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Blocking late sodium current reduces hydrogen peroxide-induced arrhythmogenic activity and contractile dysfunction

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Running title: Attenuation of arrhythmogenic actions of ROS

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Nonstandard abbreviations:

APD, action potential duration;

EAD, early afterdepolarization;

$I_{Ca(L)}$, L-type Ca^{2+} current;

$I_{Na\text{-}Ca}$, Na^{+}-Ca^{2+} exchange current;

Late I_{Na}, late sodium current;

NCX, sodium-calcium exchange(r);

ROS, reactive oxygen species;

TTX, tetrodotoxin

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Abstract

Reactive oxygen species (ROS), including hydrogen peroxide (H_2O_2), cause intracellular calcium overload and ischemia-reperfusion damage. The objective of this study was to examine the hypothesis that H_2O_2-induced arrhythmic activity and contractile dysfunction are the result of an effect of H_2O_2 to increase the magnitude of the late sodium current (late I_{Na}). Guinea pig and rabbit isolated ventricular myocytes were exposed to H_2O_2 (200 μmol/L). Transmembrane voltages and currents, and twitch shortening were measured using the whole-cell patch-clamp technique and video edge detection, respectively. Intracellular concentrations of sodium ($[Na^+]_i$) and calcium ($[Ca^{2+}]_i$) were determined by fluorescence measurements. H_2O_2 caused a persistent late I_{Na} that was almost completely inhibited by tetrodotoxin (TTX, 10 μmol/L). H_2O_2 prolonged the action potential duration (APD), slowed the relaxation rate of cell contraction, and induced early afterdepolarizations (EADs) and aftercontractions. H_2O_2 also caused increases of $[Na^+]_i$ and $[Ca^{2+}]_i$. Ranolazine (10 μmol/L), a novel inhibitor of late I_{Na}, attenuated H_2O_2-induced late I_{Na} by 51±9 %. TTX (2 μmol/L) or ranolazine (10 μmol/L) attenuated H_2O_2-induced APD prolongation and suppressed EADs. Ranolazine accelerated the twitch relaxation rate in the presence of H_2O_2 and abolished H_2O_2-induced aftercontractions. Pretreatment of myocytes with ranolazine delayed and reduced the increases of APD, $[Na^+]_i$ and $[Ca^{2+}]_i$ caused by H_2O_2. In conclusion, the results confirm the hypothesis that an increase in late I_{Na} during exposure of ventricular myocytes to H_2O_2 contributes to electrical and contractile dysfunction, and suggest that inhibition of late I_{Na} may offer protection against ROS-induced Na^+ and Ca^{2+} overload.

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Introduction

Although the exact role of hydrogen peroxide (H_2O_2) and other reactive oxygen species (ROS) in pathological processes is still under investigation, increasing evidence strongly suggests a link between ROS and ischemia-reperfusion injury (Dhalla et al, 2000; Turoczi et al, 2003; Paradies et al, 2004), “stunned myocardium”, and the development and progression of heart failure (Bolli and Marbán, 1999; Zeitz et al, 2002; Sawyer et al, 2002; Liu et al, 2005). The myocardial tissue concentration of H_2O_2 is significantly elevated in hearts exposed to ischemia-reperfusion (Slezak et al, 1995) and in the failing heart (Ide et al, 2000). ROS appear to play a major role in the dysregulation of intracellular concentrations of sodium ($[Na^+]_i$) and calcium ($[Ca^{2+}]_i$) in ischemia/reperfusion injury (Bolli and Marbán, 1999; Zeitz et al, 2002). This effect of ROS is accompanied by cellular electrical instability (e.g., arrhythmias) and contractile dysfunction characterized by a marked increase in diastolic tension (Zeitz et al, 2002; Hara et al, 1993). The cellular Ca^{2+} overload caused by ROS has been proposed to be due to a rise in $[Na^+]_i$ followed by Ca^{2+} influx via the reverse mode of the Na^+ - Ca^{2+} exchanger (NCX) (Wagner et al, 2003). As to the mechanism of the ROS-induced rise in $[Na^+]_i$, there is evidence in support of an increased entry of Na^+ via non-inactivating Na^+ channels (Ward and Giles, 1997), an enhanced Na^+-H^+ exchanger activity (Sabri et al, 1998), and an impaired Na^+-K^+-ATPase function (Kim and Akera, 1987). The amplitude of late Na^+ current (via non-inactivating Na^+ channels) and $[Na^+]_i$ and $[Ca^{2+}]_i$ are significantly increased in myocytes isolated from ischemic (Kihara et al, 1989; Haigney et al, 1994; Huang et al, 2001) or failing hearts (Pogwizd et al, 2003; Valdivia et al, 2005). It has been shown that the increase in $[Ca^{2+}]_i$ caused by ROS is attenuated by an NCX inhibitor (Zeitz et al, 2002) and is exacerbated in cells overexpressing NCX (Wagner et al, 2003). Furthermore, hypoxia-induced Na^+ and Ca^{2+} loading of cardiac myocytes can be reduced

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by blockade of I_{Na} (Haigney et al, 1994). A rise of $[Ca^{2+}]_i$ caused by hypoxia can also be prevented by inhibition of the Na^+-Ca^{2+} exchanger (Ziegelstein et al, 1992). Interestingly, verapamil does not attenuate the increase in $[Ca^{2+}]_i$ caused by ROS, indicating that the increase in Ca^{2+} entry into myocardial cells is not through L-type Ca^{2+} channels (Zeitz et al, 2002). Thus, the Ca^{2+} overload caused by ROS is likely due to a rise in $[Na^+]_i$ that in turn leads to an increased exchange of intracellular Na^+ for extracellular Ca^{2+} via NCX.

The objective of the present study was to determine the contribution of the late Na^+ current (late I_{Na}) to the rises in $[Na^+]_i$ and $[Ca^{2+}]_i$, and to the accompanying electrical and contractile dysfunctions caused by H_2O_2. To determine the role of late I_{Na}, we used low concentrations (<10 µM) of the putative Na^+ channel blocker tetrodotoxin (TTX), and of ranolazine, a cardioprotective agent that preferentially inhibits late relative to peak I_{Na} (Undrovinas et al, 2006). Ranolazine has previously been shown to markedly attenuate, in a concentration-dependent manner, the increase in left ventricular diastolic pressure caused by H_2O_2 in rat isolated, perfused hearts (Matsumura et al, 1998).

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Methods

Chemicals

H_2O_2 was purchased from Fisher (Fair Lawn, NJ) and Merck (Darmstadt, Germany). TTX was purchased from Sigma (St. Louis, MO). Ranolazine [(±)-*N*-(2,6-dimethylphenyl)-(4[2-hydroxy-3-(2-methoxyphenoxy)propyl]-1-piperazine], a piperazine derivative (Matsumura et al, 1998), was synthesized by CV Therapeutics (Palo Alto, CA).

Isolation of Ventricular Myocytes

Electrophysiological studies were performed on guinea pig ventricular myocytes at the University of Florida; intracellular Na^+ and Ca^{2+} measurements were conducted on rabbit ventricular myocytes at Georg-August-University Göttingen. Use of animals was in accordance with the Guide for the *Care and Use of Laboratory Animals* published by US National Institutes of Health (NIH Publication No. 85-23, revised 1996) and was approved by the Institutional Animal Care and Use Committee of the University of Florida. Ventricular myocytes were isolated from adult, female Hartley guinea pigs and Chinchilla bastard rabbits, with standard enzymatic procedures described previously (Wagner et al, 2003; Song et al, 2004).

Measurements of Transmembrane Potential and Current

Guinea pig ventricular myocytes were placed into a recording chamber that was bathed with pre-warmed (35-36 ^{o}C) Tyrode solution, without or with drug(s), at a rate of 2 ml/min. The Tyrode solution contained (in mmol/L) 135 NaCl, 4.6 KCl, 1.8 $CaCl_2$, 1.1 $MgSO_4$, 10 glucose and 10 HEPES, pH 7.4. The temperature of the bathing media in a given experiment did not vary more than 0.3 ^{o}C. Transmembrane voltages and currents were recorded from quiescent, rod-shaped myocytes with clear striations, using borosilicate glass pipettes (1-3 MΩ resistance when filled) in a whole-cell configuration of the patch clamp technique. An Axopatch-200 amplifier, a

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DigiData interface and a computer with pCLAMP software (Axon Instruments, Union City, CA) were used to amplify, store, and analyze the recorded signals. The electrode capacitance and whole-cell capacitance currents were maximally compensated with the amplifier. The series resistance was compensated by 60-80%. The liquid junction potential between pipette and bath medium was calculated with pCLAMP software and corrected online.

When measuring action potentials, recording pipettes were filled with a solution containing (in mmol/L) 120 K-aspartate, 20 KCl, 1 $MgSO_4$, 4 Na_2ATP, 0.1 Na_3GTP, and 10 HEPES, pH 7.2. For inducing action potentials, a 5-ms depolarizing pulse was applied at a frequency of 0.16 Hz. The duration of the action potential was measured from onset of upstroke to 50% of repolarization (APD_{50}).

To elicit late I_{Na}, myocytes were voltage-clamped at a holding potential of -90 mV, and a 300-ms depolarizing pulse to -30 mV was applied at a frequency of 0.16 Hz. In experiments to determine the effect of ranolazine on late I_{Na}, K^+ and Ca^{2+} were omitted from the bath and pipette solutions to reduce contamination of I_{Na} by K^+ and Ca^{2+} currents. In these experiments, recording pipettes were filled with (in mmol/L) 120 Cs-aspartate, 20 CsCl, 1 $MgSO_4$, 4 Na_2ATP, 0.1 Na_3GTP, and 10 HEPES, pH 7.2. The magnitude of late I_{Na} was determined by integration of the current over the last 50 ms of the -30 mV clamp pulse, using the integration (area) feature of the pCLAMP program.

Measurement of Cell Contraction

Twitch shortenings of guinea pig ventricular myocytes were elicited by field stimulation from a Grass-S88 stimulator (Quincy, MA). The amplitude of twitch shortening was determined by a video-motion detector (Crescent Electronics, Logan, UT), and was recorded on a chart recorder (Gould 2200S, Cleveland, OH). In this study, a twitch shortening denotes a normal

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systolic contraction, whereas an early aftercontraction denotes an additional contraction that occurs during relaxation and is triggered by events following the preceding normal contraction. The amplitude of twitch shortening was measured from maximal cell relaxation to peak contraction, and the rate of relaxation was calculated by dividing the amplitude (µm) with the time (s) required from peak contraction to maximal relaxation.

Measurement of Intracellular Sodium and Calcium

Rabbit ventricular myocytes on laminin-coated recording chambers were loaded with either 10 µmol/L SBFI-AM for 2 h or 10 µmol/L Indo1-AM for 30 min, in the presence of 0.02% (w/v) pluronic acid (Molecular Probes, Eugene, OR). The chambers were mounted on the stage of an inverted microscope (Nikon Eclipse TE2000-U) and superfused with normal Tyrode solution (37°C) containing (in mmol/L) 140 NaCl, 4 KCl, 1 $MgCl_2$, 5 HEPES, 10 glucose, 2 $CaCl_2$, pH 7.4. Myocytes were continuously paced at 0.5 Hz using electrical field stimulation (10-20 V). Intracellular SBFI was alternatively excited at 340 and 380 nm and emitted epifluorescence was monitored at 510 nm for both wavelengths (F_{340} and F_{380}). Intracellular Indo1 was excited at 360 nm and emitted epifluorescence was measured at 405 and 485 nm. For both dyes, fluorescence emission was recorded using IonWizard software (IonOptix Corporation, Boston, MA). After washing out the external dye for 10 min, background fluorescence was determined for each excitation or emission wavelength. The ratios of F_{340}/F_{380} and F_{405}/F_{485}, respectively, were then calculated from the background-subtracted emission intensities at each wavelength and converted to $[Na^+]_i$ and $[Ca^{2+}]_i$ with calibration curves. In situ calibration of SBFI was accomplished by exposing myocytes to bath solutions containing 0, 5, 10 and 20 mmol/L Na^+ in the presence of 10 µmol/L gramicidin D, 80 µmol/L monensin and 50 µmol/L strophanthidin (Maier et al, 1997). The solutions with various concentrations of Na^+ were

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prepared from two stock solutions of equal ionic strength containing (in mmol/L) 140 NaCl, 10 HEPES and 1 EGTA or (in mmol/L) 140 KCl, 10 HEPES and 1 EGTA, pH was adjusted to 7.2 with Tris base. In situ calibration of Indo1 was accomplished using the equation $[Ca^{2+}]_i = K_D \cdot \beta[(R - R_{min})/(R_{max} - R)]$ as previously described (Bassani et al, 1995).

Statistical Analysis

Data are expressed as mean ± SEM. Values of “n” indicate the number of cells studied. The Student’s t-test, one-way repeated measures ANOVA followed by Student-Newman-Keuls test, and two-way ANOVA were applied where it was appropriate. A difference with a p value < 0.05 was considered statistically significant.

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Results

Ranolazine and TTX inhibited H_2O_2-induced late sodium current of guinea pig ventricular myocytes.

Late I_{Na} in the absence of drug was a small inward current (Figure 1, A and B). Incubation of cells in 200 µmol/L H_2O_2 caused an increase of late I_{Na} from -3.419±0.392 to -6.215±0.471 nC (Figure 1A). Ranolazine (10 µmol/L), an inhibitor of late I_{Na}, reduced the current to -5.072±0.440 nC in the continued presence of H_2O_2 (n = 10, $p < 0.01$; Figure 1A), a 51±9% decrease of H_2O_2-induced late I_{Na}. To confirm that the H_2O_2-induced late current was indeed a Na^+ current, the specific Na^+ channel blocker TTX (10 µmol/L) was applied in the presence of H_2O_2. Late I_{Na} was increased by 200 µmol/L H_2O_2 from -0.114±0.538 to -4.423±1.384 nC, and was decreased by TTX to -0.476±0.850 nC (n = 5, $p < 0.01$; Figure 1B), a decrease of 91±5%.

Ranolazine and TTX attenuated H_2O_2-induced action potential prolongation and early afterdepolarizations in guinea pig ventricular myocytes.

An increase of late I_{Na} caused by H_2O_2 would cause prolongation of action potential duration (APD) and early afterdepolarizations (EADs), which may explain, at least in part, the arrhythmogenic effect of H_2O_2 on cardiac myocytes. On the other hand, attenuation of late I_{Na} may antagonize these effects of H_2O_2. Therefore, the interactions between H_2O_2 and both ranolazine and TTX on action potentials were examined. For these experiments, equally effective concentrations of TTX (2 µmol/L) and ranolazine (10 µmol/L) were used. At these concentrations neither drug alone had a significant effect on APD (not shown). The earliest response to H_2O_2 (200 µmol/L) was a progressive prolongation of APD. Prolonged treatment with H_2O_2 led to development of EADs, followed by spontaneous activity and membrane

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depolarization (not shown). Both ranolazine (10 µmol/L, Figure 2A) and TTX (2 µmol/L, Figure 2B) attenuated an H_2O_2-induced prolongation of APD. The APD at 50% of repolarization (APD_{50}) was increased by H_2O_2 from 204±15 to 280±18 ms; ranolazine decreased the APD_{50} to 235±13 ms and attenuated by 58±8% the prolongation of APD caused by H_2O_2 (n = 8, $p < 0.001$; Figure 2C). In another set of experiments, H_2O_2 increased APD_{50} from 177±4 to 232±9 ms, and TTX decreased APD_{50} to 198±6 ms in the continued presence of H_2O_2 (n = 5, $p < 0.001$; Figure 2D). When EADs were induced by H_2O_2, addition of either ranolazine (10 µmol/L, n = 3) or TTX (2 µmol/L, n = 4) resulted in suppression of the EADs (Figures 3 and 4, respectively).

Pretreatment of myocytes with ranolazine significantly blunted an increase of APD caused by H_2O_2 and prevented induction by H_2O_2 of EADs. In these experiments, myocytes were treated with either saline (control) or ranolazine (10 µmol/L) 3 min prior to application of H_2O_2 and throughout the exposure to H_2O_2 (200 µmol/L). Exposure of myocytes to H_2O_2 led to an increase of APD_{50} (Figure 5). APD prolongation usually became apparent after 5 min of exposure to H_2O_2 and increased progressively thereafter (Figure 5). After a 10-min exposure to H_2O_2, APD_{50} was increased by 61±7% and EADs were induced in 7 out of 10 cells (action potentials with EADs were not included in APD_{50} calculation). In contrast, APD_{50} was increased by only 11±6% ($p < 0.01$; Figure 5) in ranolazine-treated myocytes, and EADs were not observed in any of the 8 myocytes studied.

Ranolazine reversed H_2O_2-induced contractile dysfunction of guinea pig ventricular myocytes.

H_2O_2 (200 µmol/L) initially increased the amplitude of myocyte contractile shortening and induced aftercontractions that delayed contractile relaxation (Figure 6A, record b). Prolonged treatment of myocytes with H_2O_2 eventually caused spontaneous contractions and

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decreases of contractile amplitude and rate and extent of relaxation (Figure 6A, record d). Ranolazine (10 µmol/L) did not significantly change contractile amplitude in the presence of H_2O_2 (Figure 6B), but suppressed aftercontractions (Figure 6A, record c). H_2O_2 increased myocyte contractile amplitude from 4.7±0.8 to 6.3±1.1 µm and decreased the rate of contractile relaxation from 12.2±1.0 to 7.3±1.3 µm/s (Figure 6B). Ranolazine accelerated the relaxation rate from 7.3±1.3 to 15.0±1.9 µm/s (n = 5, $p < 0.05$) in the presence of H_2O_2 (Figure 6B).

Ranolazine attenuated H_2O_2-induced intracellular Na^+ and Ca^{2+} overload of rabbit ventricular myocytes.

In this series of experiments, the effects of H_2O_2 (200 µmol/L) on $[Na^+]_i$, $[Ca^{2+}]_i$ and contractions of rabbit ventricular myocytes were determined in the absence or presence of ranolazine (10 µmol/L). H_2O_2 caused time-dependent increases of $[Na^+]_i$ and $[Ca^{2+}]_i$ (Figure 7) that led to a state of hypercontracture within 14.8±1.0 min (not shown). In the presence of ranolazine, time to H_2O_2-induced hypercontracture was significantly increased to19.3±1.1 min (n = 14, $p < 0.05$). Consistent with this finding, ranolazine blunted the time-dependent increases of $[Na^+]_i$ and $[Ca^{2+}]_i$ during exposure to H_2O_2. There was a trend of reduction by ranolazine of baseline $[Na^+]_i$, from 5.0±0.8 to 2.4±1.0 mmol/L, and baseline diastolic $[Ca^{2+}]_i$, from 179±44 to 99±26 nmol/L, respectively. However, these changes were not statistically significant. After a 12-min incubation of myocytes with H_2O_2, $[Na^+]_i$ was increased to 16.7±2.8 mmol/L, whereas in the presence of ranolazine, $[Na^+]_i$ was significantly reduced to 8.0±2.2 mmol/L ($p < 0.05$; Figure 7, A and B). Similarly, H_2O_2-induced increase of $[Ca^{2+}]_i$ was significantly attenuated by ranolazine. At the end of a 12-min incubation with H_2O_2, diastolic $[Ca^{2+}]_i$ was 569±106 and 338±61 nmol/L, respectively, in the absence and presence of ranolazine ($p < 0.05$; Fig 7, C and D).

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Discussion

The major finding of this study is that blocking late I_{Na} with either ranolazine or TTX attenuates H_2O_2-induced arrhythmic activity and contractile dysfunction in cardiac myocytes. In addition, ranolazine was found to significantly reduce the rise in $[Na^+]_i$ and $[Ca^{2+}]_i$ caused by H_2O_2. The results of the study suggest that inhibition of late I_{Na} may attenuate reactive oxygen species-induced cardiac dysfunction.

The hypothesis tested in the present study can be summarized as follows (Figure 8): ROS, such as H_2O_2, increase late I_{Na} and thereby cause 1) prolongation of APD and induction of EADs, and 2) a rise in $[Na^+]_i$ which, because intracellular Na^+ is exchanged for extracellular Ca^{2+} via NCX, causes cellular Ca^{2+} overload. Ca^{2+} overload of cardiomyocytes is associated with electrical instability (i.e., arrhythmias) and contractile dysfunction (i.e., impaired relaxation). Hence, by reducing the increase in late I_{Na}, the deleterious effects of H_2O_2 on cardiomyocyte function (electrical and contractile) can be attenuated. In support of the hypothesis (Figure 8), results of other studies have shown that H_2O_2 can cause increases of late I_{Na} (Ward and Giles, 1997; Ma et al, 2005), intracellular Na^+ and Ca^{2+} (Wagner et al, 2003), and ventricular diastolic tension and pressure (Zeitz et al, 2002; Hara et al, 1993).

To verify the hypothesis just described (Figure 8), we measured late I_{Na}, action potentials, cell contractions, and intracellular concentrations of Na^+ and Ca^{2+}. We found that ranolazine and TTX effectively reduced H_2O_2-induced late I_{Na} (Figure 1), action potential prolongation (Figure 2), EADs (Figures 3-4), and cell contractile dysfunction (Figure 6). Pretreatment of myocytes with ranolazine significantly delayed or prevented action potential prolongation (Figure 5), cell contracture, and increases of $[Na^+]_i$ and $[Ca^{2+}]_i$ caused by subsequent exposure of cells to H_2O_2 (Figure 7). Thus, the hypothesis presented in Figure 8 is fully supported by the present results. In

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addition, ranolazine has been reported to reduce H_2O_2-induced contractile dysfunction of rat isolated hearts (Matsumura et al, 1998). The results of the present study using ventricular myocytes thus provide an ionic mechanism (i.e., inhibition of late I_{Na}) to explain the protective action of ranolazine against ROS-induced increases of left ventricular end-diastolic and coronary perfusion pressures (Matsumara et al, 1998).

Although the amplitude of late I_{Na} recorded in the absence of drugs is small, late I_{Na} is found to contribute to the regulation of APD (Kiyosue and Arita, 1989; Maltsev et al, 1998; Sakmann et al, 2000). Blocking late I_{Na} with TTX caused a 10-20% decrease of APD (Kiyosue and Arita, 1989; Maltsev et al, 1998; Sakmann et al, 2000) and suppressed EADs of myocytes isolated from failing hearts (Maltsev et al, 1998). We found that in the absence of TTX or ranolazine, the amplitude of late I_{Na} can be markedly enhanced by H_2O_2 by several fold (data not shown). Therefore, an increase by H_2O_2 of late I_{Na} is expected to cause a significant prolongation of APD. Consistent with previous reports (Beresewicz and Horackova, 1991), we found that the early effect of H_2O_2 on myocyte membrane potential was an increase in the duration of action potentials. This effect of H_2O_2 can be largely explained by an increase of late I_{Na}, because it was significantly attenuated by either ranolazine or TTX. Thus, blocking late I_{Na} may be the key to reduce H_2O_2-induced arrhythmic activity and contractile dysfunction.

In myocytes pretreated with ranolazine both $[Na^+]_i$ and $[Ca^{2+}]_i$ were found to be lower, prior to the addition of H_2O_2, than in untreated myocytes. Whether this observation is due to an inherent variation in the intracellular ion concentrations or to a true effect of ranolazine remains to be investigated. However, in another study of rat isolated perfused hearts in which this issue was investigated, ranolazine at a concentration of 10 µmol/L did not lower baseline systolic or diastolic $[Ca^{2+}]_i$ (Fraser et al, 2005). Regardless, a possible explanation for the lower $[Na^+]_i$ and

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$[Ca^{2+}]_i$ in myocytes pretreated with ranolazine is that this piperazine derivative by reducing basal I_{Na}, albeit small in normal myocytes, could indeed reduce basal $[Na^+]_i$ and $[Ca^{2+}]_i$. Evidence in support of this explanation is that ranolazine, like TTX, can cause small shortening of the APD. The net effect of ranolazine on ventricular APD depends on the relative contribution of delayed rectifier K^+ current and late I_{Na} to the repolarization (Song et al, 2004).

There are potential limitations to this study. Firstly, H_2O_2 might elevate $[Na^+]_i$ via enhancing Na^+-H^+ exchange (Sabri et al, 1998) or reducing Na^+-K^+-ATPase (Kim and Akera, 1987) activity. Inhibition of Na^+-K^+-ATPase may cause a transient prolongation of action potential (Levi, 1991). However, it is unlikely that modulation of Na^+-K^+-ATPase plays a significant role in H_2O_2-induced action potential prolongation and EADs, because the effect of H_2O_2 was blocked by TTX. Furthermore, ranolazine at 20 µmol/L had no effect on the Na^+-H^+ exchanger in MDCK cells (CV Therapeutics, 2003).

Secondly, our data interpretation is dependent on the selectivity of TTX and ranolazine for Na^+ channels. The late (persistent) I_{Na} is more susceptible than the peak inward I_{Na} to the inhibitory effect of TTX (Kiyosue and Arita, 1989; Maltsev et al, 1998). In the present study, TTX at a low concentration of 2 µmol/L significantly attenuated H_2O_2-induced action potential prolongation and EADs, whereas it had little effect on the basal action potentials (in the absence of H_2O_2, data not shown). This result indicates that the prolongation of APD caused by H_2O_2 can be mainly attributed to an increase of late I_{Na}. Consistent with the findings of the present study, TTX has been shown to markedly attenuate the deleterious effects (Ca^{2+}-overload, diastolic dysfunction, etc.) caused by ROS and palmitoyl-L-carnitine, known to increase late I_{Na} (Ver Donck and Borgers, 1991; Hara et al, 1997). Ranolazine has also been reported to be a rather selective (38-fold) inhibitor of late relative to peak I_{Na} (Undrovinas et al, 2006). In ventricular

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myocytes of dogs with chronic heart failure, ranolazine inhibited peak and late I_{Na} with potencies of 244 and 6.5 µmol/L, respectively (Undrovinas et al, 2006). Ranolazine at a concentration of 10 µmol/L has been shown to effectively attenuate anemone toxin II-induced late I_{Na}, prolongation of APD, and formation of EADs (Song et al, 2004). Alternative explanations for the effect of ranolazine to attenuate H_2O_2-induced action potential prolongation and EADs are an inhibition of the L-type Ca^{2+} current ($I_{Ca(L)}$) and/or Na^+-Ca^{2+} exchange current ($I_{Na\text{-}Ca}$). However, the IC_{50} values for ranolazine to inhibit $I_{Ca(L)}$ and $I_{Na\text{-}Ca}$ were reported to be around 300 µmol/L and 91µmol/L, respectively (Antzelevitch et al, 2004; Schram et al, 2004). Moreover, we found that H_2O_2 caused a decrease of $I_{Ca(L)}$ in guinea pig ventricular myocytes (data not shown). Therefore, it is unlikely that putative inhibition by ranolazine of $I_{Ca(L)}$, $I_{Na\text{-}Ca}$ and/or peak I_{Na} can explain its attenuation of the effect of H_2O_2. It is also unlikely that ranolazine reduces the effects of H_2O_2 via radical scavenging or antioxidant actions, because ranolazine does not decrease H_2O_2-induced lipid peroxidation (Matsumura et al, 1998).

In summary, block of late I_{Na} by TTX or ranolazine attenuates the deleterious effects of H_2O_2 on electrical and contractile functions and cellular Na^+ and Ca^{2+} homeostasis of cardiac myocytes. The results of the present study suggest that an increase of late I_{Na} is a major ionic mechanism underlying the cardiac actions of H_2O_2. Reducing late I_{Na} may be a critical step to attenuate ROS-induced myocardial dysfunction.

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This article has not been copyedited and formatted. The final version may differ from this version.

References

Antzelevitch C, Belardinelli L, Zygmunt AC, Burashnikov A, Di Diego JM, Fish JM, Cordeiro JM, and Thomas G (2004) Electrophysiological effects of ranolazine, a novel antianginal agent with Antiarrhythmic properties. *Circulation* **110**:904-910.

Bassani JWM, Bassani RA, and Bers DM (1995) Calibration of Indo-1 and resting intracellular $[Ca]_i$ in intact rabbit cardiac myocytes. *Biophys J* **68**:1453-1460.

Beresewicz A and Horackova M (1991) Alterations in electrical and contractile behavior of isolated cardiomyocytes by hydrogen peroxide: possible ionic mechanism. *J Mol Cell Cardiol* **23**:899-918.

Bolli B and Marbán E (1999) Molecular and cellular mechanisms of myocardial stunning. *Physiol Rev* **79**:609-634.

CV Therapeutics (2003) Ranexa (renolazine) FDA review documents. NDA 21-526.

Dhalla NS, Elmoselhi AB, Hata T, and Makino N (2000) Status of myocardial antioxidants in ischemia-reperfusion injury. *Cardiovasc Res* **47**:446-456.

Fraser H, Belardinelli L, Wang L, McVeigh JJ, and Clanahan AS (2005) Inhibition of late I_{Na} by ranolazine reduces Ca^{2+} overload and LV mechanical dysfunction in ejecting rat hearts. *Eur Heart J* **26**(Abstract Supplement):414.

Haigney MCP, Lakatta EG, Stern MD, and Silverman HS (1994) Sodium channel blockade reduces hypoxic sodium loading and sodium-dependent calcium loading. *Circulation* **90**:391-399.

Hara A, Arakawa J, Hashizume H, and Abiko Y (1997) Beneficial effects of dilazep on the palmitoyl-L-carnitine-induced derangements in isolated, perfused rat heart: comparison with tetrodotoxin. *Jpn J Pharmacol* **74**:147-153.

JPET Fast Forward. Published on March 24, 2006 as DOI: 10.1124/jpet.106.101832
This article has not been copyedited and formatted. The final version may differ from this version.

Hara A, Matsumura H, and Abiko Y (1993) Lidocaine attenuates both mechanical and metabolic changes induced by hydrogen peroxide in the rat heart. *Am J Physiol* **265**:H1478-H1485.

Huang B, El-Sherif T, Gidh-Jain M, Qin D, and El-Sherif N (2001) Alterations of sodium channel kinetics and gene expression in the postinfarction remodeled myocardium. *J Cardiovasc Electrophysiol* **12**:218-225.

Ide T, Tsutsui H, Kinugawa S, Suematsu N, Hayashidani S, Ichikawa K, Utsumi H, Machida Y, Egashira K, and Takeshita A (2000) Direct evidence for increased hydroxyl radicals originating from superoxide in the failing myocardium. *Circ Res* **86**:152-157.

Kihara Y, Grossman W, and Morgan JP (1989) Direct measurement of changes in intracellular calcium transients during hypoxia, ischemia, and reperfusion of the intact mammalian heart. *Circ Res* **65**:1029-1044.

Kim M-S and Akera T (1987) O_2 free radicals: cause of ischemia-reperfusion injury to cardiac Na^+-K^+-ATPase. *Am J Physiol* **252**:H252-H257.

Kiyosue T and Arita M (1989) Late sodium current and its contribution to action potential configuration in guinea pig ventricular myocytes. *Circ Res* **64**:389-397.

Levi AJ (1991) The effect of strophanthidin on action potential, calcium current and contraction in isolated guinea-pig ventricular myocytes. *J Physiol* **443**:1-23.

Liu H, Colavitti R, Rovira II, and Finkel T (2005) Redox-dependent transcriptional regulation. *Circ Res* **97**:967-974.

Ma J-H, Luo A-T, and Zhang P-H (2005) Effect of hydrogen peroxide on persistent sodium current in guinea pig ventricular myocytes. *Acta Pharmacologica Sinica* **26**:828-834.

Maier LS, Pieske B, and Allen DG (1997) Influence of stimulation frequency on $[Na^+]_i$ and contractile function in Langendorff-perfused rat heart. *Am J Physiol* **273**:H1246-H1254.

JPET Fast Forward. Published on March 24, 2006 as DOI: 10.1124/jpet.106.101832

Maltsev VA, Sabbah HN, Higgins RSD, Silverman N, Lesch M, and Undrovinas AI (1998) Novel, ultraslow inactivation sodium current in human ventricular cardiomyocytes. *Circulation* **98**:2545-2552.

Matsumura H, Hara A, Hashizume H, Mazuyasu K, and Abiko Y (1998) Protective effects of ranolazine, a novel anti-ischemic drug, on the hydrogen peroxide-induced derangements in isolated, perfused rat heart: comparison with dichloroacetate. *Jpn J Pharmacol* **77**:31-39.

Paradies G, Petrosillo G, Pistolese M, Venosa ND, Federici A, and Ruggiero FM (2004) Decrease in mitochondrial complex I activity in ischemic/reperfused rat heart. *Circ Res* **94**:53-59.

Pogwizd SM, Sipido KR, Verdonck F, and Bers DM (2003) Intracellular Na in animal models of hypertrophy and heart failure: contractile function and arrhythmogenesis. *Cardiovasc Res* **57**:887-896.

Sabri A, Byron KL, Samarel AM, Bell J, and Lucchesi PA (1998) Hydrogen peroxide activates mitogen-activated protein kinases and Na^+-H^+ exchange in neonatal rat cardiac myocytes. *Circ Res* **82**:1053-1062.

Sakmann BFAS, Spindler AJ, Bryant SM, Linz KW, and Noble D (2000) Distribution of a persistent sodium current across the ventricular wall in guinea pigs. *Circ Res* **87**:910-914.

Sawyer DB, Siwik DA, Xiao L, Pimentel DR, Singh K, and Colucci WS (2002) Role of oxidative stress in myocardial hypertrophy and failure. *J Mol Cell Cardiol* **34**:379-388.

Schram G, Zhang L, Derakhchan K, Ehrlich JR, Belardinelli L, and Nattel S (2004) Ranolazine: ion-channel-blocking actions and *in vivo* electrophysiological effects. *Br J Pharmacol* **142**:1300-1308.

Slezak J, Tribulova N, Pristacova J, Uhrik B, Thomas T, Khaper N, Kaul N, and Singal PK (1995) Hydrogen peroxide changes in ischemic and reperfused heart. *Am J Pathol* **147**:772-781.

Song Y, Shryock JC, Wu L, and Belardinelli L (2004) Antagonism by ranolazine of the pro-arrhythmic effects of increasing late I_{Na} in guinea pig ventricular myocytes. *J Cardiovasc Pharmacol* **44**:192-199.

Turoczi T, Jun L, Cordis G, Morris JE, Maulik N, Stevens RG, and Das DK (2003) HFE mutation and dietary iron content interact to increase ischemia/reperfusion injury of the heart in mice. *Circ Res* **92**:1240-1246.

Undrovinas AI, Belardinelli L, Undrovinas NA, and Sabbah HN (2006) Ranolazine improves abnormal repolarization and contraction in left ventricular myocytes of dogs with heart failure by inhibiting late sodium current. *J Cardiovasc Electrophysiol* (in press).

Valdivia CR, Chu WW, Pu J, Foell JD, Haworth RA, Wolff MR, Kamp TJ, and Makielski (2005) Increased late sodium current in myocytes from a canine heart failure model and from failing human heart. *J Mol Cell Cardiol* **38**:475-483.

Ver Donck L and Borgers M (1991) Myocardial protection by R 56865: a new principle based on prevention of ion channel pathology. *Am J Physiol* **261**:H1828-1835.

Wagner S, Seidler T, Picht E, Maier LS, Kazanski V, Teucher N, Schillinger W, Pieske B, Isenberg G, Hasenfuss G, and Kögler H (2003) Na^{+}-Ca^{2+} exchanger overexpression predisposes to reactive oxygen species-induced injury. *Cardiovasc Res* **60**:404-412.

Ward CA and Giles WR (1997) Ionic mechanism of the effects of hydrogen peroxide in rat ventricular myocytes. *J Physiol* **500**:631-642.

JPET Fast Forward. Published on March 24, 2006 as DOI: 10.1124/jpet.106.101832
This article has not been copyedited and formatted. The final version may differ from this version.

Zeitz O, Maass AE, Nguyen PV, Hensmann G, Kögler H, Möller K, Hasenfuss G, and Janssen PML (2002) Hydroxyl radical-induced acute diastolic dysfunction is due to calcium overload via reverse-mode Na^{+}-Ca^{2+} exchange. *Circ Res* **90**:988-995.

Ziegelstein RC, Zweier JL, Mellits ED, Younes A, Lakatta EG, Stern MD, and Silverman HS (1992) Dimethylthiourea, an oxygen radical scavenger, protects isolated cardiac myocytes from hypoxic injury by inhibition of Na^{+}-Ca^{2+} exchange and not by its antioxidant effects. *Circ Res* **70**:804-811.

JPET Fast Forward. Published on March 24, 2006 as DOI: 10.1124/jpet.106.101832
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Footnotes

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Figure Legends

Figure 1. Ranolazine (10 µmol/L, panel A) and tetrodotoxin (TTX, 10 µmol/L, panel B) attenuated hydrogen peroxide (H_2O_2, 200 µmol/L)-induced late sodium current of guinea pig ventricular myocytes. Sodium current was elicited by 300-ms voltage-clamp pulses from -90 to -30 mV. Myocytes were sequentially treated with a) no drug (control), b) H_2O_2, and c) H_2O_2 plus either ranolazine or TTX.

Figure 2. Attenuation by ranolazine (Ran, 10 µmol/L) and tetrodotoxin (TTX, 2 µmol/L) of hydrogen peroxide (H_2O_2, 200 µmol/L)-induced prolongation of action potential duration of guinea pig ventricular myocytes. Panels A and B: superimposed action potentials recorded from a single cell in the presence of a) no drug (control), b) H_2O_2, and c) H_2O_2 plus Ran (panel A) or TTX (panel B). Panels C and D: summary of data from experiments similar to those shown in Panels A and B, respectively. * and **, $p < 0.001$ vs. control and H_2O_2 alone, respectively.

Figure 3. Inhibition by ranolazine (10 µmol/L) of hydrogen peroxide (H_2O_2, 200 µmol/L)-induced early afterdepolarizations (EADs) of a guinea pig ventricular myocyte. The myocyte was sequentially treated with a) no drug (control), b) H_2O_2, c) H_2O_2 plus ranolazine, and d) H_2O_2 alone (to wash out ranolazine). Each panel shows five superimposed, consecutive action potentials. Arrows indicate EADs.

Figure 4. Inhibition by tetrodotoxin (TTX, 2 µmol/L) of hydrogen peroxide (H_2O_2, 200 µmol/L)-induced early afterdepolarizations (EADs) of a guinea pig ventricular myocyte. The myocyte was treated with a) no drug (control), b) H_2O_2, c) H_2O_2 plus TTX, and d) H_2O_2 alone (to wash

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out TTX). Each panel shows three superimposed, consecutive action potentials. Arrows indicate EADs.

Figure 5. Pretreatment of guinea pig ventricular myocytes with ranolazine (10 µmol/L) prevented hydrogen peroxide (H_2O_2, 200 µmol/L)-induced prolongation of action potential duration. H_2O_2 was applied in the absence (closed circle) or presence (open circle) of ranolazine. The action potential duration at 50% of repolarization (APD_{50}) was normalized as percentage of control (before application of H_2O_2), and was plotted against H_2O_2 exposure time. Each point represents data collected from 3-10 cells. *, $p < 0.01$ vs. ranolazine-treated group.

Figure 6. Ranolazine (Ran, 10 µmol/L) attenuates hydrogen peroxide (H_2O_2, 200 µmol/L)-induced contractile dysfunction of guinea pig ventricular myocytes. Panel A: amplitudes of twitch shortening of a single myocyte. Records were obtained in the absence (a) and presence (b-d) of drugs as indicated. H_2O_2 (b) increased the amplitude of twitch shortening and induced aftercontractions (additional contractions during relaxation). Ran (c) suppressed the aftercontractions, but had little effect on the twitch amplitude. During washout of Ran in the continued presence of H_2O_2 (d), the aftercontractions resumed and eventually spontaneous contractions with a decreased twitch amplitude appeared. Panel B: summary of experiments similar to those shown in Panel A. Each bar represents data collected from five cells. * and **, $p < 0.05$ vs. control and H_2O_2 alone, respectively; NS, no significant difference vs. H_2O_2 alone.

Figure 7. Ranolazine (10 µmol/L) slows H_2O_2 (200 µmol/L)-induced increases of $[Na^+]_i$ and diastolic $[Ca^{2+}]_i$ in rabbit ventricular myocytes. Panels A, B and C: effect of H_2O_2 on $[Na^+]_i$. A:

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original traces of SBFI fluorescence in the absence and presence of ranolazine before (0 min) and after a 12-min exposure to H_2O_2 (in gray). B: time-course of changes in $[Na^+]_i$. Mean values for calibrated signals are normalized to baseline. C: concentrations of Na^+ measured at the end of a 12-min exposure to H_2O_2. Panels D, E and F: effect of H_2O_2 on $[Ca^{2+}]_i$. D: original traces of Ca^{2+} transient in the absence and presence of ranolazine before (0 min) and after a 12-min exposure to H_2O_2 (in gray). Please note that in the absence of ranolazine, at the end of 12-min H_2O_2 treatment the Ca^+ transients had disappeared, consistent with an early hypercontracture. E: time-course of changes in diastolic $[Ca^{2+}]_i$. Mean values for diastolic $[Ca^{2+}]_i$ are normalized to baseline. F: diastolic $[Ca^{2+}]_i$ determined after a 12-min exposure to H_2O_2.

Figure 8. An action of H_2O_2 to increase late I_{Na} is a potential mechanism by which H_2O_2 prolongs action potential duration (APD) and causes early afterdepolarizations (EADs) and increases of intracellular $[Na^+]$ and $[Ca^{2+}]$. TTX and ranolazine inhibit H_2O_2-induced late I_{Na} (dashed arrow). ROS, reactive oxygen species; TTX, tetrodotoxin; Ran, ranolazine; NCX, Na^+-Ca^{2+} exchanger.

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Figure 1

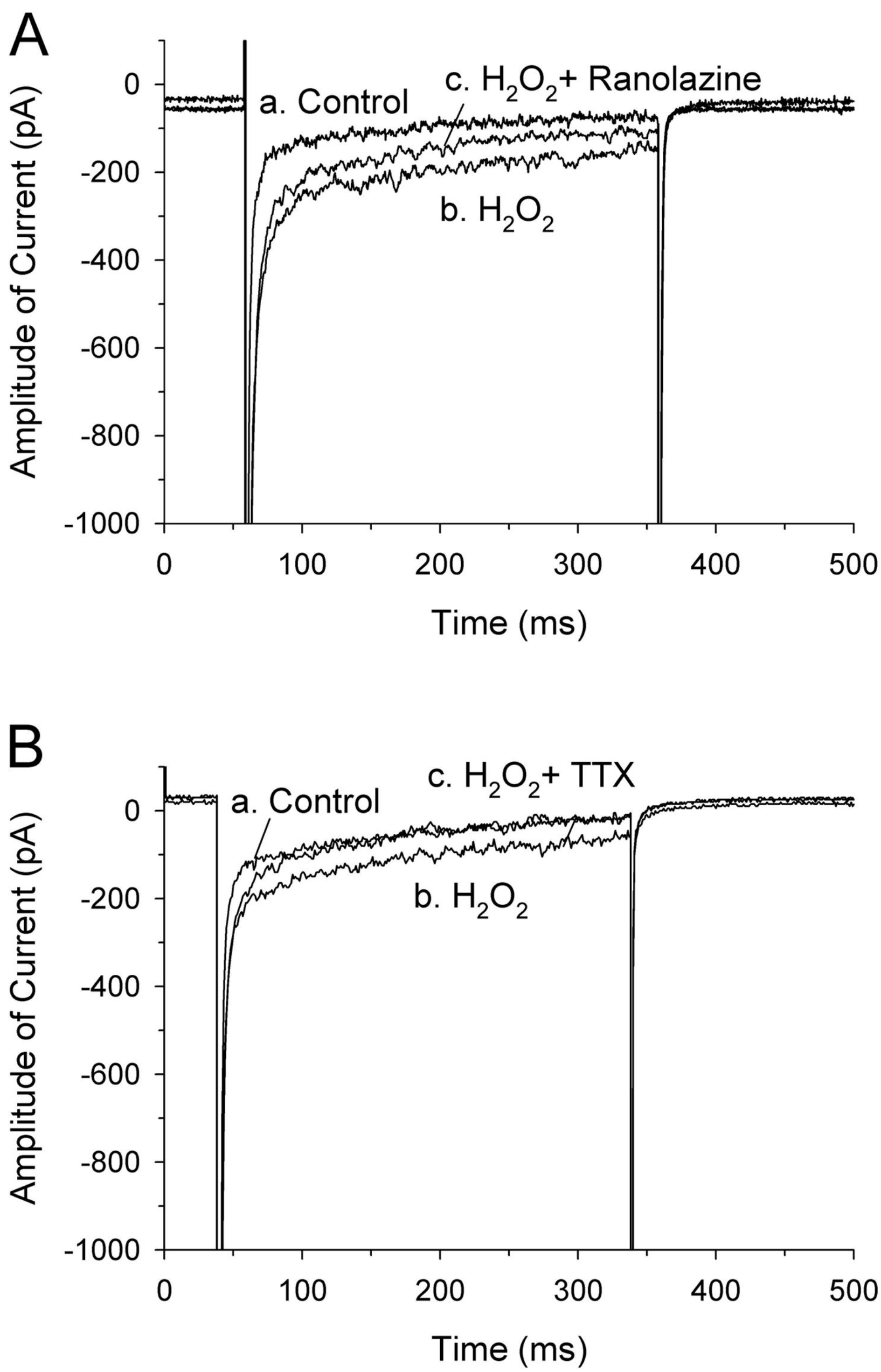
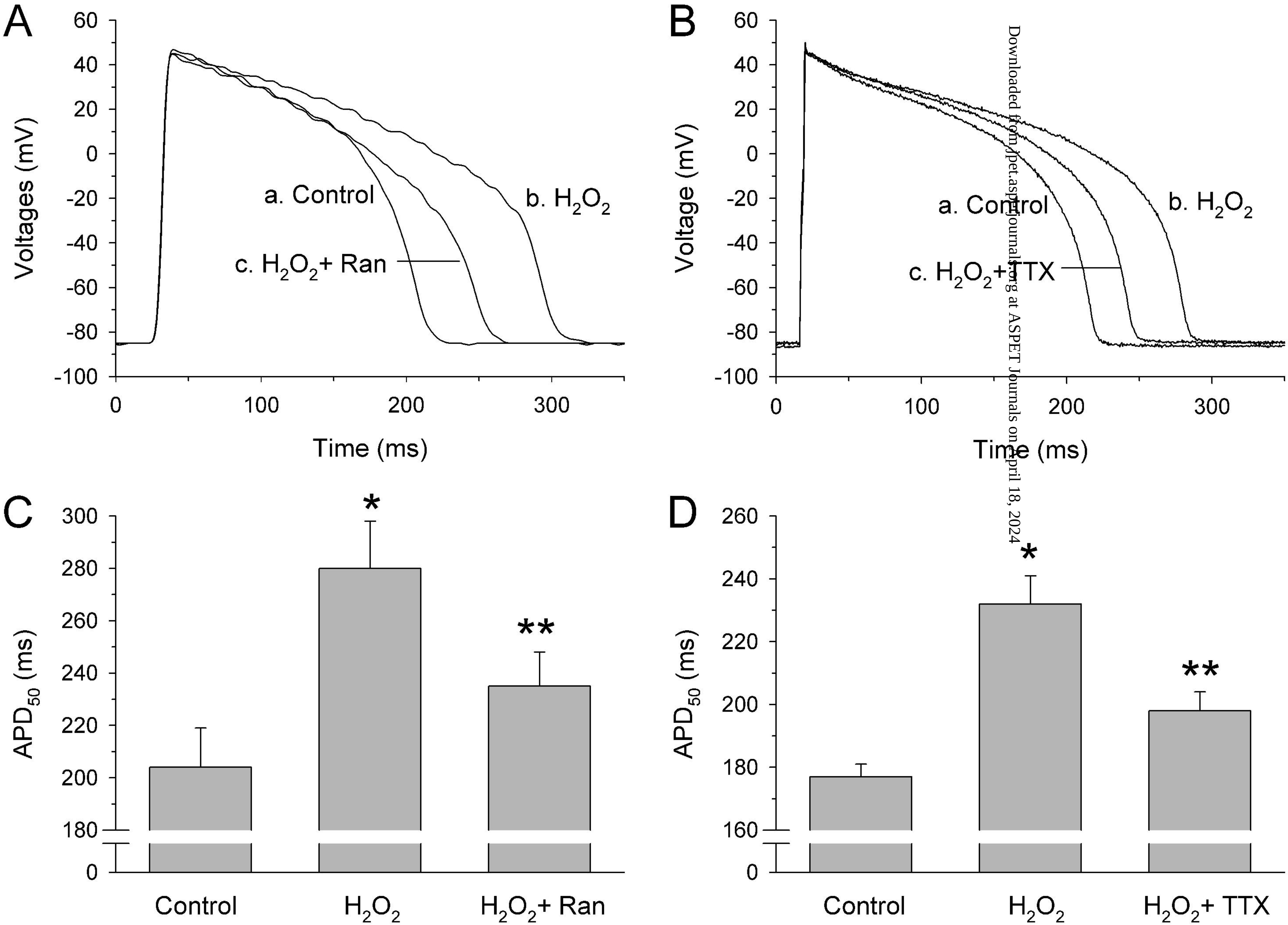


Figure 2



A
Voltages (mV)
a. Control
b. H_2O_2
c. H_2O_2+ Ran
Time (ms)
B
Voltage (mV)
a. Control
b. H_2O_2
c. H_2O_2+TTX
Time (ms)
C
APD_{50} (ms)
Control
H_2O_2
H_2O_2+ Ran
D
APD_{50} (ms)
Control
H_2O_2
H_2O_2+ TTX

Figure 3

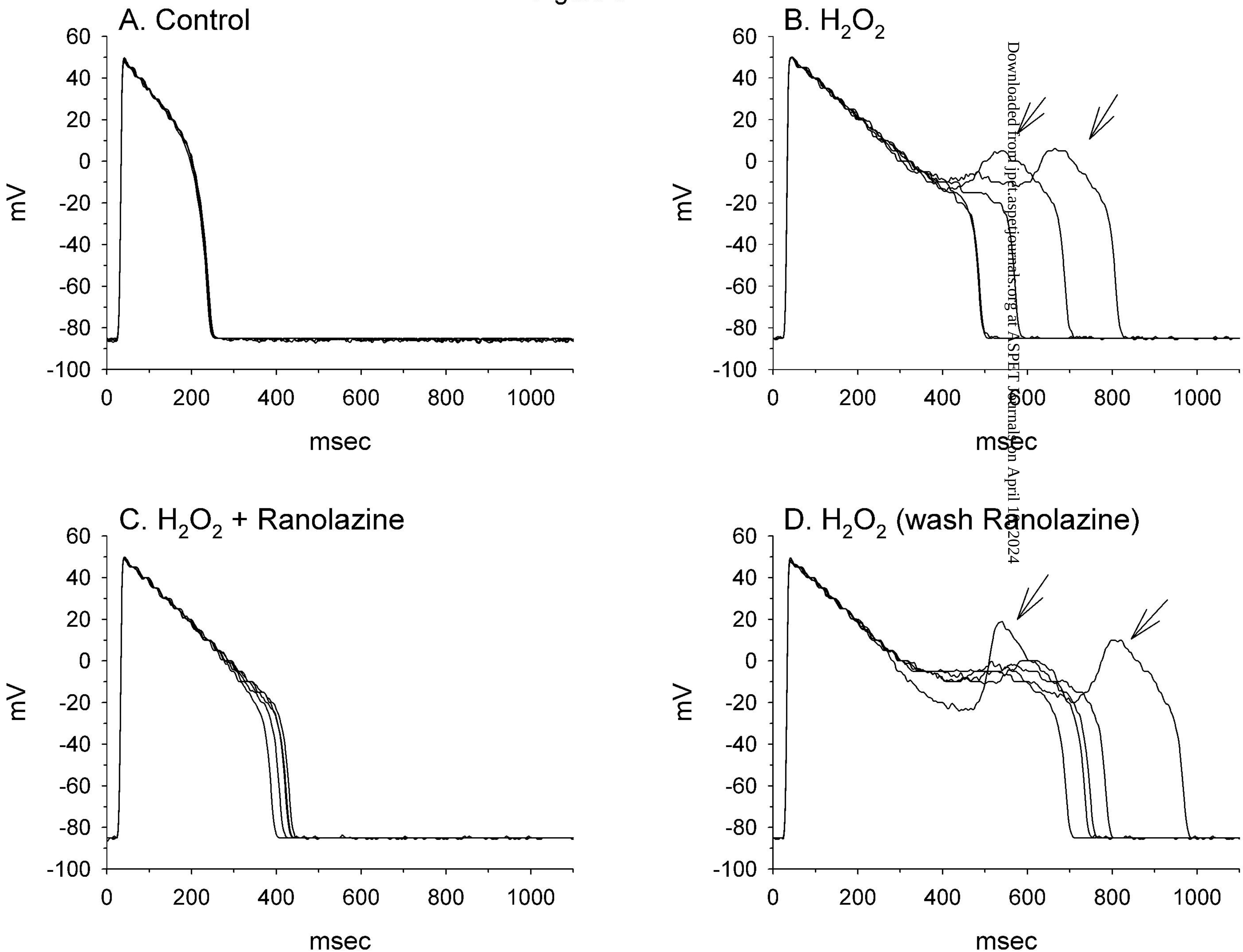

A. Control
B. H_2O_2
C. H_2O_2 + Ranolazine
D. H_2O_2 (wash Ranolazine)
mV
msec
60
40
20
0
-20
-40
-60
-80
-100
0
200
400
600
800
1000

Figure 4

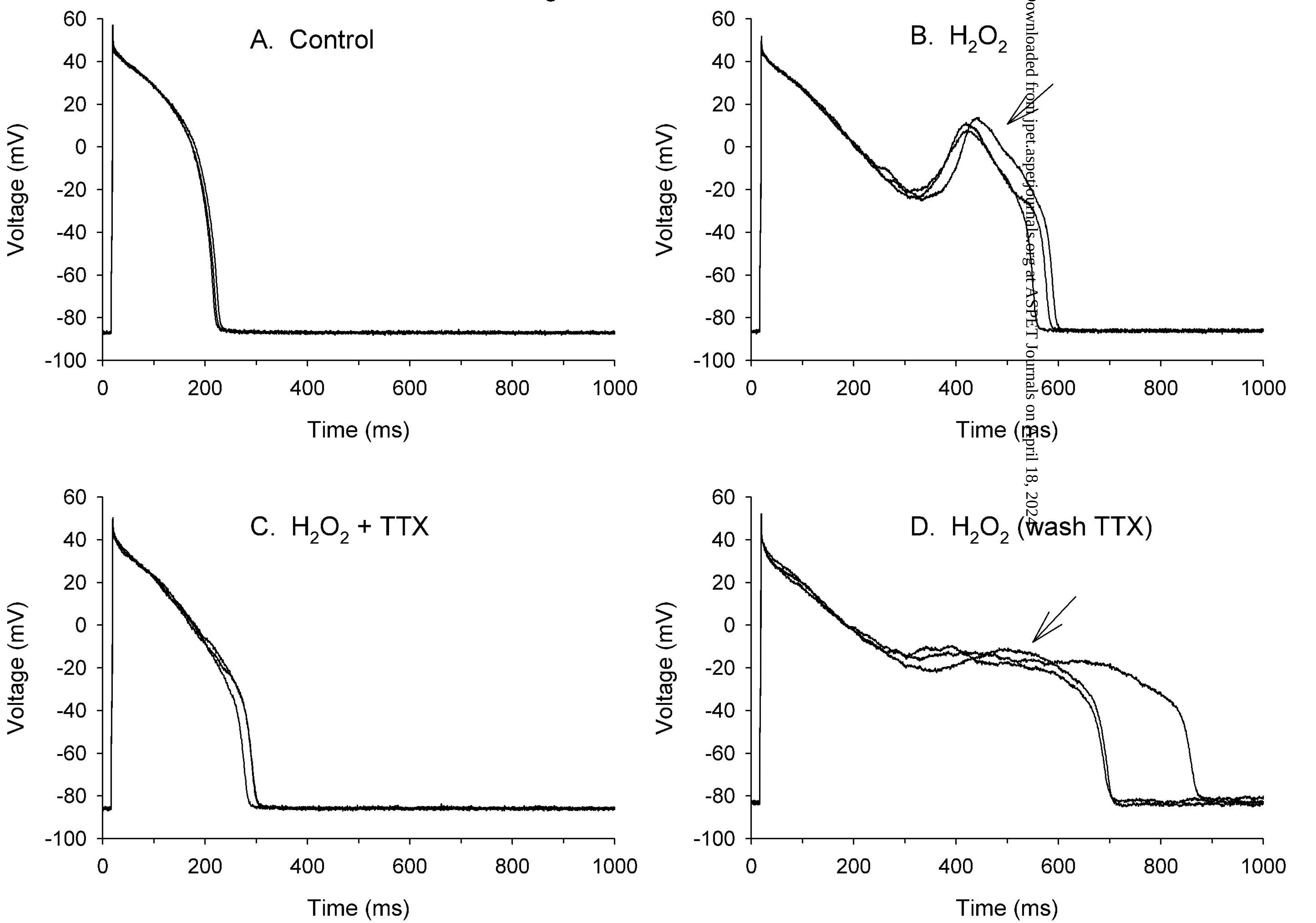

A. Control
B. H_2O_2
C. H_2O_2 + TTX
D. H_2O_2 (wash TTX)
Voltage (mV)
Time (ms)
60
40
20
0
-20
-40
-60
-80
-100
0
200
400
600
800
1000

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Figure 5

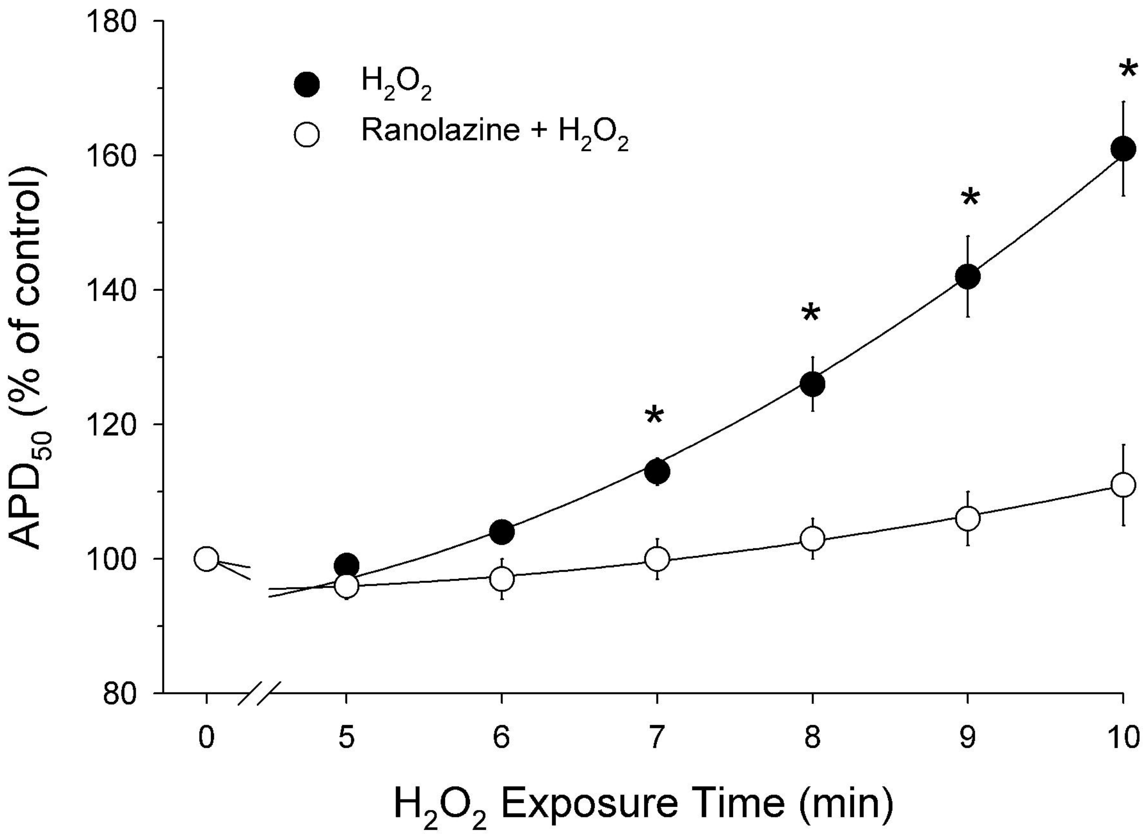
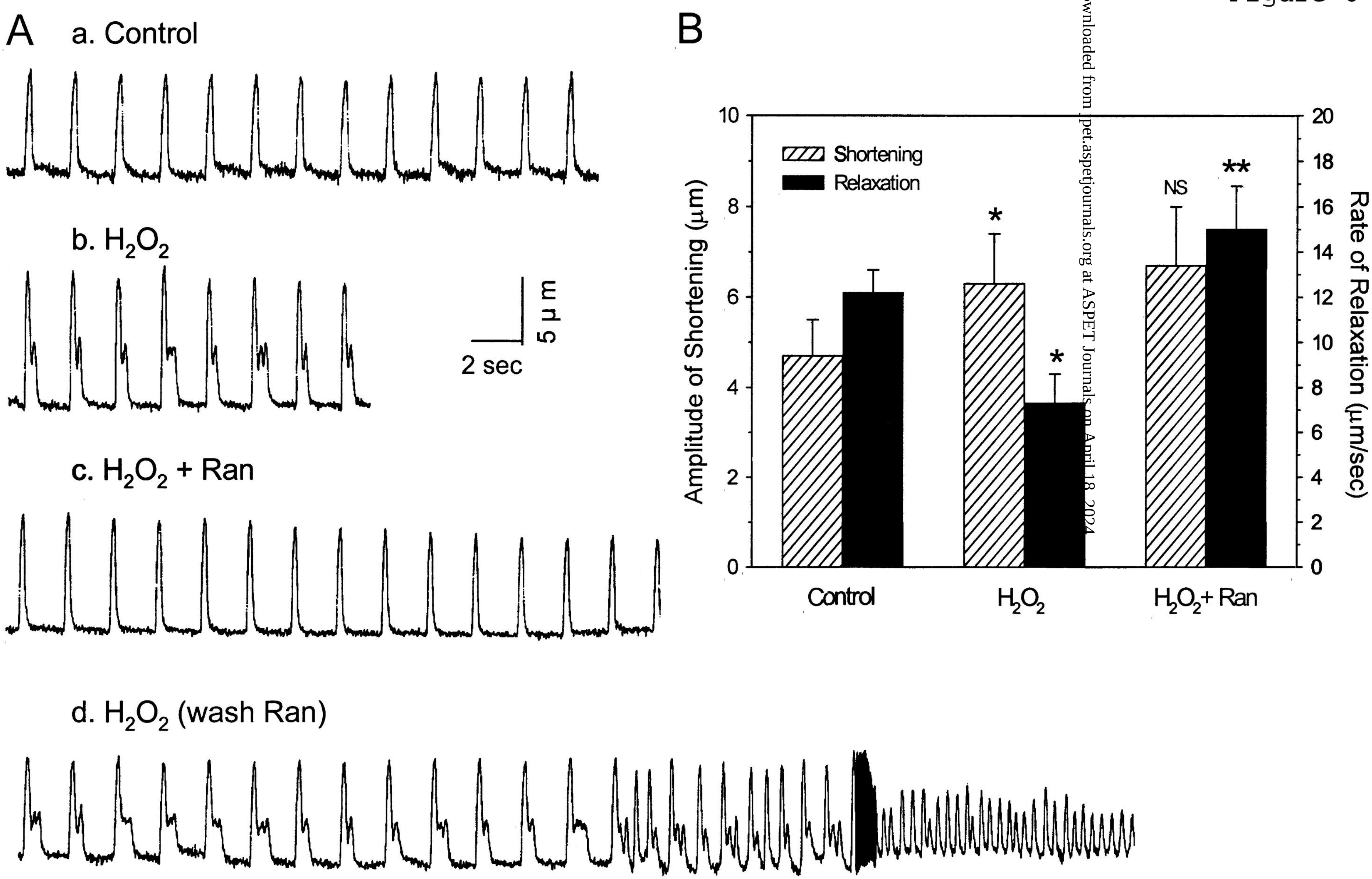
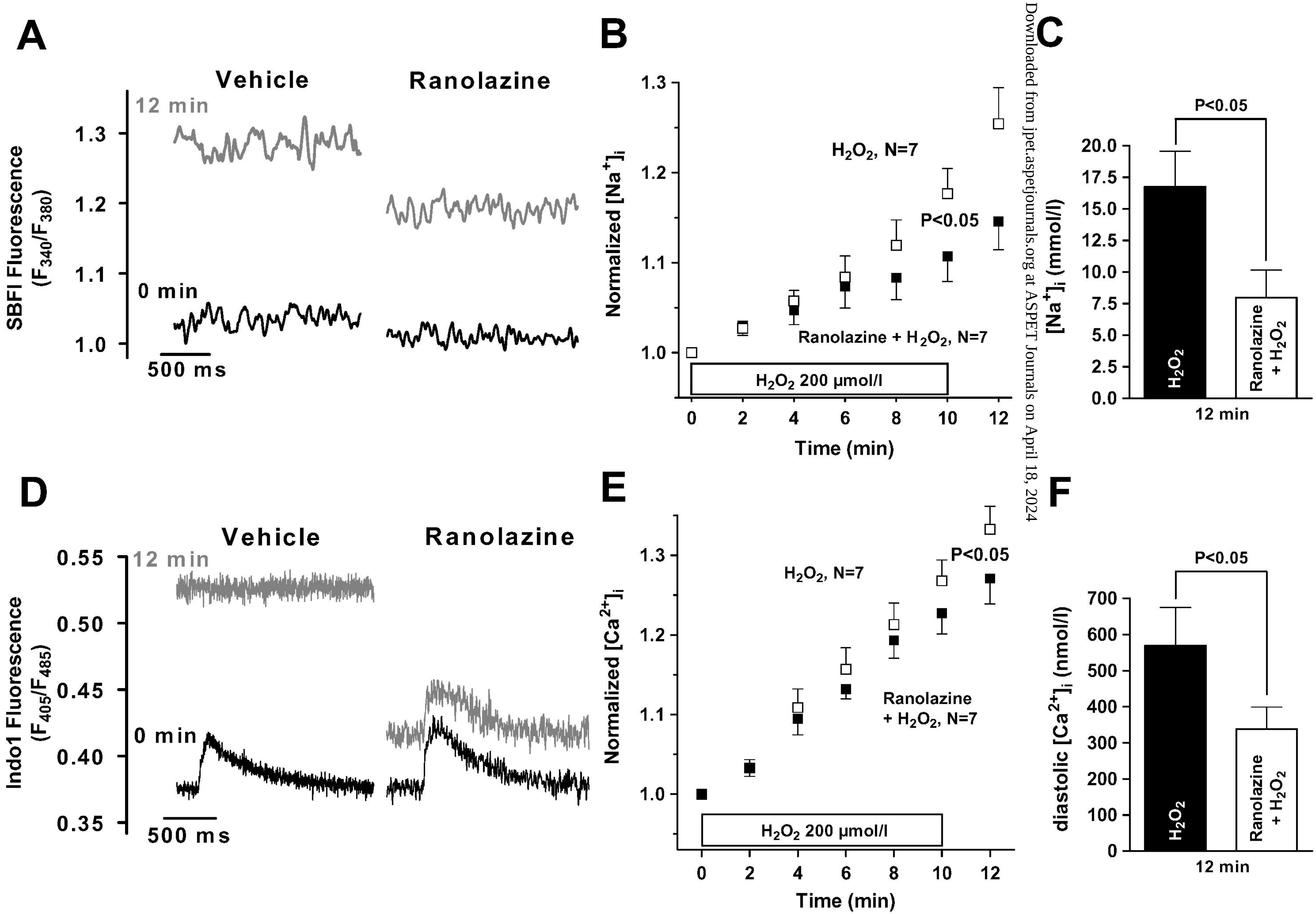


Figure 6



A
a. Control
b. H_2O_2
5 μ m
2 sec
c. H_2O_2 + Ran
d. H_2O_2 (wash Ran)
B
Shortening
Relaxation
Amplitude of Shortening (μm)
Rate of Relaxation (μm/sec)
NS
**
*
*
Control
H_2O_2
H_2O_2+ Ran
0
2
4
6
8
10
12
14
16
18
20

Figure 7



A
Vehicle
Ranolazine
12 min
0 min
SBFI Fluorescence (F_{340}/F_{380})
1.3
1.2
1.1
1.0
500 ms
B
Normalized $[Na^+]_i$
H_2O_2, N=7
P<0.05
Ranolazine + H_2O_2, N=7
H_2O_2 200 µmol/l
Time (min)
C
P<0.05
$[Na^+]_i$ (mmol/l)
H_2O_2
Ranolazine + H_2O_2
12 min
D
Vehicle
Ranolazine
12 min
0 min
Indo1 Fluorescence (F_{405}/F_{485})
500 ms
E
Normalized $[Ca^{2+}]_i$
H_2O_2, N=7
P<0.05
Ranolazine + H_2O_2, N=7
H_2O_2 200 µmol/l
Time (min)
F
P<0.05
diastolic $[Ca^{2+}]_i$ (nmol/l)
H_2O_2
Ranolazine + H_2O_2
12 min

Figure 8