) I/R, (c) NPPB+I/R, (d) DIDS+I/R, (e) 90 Cl+I/R, (f) CaP, (g) NPPB+CaP, (h) DIDS+CaP, (i) 90 Cl+CaP. Scale bar, 20 μ m. B. The group results regarding the percentage of μ -calpain membrane-positive cells. Each bar represents the mean $\pm$ SEM, n=6. ***P* < 0.01 vs. Control, ##*P* < 0.01 vs. the corresponding group without drugs. Please refer to Figures 2 and 5 for the abbreviations.

Fig. 9. The blockade of transmembrane Cl⁻ flux and the inhibition of calpain with MDL 28170 (MDL) reduced the percentage of cleaved 150-kDa fragments of α -fodrin in the hearts subjected to either I/R (A) or Ca²⁺ paradox (B). Top, representative blots. Bottom, the group results regarding the densitometric analysis. GAPDH was used as a loading control. The values were expressed as arbitrary units relative to the control group, which was given a value of 1. Each bar represents the mean $\pm$ SEM, n=6. ***P* < 0.01 vs. Control, #*P* < 0.05 vs. I/R, ##*P* < 0.01 vs. CaP. Please refer to Figures 2 and 5 for the abbreviations.

Discussion

We evaluated the effects of NPPB, DIDS, and a low-Cl⁻ solution on myocardial I/R injury, observed the drugs' effects on the Ca²⁺ overload-induced injury, which was induced via Ca²⁺ paradox, and also measured the activity of calpain, an endogenous Ca²⁺-dependent protease. The data revealed that as was the case with MDL 28170, an inhibitor of calpain, the blockade of Cl⁻ transmembrane flux protected against the injury, both in ischemia and reperfusion and in Ca²⁺ paradox, findings accompanied by a decrease in calpain activity. Therefore, this study was the first to provide evidence that the activation of calpain represents a new pathway that mediates Cl⁻-induced heart injury. More importantly, it also suggests that intracellular Ca²⁺ overload regulates calpain activation.

NPPB, DIDS, 9-AC, and Cl⁻ substitutes such as gluconate have been widely used to investigate the function of Cl⁻ in the heart [26]. However, the data should be viewed with caution as controversy exists regarding the specifics of this process; for example, previous studies have provided evidence that 100 μM NPPB stimulated PKA [27], which inhibits calpain activity [28]; 10 μM NPPB blocked the L-type Ca²⁺ channels in the cerebral arteries of rats [29]. In this study, two types of channel blockers (NPPB and DIDS) were utilized at a low concentration (5 μM), as was the substitution of gluconate for Cl⁻; the data revealed that each of the agents significantly reduced the incidence of cell death and facilitated an improved hemodynamic recovery from both I/R and Ca²⁺ paradox exposure, which were accompanied by the inhibition of calpain activity. Therefore, although another pathway may be involved in the cardioprotection induced by NPPB, DIDS, and 90 mM Cl⁻, we believe that the blockade of transmembrane Cl⁻ flux acts as a key upstream executor of this process.

We observed that both Cl⁻ channel blockers (NPPB and DIDS) and a low-Cl⁻ solution not only reduced the size of myocardial injury area and LDH release but also reduced caspase 3 activity and the apoptotic indices. These data are consistent with those of previous studies, suggesting that both necrosis and apoptosis are involved in Cl⁻-induced cell death in the setting of myocardial ischemia and reperfusion [30]. Additionally, our data revealed that increases in both the apoptotic indices and caspase 3 activity were very small (Fig. 3 & Fig. 6), whereas the increases in myocardial injury area and LDH release were each very prominent (Fig. 2 & Fig. 5). Therefore, it is highly likely that necrosis, rather than apoptosis, was the predominant pathway in the cell death observed in these experiments.

In the present study, we observed that Cl⁻ channel blockers (NPPB and DIDS) elicited a transient increase in LVDP, increases accompanied by a decrease in heart rate in the control hearts (Table 2). As contractility is dependent on both pacing frequency and Ca²⁺ [31], our findings suggest that Cl⁻ may regulate heart performance via heart rate and Ca²⁺. Additionally, compared with both the I/R and the Ca²⁺ paradox groups, which exhibited no changes in heart performance, treatment with NPPB, DIDS, and 90 mM Cl⁻ significantly improved LVDP recovery and decreased LVEDP following reperfusion. Given the data regarding cell death, our results suggest that the blockade of transmembrane Cl⁻ flux decreases the incidence of cardiac cell death and improves the recovery of hemodynamic performance following ischemia and reperfusion.

In this study, we observed that as was the case for MDL 28170, the blockade of transmembrane Cl⁻ flux not only rescued heart tissue but also decreased both the translocation of calpain and the proteolysis of fodrin. These results suggest that calpain is located downstream of Cl⁻-induced heart injury. It is known that the activity of calpain is regulated by Ca²⁺, pH, the endogenous inhibitor calpastatin, and self-phosphorylation [14, 15]. To test the hypothesis that calpain activation is directly dependent on Ca²⁺ overload in the setting of Cl⁻-induced heart injury, we performed experiments with Ca²⁺ paradox, a well-known experimental model used for studying Ca²⁺ overload without altering cellular pH [21]. As was the case in the setting of ischemia and reperfusion, the blockade of transmembrane Cl⁻ flux with NPPB, DIDS, and 90 mM Cl⁻ not only rescued cardiac tissue and improved cardiac function but also decreased the percentages of the membrane translocation of calpain and the numbers of cleaved 150-kDa fragments by Ca²⁺ paradox. Therefore, it is reasonable to

suggest that during reperfusion, the blockade of Cl⁻ transmembrane flux directly reduces intracellular Ca²⁺ overload, inhibits calpain activity, and mitigates heart injury. Additionally, a previous study demonstrated that Cl⁻ transport exacerbates the decline in pH in ischemic cardiac cells [5], suggesting that ischemic treatment before reperfusion may also be a target time period for the utilization of the rescue capabilities of NPPB, DIDS and low Cl⁻.

Our data are inconsistent with those previous studies that observed that inhibiting CFTR caused a transient diastolic Ca²⁺ concentration increase [32]. The discrepancy may be because various Cl⁻ channels, which are regulated by protein kinase A, protein kinase C, cell volume, and purinergic receptors, are present in the heart [2]. Therefore, additional studies are necessary to investigate which Cl⁻ channel is involved in regulating intracellular Ca²⁺. Additionally, previous studies have suggested that Cl⁻ modulates the L-type Ca²⁺ channels found in normal cardiac cells [7]; however, we demonstrated previously that a sodium/calcium exchanger is a major contributor to diastolic Ca²⁺ overload in the setting of both ischemia and reperfusion and Ca²⁺ paradox [22, 33]. Therefore, whether transmembrane Cl⁻ flux regulates transmembrane Ca²⁺ flux via the sodium/calcium exchanger warrants further study.

Mitigating diastolic Ca²⁺ overload has been accepted as a potential strategy for protecting the heart in the setting of ischemia and reperfusion; however, the existing interventional agents are both very limited and unsatisfactory [34, 35]. In the ESCAMI trials, inhibiting Na⁺/H⁺ exchanger activity with eniporide failed to modify infarct size and improve clinical outcomes [36]. No Na⁺/Ca²⁺ inhibitors have been approved for human use [34, 35]. Calpain is a Ca²⁺-dependent protease; we observed that Cl⁻ transporter blockers significantly rescued the hearts subjected to both ischemia and reperfusion and Ca²⁺ paradox via the inhibition of calpain, suggesting that the blockade of transmembrane Cl⁻ flux represents a new pathway by which to attenuate intracellular Ca²⁺ overload. As postoperative atrial fibrillation is associated with a high serum chloride concentration [10], we believe that controlling plasma Cl⁻ concentrations may attenuate intracellular Ca²⁺ overload and minimize the damage inflicted by cardiac surgery.

In conclusion, this study was the first to provide evidence that the blockade of transmembrane Cl⁻ flux via the inhibition of calpain activity mitigates I/R-induced cardiac injury. Our findings indicate that controlling plasma Cl⁻ concentrations may be an effective approach for attenuating reperfusion injury via the reduction of Ca²⁺ overload during heart surgery. Additional studies are necessary to clarify the roles of the different Cl⁻ channels in Ca²⁺ overload and the mechanisms underlying this process.

Abbreviations

I/R (ischemia/reperfusion); LVDP (left ventricular developed pressure); LVEDP (left ventricular end-diastolic pressure).

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Disclosure Statement

The authors declare that there are no conflicts of interest regarding the publication of this paper.

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