Late Sodium Current Inhibition Alone with Ranolazine Is Sufficient to Reduce Ischemia- and Cardiac Glycoside-Induced Calcium Overload and Contractile Dysfunction Mediated by Reverse-Mode Sodium/Calcium Exchange

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ABSTRACT

Excessive reverse-mode (RM) sodium/calcium exchanger 1.1 (NCX1.1) activity, resulting from intracellular sodium accumulation caused by reduced Na+/K+-ATPase activity, increased Na-H exchanger 1 activity. The induction of the voltage-gated sodium channel late current component (late INa), is a major pathway for intracellular calcium (Ca2+) loading in cardiac ischemia-reperfusion (IR) injury and cardiac glycoside toxicity. Inhibition of late INa with the antianginal agent ranolazine is protective in models of IR injury and cardiac glycoside toxicity. However, whether inhibition of late INa alone is sufficient to provide maximal protection or additional inhibition of RM NCX1.1 provides further benefit remains to be determined conclusively. Therefore, the effects of ranolazine were compared with the INa inhibitor lidocaine in models of IR injury and ouabain toxicity, RM NCX1.1-mediated Ca2+ overload, and patch-clamp assays of RM NCX1.1 currents. Ranolazine and lidocaine (10 μM) similarly reduced Ca2+ overload and improved left ventricle work recovery in whole-heart models of IR injury or exposure to ouabain (80 μM). Ranolazine (10 μM), but not lidocaine (10 μM), reduced RM NCX1.1-mediated Ca2+ overload in ventricular myocytes. Furthermore, ranolazine inhibited RM NCX1.1 currents (IC50 1.7 μM), without affecting forward mode currents, revealing that ranolazine has novel RM NCX1.1 inhibitory actions. However, because lidocaine provides similar protection to ranolazine in whole-heart models but does not inhibit RM NCX1.1, we conclude that induction of late INa is upstream of RM NCX1.1 activity and selective inhibition of late INa alone is sufficient to reduce Ca2+ overload and contractile dysfunction in IR injury and cardiac glycoside toxicity.

Introduction

Intracellular calcium (Ca2+) overload is a key contributor to the mechanical and electrical dysfunction observed in cardiac ischemia-reperfusion (IR) injury and cardiac glycoside toxicity (Barry, 1987; Tani, 1990; Ferrari et al., 1993; Silverman and Stern, 1994; Piper et al., 2003; Ruch et al., 2003). Excessive Ca2+ influx mediated by reverse mode (RM) operation of the cardiac Na+-Ca2+ exchanger 1.1 (NCX1.1) is thought be the major mediator of the observed Ca2+ overload. Deleterious RM NCX1.1 activity occurs as a consequence of intracellular sodium (Na+) loading in response to 1) increased Na+ influx by Na+/H+ exchanger 1 (Eigel and Hadley, 1999; Fliegel, 2009), 2) impaired ATP availability to support Na+/K+ ATPase-mediated Na+ extrusion (Ju et al., 1996; Noble and Noble, 2006) in ischemia or direct inhibition by cardiac glycosides, and, more recently, 3) augmentation of a late and persistent Na+ current mediated by voltage-gated Na+ channels (late INa). Therefore, RM NCX1.1, Na+-H+

ABBREVIATIONS: Ca2+, intracellular calcium; IR, ischemia-reperfusion; lINa, sodium current; NCX1.1, sodium/calcium exchanger 1.1; RM, reverse mode; FM, forward mode; Na+, intracellular sodium; NRVM, neonatal rat ventricular myocyte; DMSO, dimethyl sulfoxide; AUC, area under the curve; shRNA, short hairpin RNA; ANOVA, analysis of variance; LV, left ventricle; TTX, tetrodotoxin; CaMKII, Ca2+/calmodulin-dependent kinase II; TEA, tetraethylammonium; WT, wild type; F405/F585, fluorescence at 405 nm/fluorescence at 485 nm; Kbr-R7943, 2-(2-(4-(4-nitrobenzyllox)-phenyl)ethyl)isothiourea methanesulfonate.
exchanger 1, and late INa have been investigated extensively as therapeutic targets to limit Ca\(^{2+}\), overload and elicit anti-

andric actions (Karmazyn et al., 2001; Lee et al., 2005; Saint, 2008; Moreno and Clancy, 2012). However, the relative importance of late INa and RM NCX1.1 inhibition in reducing IR injury and cardiac glycoside toxicity remains to be clearly delineated.

With respect to late INa, ranolazine (Ranexa; Gilead Sciences, Foster City, CA) is an approved drug for the treatment of angina pectoris. At therapeutic plasma concentrations (1–10 \(\mu M\)), ranolazine’s anti-ischemic action is thought to arise by inhibition of late INa (Antzelevitch et al., 2004a,b), reducing Na\(^+\) loading and thus attenuating RM NCX1.1-mediated Ca\(^{2+}\) overload to decrease contractile and electrical dysfunction caused by ischemia or selective augmentation of late INa, by sea anemone toxin II (Fraser et al., 2006; Sossalla et al., 2008; Wasserstrom et al., 2009). Furthermore, ranolazine has recently been shown to reduce Ca\(^{2+}\) overload and contractile dysfunction in a model of cardiac glycoside-induced toxicity, presumably by the inhibition of late INa (Hoyer et al., 2011). However, it is not known whether the inhibition of Na\(^+\)/K\(^+\) ATPase with cardiac glycosides involves augmentation of late INa. Although NCX1.1 has been previously excluded as a target, because ranolazine has little or no effect on the physiological forward mode (FM) activity of NCX1.1 (Antzelevitch et al., 2004a,b), the effect of ranolazine on the pathophysiological RM NCX1.1 activity has not been examined directly. Because RM NCX1.1 activity is the key mediator of Ca\(^{2+}\) overload caused either by ischemia or cardiac glycoside toxicity, the possibility remains that the beneficial effects of ranolazine may be caused, in part, by the direct inhibition of RM NCX1.1 activity.

Accordingly, we examined the effects of ranolazine and lidocaine, a known late INa inhibitor (Fedida et al., 2006) in 1) whole-heart models of IR injury and cardiac glycoside toxicity, 2) RM NCX1.1-mediated cellular Ca\(^{2+}\) overload independent of late INa, and 3) electrogenic FM and RM currents recorded from recombinant human cardiac NCX1.1.

Our findings indicate that ranolazine is an effective inhibitor of pathophysiological RM NCX1.1 at therapeutic concentrations, but this novel effect does not confer any additional protection over selective inhibition of late INa alone with lidocaine and suggests that induction of late INa is upstream of deleterious RM NCX1.1 activity in both IR injury and cardiac glycoside toxicity.

**Materials and Methods**

**Heart Perfusions and Measurements of LV Mechanical Function in Working Mode.** Adult male Sprague-Dawley rats (300–400 g) (Charles River Laboratories, Wilmington, MA) were anesthetized with pentobarbital (150 mg/kg i.p.) in accordance with the Canadian Council on Animal Care guidelines and the National Institutes of Health’s Guide for the Care and Use of Laboratory Animals (Institute of Laboratory Animal Resources, 1996) and approved by the University of Alberta Animal Policy and Welfare Committee (protocols 027 and 152). Hearts were excised, the aorta was cannulated, and a nonworking (Langendorff) perfusion was initiated within 30 s with Krebs-Henseleit solution (Ju et al., 1996; Karmazyn et al., 2001; Fraser et al., 2006). After 10 min, working-mode perfusion was initiated with recirculating perfusate (volume 100 ml; 37°C; pH 7.4; gassed with a 95% O\(_2\)/CO\(_2\) mixture) consisting of a modified Krebs-Henseleit solution containing 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 1.2 mM MgSO\(_4\), 2.5 mM CaCl\(_2\), 25 mM NaHCO\(_3\), 11 mM glucose, 1.2 mM palmitate, and 100 U/liter insulin. Palmitate was prebound to bovine serum albumin (final concentration of 3% wt/vol).

Heart perfusions were conducted at a constant workload (preload, 11.5 mm Hg; after load, 80 mm Hg; pacing rate of 5 Hz).

Systolic and diastolic aortic pressures (mm Hg) were measured with a pressure transducer (ADInstruments, Colorado Springs, CO) attached to the aortic outflow line. Cardiac output (ml/min) and aortic flow (ml/min) was measured by using ultrasonic flow probes (Transonic T206; Transonic Systems Inc., Ithaca, NY) placed in the left atrial inflow line and the aortic outflow line, respectively. All parameters were acquired by using Chart 5.0 software (ADInstruments). LV minute work (LV work, l/min · mm Hg) was used as a continuous index of LV mechanical function and calculated as cardiac output × LV developed pressure (aortic systolic pressure − preload pressure). Coronary flow (ml/min) was calculated as the difference between cardiac output and aortic flow, and coronary vascular conductance (ml/min/mm Hg) was measured as coronary flow divided by aortic diastolic perfusion pressure (Fraser et al., 2006).

**Heart Perfusions and Measurements of LV Intracellular Ca\(^{2+}\).** Hearts were loaded with the fluorescent Ca\(^{2+}\) indicator indo-1/acetoxymethyl ester (5 \(\mu M\)), and fluorescence was measured in a −0.5-cm\(^2\) area of the epicardial surface of the LV free wall by using a bifurcated fiber optic cable and spectrophotofluorometer (Photon Technology International, London, ON, Canada) (Wang et al., 2001). Fluorescent signals were sampled at 500 Hz, and the ratio of indo-1 fluorescence emitted at 405 and 485 nm was calculated to give an index of heart-to-beat Ca\(^{2+}\) (Fraser et al., 2006; Wang et al., 2008).

**Experimental Protocols for Heart Perfusions.** After the indo-1 loading procedure, normal aerobic perfusion of the hearts was conducted for 15 min to obtain baseline diastolic Ca\(^{2+}\), systolic Ca\(^{2+}\), and LV work. After the 15-min baseline aerobic period, hearts were either 1) subjected to an IR protocol comprised of 20 min of global, no-flow ischemia (no pacing), followed by 30 min of aerobic reperfusion (5-Hz pacing restarted within 2 min of reperfusion) (ranolazine (10 \(\mu M\), lidocaine (10 \(\mu M\), or vehicle (control)) were added 5 min before ischemia and remained present throughout ischemia and reperfusion), or 2) exposed to ouabain (80 \(\mu M\)) during normal aerobic perfusion for 30 min in the absence (control) or presence of either ranolazine (10 \(\mu M\)) or lidocaine (10 \(\mu M\)) that was administered 10 min after ouabain.

**Cellular Model of RM NCX1.1-Mediated Ca\(^{2+}\) Overload.** Neonatal rat ventricular myocytes (NRVMs) were isolated and cultured as described previously (Kovic et al., 2003). After 48 to 72 h of isolation, NRVMs were loaded with calcium green-1/acetoxymethyl ester (2 \(\mu M\)) for 30 min at room temperature (22 ± 1°C) and 30 min at 37°C, and then subjected to the protocol below as detailed previously (Eigel and Hadley, 2001; Soliman et al., 2009). Cells were excited with 488 ± 10-nm wavelength light, and fluorescent signals emitted at 520 ± 10 nm were measured by using a photomultiplier detection system (Photon Technology International). Data were analyzed by using Felix32 (Photon Technology International) and Clampfit 10.1 (Molecular Devices, Sunnyvale, CA) software.

To assess Ca\(^{2+}\) influx via RM NCX1.1 activity, NRVMs were superfused for 2 min with a solution containing 140 mM NaCl, 4 mM KCl, 10 mM HEPES, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 mM dextrose. Cells were then superfused with a K\(^{+}\)-free solution (144 mM NaCl, 10 mM HEPES, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 mM dextrose) for 5 min, followed by 5-min superfusion with Na\(^+\)-free solution (140 mM LiCl, 4 mM KCl, 10 mM HEPES, 2.5 mM CaCl\(_2\), 1 mM MgCl\(_2\), and 10 mM dextrose). Tetrodotoxin (TTX; 10 \(\mu M\)) was added to both K\(^{+}\)-free and Na\(^+\)-free solutions to inhibit cardiac Na\(^+\) channels. Thapsigargin (2 \(\mu M\)) was added to the Na\(^+\)-free solution to reduce Ca\(^{2+}\) sequestration by sarcoplasmic reticulum Ca\(^{2+}\)-ATPase 2a. 2-(2-(4-(4-Nitrobenzyloxy)phenyl)ethyl)isothiourrea methanesulfonate (KB-R7943) (5 \(\mu M\)), ranolazine (10 \(\mu M\), or lidocaine (10 \(\mu M\), or...
NiCl₂ (5 mM) was applied during the Na⁺-free perfusion period to measure their effects on RM NCX1 activity. To silence NCX1 expression in NRVMs, cells were infected with an adenoviral vector encoding short hairpin RNA for NCX1 (AdshRNA-NCX1) 72 h before experiments as described previously (Soliman et al., 2009). The AdshRNA-NCX1 construct was generously provided by Dr. Grant Pierce (University of Manitoba, Winnipeg, Canada) (Hurtado et al., 2007).

**Molecular Biology, Transfection, and Electrophysiology of NCX1.1.** Mammalian expression vector plasmids containing either human NCX1.1, rat NCX1.1-F255E, or human NCX1.1-K264Q constructs were transfected into cultured tsA201 cells, and the whole-cell or excised inside-out patch-clamp techniques were used to measure macroscopic RM and FM NCX1.1 currents. Whole-cell patch-clamp RM NCX1.1 currents were recorded by using a similar procedure to that described previously (Su et al., 1999), with the exception that pipettes were pulled from borosilicate glass to yield resistances of 2 to 4 MΩ when filled with pipette solution. The pipette solution contained 20 mM NaCl, 130 mM CsCl, 10 mM HEPES, 5.5 mM glucose, 3.3 mM MgCl₂, 1 mM CaCl₂, and 15 mM EGTA. pH was adjusted to 7.4 with CsOH. Outward currents were elicited by rapidly switching from Na⁺-containing extracellular solution (138 mM NaCl, 12 mM HEPES, 11 mM glucose, 1 mM MgCl₂, and 1 mM CaCl₂) to a Na⁺-free solution containing 138 mM CsCl, 12 mM HEPES, 11 mM glucose, 1 mM MgCl₂, and 1 mM CaCl₂. Holding potential was −40 mV, and outward RM NCX1.1 currents were measured and analyzed by using an Axopatch 200B amplifier and Clampex 10.1 software (Molecular Devices). Experiments were performed at room temperature (22 ± 1°C).

To study the inactivation kinetics, the excised inside-out patch-clamp technique was used to record RM and FM NCX1.1 currents as described previously (Hamming et al., 2008; Soliman et al., 2009). For RM NCX1.1 currents, the pipette (extracellular) solution contained 140 mM CsCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, and 4 mM CaCl₂. pH was adjusted to 7.4 with CsOH. Outward currents were elicited by rapidly switching from an intracellular cesium-based solution containing 120 mM CsCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, and 4.28 mM CaCl₂ to an intracellular sodium-based solution containing 30 mM CsCl, 90 mM NaCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, and 4.28 mM CaCl₂. Free calcium concentrations were buffered to 800 nM with 5 mM EGTA, and pH was adjusted to 7.2 with CsOH.

For FM NCX1.1 inward current measurements, the pipette (extracellular) solution contained 30 mM CsCl, 90 mM NaCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, and 4.28 mM CaCl₂. Free calcium concentrations were buffered to 800 nM with 5 mM EGTA, and pH was adjusted to 7.4 with CsOH. FM inward currents were activated by rapidly changing the intracellular solution from a cesium-based low-calium solution containing 120 mM CsCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, 4.28 mM CaCl₂, and 5 mM EGTA to a cesium-based high-sodium solution containing 140 mM CsCl, 20 mM TEA, 5 mM HEPES, 2 mM MgATP, 10 mM glucose, 1.4 mM MgCl₂, and 4 mM CaCl₂. The pH of all intracellular solutions was adjusted to 7.2 with CsOH. NCX1 currents were activated for 60 s. Excised patches were held at 0 mV, and NCX1.1 currents were measured by using an Axopatch 200B amplifier and Clampex 10.1 software (Molecular Devices). All experiments were performed at room temperature (22 ± 1°C).

**Experimental Compounds.** Ouabain, lidocaine, thapsigargin, and NiCl₂ were purchased from Sigma-Aldrich (Oakville, ON, Canada). KB-R7943 and ranolazine were purchased from Torcix Biosciences (Ellisville, MO). Tetrodotoxin citrate was obtained from Alomone Labs (Jerusalem, Israel). DMSO concentration did not exceed 0.25% (v/v) in heart perfusion experiments, 0.3% (v/v) in NRVM cellular imaging, and 0.1% (v/v) in patch-clamp experiments. DMSO was present in all control solutions as vehicle control.

**Data Analysis and Statistical Methods.** Data are displayed as mean ± S.E.M. Late NCX1.1 current amplitudes were measured and normalized to peak current. The time constant of RM NCX1.1 current inactivation (τ) was obtained by fitting currents with a best-fit single exponential function. Statistical significance was assessed by using the unpaired or paired Student’s t test or one-way ANOVA with a Tukey’s post hoc analysis where indicated. Differences were considered significant at p < 0.05.

**Results**

**Attenuation of IR-Induced LV Dysfunction and Ca²⁺,i Overload by Ranolazine or Lidocaine.** During baseline aerobic perfusion of isolated rat hearts, indices of LV mechanical function and diastolic Ca²⁺,i were within normal ranges (Figs. 1A and 2, A and C). During reperfusion, LV work partially recovered and after 30-min reperfusion of vehicle-treated hearts was 28% (n = 14; p < 0.001) of preischemic baseline values (Fig. 2A). In contrast, ranolazine or lidocaine enhanced the recovery of postischemic function as LV work recovered to 78% (n = 8; p < 0.001) and 70% (n = 5; p < 0.001), respectively, of preischemic baseline values (Fig. 2, A and B). Recovery of coronary flow and coronary vascular conductance were also improved by ranolazine or lidocaine (data not shown). During global ischemia, all measurable LV work ceased, and diastolic Ca²⁺,i rose steadily in vehicle-treated hearts, increasing by 54% relative to preischemic values after 25 min (n = 14; p < 0.001; Figs. 1B and 2, C and D). In contrast, ranolazine (10 μM) or lidocaine (10 μM) reduced the increases in diastolic Ca²⁺,i during ischemia to 29% (n = 8; p < 0.01) and 70% (n = 5; p < 0.01), respectively (Fig. 2, C and D). In contrast, systolic Ca²⁺,i was not affected by either ranolazine or lidocaine (Fig. 2, E and F), despite the reduction in diastolic Ca²⁺,i.

To further investigate the underlying mechanisms for cardioprotection afforded by ranolazine or lidocaine, the Na⁺/K⁺-ATPase inhibitor ouabain was used to increase Na⁺, and thereby enhance RM NCX1.1-mediated Ca²⁺,i accumulation by mechanisms independent of IR injury. Although LV work was depressed after the 30-min exposure to ouabain (80 μM) by 67% relative to baseline values (n = 6; p < 0.01; Fig. 3, A and B), ouabain-induced depression of LV work was significantly attenuated by ranolazine or lidocaine, and LV work reduced from such recordings. Dashed lines represent the systolic and diastolic calcium levels at aerobic baseline to illustrate the changes occurring during global ischemia of ouabain treatment.
was depressed by only 23% \((n = 5; p < 0.01)\) or 31% \((n = 6; p < 0.01)\) (Fig. 3, A and B). Ouabain (80 \(\mu M\)) significantly increased diastolic Ca\(^{2+}\) \((p < 0.001; n = 6;\) Fig. 3, C and D). Application of ranolazine (10 \(\mu M\)) or lidocaine (10 \(\mu M\)) 10 min after ouabain significantly reduced ouabain-induced diastolic Ca\(^{2+}\) accumulation relative to vehicle-treated hearts to 14% \((n = 5; p < 0.001)\) or 16% \((n = 6; p < 0.001)\), respectively (Fig. 3, C and D). However, systolic Ca\(^{2+}\) was not affected by either ranolazine or lidocaine (Fig. 3, E and F), despite the reduction in diastolic Ca\(^{2+}\).

**Ranolazine Inhibits Activity in a Cellular Model of RM NCX1.1-Induced Ca\(^{2+}\) Overload.** Because the effects of ranolazine on RM NCX1.1 activity have not been tested previously, yet may account for some of the protective actions of ranolazine, we sought to determine whether ranolazine directly inhibits RM NCX1.1 activity in an intact cellular model (Fig. 4). We used NRVMs in which RM NCX1.1 was activated by an initial superfusion (5 min) with a K\(^{-}\)-free solution to uncouple the Na\(^{+}\)/K\(^{-}\)-ATPase to increase Na\(^{+}\) accumulation followed by superfusion with a Na\(^{+}\)-free solution (5 min) to induce RM NCX1.1 activity. Under these conditions, RM NCX1.1 exchanges Na\(^{+}\) for extracellular Ca\(^{2+}\), resulting in Ca\(^{2+}\) accumulation that can be quantified by using Ca\(^{2+}\) imaging. The Na\(^{+}\) channel toxin TTX (10 \(\mu M\)) was used throughout the experiments to eliminate any involvement of INa. To verify the participation of RM NCX1.1 activity in this cellular model, the NCX1 inhibitor Ni\(^{2+}\) (5 mM) was applied during Na\(^{+}\)-free superfusion and almost completely abolished the increase in Ca\(^{2+}\) (92.1 \pm 1.7% reduction; \(p < 0.01\); Fig. 4, A and C). Likewise, molecular knockdown of NCX1.1 by adenosiviral delivery of NCX1 shRNA (AdshRNA-NCX1) also resulted in a marked decrease in the observed Ca\(^{2+}\) signal (73.9 \pm 13.6% reduction; \(p < 0.01\); Fig. 4, A and C). These data confirm that the induced Ca\(^{2+}\) overload occurring during Na\(^{+}\)-free conditions is mediated predominantly by RM NCX1.1 activity.

Using this validated cellular model of evoked RM NCX1.1 activity, the effects of ranolazine, the Na\(^{+}\) channel inhibitor lidocaine, and the NCX inhibitor KB-R7943 were compared. Ranolazine (10 \(\mu M\)) decreased Ca\(^{2+}\) overload by 23.6 \pm 2.6% \((p < 0.001)\) (Fig. 4, B and C). In direct contrast, lidocaine (10 \(\mu M\)) had no significant effect on reducing Ca\(^{2+}\) overload (Fig. 4, B and C). KB-R7943 (5 \(\mu M\)) had an effect similar to ranolazine and decreased Ca\(^{2+}\) overload by 20.7 \pm 3.9% \((p < 0.01)\). These data indicate that ranolazine, but not lidocaine,
inhibits RM NCX1.1-dependent \( \text{Ca}^{2+} \) overload independently of peak or late \( I_{nS} \) inhibition.

**Ranolazine Inhibits Reverse-Mode Current in Recombinant Human NCX1.1.** To directly assess the effects of ranolazine on NCX1.1 activity, the whole-cell patch-clamp technique was used to measure \( \text{Ni}^{2+} \)-sensitive RM currents (Fig. 5, A and F) in tsA201 cells transiently expressing the human heart NCX1.1 splice variant. Extracellular ranolazine elicited a concentration-dependent inhibition of RM NCX1.1 current with a maximum inhibition of \( \sim 80\% \) occurring at a concentration of 100 \( \mu \text{M} \) (IC\(_{50} = 1.7 \mu \text{M}\)) (Fig. 5, B–D). The inhibitory effect of ranolazine (10 \( \mu \text{M}\)) was readily reversible after 1 min of drug washout (99.1 \pm 2.3\% versus control). In direct contrast, lidocaine (10 \( \mu \text{M}\)) did not significantly inhibit RM NCX1.1 currents (Fig. 5, E and F). Upon reintroduction of extracellular \( \text{Na}^+ \), NCX1.1 switches to FM operation to extrude \( \text{Ca}^{2+} \) from the cell, resulting in a small but measurable transient inward current (Fig. 5B). No inhibition of this FM NCX1.1 current was observed with the application of either ranolazine or lidocaine (Fig. 5, B–E).

Because many NCX1.1 inhibitors have been demonstrated to display a selectivity for RM NCX1.1 currents displaying \( \text{Na}^+ \)-dependent \( I_1 \) inactivation (Iwamoto, 2004; Iwamoto and Kita, 2004), the excited inside-out patch technique was used to measure RM and FM NCX1.1 currents where the inactivation kinetics are more readily observed. Whereas ranolazine (10 \( \mu \text{M}\)) reversibly inhibited the activating RM NCX1.1 currents, even a high concentration of ranolazine (100 \( \mu \text{M}\)) failed to inhibit the nonactivating FM NCX1.1 currents (Fig. 6) as reported previously (Antzelevitch et al., 2004b). To gain further insights into the mechanism of RM NCX1.1 inhibition and the relationship to the inactivation process, the effects of ranolazine on NCX1.1 K264Q and F255E mutants were assessed. RM NCX1.1-K264Q currents do not exhibit the normal characteristic \( I_1 \) inactivation, and ranolazine was unable to inhibit these mutant nonactivating RM currents (Fig. 7A). A similar lack of effect was observed in the nonactivating FM NCX1.1-WT currents (Fig. 7B). Conversely, NCX1.1-F255E RM currents exhibit a faster inactivation process (\( \tau = 519 \pm 42 \text{ ms} \)), and this was significantly accelerated by ranolazine (10 \( \mu \text{M}\); \( \tau = 404 \pm 29 \text{ ms}\); \( p < 0.05\); Fig. 7, C and D).

**Discussion**

**Late \( I_{nS} \), IR Injury and Cardiac Glycoside Toxicity.** Late \( I_{nS} \) inhibition has been suggested to be a useful ap-
near preischemic values (Fig. 2, C and D), presumably via the inhibition of late INa.

Cardiac glycosides such as ouabain are useful clinically to increase inotropy of the failing heart (Kjeldsen and Bundgaard, 2003; Schoner and Scheiner-Bobis, 2007) and are thought to act via inhibition of Na\(^+\)/K\(^+\)-ATPase activity, resulting in Na\(^+\) accumulation that favors RM NCX1.1-mediated elevations in Ca\(^{2+}\), and enhanced myocardial contractility (Rocchetti et al., 2003; Saini and Dhalla, 2007). In contrast, higher concentrations of ouabain are cardiotoxic and impair LV mechanical function because of excessive Na\(^+\) accumulation that promotes NCX1.1-mediated Ca\(^{2+}\) overload. Studies have suggested that ouabain may induce late I\(_{Na}\) through voltage-gated Na\(^+\) channel phosphorylation by Ca\(^{2+}\)/calmodulin-dependent kinase II (CaMKII) (Maltsev et al., 2008; Hoyer et al., 2011), thus providing a mechanistic rationale for the protective effects of ranolazine reported previously in a cardiac glycoside toxicity model of ouabain-induced cardiac dysfunction (Hoyer et al., 2011). Indeed, we found that ranolazine and lidocaine are equally effective in reducing ouabain-induced LV dysfunction and Ca\(^{2+}\) accumulation in whole hearts (Fig. 3).

Taken together, our whole heart data support the notion that the induction of late I\(_{Na}\) is a major upstream mediator of Na\(^+\) accumulation and subsequent RM NCX1.1-mediated Ca\(^{2+}\) overload observed in these two pathologies. This conclusion is based on several assumptions: 1) early induction of late I\(_{Na}\) and/or elevations in Ca\(^{2+}\) are sufficient to lead to CaMKII activation and subsequent sodium channel phosphorylation and the further augmentation of late I\(_{Na}\) that leads to Na\(^+\) loading and RM NCX1.1 activity and 2) ranolazine and lidocaine have no effect on RM NCX1.1 activity that is a major mediator of Ca\(^{2+}\) overload. Indeed, with respect to CaMKII, it has recently been shown that late I\(_{Na}\) activates CaMKII and leads to sodium channel phosphorylation and increased late I\(_{Na}\) (Yao et al., 2011). However, with respect to the lack of RM NCX1.1 inhibition, the effects of these cardioprotective agents on RM NCX1.1 activity have not been tested directly until this study.

**Ranolazine Inhibition of RM NCX1.1 Activity.** To test whether ranolazine or lidocaine inhibits RM NCX1.1 activity directly and independently of late I\(_{Na}\), we used an isolated cardiomyocyte model in which TTX was included to inhibit both peak and late I\(_{Na}\) to ensure that any drug-induced alteration of NCX1.1-mediated Ca\(^{2+}\) overload would be independent of I\(_{Na}\) inhibition. Under these conditions, ranolazine and the NCX inhibitor KB-R7943 (Iwamoto and Kita, 2004; Iwamoto, 2004) inhibited evoked RM NCX1.1 activity by \(~\)21 to 24%, respectively (Fig. 4). It is noteworthy that the I\(_{Na}\) inhibitor lidocaine was without effect in this cellular model.

Previous studies have characterized the cardiac ion channel and exchanger pharmacology of ranolazine, including peak and late I\(_{Na}\), I\(_{K1}\), I\(_{Ks}\), I\(_{to}\) L-type Ca\(^{2+}\) channels, and NCX1.1 (Antzelevitch et al., 2004a,b). At therapeutic concentrations of 1 to 10 \(\mu M\), ranolazine inhibits late I\(_{Na}\) more effectively than any other cardiac ion channel. It is noteworthy that ranolazine has been reported to be only a weak inhibitor of NCX1 currents (IC\(_{50}\) 91 \(\mu M\)) (Antzelevitch et al., 2004b). However, in that study, NCX currents were elicited by FM NCX1.1 exchange activity, and the effects on RM NCX1 activity were not characterized. Our comparison of ranolazine on FM and RM NCX1.1 activity provides evidence...
that ranolazine is an effective inhibitor of RM NCX1.1 activity at therapeutically relevant concentrations (IC50 1.7 μM; Figs. 5, B-D). Furthermore, ranolazine is a selective RM inhibitor because it is unable to inhibit FM NCX1.1 activity, even at higher than therapeutic concentrations (100 μM) (Figs. 5D and 6B). Indeed, RM-selective inhibition of NCX1.1 probably would be a desirable therapeutic property because it reduces the pathophysiological Ca2+ influx that leads to Ca2+ overload, while sparing Ca2+ efflux resulting from FM activity.

RM selectivity of NCX1.1 inhibitors is thought to be caused by the drug binding to exchangers undergoing the I1 inactivation process, a characteristic of RM, but not FM NCX1.1 currents (Elias et al., 2001; Bouchard et al., 2004; Hamming et al., 2008). To assess whether the RM selectivity of ranolazine similarly depends on the I1 inactivation process, we measured the effects of ranolazine on RM currents arising from NCX1.1 with altered I1-inactivation properties (Mat-suoka et al., 1997). Although ranolazine did not inhibit the nonactivating RM current of the K264Q mutant NCX1.1, it further accelerated the rate of current inactivation in NCX1.1 containing the F255E mutant that displays an increased rate of inactivation (Fig. 7). Taken together, these findings support our conclusion that ranolazine is a RM-selective inhibitor of NCX1.1 activity and possesses a mechanism of action similar to other known NCX1.1 inhibitors.

In summary, our results indicate that ranolazine and lidocaine are equally effective in reducing contractile dysfunction and Ca2+ loading in whole-heart models of IR injury and cardiac glycocide toxicity. In addition, ranolazine, but not lidocaine, has a direct inhibitory effect on cardiac RM NCX1.1 activity. Taken together, these results suggest that late Iiso is involved in both IR injury and cardiac glycocide toxicity and the observed RM NCX1.1 inhibitory effects of ranolazine do not confer any additional protection in these models. Therefore, induction of late Iiso is probably upstream of RM NCX1.1-mediated Ca2+ over-load, and the observed inhibition of RM NCX1.1 activity with ranolazine does not confer any additional protection over late Iiso inhibition alone in models of IR injury and cardiac glycocide toxicity.

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Authorship Contributions

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