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Plasma-membrane K_{ATP} channel-mediated cardioprotection involves posthypoxic reductions in calcium overload and contractile dysfunction: mechanistic insights into cardioplegia

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ABSTRACT

Our recent data demonstrate that activation of *pm*K_{ATP} channels polarizes the membrane of cardiomyocytes and reduces Na^+/Ca^{2+} exchange-mediated Ca^{2+} overload. However, it is important that these findings be extended into contractile models of hypoxia/reoxygenation injury to further test the notion that *pm*K_{ATP} channel activation affords protection against contractile dysfunction and calcium overload. Single rat heart right ventricular myocytes were enzymatically isolated, and cell contractility and Ca^{2+} transients in field-stimulated myocytes were measured in a cellular model of metabolic inhibition and reoxygenation. Activation of *pm*K_{ATP} with P-1075 (5 μM) or inhibition of the Na^+/Ca^{2+} exchanger with KB-R7943 (5 μM) reduced reoxygenation-induced diastolic Ca^{2+} overload and improved the rate and magnitude of posthypoxic contractile recovery during the first few minutes of reoxygenation. Moreover, diastolic Ca^{2+} overload and posthypoxic contractile dysfunction were aggravated in ventricular myocytes either subjected to specific blockade of *pm*K_{ATP} with HMR1098 (20 μM) or expressing the dominant-negative *pm*K_{ATP} construct Kir6.2(AAA) in the presence of P-1075. Our results suggest that a common mechanism, involving resting membrane potential-modulated increases in diastolic $[Ca^{2+}]_i$, is responsible for the development of contractile dysfunction during reoxygenation following metabolic inhibition. This novel and highly plausible cellular mechanism for *pm*K_{ATP}-mediated cardioprotection may have direct clinical relevance as evidenced by the following findings: a hypokalemic polarizing cardioplegia solution supplemented with the *pm*K_{ATP} opener P-1075 improved Ca^{2+} homeostasis and recovery of function compared with hyperkalemic depolarizing St. Thomas' cardioplegia following contractile arrest in single ventricular myocytes and working rat hearts. We therefore propose that activation of *pm*K_{ATP} channels improves posthypoxic cardiac function via reductions in abnormal diastolic Ca^{2+} homeostasis mediated by reverse-mode Na^+/Ca^{2+} exchange.

Key words: ischemia/reperfusion • plasma membrane ATP-sensitive potassium channel • Na^+/Ca^{2+} exchanger • diastolic $[Ca^{2+}]_i$ • potassium channel opener

Since the discovery of plasma-membrane (*pm*) K_{ATP} channels in cardiac myocytes 21 years ago (1), these channels have been shown to be important in the control of cardiac excitability during ischemic episodes, and many studies have demonstrated that activation of *pm*K_{ATP} channels under hypoxic or ischemic conditions is cardioprotective (2–5). *Pm*K_{ATP} channels have also been implicated in the powerful cardioprotective phenomenon of ischemic preconditioning (IPC) (6, 7).

Initially, it was thought that the negative inotropy caused by *pm*K_{ATP} channel-mediated action potential shortening would conserve energy and be protective, but this was challenged by the finding that K_{ATP} channel openers protected the heart in concentrations that did not shorten the cardiac action potential (8, 9). Subsequently, another distinct population of K_{ATP} channels found in the inner mitochondrial membrane (*mito*K_{ATP}) was suggested as an important mediator of the cardioprotective effects of K_{ATP} channel openers (10–13). However, our own recent work (14), in which we used electrically quiescent myocytes and still observed *pm*K_{ATP}-mediated protection in the absence of any action potential generation, suggests that *pm*K_{ATP} channel activation can be protective via a mechanism other than action potential shortening. Recent transgenic animal experiments have provided strong evidence that activation of *pm*K_{ATP} channels is a major player in the observed cardioprotection. For example, the hearts from *pm*K_{ATP} channel knockout mice failed to precondition and had poor postischemic functional recovery—an effect mimicked in wild-type mice by the *pm*K_{ATP} channel selective inhibitor HMR1098 (15). From the available evidence, it is likely that activation of both *pm* and *mito*K_{ATP} channels can be protective via distinct mechanisms and have central roles to play in IPC and cardioprotection (5).

It is perhaps difficult to reconcile the purpose of *pm*K_{ATP} channels, despite their high density, if they have no role to play during periods of metabolic stress such as intense exercise or ischemia. Clearly this is not the case, as there is excellent evidence to support the protective effects of *pm*K_{ATP} channel activation in the ischemic myocardium. Therefore, a separate and as yet unidentified *pm*K_{ATP} channel-mediated protective mechanism likely exists. In this regard, recent evidence from our and other laboratories suggests that Ca^{2+} handling is regulated by *pm*K_{ATP} channel activity (14, 16–18).

One of the major clinical correlates of ischemia/reperfusion injury is contractile dysfunction, in which Ca^{2+} overload via increased Na^+/Ca^{2+} exchange is a major contributor (19, 20). Based on our recent data (21, 14), we postulated that a major function of *pm*K_{ATP} channels is to favor a polarized membrane potential and therefore reduce the magnitude of reverse-mode Na^+/Ca^{2+} exchange decreasing intracellular Ca^{2+} overload during periods of metabolic stress such as ischemia/reperfusion or intense cardiac work. This would diminish the likelihood of cellular injury such as hypercontracture/death and contractile stunning (22) or the development of arrhythmias.

In this present study, we investigate the relationship between *pm*K_{ATP} activation, reverse-mode Na^+/Ca^{2+} exchanger activity, intracellular calcium transients, and contractile dysfunction during recovery following a brief period of metabolic inhibition in field-stimulated rat ventricular myocytes. In addition, we then investigate the implications of our initial findings in the setting of

experimental cardioplegia using hyperkalemic-depolarizing and hypokalemic-polarizing solutions in single-cell and whole-heart models.

MATERIALS AND METHODS

Myocyte isolation

Adult male Sprague-Dawley rats were killed with pentobarbital (150 mg/kg, i.p.) according to the University of Alberta Animal Policy and Welfare Committee and the Canadian Council on Animal Care (CCAC) Guidelines. The hearts were then removed, and ventricular myocytes were obtained by enzymatic dissociation using the standard protocols described previously (23, 24). After 1 h, cells were placed on coverslips for observation at ×200 with an inverted microscope and were superfused with control solution containing (in mM): NaCl 140, KCl 5, HEPES 10, $CaCl_2$ 2.0, $MgCl_2$ 1.4, and glucose 10.

Measurement of cell shortening and Ca^{2+} transients

Cell shortening was measured using a video edge detector system (Crescent Electronics, Salt Lake City, UT) at 60 Hz frame rate. Myocytes were field-stimulated with 2 ms square pulses at a constant current 20% above threshold value. Cell shortening was expressed as fractional shortening ($\Delta L/L_0$, where L_0 is resting cell length).

Ventricular myocytes from adult rats were loaded for 30 min at room temperature and for 30 min at 37°C with the Ca^{2+}-sensitive fluorescent probe Calcium Green-1AM (4 μM, in a 1:1 v/v dimethyl sulfoxide:pluronic acid mixture, Molecular Probes, Eugene, OR). After loading, cells were washed and placed on coverslips for observation at ×200. Cells were then superfused as described above. A Photon Technology International Photomultiplier Detection System (PTI, Lawrenceville, NJ) with Clampex software (version 8.1) was used for data acquisition and analysis. Calcium Green-1AM was excited with 480 nm light, and the emitted light intensity at 520 nm was digitized and stored. The duration of the Ca^{2+} transient at 90% recovery was obtained by measuring the interval between the onset of the Ca^{2+} transient (10% above the average diastolic value) to the time point where the transient returned to 90% of average diastolic value. Cell shortening and Ca^{2+} transient experiments were performed at room temperature (22±1°C).

In vivo adenoviral cardiomyocyte infection with the dominant-negative Kir6.2(AAA) construct

The dominant-negative triple point mutation GFG to AAA in the pore region of the K_{ATP} channel subunit Kir6.2 (24) was created using the protocol outlined in the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). GFG→AAA, forward primer: 5′ caggtgagcattgctgccgccgggcgcatggtgac 3′; reverse, 5′ ccatgcgcccggcggcagcaatggtcacctgg 3′. The sequenced Kir6.2(AAA) insert was then excised from pCDNA3 vector and reinserted into pAdTrack-CMV transfer vector and the bicistronic adenoviral vector designed to coexpress Kir6.2(AAA), and GFP was then constructed as per the manufacturer's instructions (PAdEasy, Stratagene). The bicistronic adenoviral design is illustrated in Fig. 3*A*. Cardiomyocytes were infected in vivo to overexpress either the green fluorescent protein (GFP) (control) or GFP in

combination with Kir6.2(AAA). Following left lateral thoracotomy, a solution containing 4×10^{11} pfu/ml viral titer (10 μl/injection site, 10 sites) was injected into the lateral left ventricular wall and into the apex of the heart. The animals were allowed to recover for 5 days, and then single ventricular myocytes were isolated from the infected region by visual identification of GFP expression.

Measurement of whole-cell current in tsA201 cells expressing the rat NCX1.1

Infection of tsA201 cells with 30 pfu/cell of an adenoviral rat heart NCX1.1 construct was performed, and whole-cell patch clamp recordings were undertaken as described previously (26). The NCX1.1 construct was generously provided by Dr. J. Lytton (University of Calgary, Calgary, Alberta, Canada) and the adenovirus construct by Dr. J. Y. Cheung (Geisinger Medical Center, Danville, PA) (27).

Cardioplegia in isolated rat ventricular myocytes

Isolated rat ventricular myocytes were subjected to the following protocol: 2 min with control, 4 min with a cardioplegia solution (either St. Thomas' cardioplegia [STC] or hyperpolarizing cardioplegia solution [HPC]), 6 min cardioplegia with metabolic inhibition (MI; 5 mM 2-deoxy-glucose + 4 mM NaCN), and 12 min recovery with control solution. Table 1 shows the composition of solutions used in the cardioplegia experiments. All solutions had a pH of 7.4. Fractional cell shortening and Ca^{2+} transients were recorded continuously during the protocol as described above.

Cardioplegia in perfused rat hearts

Male Sprague-Dawley rats (300–350 g) were anesthetized with sodium pentobarbital, and their hearts were excised and perfused in Langendorff (nonworking) mode at a constant perfusion pressure of 60 mmHg with Krebs-Henseleit solution at 37°C for 10 min. Hearts were then randomly assigned to groups that received either 25 ml of ice-cold STC (n=8) or 25 ml of ice-cold HPC (n=7) solution (Table 1) administered at constant perfusion pressure of 60 mmHg (Fig. 7*A*). Hearts were then removed from the perfusion apparatus and stored in their respective cardioplegia solution at 3°C for 6 h. Following storage, hearts were rewarmed to 37°C during 10 min of Langendorff mode perfusion. Recovery of mechanical function was assessed during a subsequent 30-min period of working mode during which perfusate entered the left atrium at a constant preload (11.5 mmHg) and was ejected by the left ventricle against a constant afterload (80 mmHg). A group of hearts that were perfused under identical conditions, but not subjected to cardioplegia or storage, served as nonstored controls (n=8). Coronary flow (ml/min) was measured during Langendorff perfusion periods before and after cardioplegia. During working mode perfusion, systolic and diastolic aortic pressures, cardiac output (CO), and aortic flow were measured. Left ventricular minute work (l/min.mmHg) was calculated as CO × (peak systolic pressure – preload pressure) as described previously (28) and served as an index of cardiac mechanical function.

Drugs and chemicals

HMR 1098 (1-[[5-[2-(5-chloro-*o*-anisamido)ethyl]-2-methoxyphenyl]sulfonyl]-3-methylthiourea, sodium salt) (Aventis, Frankfurt, Germany) was dissolved as a 5 mM stock solution in distilled water. KB-R7943 (Tocris, Ellisville) and P-1075 (Tocris) were made up as 10 and 20 mM stock solutions in dimethyl sulfoxide, respectively. Pluronic F-127 (Molecular Probes) was prepared as a 20% w/v stock solution in dimethyl sulfoxide. Each stock solution was diluted to the required concentration immediately before use.

Statistics

All data are presented as mean ± SE. Statistical analyses of data were performed using Student's paired and unpaired *t* test and ANOVA as appropriate. A level of $P < 0.05$ was considered statistically different.

RESULTS

Activation of *pm*K_{ATP} and inhibition of the Na^+/Ca^{2+} exchanger reduce the duration of post-MI contractile dysfunction in rat ventricular myocytes

Previous experiments have demonstrated that activation of *pm*K_{ATP} channels in electrically quiescent myocytes reduced Ca^{2+} loading and cellular hypercontracture during reoxygenation (14). However, in order to translate these findings to dynamic changes in contractility, a field-stimulated working myocyte model was adopted. Reoxygenation of ventricular myocytes following 6 min of metabolic inhibition resulted in a transient decrease in contractile amplitude during reoxygenation (Fig. 1*A*). Myocytes reoxygenated with the *pm*K_{ATP} opener P-1075 (5 μM) (Fig. 1*C*), the Na^+/Ca^{2+} exchanger inhibitor KB-R7943 (5 μM) (Fig. 1*B*), or their combination (Fig. 1*D*) exhibited a similar transient decrease in contractile amplitude. However, the duration of the decrease in contractile amplitude was significantly less in the P-1075 and KB-R7943 groups compared with control (see Fig. 1*E*). The *pm*K_{ATP} inhibitor HMR 1098 (20 μM) blocked the effect of 5μM P-1075 on duration of dysfunction (Fig. 1*E*). When applied alone, HMR 1098 did not increase the duration of dysfunction (Fig. 1*E*) but caused a significant reduction in contractile recovery at the end of the reoxygenation period (Fig. 1*F*).

The contractile performance of isolated rat ventricular myocytes during baseline steady-state conditions and at 2 min of reoxygenation following MI is summarized in Fig. 2*A–D*. The myocyte cell length at basal conditions, ranging from 98.6 ± 10.0 to 112.7 ± 7.9 μm (Table 2) (mean=104.8±2.4 μm, n=47 cells), was not significantly different among groups. In addition, fractional cell shortening and ±d*L*/d*t* values at basal conditions were not significantly different among groups (Table 2). There was a reduction in fractional cell shortening and its first derivative (±d*L*/d*t*) in all groups at 2 min reoxygenation (Table 2). However, fractional cell shortening, maximum rate of shortening (+d*L*/d*t*), and maximum rate of relaxation (–d*L*/d*t*) were significantly higher in groups superfused with the *pm*K_{ATP} opener P-1075 (5 μM), Na^+/Ca^{2+} exchanger inhibitor KB-R7943 (5 μM), and their combination, indicating improved contractility and relaxation at the early stages of reoxygenation in these groups (Table 2 and Fig. 2*E*). In myocytes reoxygenated with solutions containing the specific *pm*K_{ATP} inhibitor HMR 1098, fractional cell shortening and ±d*L*/d*t* values were similar to those of the control group but were

significantly lower than in the P-1075 group at 2 min reoxygenation (Table 2 and Fig. 2*E*). The maximum rate of shortening and relaxation fully recovered by the end of the reoxygenation period in the control, P-1075, and KB-R7943 + P-1075 groups, but were significantly reduced in the HMR 1098, HMR 1098 + P-1075, and KB-R7943 groups (Table 2). These results confirm the protective effect of activation of *pm*K_{ATP} channels but also indicate that by the end of the reoxygenation period, when cardiac myocytes have recovered from metabolic inhibition, the blockade of the Na^+/Ca^{2+} exchanger is no longer beneficial. Our data are in agreement with a recent study showing that KB-R7943 is cardioprotective but can also have negative inotropic effects (29).

There were no significant differences in time to peak shortening (TPS) and duration of contraction values among the experimental groups in basal conditions and at 2 min reoxygenation (Table 2). In the KB-R7943 group, however, both TPS and the length of contraction were significantly longer than in the control group, indicating slower contractions during field stimulation (Table 2).

Functional *pm*K_{ATP} channels are needed for improved recovery during early reoxygenation

While these studies provide evidence for the protective role of pharmacological *pm*K_{ATP} channel activation with P-1075, it is necessary to confirm and extend these findings using a molecular approach to functionally silence endogenous *pm*K_{ATP} channel activity. The next set of experiments was performed with the specific aim of studying the cardioprotective efficacy of P-1075 in the presence and absence of functional *pm*K_{ATP} channels during reoxygenation following metabolic inhibition. To achieve this, we constructed a dominant-negative adenoviral construct of the *pm*K_{ATP} channel subunit Kir6.2, AdVKir6.2(AAA), that also coexpresses GFP (30).

Single-cell contractility experiments were performed on myocytes infected in vivo with AdVKir6.2(AAA) 96 h before enzymatic dissociation. This infection period is sufficient to allow incorporation of the mutated Kir6.2(AAA) subunit into the endogenous K_{ATP} channel complex and suppress functional K_{ATP} current (30). Visually identified ventricular myocytes (Fig. 3*A*) were then subjected to the previously described MI/reoxygenation protocol. There was no difference in basal cell length and fractional cell shortening between the AdVKir6.2(AAA)/P-1075 and control groups (100.2±11.6 vs. 112.7±8.0 μm and 13.0±1.6 vs. 12.8±1.3%, respectively; $P>0.05$). However, after the development of reoxygenation-induced contractile dysfunction, myocytes expressing the Kir6.2(AAA) construct, in the presence of P-1075, failed to recover (Fig. 3*B*, 3*C*). Note that in the presence of nonfunctional *pm*K_{ATP} channels, P-1075 (5 μM) did not offer any beneficial effects on contractile function in these myocytes in early reoxygenation. These results confirm a critical role of *pm*K_{ATP} in posthypoxic cardiac functional recovery and are in good agreement with the studies of Suzuki et al. (15) on Kir6.2 KO mouse hearts.

Reduction of post-MI diastolic $[Ca^{2+}]_i$ following *pm*K_{ATP} activation and inhibition of the Na^+/Ca^{2+} exchanger: possible mechanism for the amelioration of contractile dysfunction

Abnormal calcium homeostasis in the form of diastolic Ca^{2+} overload is a major contributor to the contractile and electrical dysfunction observed during reperfusion/reoxygenation (31). Reverse-mode Na^+/Ca^{2+} exchange activity is thought to play an important role in the

development of calcium overload (19, 20). Therefore, we set out to determine the effects of *pm*K_{ATP} channel activation and Na^+/Ca^{2+} exchange inhibition on dynamic changes in intracellular calcium.

Field-stimulated ventricular myocytes were subjected to the MI/reoxygenation protocol, and Ca^{2+} transients were continuously recorded every 1 s for the duration of the experiment. There was a substantial increase in diastolic $[Ca^{2+}]_i$ during reoxygenation with the control solution, followed by a recovery to basal values (Fig. 4*A*). Application of the Na^+/Ca^{2+} exchanger inhibitor KB-R7943 (5 μM), the *pm*K_{ATP} opener P-1075 (5 μM), and their combination in the reoxygenation solution significantly reduced elevations in $[Ca^{2+}]_i$ (Fig. 4). The *pm*K_{ATP} inhibitor HMR 1098 prevented the beneficial effects of P-1075 on diastolic $[Ca^{2+}]_i$ but did not cause a further increase when applied alone (Fig. 4*E*). These data are in agreement with our previous results from quiescent rat ventricular myocytes (14). The time course of changes in diastolic $[Ca^{2+}]_i$ and development of contractile dysfunction were similar in the present study, suggesting a close relationship between diastolic Ca^{2+} levels and myocardial function in reoxygenation following metabolic inhibition.

Detailed analysis revealed no significant differences in the duration of the Ca^{2+} transient at 90% recovery level, the time to peak, and the amplitude of the Ca^{2+} transients in any of the experimental groups in basal conditions and during reoxygenation (Table 3). These results suggest that in our cellular model of stunning, increases in diastolic $[Ca^{2+}]_i$ and not alterations in calcium handling are responsible for the development of postischemic contractile dysfunction.

To confirm the inhibitory effect of 5 μM KB-R7943 on reverse-mode Na^+/Ca^{2+} exchange, some whole-cell patch-clamp experiments were carried out on tsA201 cells expressing rat heart NCX1.1. Large outward currents (due to reverse-mode Na^+/Ca^{2+} exchanger activity) were recorded from these cells when a Ca^{2+}-free extracellular solution was changed to a solution containing 2 mM Ca^{2+} at 0 mV holding potential as described previously (26). A representative trace from those experiments demonstrates that 5 μM KB-R7943 almost completely inhibited this outward current (Fig. 4*F*).

Diastolic resting membrane potential of ventricular myocytes is critically important in the recovery of function and calcium homeostasis during simulated cardioplegia

Postoperative cardiac dysfunction is a common problem associated with the use of hyperkalemic cardioplegic solutions (32, 33). Our data suggest that maneuvers designed to hyperpolarize the resting membrane potential of cardiomyocytes may afford a marked degree of protection from ischemia/reperfusion injury in the setting of cardioplegia. Therefore, we compared the contractility of cardiac myocytes subjected to cardioplegia with modified depolarizing STC solution (containing 16 mM K^+) and an HPC solution (containing 3.2 mM K^+). During superfusion with STC, in some ventricular myocytes large contractions were observed, this behavior was not detected in cells in the HPC group (Fig. 5*A*, 5*B*). The improved recovery of function (ΔL and d*L*/dt) in the HPC group compared with the STC group is illustrated in Fig. 5*A* and 5*B*. Fractional cell shortening was significantly better at 6 and 12 min of recovery following cardioplegia in the HPC group compared with STC (Fig. 5*C*). Diastolic cell length was significantly reduced in the STC group and was significantly increased in the HPC group compared with basal values in the same group. Diastolic cell length was also significantly shorter

in the STC group compared with HPC during cardioplegia + MI and did not return to basal cell length as opposed to the HPC group (Fig. 5*D*).

Calcium transients were recorded from another two groups of ventricular myocytes subjected to the same cardioplegia protocol, with either STC or HPC. Representative recordings show Ca^{2+} transients from two myocytes from the STC and the HPC groups in Fig. 6*A* and 6*B*, respectively. Note the increase in diastolic $[Ca^{2+}]_i$ during recovery following STC and lack of change in diastolic $[Ca^{2+}]_i$ in recovery following HPC. Grouped data show that superfusion of ventricular myocytes with a depolarizing cardioplegia solution and subsequent metabolic inhibition resulted in a significantly elevated diastolic $[Ca^{2+}]_i$ during recovery (Fig. 6*D*). These data further suggest a crucial role for resting membrane potential in the modulation of diastolic $[Ca^{2+}]_i$ and cardiomyocyte function during recovery following cardioplegia. These results are also in agreement with our previous observations showing a close relationship between $[K^+]_o$, resting membrane potential, and Na^+/Ca^{2+} exchanger-mediated Ca^{2+} loading (21).

HPC with activation of *pm*K_{ATP} improves cardiac function in isolated working rat hearts

To determine whether the beneficial effects of the HPC solution on Ca^{2+} homeostasis of ventricular myocytes directly translates into improved cardiac recovery, we carried out another series of experiments on isolated rat hearts. Hearts were randomly assigned to three groups: 1) 6 h of depolarizing cardioplegia with STC solution, 2) 6 h of hyperpolarizing cardioplegia with HPC solution, and 3) a group of hearts not subjected to cold cardioplegic storage with a time-matched recovery perfusion protocol of 10 min Langendorff and 30 min of working heart mode (Fig. 7*A*). Coronary artery flow significantly improved in the HPC group compared with the STC group at 10 min Langendorff perfusion following 6 h of cardioplegia (Fig. 7*B*). After 30 min of perfusion in working heart mode, cardiac function of rat hearts subjected to 6 h of STC was significantly impaired compared with both the HPC group and the control group not subjected to cold cardioplegic storage (Fig. 7*C*). Note that these experiments did not include subjecting the heart to metabolic inhibition and showed a similarly more beneficial effect of HPC on cardiac function compared with STC as observed in the single-cell model.

DISCUSSION

Overview

In the present study, we confirm that activation of *pm*K_{ATP} channels is cardioprotective in a cellular model of contractility: addition of the *pm*K_{ATP} opener P-1075 to the reoxygenation solution 1) significantly reduced the duration of post-MI contractile dysfunction (Fig. 1) and 2) improved contractility ($\pm dL/dt$) during the period of dysfunction (Fig. 2). These effects were prevented by the selective *pm*K_{ATP} blocker HMR 1098 (Fig. 1, 2) and did not develop when ventricular myocytes expressing the dominant-negative Kir6.2(AAA) *pm*K_{ATP} construct were superfused with P-1075-containing solution (Fig. 3). Our results show that improved Ca^{2+} homeostasis is responsible for the beneficial effects of *pm*K_{ATP} channel activation in early reoxygenation. Specifically, activation of *pm*K_{ATP} reduced the increase in diastolic $[Ca^{2+}]_i$ during reoxygenation (Fig. 4*C–E*). This effect could be reversed by adding the *pm*K_{ATP} inhibitor HMR 1098 to the reoxygenation solution, resulting in a diastolic $[Ca^{2+}]_i$ increase similar to the control group (Fig. 4*E*).

pmK_{ATP} channel pharmacology

The purpose of this study was to investigate whether activation of *pm*K_{ATP} channels can improve contractile function and Ca^{2+} homeostasis after metabolic inhibition/reoxygenation and experimental cardioplegia. Therefore, it was necessary to selectively activate and block *pm*K_{ATP} as opposed to *mito*K_{ATP} channels. Functional K_{ATP} channels are formed by four inwardly rectifying potassium channel subunits (Kir6.2) and four regulatory sulphonylurea receptor subunits (SUR) (34). The distinct isoforms of SUR expressed in different tissues confer pharmacological characteristics to K_{ATP} channels, and SUR2A is the isoform found in the plasma membrane of cardiac myocytes (35). The *pm*K_{ATP} channel has been identified as Kir6.2/SUR2A, while the pharmacological profile of the *mito*K_{ATP} is very similar to Kir6.1/SUR1 (36). In the present experiments, the pinacidil derivative P-1075 was used for *pm*K_{ATP} channel activation (36) and the cardioselective sulphonylurea HMR 1098 was used for inhibition of *pm*K_{ATP} channels (37). Recent work from our laboratory shows that HMR 1098 is a selective inhibitor of Kir6.2/SUR2A and has no effect on the strong inward rectifier Kir2.1 current (38).

Previous studies on guinea pig ventricular myocytes and canine Purkinje fibers have reported a strong action potential duration (APD) shortening effect of P-1075 at micromolar concentrations (39, 40). In a set of preliminary experiments on normoxic rat ventricular myocytes, a 10 min superfusion of 5 μM P-1075 caused a significant outward current in whole-cell perforated patch recordings as well as a mild negative inotropic effect (data not shown). These initial findings are in agreement with the data in Table 2 and suggest a relatively smaller reduction in APD in rat ventricular myocytes as opposed to species with a longer APD possessing pronounced plateaus. We have previously shown that the K_{ATP} opener cromakalim (50 μM) has minimal effects on the rat ventricular myocyte APD (24, 41). However, when chronic disease states (diabetes and hypothyroidism) are induced and a significant increase in APD is observed, cromakalim has a much greater APD shortening effect (24, 41). These data suggest that 5 μM P-1075 affects APD in rat ventricular myocytes, although certainly not enough to severely impair contractility.

P-1075 had no beneficial effect on contractile recovery in ventricular myocytes expressing the dominant-negative Kir6.2(AAA) *pm*K_{ATP} construct (Fig. 3) as opposed to those expressing the endogenous wild-type *pm*K_{ATP} channel (Fig. 1, 2). These results suggest that the effects of P-1075 and HMR 1098 observed during reoxygenation are linked to *pm*K_{ATP} and not to *mito*K_{ATP}.

The suggestion that only activation of *mito*K_{ATP} as opposed to *pm*K_{ATP} results in protection of the myocardium (10) is largely based on pharmacological studies using diazoxide for opening and 5-hydroxydecanoate (5-HD) for blocking *mito*K_{ATP} channels (11, 12). Diazoxide has been shown to 1) cause flavoprotein oxidation independent of *mito*K_{ATP} effects (42, 43); 2) activate *pm*K_{ATP} channels even at low concentrations in the presence of ADP, which is likely to be present in ischemia and early reperfusion (44); and 3) protect the myocardium without depolarizing mitochondria or oxidizing flavoproteins (45). 5-HD can be a substrate or inhibitor of fatty acid β-oxidation (46, 47), opposing its use as a specific *mito*K_{ATP} inhibitor.

We and others suggest that activation of both subpopulations of K_{ATP} channels have important cardioprotective effects (5, 10). Several papers published recently provide evidence that *pm*K_{ATP} has a critical role in protecting the myocardium in ischemia/reperfusion experiments. The well-characterized infarct size decreasing effect of ischemic preconditioning was abolished in Kir6.2-

deficient mice, and this result could be reproduced by specific blockade of *pm*K_{ATP} channels in their wild-type counterparts (15). Recently, diazoxide has been shown to exert its cardioprotective effects only in mice hearts expressing *pm*K_{ATP} channels, not in Kir6.2 knockout hearts (48). These studies strongly support a critical cardioprotective role for *pm*K_{ATP} channels in the setting of ischemia/reperfusion. To date, however, researchers have not identified the precise ionic mechanisms responsible for this protection.

Intracellular mechanisms for *pm*K_{ATP} channel-mediated protection

Ischemia and subsequent reperfusion of the heart can cause elevated $[Ca^{2+}]_i$ in ventricular myocytes leading to 1) the development of cardiac arrhythmias and sudden cardiac death; 2) reversible contractile dysfunction and myocardial stunning (31); 3) the opening of the mitochondrial permeability transition pore, mitochondrial swelling, and eventually cell necrosis; and 4) apoptosis (49). Therefore, strategies that decrease pathological increases in $[Ca^{2+}]_i$ in ventricular myocytes potentially reduce reversible and irreversible myocyte injury and result in improved survival and function of the myocardium. The mechanism by which altered calcium homeostasis causes contractile dysfunction is still not clearly established. Calcium can activate proteases (e.g., calpain I) which in turn modifies the Ca^{2+} sensitivity or degradation of myofilaments, such as troponin I (50). It has also been shown that a species-dependent altered Ca^{2+} handling can significantly contribute to postischemic dysfunction (51). Our results confirm that increased $[Ca^{2+}]_i$ causes contractile dysfunction in reoxygenation following a brief period of metabolic inhibition. In the present study, no significant differences were detected in the duration, amplitude, and time to peak of the calcium transient in the test groups compared with the control group during reoxygenation (Table 3), suggesting that instead of altered calcium handling, increased basal diastolic $[Ca^{2+}]_i$ plays a critical role in the development of contractile dysfunction in our experimental model.

The Na^+/Ca^{2+} exchanger is a major regulator of intracellular calcium homeostasis in cardiac myocytes, with the primary function of removing Ca^{2+} from the cytosol during diastole, and it can also play a significant role in excitation-contraction coupling (52). However, the exchanger can be a significant contributor to increased $[Ca^{2+}]_i$ during hypoxia/reoxygenation (20), when the elevated $[Na^+]_i$ and depolarization of the plasma membrane (21) can cause the exchanger to work in reverse mode. Accordingly, antisense inhibition (19) or pharmacological block (21) (Fig. 1*G*, Fig. 4*B*, 4*E*) of the Na^+/Ca^{2+} exchanger decreases reoxygenation-induced calcium overload in ventricular myocytes. The results from this present study (Fig. 1, 2, and 4) further confirm a central role for reverse-mode Na^+/Ca^{2+} exchanger activity in elevating diastolic $[Ca^{2+}]_i$ and causing contractile dysfunction in ventricular myocytes after a metabolic insult.

In two recent studies using quiescent rat ventricular myocytes, we have shown that calcium overload via reverse-mode Na^+/Ca^{2+} exchanger activity is critically dependent on diastolic membrane potential (21) and that pharmacological activation of *pm*K_{ATP} channels causes hyperpolarization of the diastolic membrane potential and prevents calcium overload (14). Based on the findings of these previous studies and the results of the present experiments, we conclude that 1) depolarization of the diastolic membrane potential, either in the setting of myocardial ischemia/reperfusion or by depolarizing cardioplegia, leads to increased diastolic $[Ca^{2+}]_i$ and contractile dysfunction and 2) the activation of *pm*K_{ATP} channels protects the myocardium by hyperpolarization of the diastolic membrane potential that leads to the reduction of calcium

overload and contractile dysfunction via membrane potential-dependent reverse-mode Na^+/Ca^{2+} exchanger activity.

Implications for cardioplegia

During cardiac surgery, the heart is subjected to global ischemia. Many cardioplegic solutions used to arrest the heart in an attempt to preserve its function are hyperkalemic and contain >15 mM $[K^+]$. Although these solutions are generally effective, several studies suggest that the observed postoperative cardiac dysfunction observed in higher-risk patients can be due to, at least in part, the use of elevated $[K^+]$ (32, 33). Our results from isolated ventricular myocytes and working rat hearts provide evidence that the use of a hyperkalemic cardioplegic solution leads to inferior contractile function upon recovery compared with a hypokalemic solution.

New strategies have been tested to reduce cardiac dysfunction following cardioplegia, including the addition of K_{ATP} channel openers to the arresting solutions, yielding inconsistent results. While the nonspecific K_{ATP} opener pinacidil has been found to enhance cardioprotection (53, 54), lemakalim lost its ability to improve postischemic recovery when added to a hyperkalemic cardioplegic solution (55). Based on our findings, it is likely that lemakalim's beneficial effects on cardiac function were diminished by membrane depolarization in the hyperkalemic milieu, as opening of K_{ATP} channels in this setting would merely clamp the resting membrane potential to a depolarized Nernst potential for potassium ions.

We demonstrate here that an HPC solution supplemented with a specific *pm*K_{ATP} opener offers superior postischemic recovery compared with a depolarizing, hyperkalemic cardioplegia solution both in isolated ventricular myocytes (Fig. 5) and working rat hearts (Fig. 7). Our recent study indicates that increased $[K^+]$ depolarizes myocytes, leading to calcium overload due to reverse-mode Na^+/Ca^{2+} exchange activity (21). Our findings confirm that solutions that hyperpolarize the diastolic membrane potential are effective during cardioplegia (56) and infer a mechanism for membrane potential-dependent changes in myocyte contractility as diastolic $[Ca^{2+}]_i$ is increased when the membrane potential is depolarized in ventricular myocytes (Fig. 6). The present study is also in good agreement with a recent study showing impaired contractile function following depolarizing cardioplegia compared with hyperpolarizing cardioplegia with adenosine and lidocaine in rat hearts (57).

Kobayashi et al. (58) have recently shown that a cardioplegia solution containing nicorandil provided significantly better myocardial oxygen consumption during recovery compared with a cardioplegia solution with 30 mM KCl. The present study and previous work from our laboratory (14, 21) provide evidence that reverse-mode Na^+/Ca^{2+} exchanger activity is involved in resting membrane potential depolarization-induced increases in diastolic $[Ca^{2+}]_i$ of ventricular myocytes and leads to the development of impaired contractile recovery after cardiac arrest with solutions containing elevated $[K^+]$.

Summary

The results of the present study show that activation of *pm*K_{ATP} channels and inhibition of the Na^+/Ca^{2+} exchanger reduces the duration of contractile dysfunction upon reoxygenation following metabolic inhibition. This *pm*K_{ATP} channel-mediated mechanism involves

hyperpolarization of the resting membrane potential and a consequent reduction in Na^+/Ca^{2+} exchanger-mediated elevation of diastolic $[Ca^{2+}]_i$. We propose that it is a novel and plausible mechanism by which activation of *pm*K_{ATP} offers cardioprotection against reperfusion injury and has direct clinical relevance in the setting of cardioplegia.

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Table 1

Composition of modified St. Thomas' cardoplegia (STC) and hyperpolarizing cardioplegia (HPC) solutions (in mM)

	STC	HPC
KCl	16	3.2
$MgCl_2$	16	1
P-1075	not present	0.05
NaCl	110	140
$CaCl_2$	2	2
HEPES	10	10
Glucose	10	10
Lidocaine	1	1

Table 2

Cardiomyocyte contractile function after MI/reoxygenation[a]

				Reoxygenation	
		n	Basal	2 min	10 min
Diastolic cell length (μm)	Control	7	112.7 ± 8.0	110.1 ± 9.1	109.3 ± 8.5
	KB-R	8	109.4 ± 3.8	108.1 ± 3.5	106.2 ± 3.4
	P-1075	8	98.9 ± 4.0	99.8 ± 4.2	99.9 ± 4.3
	KB-R + P-1075	7	98.6 ± 10.0	92.6 ± 9.94	94.2 ± 9.8
	HMR	7	104.6 ± 4.5	92.7 ± 3.2	87.3 ± 5.9*
	HMR + P-1075	10	108.2 ± 3.8	103.7 ± 7.3	103.2 ± 4.9
Cell shortening (%)	Control	7	12.8 ± 1.3	1.3 ± 0.6	11.5 ± 1.8
	KB-R	8	11.5 ± 0.8	6 ± 1.5*	7.3 ± 1.0
	P-1075	8	11.7 ± 0.7	5.8 ± 1.4*	8.7 ± 1.0
	KB-R + P-1075	7	13.9 ± 1.4	8.4 ± 2.0**	11.9 ± 1.4
	HMR	7	14.7 ± 1.6	2.2 ± 1.5	6.2 ± 2.5*
	HMR + P-1075	10	12.8 ± 1.0	2.7 ± 1.1	6.9 ± 1.3*
Maximum rate of shortening (+d*L*/d*t*)	Control	7	171 ± 18.4	24 ± 8.3	151 ± 24.2
	KB-R	8	141 ± 16.7	80 ± 19.4	81 ± 14.6*
	P-1075	8	151 ± 17.3	85 ± 21.4	110 ± 23.4
	KB-R + P-1075	7	172 ± 11.1	95 ± 19.2	123 ± 15.2
	HMR	7	188 ± 19.2	16 ± 14.4[#]	60 ± 23.7*
	HMR + P-1075	10	148 ± 13.6	34 ± 13.3[#]	86 ± 14.4*
Maximum rate of relengthening (–d*L*/d*t*)	Control	7	142 ± 15.5	15 ± 5.1	119 ± 21.7
	KB-R	8	113 ± 16.4	76 ± 19.8*	57 ± 12.4*
	P-1075	8	121 ± 20.7	68 ± 19.5*	79 ± 23.6
	KB-R + P-1075	7	159 ± 13.5	82 ± 15.6*	104 ± 17.3
	HMR	7	145 ± 17.7	12 ± 11.1[#]	43 ± 15.8*
	HMR + P-1075	10	113 ± 12.7	24 ± 10.7[#]	55 ± 13.7*
TPS (ms)	Control	7	165.1 ± 9.8	111.9 ± 14.6	162.2 ± 6.3
	KB-R	8	177.3 ± 11.0	180.1 ± 22.2	239.7 ± 8.2***
	P-1075	8	182.4 ± 17.2	157.3 ± 15.3	174.4 ± 14.1
	KB-R + P-1075	7	156.8 ± 7.4	129.2 ± 6.6	186.7 ± 11.4
	HMR	7	183.7 ± 7.8	168.5 ± 51.9	180.3 ± 12.3
	HMR + P-1075	10	185 ± 6.5	150.2 ± 13.6	180.1 ± 5.8
Contraction length, 90% (ms)	Control	7	313.1 ± 20.2	401.5 ± 30.8	338.8 ± 41.0
	KB-R	8	391.8 ± 29.5	458.6 ± 64.9	531.7 ± 43.7*
	P-1075	8	389.7 ± 47.5	397.5 ± 29.9	415.5 ± 41.1
	KB-R + P-1075	7	340 ± 13.6	359.4 ± 32.2	400.8 ± 22.2
	HMR	7	376.3 ± 21.9	468.1 ± 69.6	444 ± 73.9
	HMR + P-1075	10	396.9 ± 23.6	409.3 ± 40.0	478.7 ± 50.2

[a]KB-R, KB-R7943; HMR, HMR 1098; *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$ vs. control, [#]$P < 0.05$ vs. P-1075 group.

Table 3

TABLE 3. Properties of $[Ca^{2+}]_i$ transients in cardiomyocytes after MI/reoxygenation

				Reoxygenation	
		n	Basal	2 min	10 min
CaT_{90} (ms)	Control	9	539±31.3	587±54.0	524±18.8
	KB-R	11	511±16.4	512±28.5	546±28.5
	P-1075	11	567±17.4	574±26.1	556±15.3
	KB-R + P-1075	8	563±20.5	543±18.1	524±12.4
	HMR	6	526±35.8	649±89.5	576±39.7
	HMR + P-1075	7	542±14.7	727±142.2	554±10.2
Time to peak (ms)	Control	9	119±11.0	132±6.5	110±6.8
	KB-R	11	112±4.5	106±5.3	123±7.4
	P-1075	11	117±8.5	117±7.3	103±5.1
	KB-R + P-1075	8	106±5	109±6.9	104±5.0
	HMR	6	98±7.3	159±62.9	159±65.0
	HMR + P-1075	7	125±5.4	212±77.0	119±3.8
Amplitude (% of basal)	Control	9	100	64.3±13.2	93±8.1
	KB-R	11	100	93.9±11.3	94±4.1
	P-1075	11	100	64.6±9.8	85±7.1
	KB-R + P-1075	8	100	86±12.2	86.5±4.5
	HMR	6	100	87.7±19.0	93.3±8.0
	HMR + P-1075	7	100	53±8.6	90.8±7.2

Fig. 1

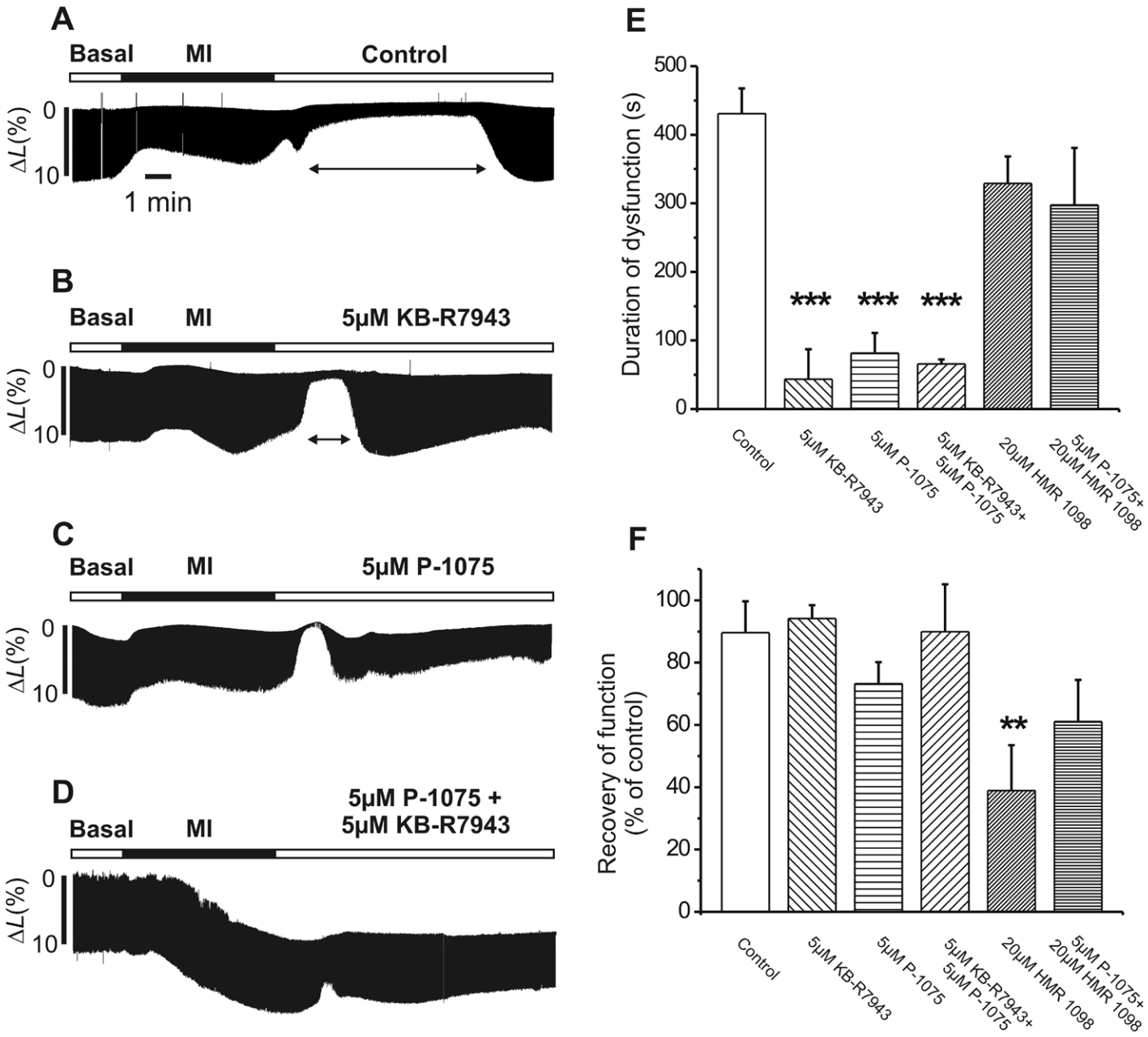

Figure 1. Representative cell shortening recordings from field-stimulated (1 Hz) rat ventricular myocytes subjected to 2 min of superfusion with control solution (basal), 6 min metabolic inhibition (MI), and 10 min reoxygenation with solution containing control vehicle (0.05% DMSO) (***A***), KB-R7943 (5 μM) (***B***), P-1075 (5 μM) (***C***), and P-1075 (5 μM) + KB-R7943 (5 μM) (***D***). ***E)*** Grouped data show that inhibition of the Na^+/Ca^{2+} exchanger (KB-R7943) as well as activation of *pm*K_{ATP} channels (P-1075) decrease the duration of post MI contractile dysfunction. ***F)*** Significant impairment of cell shortening was observed in cardiac myocytes superfused with the *pm*K_{ATP} channel blocker HMR 1098 at the end of the recovery period. *ΔL* (%): % change in cell length. ***A, B)*** Arrows denote duration of post-MI contractile dysfunction. ***A)*** Horizontal bar represents a 1 min interval. The duration of dysfunction was defined as the time period when the cell exhibited <50% of basal fractional cell shortening value in the reoxygenation period. n = 7–10 cells/group, $^{**}P < 0.01$, $^{***}P < 0.001$ vs. control group.

Fig. 2

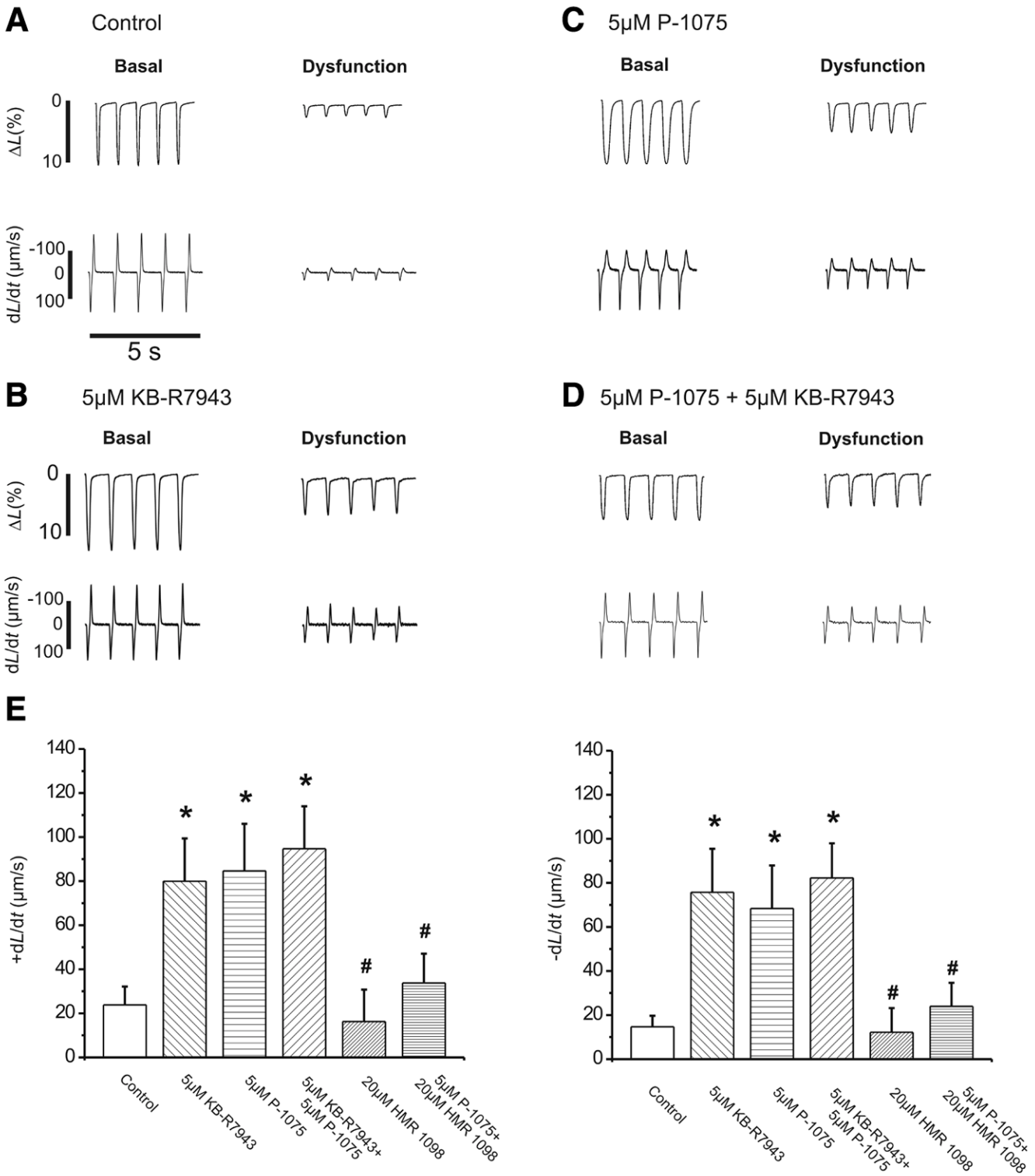

Figure 2. Pharmacological activation of *pm*K_{ATP} channels and inhibition of the Na^+/Ca^{2+} exchanger improve contractility of rat ventricular myocytes during early reoxygenation following metabolic inhibition (MI). Representative traces show changes in cell length (ΔL) and its first derivative (dL/dt) at the end of basal conditions and during contractile dysfunction in ventricular myocytes superfused with solution containing control vehicle (0.05% DMSO) (***A***), KB-R7943 (5 μM) (***B***), P-1075 (5 μM) (***C***), and KB-R7943 (5 μM) + P-1075 (5 μM) (***D***). ***E***) Grouped data show maximal rate of shortening and relengthening ($\pm dL/dt$) at 2 min reoxygenation following MI. n = 7–10 cells/group, $^*P < 0.05$ vs. control group, $^{\#}P < 0.05$ vs. P-1075 group.

Fig. 3

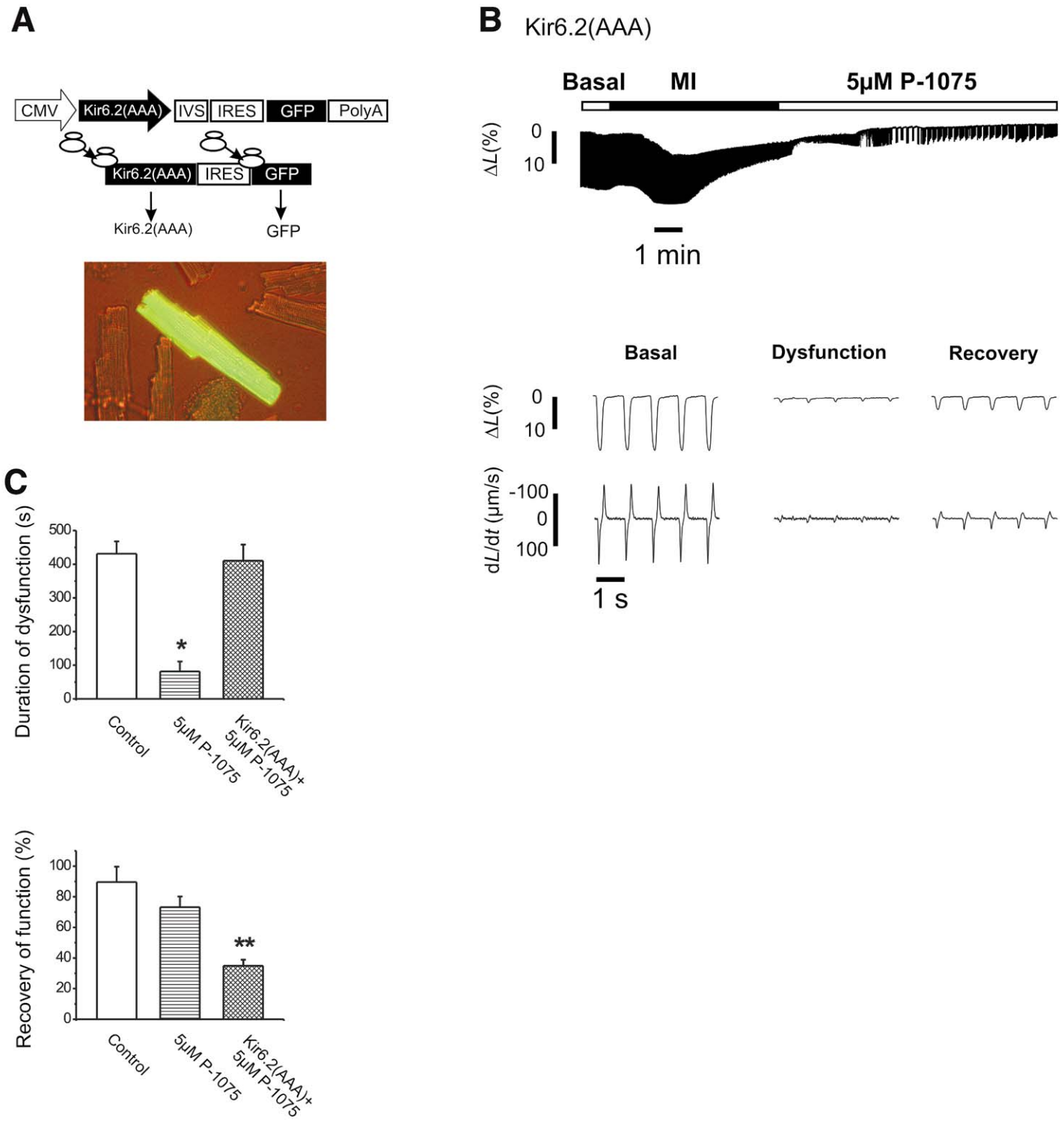

Figure 3. ***A*****)** Bicistronic adenoviral vector design and image of a GFP-tagged rat ventricular myocyte 96 h after in vivo infection with the dominant-negative Kir6.2(AAA) *pm*K_{ATP} channel adenoviral construct. ***B*****)** Representative cell shortening recording from a cardiac myocyte with the Kir6.2(AAA) construct subjected to 6 min metabolic inhibition (MI) and 10 min reoxygenation with control solution containing P-1075 (5 μM). ***C*****)** Expanded cell shortening traces and their first derivative as well as grouped data show that in cardiac myocytes with the Kir6.2(AAA) construct the *pm*K_{ATP} opener P-1075 failed to reduce the duration of post-MI contractile dysfunction and recovery of function was impaired. n = 4–7 cells/group, $*P < 0.05$, $**P < 0.01$ vs. control group.

Fig. 4

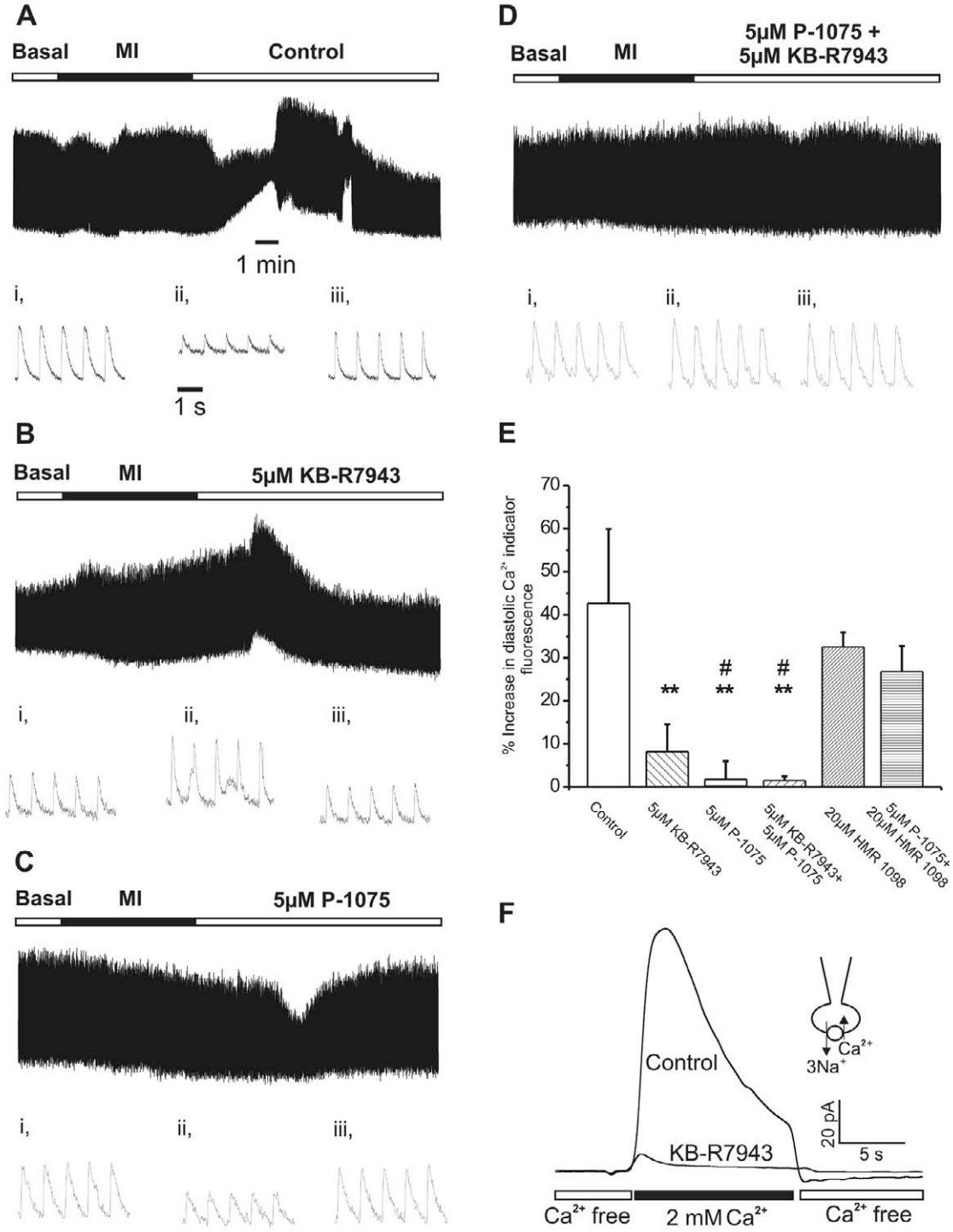

Figure 4. Representative Ca^{2+} transient recordings from field-stimulated (1 Hz) rat ventricular myocytes subjected to 6 min metabolic inhibition (MI) and 10 min reoxygenation with solution containing control vehicle (0.05% DMSO) (***A***), KB-R7943 (5 μM) (***B***), P-1075 (5 μM) (***C***), and P-1075 (5 μM) + KB-R7943 (5 μM) (***D***). Expanded Ca^{2+} transient traces were taken from the end of control (***i***), during peak dysfunction (***ii***), and at the end of reoxygenation periods (***iii***). ***E)*** Grouped data show significant reduction of reoxygenation-induced maximum increases in diastolic Ca^{2+} after activation of *pm*K_{ATP} channels (P-1075) as well as inhibition of the Na^+/Ca^{2+} exchanger (KB-R7943). ***F)*** Representative trace from a tsA201 cell expressing rat heart NCX1.1 shows block of NCX1.1 outward current by 5 μM KB-R7943 in a whole-cell patch-clamp configuration at a holding potential of 0 mV. Drugs were added to the reoxygenation solution only. ***A)*** Horizontal bars represent 1 min and 1 s intervals, respectively. Maximum increase in diastolic $[Ca^{2+}]_i$ was normalized to the amplitude of the Ca^{2+} transient at the end of control period in each cell. n = 7–11 cells/group, $^{**}P < 0.01$ vs. control group, $^{\#}P < 0.05$ vs. HMR 1098 group.

Fig. 5

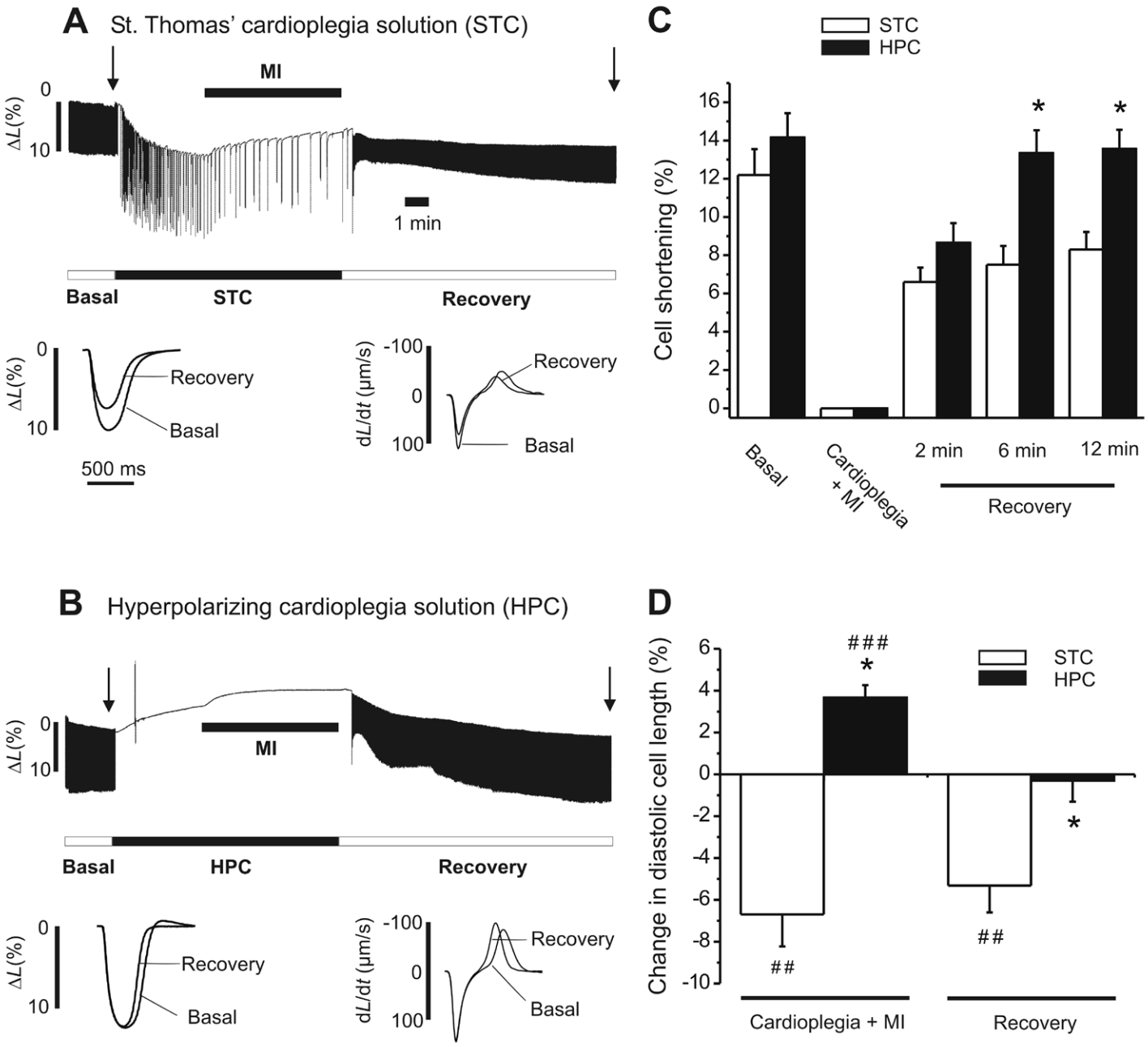

Figure 5. Representative cell shortening recordings from field-stimulated rat ventricular myocytes superfused with depolarizing St. Thomas' cardioplegia (STC), metabolic inhibition (MI), and reoxygenation solutions (***A***) and with hyperpolarizing cardioplegia (HPC), MI, and reoxygenation solutions (***B***). Myocytes in both groups were superfused with control solution during the recovery period. Cardiomyocytes in the STC group exhibited occasional large contractions during superfusion with the cardioplegia solution. This behavior was not observed in the HPC group. Note the improved post-MI contractile recovery in the HPC group (***B***) compared with the reduced contractility and rate of relaxation in the STC group (***A***) as demonstrated by individual cell shortening, rate of cell shortening traces (averaged for 10 steady-state beats), and grouped data (***C***). ***D)*** Diastolic cell length was significantly shorter in the STC group during cardioplegia + MI and at the end of the 12 min recovery periods. In the HPC group, diastolic cell length significantly increased during cardioplegia + MI and returned to control by the end of the recovery period. ***A, B)*** Arrows indicate time points where cell shortening traces were used to construct individual traces. $n = 7$ in all groups, $^{*}P < 0.05$ vs. STC group. $^{\#\#}P < 0.01$, and $^{\#\#}$ $^{\#}P < 0.001$ vs. control period in the same group.

Fig. 6

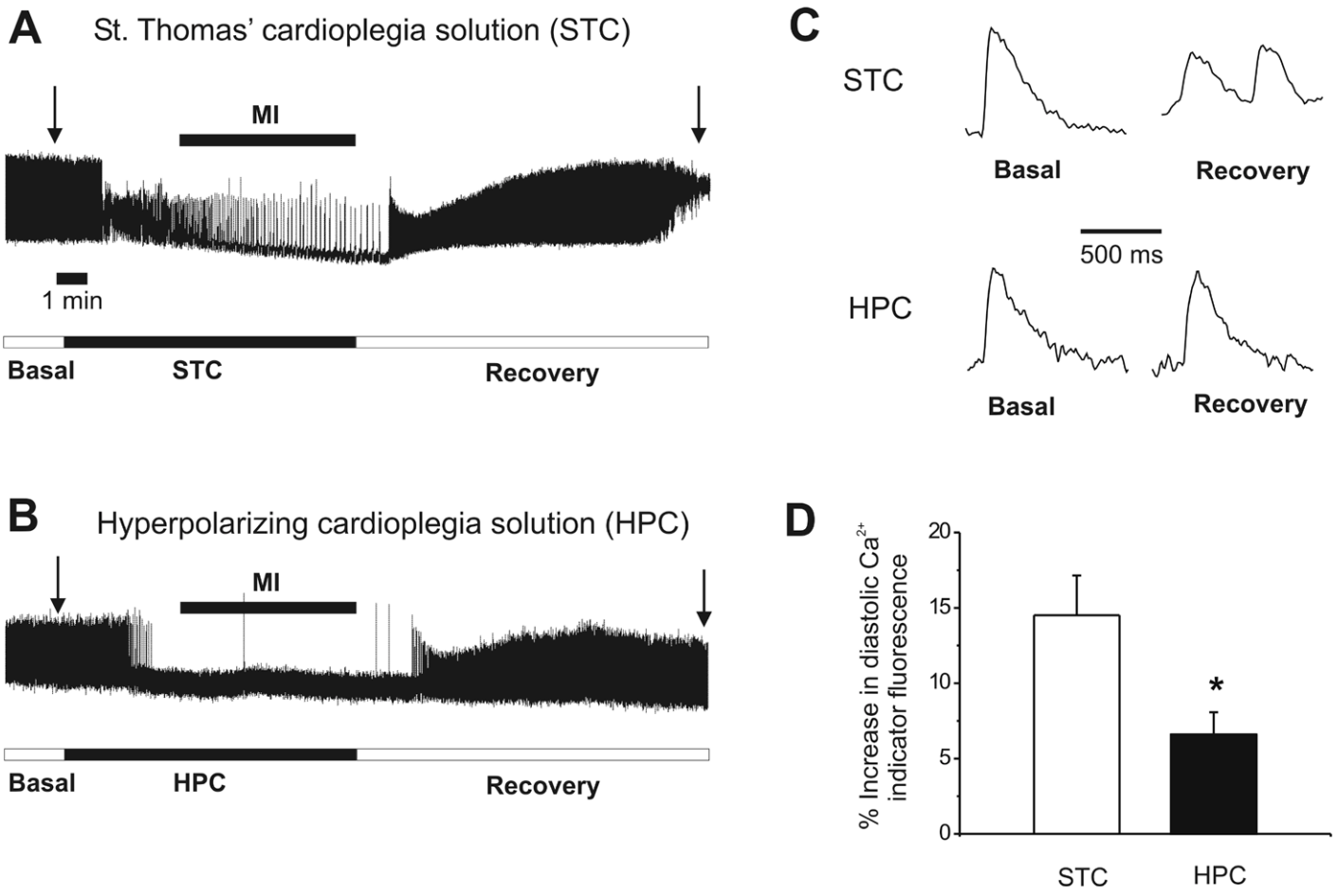

Figure 6. Representative Ca^{2+} transient recordings from field-stimulated rat ventricular myocytes superfused with St. Thomas' cardioplegia (STC) (***A***) or hyperpolarizing cardioplegia (HPC) (***B***) solutions before and during metabolic inhibition (MI). A marked increase in diastolic $[Ca^{2+}]_i$ was observed in myocytes superfused with STC during recovery as demonstrated by the full 24 min recordings (***A***, ***B***), individual Ca^{2+} transients (***C***), and grouped data (***D***). Arrows denote time points where individual Ca^{2+} transients were used to construct ***panel C***. $n = 8$ cells in all groups. $^*P < 0.05$ vs. STC group.

Fig. 7

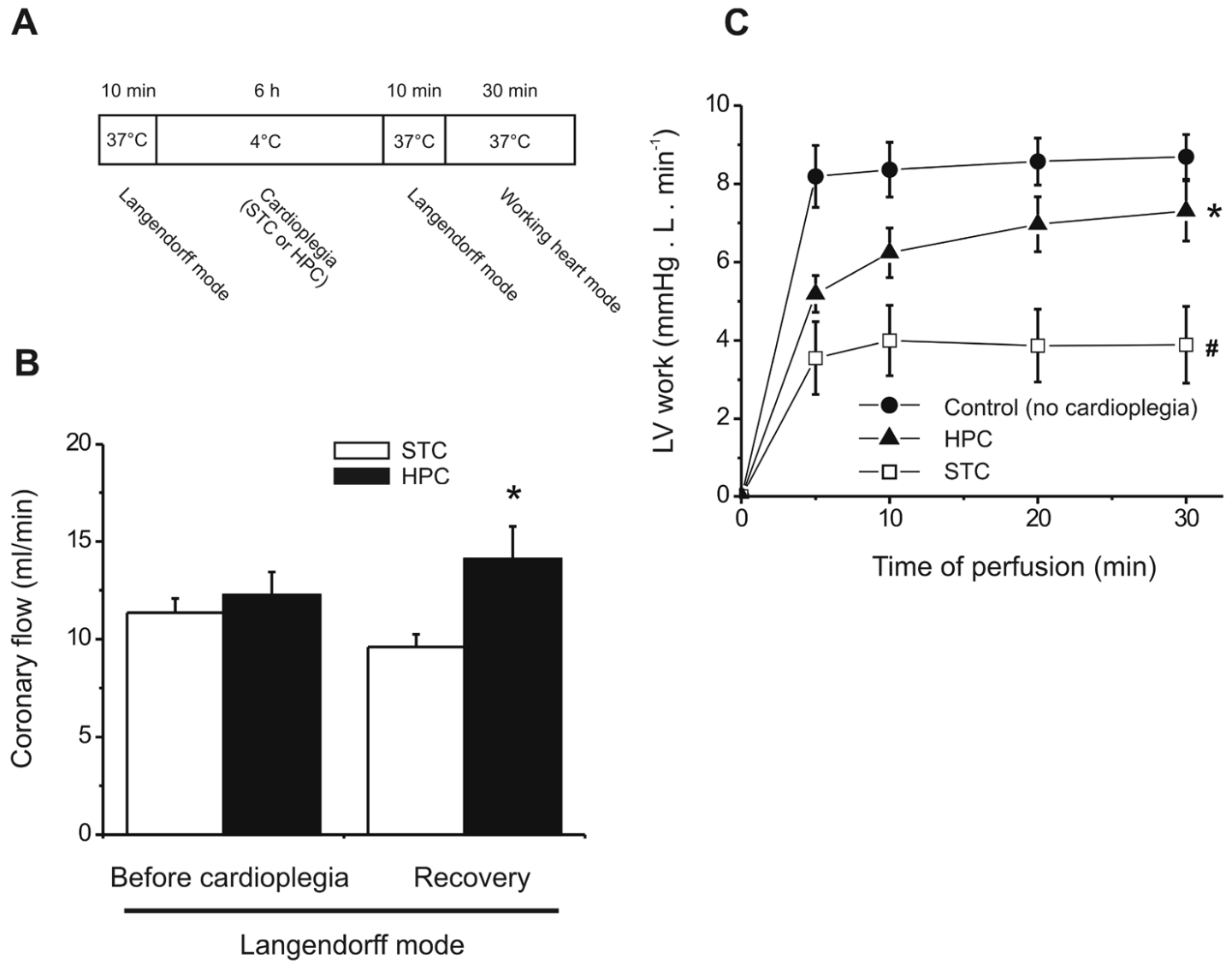

Figure 7. ***A*)** Heart perfusion protocol. Rat hearts underwent an initial 10 min Langendorff perfusion then were subjected to 6 h St. Thomas' cardoplegia (STC) or hyperpolarizing cardioplegia (HPC) and 10 min Langendorff reperfusion followed by 30 min working mode reperfusion. Grouped data show significantly increased coronary artery flow at 10 min recovery in the HPC group compared with the STC group (during Langendorff mode) (***B***), and significantly improved left ventricular (LV) work in the HPC group at the end of 30 min recovery period in working heart configuration (n=7–8 hearts/group) (***C***). *$P < 0.05$ vs. STC group. $^{\#}P < 0.05$ vs. control group.