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L-Arginine ameliorates effects of ischemia and reperfusion in isolated cardiac myocytes

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Abstract

We determined effects of the nitric oxide (NO) precursor L-arginine, on isolated guinea pig ventricular myocytes under normoxic conditions and simulated ischemia and reperfusion. Currents and contractions were recorded with voltage clamp and a video edge detector, respectively. In normoxia, L-arginine (50–200 μM) had little effect on Ca^{2+} current, but significantly decreased contraction. Ischemia in the absence of L-arginine reduced Ca^{2+} current and abolished contractions. In reperfusion, the arrhythmogenic transient inward current was induced and cells exhibited sustained contractile depression (stunning). With L-arginine (100 μM) in ischemia, Ca^{2+} current did not decline and recovery of contraction was potentiated in reperfusion. L-Arginine had no effect on transient inward current. Inhibition of nitric oxide synthase reversed effects of L-arginine on contractions but not Ca^{2+} current. Thus, NO contributes to beneficial effects of L-arginine in reperfusion, although effects on $I_{Ca\text{-}L}$ are independent of NO. Further, L-arginine effects differ under normoxic and ischemic conditions.

Keywords: Ca^{2+} current, L-type; Nitric oxide (NO); Contraction; Excitation–contraction coupling

1. Introduction

Nitric oxide (NO) is one of the endogenous substances released in myocardial ischemia and reperfusion (Depré et al., 1996; Zweier et al., 1995). Many studies have examined effects of NO supplementation, in the form of NO donors or the NO precursor L-arginine, in isolated perfused hearts and in in situ hearts in ischemia and reperfusion. These studies have shown that NO supplementation in ischemia and reperfusion reduces the area of cardiac necrosis, augments recovery of contractile function and improves metabolic function in reperfusion (Draper and Shah, 1997; Li et al., 1996; Node et al., 1996; Schulz and Wambolt, 1995; Weyrich et al., 1992; Williams et al., 1995). NO also has been shown to reduce the incidence of arrhythmias in ischemia and reperfusion (Pagliaro et al., 2001). Thus, NO is thought to be protective in myocardial ischemia and reperfusion.

Paradoxically, inhibition of nitric oxide synthase, the enzyme responsible for synthesis of NO, also has been shown to be cardioprotective in ischemia and reperfusion (Patel et al., 1993; Schulz and Wambolt, 1995; Zweier et al., 1995). Indeed, hearts treated with nitric oxide synthase inhibitors show improved recovery of mechanical function and reduced infarct size in reperfusion (Patel et al., 1993; Schulz and Wambolt, 1995; Zweier et al., 1995). Detrimental effects of NO are believed to be due to oxygen-free radical production. Mitochondria are known to produce the superoxide anion in vivo (Packer and Murphy, 1995). NO can react with the superoxide anion to form peroxynitrite anion, a strong oxidant that can cause lipid peroxidation and cell damage (Beckman et al., 1990). Thus, NO is believed to have both detrimental and beneficial effects in myocardial ischemia and reperfusion.

The beneficial effects of NO in ischemia and reperfusion are generally believed to be the result of its actions on non-cardiac cells. NO supplementation increases coronary vasodilatation, inhibits platelet aggregation, and inhibits platelet and neutrophil adhesion to the endothelium (Pabla et al., 1996). However, NO also may exert direct effects on cardiac myocytes. The NO precursor L-arginine improves cell survival in a model of anoxia and reoxygenation in isolated

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human ventricular myocytes (Shiono et al., 2002). In addition, NO is believed to have direct effects on electrophysiologic and contractile properties of cardiac myocytes. NO supplementation has minimal effects on L-type Ca^{2+} current ($I_{Ca\text{-}L}$) under basal conditions, but inhibits $I_{Ca\text{-}L}$ in the presence of ß-adrenoceptor stimulation (Wahler and Dollinger, 1995). In addition, NO supplementation inhibits $I_{Ca\text{-}L}$ in myocytes isolated from transplanted hearts undergoing rejection (Ziolo et al., 2001a). NO also inhibits cell shortening in isolated cardiac myocytes (Brady et al., 1992, 1993; Kojda et al., 1996). Therefore, direct effects of NO on cardiac myocytes might contribute to the beneficial or detrimental effects of NO in ischemia and reperfusion. However, whether NO impacts upon electrophysiologic or contractile properties of cardiac myocytes in myocardial ischemia and reperfusion has not been investigated.

The objective of this study was to determine whether the NO precursor, L-arginine, affects membrane currents and contractions in isolated cardiac myocytes exposed to ischemia and reperfusion. We utilized an isolated cell model of simulated ischemia and reperfusion which we developed previously (Cordeiro et al., 1994; Louch et al., 2000, 2002) to examine effects of L-arginine on ionic currents and contractions in guinea pig ventricular myocytes under normoxic conditions and in cells exposed to simulated ischemia and reperfusion.

2. Materials and methods

2.1. Cell isolation

Experiments were conducted on male and female guinea pigs (325–425 g) purchased from Charles River (St. Constant, Quebec). The animals were cared for by the Dalhousie University Animal Care Facility, in accordance with the guidelines on the Care and Use of Experimental Animals set by the Canadian Council on Animal Care (Two Volumes, Ottawa, Ontario: Canadian Council on Animal Care, Volume 1, 1993; Volume 2, 1984). The protocol was approved by the Dalhousie University Committee for Laboratory Animals. Ventricular myocytes were obtained by enzymatic dissociation with techniques similar to those described previously (Cordeiro et al., 1994; Louch et al., 2000, 2002). Briefly, animals were weighed, injected with heparin (3.3 IU/g) and anesthetized with sodium pentobarbital (80 mg/kg). The heart was cannulated through the aorta and perfused retrogradely for 7 min with oxygenated (100% O_2) Ca^{2+}-free solution (36 °C) of the following composition (mM): 120 NaCl, 4 KCl, 1.2 KH_2PO_4, 1.2 $MgSO_4$, 10 HEPES and 12 glucose (pH 7.4 with NaOH). The perfusate then was changed to the solution described above supplemented with collagenase A (20–25 mg; Boehringer Mannheim) and protease type XIV (4.8 mg; Sigma) and the heart was perfused for an additional 5 min. The ventricles then were removed and washed with a high-K^+ substrate-rich solution of the following composition (mM): 80 KOH, 30 KCl, 30 KH_2PO_4, 3 $MgSO_4$, 50 glutamic acid, 20 taurine, 0.5 EGTA, 10 HEPES and 10 glucose (pH 7.4 with KOH). Finally, the ventricles were minced, and single ventricular myocytes were obtained by swirling the minced ventricular tissue in high-K^+ solution.

Myocytes in high potassium substrate rich solution were placed in a modified Petri dish in an open perfusion microincubator (PDMI, Medical Systems) mounted on the stage of an inverted microscope. The microscope was located on a vibration-isolated workstation. Cells were allowed to adhere to the bottom of the chamber for 30 min before superfusion (3 ml min^{-1}, 37 °C) with Tyrode's solution of the following composition (mM): NaCl, 129; $NaHCO_3$, 20; NaH_2PO_4, 0.9; KCl, 4; $MgSO_4$, 0.5; $CaCl_2$, 2.5; glucose, 5.5; pH = 7.4, gassed with 95% O_2, 5% CO_2. A bipolar temperature controller (Model TC-202, Medical Systems) was used to maintain temperature between 36 and 37 °C in all experiments.

2.2. Experimental methods

Discontinuous single electrode voltage clamp recordings (sample rate 7–9 kHz) were made with an Axoclamp 2B amplifier (Axon Instruments, Foster City, CA). Recordings were made with high resistance microelectrodes (18–23 MΩ, filled with 2.7 M KCl) to reduce cell dialysis and to avoid buffering intracellular Ca^{2+} levels. Current and transmembrane voltage were recorded in all experiments. Cells were visualized with a closed circuit television camera and were displayed on a video monitor. Unloaded cell shortening was sampled at 120 Hz with a video edge detector (Crescent Electronics, Sandy, UT, USA) coupled to the camera. pClamp 6.1 software (Axon Instruments) was used

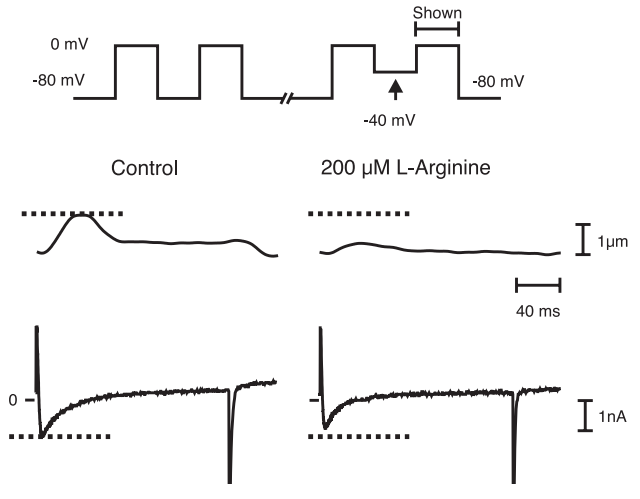

Fig. 1. L-Arginine attenuates contraction under normoxic conditions. Representative original recordings of $I_{Ca\text{-}L}$ and contraction from a myocyte in the absence of drug (left) and in the presence of 200 µM L-arginine (right). The voltage clamp protocol is illustrated at the top. L-Arginine caused a slight reduction in the amplitude of $I_{Ca\text{-}L}$ and a marked reduction in amplitude of contraction.

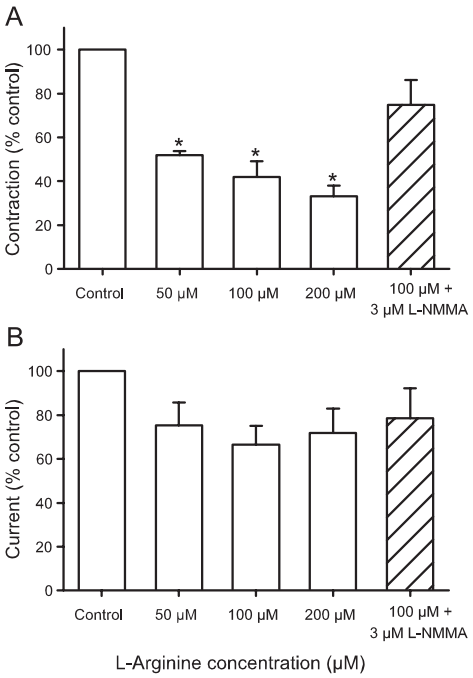

Fig. 2. L-Arginine significantly decreases contractions in isolated myocytes under normoxic conditions. (Panel A) Mean data demonstrate that the effect of L-arginine on contractions is statistically significant. The effect of L-arginine on contractions was prevented by the nitric oxide synthase inhibitor, L-NMMA. (Panel B) Mean data illustrate that L-arginine slightly reduced $I_{Ca\text{-}L}$, although this effect was not statistically significant. L-NMMA had no effect on $I_{Ca\text{-}L}$ ($n=3$ cells per group, paired data; * denotes $P<0.05$).

to generate voltage clamp protocols and to acquire and analyze data on a computer. Current, voltage and contractions were digitized with a Labmaster A/D interface at 125 kHz (TL1-125, Axon Instruments) and stored for subsequent analysis.

For experiments on cells under normoxic conditions, cells were superfused with Tyrode's solution in the absence and presence of increasing concentrations of L-arginine (50–200 μM). Drug concentration was increased at 5-min intervals. In a few experiments, cells were exposed to L-arginine (100 μM) plus the nitric oxide synthase inhibitor, N^G-monomethyl-L-arginine (L-NMMA, 3 μM). For experiments under simulated "ischemic" conditions, the following protocol was followed. After 5–10 min of control recordings in Tyrode's solution, cells were exposed to simulated ischemia for 20 min. The ischemic solution had the following composition (mM): NaCl, 123; $NaHCO_3$, 6; NaH_2PO_4, 0.9; KCl, 8; $MgSO_4$, 0.5; $CaCl_2$, 2.5; Na-lactate, 20; gassed with 90% N_2/10% CO_2, pH = 6.8. In addition, a 90% N_2/10% CO_2 gas phase was layered over the micro-incubator throughout simulated ischemia. The gas phase was delivered through an inlet located on the top of the micro-incubator (PDMI, Medical Systems). The gas was delivered to outlets around the upper edge of the incubator, which directed a layer of gas across the surface of the solution. Reperfusion was simulated by return to Tyrode's solution for 30 min. Cells received either no drug or were exposed to 100 μM L-arginine in ischemia only. In some experiments, cells were exposed to 100 μM L-arginine plus the nitric oxide synthase inhibitor L-NMMA (3 μM) in ischemia. Cells were exposed to only one cycle of ischemia and reperfusion.

Cells were voltage clamped at a holding potential of − 80 mV, as described below. The following protocols were run: *Protocol* 1. After ten 200–ms conditioning pulses from the holding potential to 0 mV separated by 150-ms intervals, a 180-ms test step was made from a post-conditioning potential of − 40 mV to a test potential of 0 mV before returning to − 80 mV. This protocol was used to elicit contraction and $I_{Ca\text{-}L}$. Current–voltage relations were then determined with

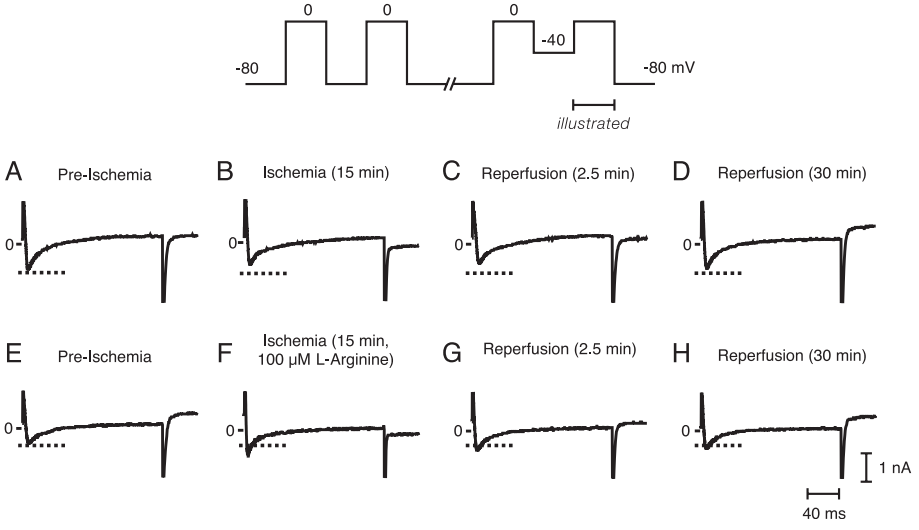

Fig. 3. Representative original recordings of $I_{Ca\text{-}L}$ from cells exposed to simulated ischemia and reperfusion in the absence and presence of L-arginine in ischemia. Voltage clamp protocol is illustrated at the top. In the absence of L-arginine, $I_{Ca\text{-}L}$ decreased slightly in ischemia (Panels A and B), with little recovery in reperfusion (Panels C and D). In the presence of L-arginine, $I_{Ca\text{-}L}$ was preserved in ischemia (Panel E and F) and reperfusion (Panels G and H). Dotted line denotes amplitude of $I_{Ca\text{-}L}$ prior to ischemia.

a similar protocol, but the test potential was changed in 10-mV steps from −40 to +80 mV. This protocol was repeated at 5-min intervals throughout the experiment. *Protocol* 2. From the holding potential, sequential steps were made to −40 and +20 mV for 300 ms each, followed by a 900-ms hyperpolarization to test potentials between −100 and −30 mV before returning to −80 mV. This protocol was used to detect the occurrence of the arrhythmogenic transient inward current (I_{TI}) and was repeated at 2.5-min intervals in early reperfusion.

2.3. Data measurement and analyses

Cell shortening was measured as the difference between the peak contraction and the baseline preceding contraction. Magnitude of $I_{Ca\text{-}L}$ was measured as the difference between peak inward current and net current 200 ms later on the same test step. I_{TI} incidence was counted as the number of cells in which I_{TI} was observed; incidence of aftercontractions was the number of cells in which aftercontractions were observed. Data other than incidence are presented as means ± S.E.M. Differences between means were assessed with a Student's *t*-test or with either one-or two-way analysis of variance (SYSTAT v7.0.1, SPSS). The non-parametric Chi-square test was used to determine whether the incidences of I_{TI} or aftercontractions were affected by drug treatment in ischemia and reperfusion (Sigmastat, Jandel Scientific). Differences were considered significant for $P<0.05$. No more than two cells from the same heart were used for any experiment.

2.4. Chemicals

L-Arginine was purchased from the Sigma (St. Louis, MO). L-NMMA was purchased from Calbiochem-Novabiochem (La Jolla, CA). Chemicals for buffer solutions were purchased from BDH (Toronto, ON), Fisher Scientific (Nepean, ON) and Sigma.

3. Results

Initially, we determined whether L-arginine had effects on $I_{Ca\text{-}L}$ and contractions under our experimental conditions in the absence of ischemia. In these experiments, cells were voltage clamped with the protocol shown at the top of Fig. 1. Following a train of 10 conditioning pulses from −80 to 0 mV, cells were repolarized to −40 mV to maintain inactivation of sodium current. A test step to 0 mV was then utilized to activate $I_{Ca\text{-}L}$ and contraction. Fig. 1 shows representative original recordings of $I_{Ca\text{-}L}$ and contraction in a ventricular myocyte under control conditions and in the presence of 200 μM L-arginine. L-Arginine caused a slight reduction in magnitude of $I_{Ca\text{-}L}$ and a substantial reduction in the amplitude of contraction. The mean data illustrated in Fig. 2A show that L-arginine caused a significant, concentration-dependent decrease in the amplitude of contraction. L-Arginine also slightly decreased the amplitude of $I_{Ca\text{-}L}$, although this effect was not statistically significant (Fig. 2B). Next, we utilized the nitric oxide synthase inhibitor, L-NMMA, to determine whether the effects of L-arginine were mediated by NO. When cells were superfused with 100 μM L-arginine plus 3 μM L-NMMA, the effects of L-arginine on contraction were prevented (Fig. 2A). The combination of L-NMMA plus L-arginine had little effect on

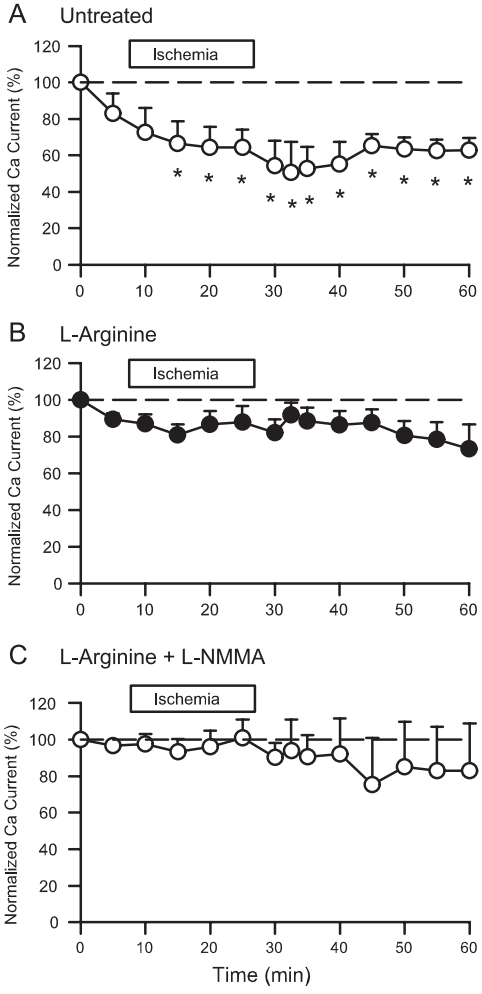

Fig. 4. The reduction in magnitude of $I_{Ca\text{-}L}$ in ischemia and reperfusion is abolished by L-arginine in the absence or presence of L-NMMA. $I_{Ca\text{-}L}$ was elicited with test voltage steps from −40 to 0 mV, as shown in Figs 1 and 3. (Panel A) Mean magnitudes of $I_{Ca\text{-}L}$ measured during simulated ischemia and reperfusion in untreated cells. $I_{Ca\text{-}L}$ was reduced throughout ischemia and reperfusion in untreated cells. (Panel B) Mean magnitudes of $I_{Ca\text{-}L}$ during ischemia and reperfusion in cells exposed to 100 μM L-arginine in ischemia. $I_{Ca\text{-}L}$ did not decline significantly in cells treated with L-arginine. (Panel C) Mean magnitudes of $I_{Ca\text{-}L}$ during ischemia and reperfusion in cells exposed to 100 μM L-arginine plus 3 μM L-NMMA in ischemia. Inclusion of L-NMMA did not abolish the effect of L-arginine on $I_{Ca\text{-}L}$. In all panels, data are expressed as the mean ± S.E.M. and are normalized to the value at time 0. The mean values for raw data values at time 0 are −1.21 ± 0.06, −1.15 ± 0.14 and −1.03 ± 0.15 nA for data in panels A, B and C, respectively. Asterisks illustrate points that are significantly different from preischemic responses ($P<0.05$; $n=10–19$ cells per group).

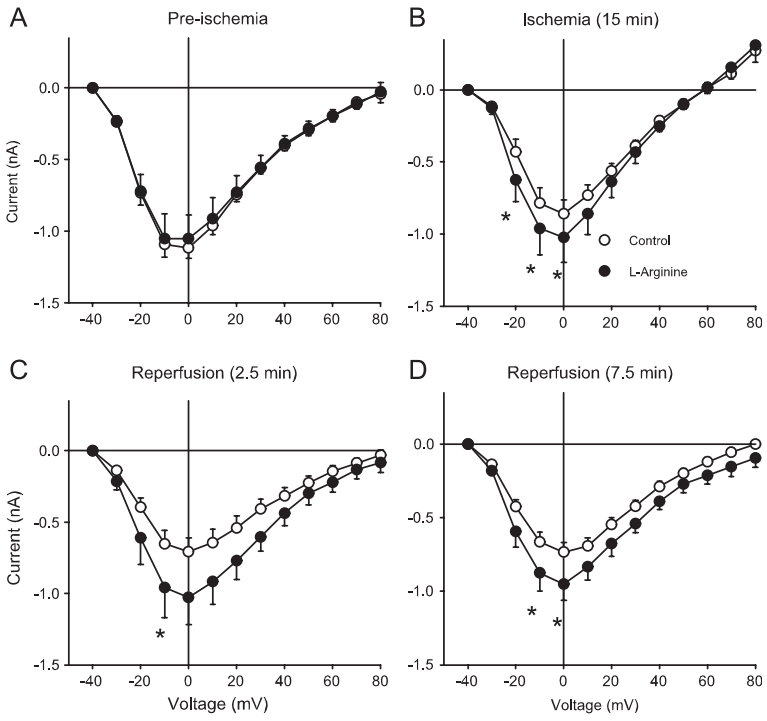

Fig. 5. Mean current–voltage relations for peak $I_{Ca\text{-}L}$ demonstrate that ischemia reduced $I_{Ca\text{-}L}$ in control cells but not in L-arginine treated cells. (Panel A) Current–voltage curves were similar in the two groups prior to ischemia. (Panel B) Ischemia reduced the magnitude of $I_{Ca\text{-}L}$ in control cells but not in cells treated with 100 μM L-arginine during ischemia. (Panels C and D) $I_{Ca\text{-}L}$ remained depressed in control cells in early reperfusion, but not in cells exposed to L-arginine in ischemia (* denotes $P < 0.05$; $n = 10–19$ cells per group).

the magnitude of $I_{Ca\text{-}L}$ (Fig. 2B). To ensure that a time-dependent decline in amplitudes of currents and contractions did not contribute to effects of L-arginine, responses also were recorded in the absence of drug. Amplitudes of contractions remained 96 ± 4% of starting values after 20 min of recording and amplitudes of currents were 96.6 ± 9% of starting values after 20 min.

Fig. 3 shows representative original recordings of $I_{Ca\text{-}L}$ in cells exposed to simulated ischemia and reperfusion in the absence and presence of L-arginine in ischemia. The voltage

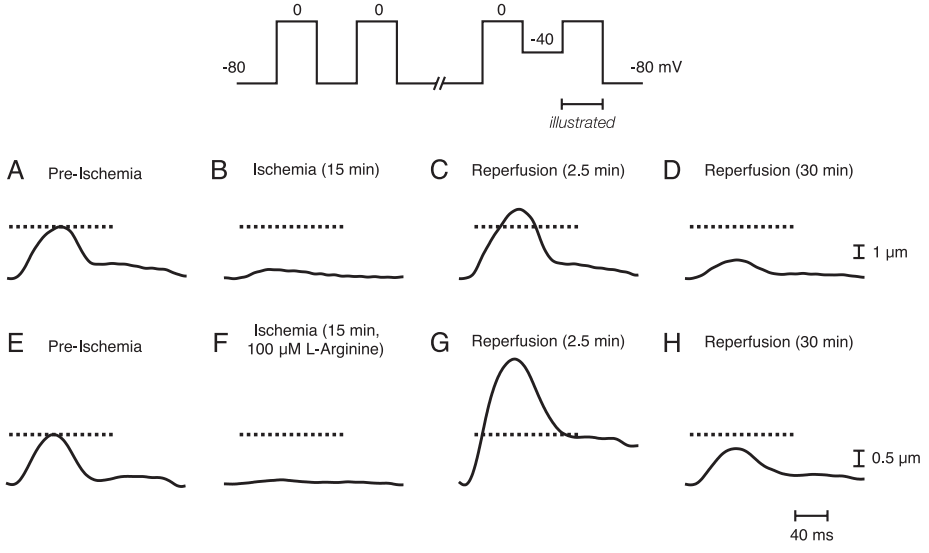

Fig. 6. Representative original recordings of contractions from cells exposed to ischemia and reperfusion in the absence and presence of L-arginine in ischemia. Voltage clamp protocol is illustrated at the top. In the absence of L-arginine, contractions were abolished during ischemia (Panels A and B), recovered in early reperfusion (Panel C) and then exhibited contractile depression in late reperfusion (Panel D). When L-arginine was included in the ischemic solution, contractions were abolished during ischemia (Panels E and F). However, contractions were potentiated in early reperfusion (Panel G) and there was little evidence for contractile depression in late reperfusion (Panel H). Dotted line denotes amplitude of contractions prior to ischemia.

clamp protocol is shown at the top of the figure. Initially, we determined the effects of ischemia and reperfusion on $I_{Ca\text{-}L}$ in the absence of L-arginine. Fig. 3 shows recordings of $I_{Ca\text{-}L}$ prior to ischemia (Fig. 3A), after 15 min of ischemia (Fig. 3B), at 2.5 min of reperfusion (Fig. 3C) and at 30 min of reperfusion (Fig. 3D). $I_{Ca\text{-}L}$ appeared to decline during ischemia and remained depressed in reperfusion. Next, we determined the effects of ischemia and reperfusion on $I_{Ca\text{-}L}$ when 100 μM L-arginine was included in the ischemic solution. Fig. 3E–H shows original recordings of $I_{Ca\text{-}L}$ in a myocyte exposed to ischemia and reperfusion in the presence of L-arginine in ischemia. There was no reduction in magnitude of $I_{Ca\text{-}L}$ in either ischemia or reperfusion when L-arginine was present (Fig. 3G,H).

Mean magnitudes of $I_{Ca\text{-}L}$ in the absence and presence of L-arginine were plotted as a function of time throughout ischemia and reperfusion as shown in Fig. 4. Fig. 4A shows that $I_{Ca\text{-}L}$ decreased significantly during ischemia in the absence of L-arginine, and did not recover to pre-ischemic levels in reperfusion. However, when L-arginine was included in the superfusate during ischemia, $I_{Ca\text{-}L}$ remained close to pre-ischemic levels throughout the entire cycle of ischemia and reperfusion (Fig. 4B). To determine whether effects of L-arginine on $I_{Ca\text{-}L}$ were mediated by NO, additional experiments were conducted in the presence of the nitric oxide synthase inhibitor, L-NMMA. Fig. 3C shows that the effects of L-arginine on $I_{Ca\text{-}L}$ were not abolished when both L-arginine and L-NMMA were included in the superfusate during ischemia. Inclusion of 3 μM L-NMMA alone in ischemia had no effect on the changes in magnitude of $I_{Ca\text{-}L}$ throughout ischemia and reperfusion (data not shown).

To determine whether L-arginine altered the voltage-dependence of $I_{Ca\text{-}L}$, we examined current–voltage relations for $I_{Ca\text{-}L}$ throughout ischemia and reperfusion in the absence and presence of L-arginine in ischemia. Fig. 5 shows current–voltage relations for $I_{Ca\text{-}L}$ in the absence and presence of L-arginine. Prior to ischemia, current–voltage relations were similar in the two groups (Fig. 5A). After 15 min of ischemia, peak $I_{Ca\text{-}L}$ was decreased across most of the voltage range in untreated cells, but not in cells exposed to L-arginine in ischemia (Fig. 5B). Peak $I_{Ca\text{-}L}$ did not recover in reperfusion in untreated cells (Fig. 5C,D). These results show that L-arginine prevented depression of $I_{Ca\text{-}L}$ without altering the shape of the current–voltage relation and without altering its voltage-dependence.

Fig. 6 shows representative original recordings of contractions recorded from cells exposed to simulated ischemia and reperfusion in the absence and presence of L-arginine in ischemia. Contractions were activated by a voltage step from −40 to 0 mV, as shown in the protocol at the top of the figure. We first determined the effects of ischemia and reperfusion on contraction in the absence of L-arginine. Fig. 6 shows contractions recorded prior to ischemia (Fig. 6A), after 15 min of ischemia (Fig. 6B), at 2.5 min of reperfusion (Fig. 6C) and at 30 min of reperfusion (Fig. 6D). This example shows that contractions were abolished in ischemia, increased in early reperfusion and then were markedly depressed in late reperfusion. We next determined effects of ischemia and reperfusion on contractions in cells exposed to 100 μM L-arginine in ischemia. Fig. 6E–H shows contractions recorded from a myocyte exposed to simulated ischemia and reperfusion when L-arginine was included in the ischemic solution. In the presence of L-arginine, contractions also were abolished in ischemia (Fig. 6F). However, in the cell exposed to L-arginine, contractions in early reperfusion exhibited a rebound to amplitudes which greatly exceeded

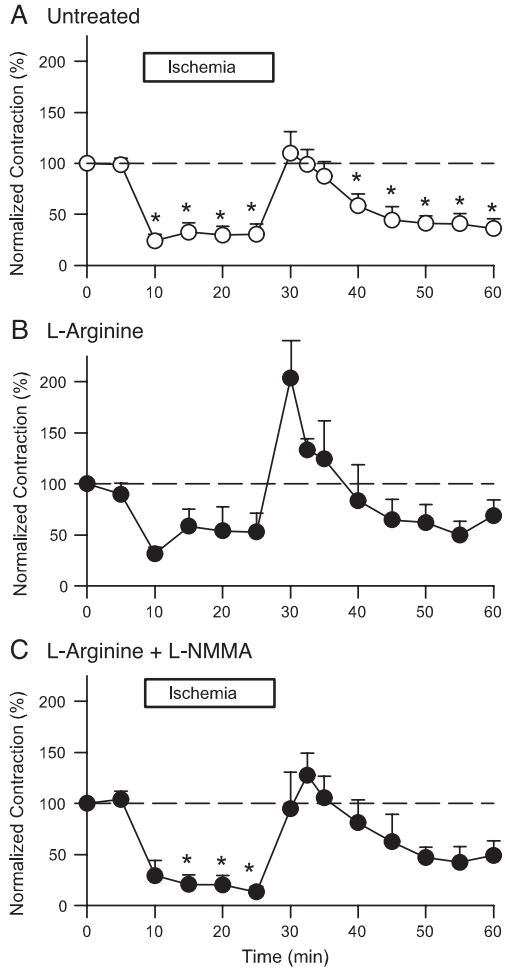

Fig. 7. Recovery of contraction in reperfusion is augmented in cells exposed to L-arginine in ischemia. Contractions were elicited with test voltage steps from −40 to 0 mV, as shown in Figs 1 and 3. (Panel A) In untreated cells, contractions were inhibited during ischemia, recovered briefly and then exhibited sustained contractile depression known as stunning in reperfusion. (Panel B) In cells treated with 100 μM L-arginine during ischemia, there was an overshoot in contraction in early reperfusion and recovery of contraction was improved in late reperfusion. (Panel C) The overshoot in contraction in early reperfusion was abolished when cells were exposed to 100 μM L-arginine plus 3 μM L-NMMA in ischemia. Data are expressed as the mean ± S.E.M. and are normalized to the value at time 0. The mean values for raw data at time 0 are 1.9 ± 0.2, 1.6 ± 0.2 and 1.5 ± 0.3 μm for data in panels A, B and C, respectively. Asterisks illustrate points that are significantly different from preischemic responses ($P<0.05$; $n=10–19$ cells group).

pre-ischemic levels (Fig. 3G) and contractile depression later in reperfusion was minimal (Fig. 3H).

The mean magnitude of contraction in the absence and presence of L-arginine was plotted as a function of time throughout ischemia and reperfusion as shown in Fig. 7. Fig. 7A shows that, in the absence of L-arginine, contractions decreased in ischemia, recovered transiently in reperfusion and then exhibited contractile depression or stunning (Louch et al., 2000, 2002). Fig. 7B shows that contractions also were depressed in ischemia when L-arginine was included in the superfusate. However, recovery of contractions in early reperfusion was greatly potentiated in cells exposed to L-arginine, and stunning was not significant in reperfusion (Fig. 7B). To determine whether effects of L-arginine on contraction were mediated by NO, additional experiments included the nitric oxide synthase inhibitor, L-NMMA. When both L-arginine and L-NMMA were included in the superfusate during ischemia, the overshoot in contractions in early reperfusion was abolished (Fig. 7C). In addition, contractile depression was observed in late reperfusion although this was not statistically significant (Fig. 7C). Inclusion of 3 μM L-NMMA alone in ischemia had no effect on the changes in amplitudes of contraction in ischemia and reperfusion (data not shown).

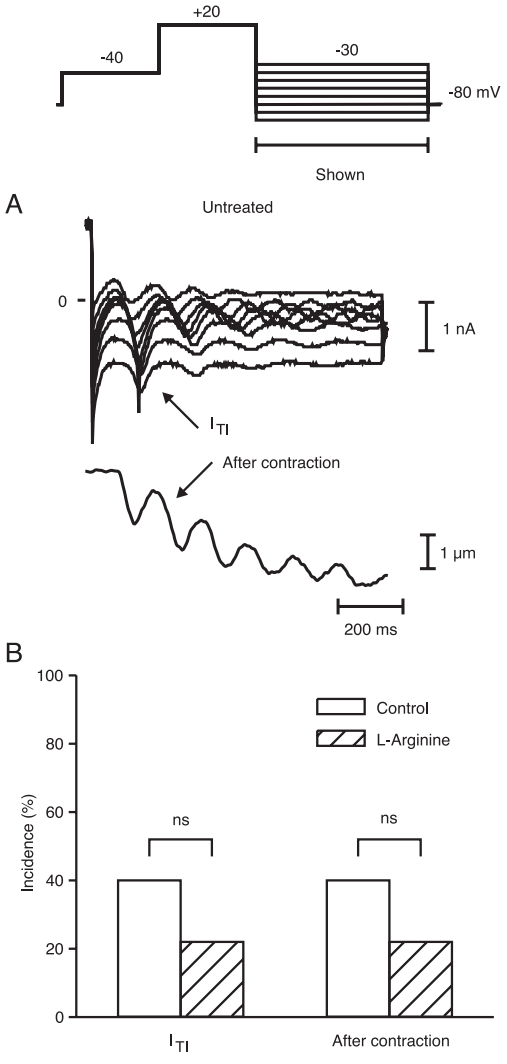

Fig. 8. L-Arginine does not affect induction of I_{TI} and aftercontractions in early reperfusion. Voltage clamp protocol is illustrated at the top. (Panel A) Representative recordings of current and contraction from a cell in early reperfusion in the absence of drug. I_{TI} (top) and aftercontractions (bottom) were observed. (Panel B) Incidences of I_{TI} and aftercontractions were similar in control cells and in cells exposed to L-arginine in ischemia ($n = 9–20$ myocytes per group). n.s. denotes not significantly different from control by Chi square analysis.

The arrhythmogenic current, I_{TI}, can be induced in isolated myocytes exposed to simulated ischemia and reperfusion (Cordeiro et al., 1994; Louch et al., 2000). Here we determined whether the incidence of I_{TI} in early reperfusion was altered in cells superfused with L-arginine in ischemia. The voltage clamp protocol is shown at the top of Fig. 8. From the holding potential of −80 mV, voltage steps were made to −40 and then +20 mV. Cells were then hyperpolarized for 900 ms to potentials between −100 and −30 mV to observe I_{TI}. Fig. 8A shows representative recordings of I_{TI} (top) and aftercontractions (bottom) at 2.5 min of reperfusion in the absence of L-arginine. The recording of I_{TI} illustrates the typical wave-like pattern of this current, which is time-locked to repolarization at different test potentials. The contraction recording below shows aftercontractions associated with I_{TI} upon repolarization to −60 mV. Fig. 8B shows the incidence of I_{TI} within the first 5 min of reperfusion in the absence and presence of L-arginine in ischemia. The incidence of I_{TI} was slightly reduced in the presence of L-arginine, but this was not statistically significant. Thus, inclusion of L-arginine in ischemia did not promote this arrhythmogenic current in reperfusion.

4. Discussion

The objective of this study was to determine whether the NO precursor, L-arginine, affects membrane currents and contractions in isolated cardiac myocytes exposed to ischemia and reperfusion. Our results showed that, under normoxic conditions, L-arginine had only minimal effects on the magnitude of $I_{Ca\text{-}L}$, but markedly reduced magnitudes of contractions in guinea pig ventricular myocytes. This inhibitory effect of L-arginine on contraction was largely prevented by the nitric oxide synthase inhibitor L-NMMA under normoxic conditions. However, effects of L-arginine on contractions and $I_{Ca\text{-}L}$ were very different in ischemia and reperfusion. When cells were exposed to ischemia plus L-arginine, L-arginine prevented the decrease in $I_{Ca\text{-}L}$ typically observed in ischemia and reperfusion. Interestingly, this effect was not attenuated by L-NMMA. Thus, effects of L-arginine on $I_{Ca\text{-}L}$ in ischemia and reperfusion appear to be independent of NO production. Inclusion of L-arginine in ischemia also augmented contractile recovery in early reperfusion and attenuated stunning in late reperfusion, with no effect on the incidence of I_{TI} in early reperfusion. The

effect of L-arginine on contractile recovery in early reperfusion was reversed by L-NMMA. These observations suggest that effects of L-arginine on recovery of contraction in ischemia and reperfusion are likely mediated, at least in part, by NO.

In the present study, we found that L-arginine caused only a slight decrease in the magnitude of $I_{Ca\text{-}L}$ and a large decrease in amplitudes of contractions under normoxic conditions. Further, we found that the effect of L-arginine on contractions could be reversed by L-NMMA, which suggests that this effect is mediated by NO production. These observations are in general agreement with the results of earlier studies of the effects of NO supplementation on isolated cardiac myocytes. Previous studies have reported that NO inhibits cell shortening in isolated cardiac myocytes (Brady et al., 1992, 1993; Kojda et al., 1996). In addition, although previous studies have shown that NO supplementation inhibits $I_{Ca\text{-}L}$ when current is augmented by ß-adrenoceptor stimulation and in cells from transplanted hearts undergoing rejection (Wahler and Dollinger, 1995; Ziolo et al., 2001a), it has little effect on $I_{Ca\text{-}L}$ under basal conditions (Wahler and Dollinger, 1995). Interestingly, our results demonstrate that L-arginine markedly reduced amplitudes of contractions with only minimal inhibition of $I_{Ca\text{-}L}$. $I_{Ca\text{-}L}$ is believed to initiate contraction in heart by release of Ca^{2+} from the sarcoplasmic reticulum (Bers, 2001). As the effect of L-arginine on contractions is much larger than its effect on $I_{Ca\text{-}L}$, our results suggest that L-arginine might alter the coupling between $I_{Ca\text{-}L}$ and sarcoplasmic reticulum Ca^{2+} release, at least under normoxic conditions. However, this remains to be demonstrated conclusively.

Previous studies have shown that the magnitude of $I_{Ca\text{-}L}$ is decreased in ischemia and reperfusion (Cordeiro et al., 1994; Louch et al., 2000, 2002) and by metabolic inhibition (Lederer et al., 1989). In the present study, we found that inclusion of L-arginine in the ischemic solution prevented this decrease in $I_{Ca\text{-}L}$ in ischemia and reperfusion. This is surprising, as L-arginine actually caused a slight decrease in $I_{Ca\text{-}L}$ under normoxic conditions. Thus, the results of this study show that effects of L-arginine on $I_{Ca\text{-}L}$ in ischemia differ from effects under normoxic conditions.

It is unlikely that the effects of L-arginine in ischemia are mediated by production of NO, as the actions of L-arginine on $I_{Ca\text{-}L}$ were not affected by the nitric oxide synthase inhibitor L-NMMA. Therefore, our results demonstrate that L-arginine has effects on $I_{Ca\text{-}L}$ that are independent of NO synthesis. The mechanism by which L-arginine prevents the decline in $I_{Ca\text{-}L}$ in ischemia and reperfusion is not known. Cytosolic Ca^{2+} levels increase in ischemia (Nayler et al., 1979; Tani and Neely, 1989) and elevated free intracellular Ca^{2+} inhibits $I_{Ca\text{-}L}$ (Hofer et al., 1997; Schuhmann et al., 1997). Thus, it is possible that L-arginine prevents depression of $I_{Ca\text{-}L}$ by reducing cytosolic Ca^{2+} levels in ischemia, although there is no direct evidence for this at the present time.

Earlier studies have shown that contractions associated with activation of $I_{Ca\text{-}L}$ rapidly decline in ischemia, recover transiently in reperfusion and then exhibit contractile depression later in reperfusion (Cordeiro et al., 1994; Louch et al., 2000, 2002). Here we found that contractions also were inhibited in ischemia in the presence of L-arginine. However, recovery of contractions in early reperfusion was greatly potentiated in cells exposed to L-arginine in ischemia. In addition, L-arginine attenuated stunning in reperfusion. Interestingly, under normoxic conditions, L-arginine significantly reduced the amplitudes of contractions. Thus, the results of this study demonstrate that the effects of L-arginine on contraction in ischemia cannot be predicted from its effects in the absence of ischemia.

The mechanism by which L-arginine potentiates recovery of contractile function in reperfusion is not yet known. It is possible that L-arginine improves contractile recovery, at least in part, because it prevents the decrease in $I_{Ca\text{-}L}$ in ischemia. If there is an increase in availability of $I_{Ca\text{-}L}$ in ischemia and reperfusion, there would be more current available to trigger sarcoplasmic reticulum Ca^{2+} release (Bers, 2001). However, effects of L-arginine on $I_{Ca\text{-}L}$ were independent of NO synthesis, while effects of L-arginine on the overshoot in contraction are abolished by L-NMMA and likely involve NO. It is possible that NO might sensitize one or more components involved in excitation–contraction coupling in the cell to augment contractile recovery in reperfusion. Indeed, there is some evidence that NO might affect sarcoplasmic reticulum Ca^{2+} release. Studies have shown that NO can increase or decrease sarcoplasmic reticulum Ca^{2+} release depending upon the concentration of NO and the ambient level of ß-adrenoceptor activation (Zahradnikova et al., 1997; Ziolo et al., 2001b). Thus, modulation of sarcoplasmic reticulum Ca^{2+} release by NO also might augment recovery of contraction in reperfusion.

In earlier studies with our cellular model of simulated ischemia and reperfusion, we reported that both I_{TI} and aftercontractions can occur in early reperfusion (Cordeiro et al., 1994; Louch et al., 2000). I_{TI} is thought to arise as a consequence of intracellular Ca^{2+}-overload (Lederer and Tsien, 1976; Kass et al., 1978), which gives rise to the oscillatory release of Ca^{2+} from the sarcoplasmic reticulum and triggers cardiac arrhythmias (Ferrier et al., 1973). In the present study, we found that L-arginine had little effect on the incidence of I_{TI} or aftercontractions observed in early reperfusion. Indeed, the incidence of I_{TI} was slightly decreased in the presence of L-arginine, despite increased contractile activity in reperfusion. Thus, L-arginine preserves contractile activity without promoting this mechanism of arrhythmia. A number of previous studies have demonstrated that NO has significant antiarrhythmic effects in various models of ischemia and reperfusion (reviewed by Pagliaro et al., 2001). The results of the present study demonstrate that antiarrhythmic effects of NO in ischemia and reperfusion are not likely due to inhibition of I_{TI}.

In summary, the results of this study demonstrate that inclusion of L-arginine in ischemia prevents the decrease in $I_{Ca\text{-}L}$ typically observed in ischemia and reperfusion, and improves recovery of contractile function in reperfusion. However, L-arginine had no effect on the incidence of I_{TI} in early reperfusion. In contrast, under normoxic conditions, L-arginine slightly inhibited $I_{Ca\text{-}L}$ and markedly reduced magnitudes of contractions in guinea pig ventricular myocytes. Thus, an important finding in the present study is that the effects of L-arginine under normoxic conditions are very different from its effects in ischemia and reperfusion. In addition, our results demonstrate that effects of L-arginine on recovery of contraction in reperfusion appear to be mediated, at least in part, by NO. However, L-arginine has marked effects on $I_{Ca\text{-}L}$ in ischemia and reperfusion that are independent of NO synthesis.

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