Late INa 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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Running title page

**Running title:** Ranolazine, Late $I_{Na}$, and cardioprotection

**Commonly used abbreviations:**

IR injury; ischemia reperfusion injury
Late $I_{Na}$; late or persistent current of the voltage-gated sodium channel
NCX1.1; cardiac sodium/calcium exchanger splice variant NCX1.1
RM; reverse-mode operation of NCX1.1
FM; forward-mode operation of NCX1.1
$Ca^{2+}_{i}$; intracellular calcium
$Na^{+}_{i}$; intracellular sodium

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Abstract.

Excessive reverse-mode sodium/calcium exchanger (RM NCX1.1) activity, resulting from Na\(^{+}\)\(_i\) accumulation due to reduced Na\(^{+}\)/K\(^{+}\)-ATPase activity, increased NHE1 activity and the induction of the voltage-gated sodium channel late current component (late I\(_{Na}\)), is a major pathway for Ca\(^{2+}\)\(_i\) loading in cardiac ischemia-reperfusion (IR) injury and cardiac glycoside toxicity. Inhibition of late I\(_{Na}\) with the anti-anginal agent ranolazine is protective in models of IR injury and cardiac glycoside toxicity. However, whether inhibition of late I\(_{Na}\) alone is sufficient to provide maximal protection or if additional inhibition of RM NCX1.1 provides further benefit remains to be determined conclusively. Therefore, the effects of ranolazine were compared to the I\(_{Na}\) inhibitor lidocaine in models of IR injury and ouabain toxicity, RM NCX1.1-mediated Ca\(^{2+}\) overload and in patch-clamp assays of RM NCX1.1 currents. Ranolazine and lidocaine (10 µM) similarly reduced Ca\(^{2+}\)\(_i\) overload and improved LV 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 Ca\(^{2+}\)\(_i\) overload in ventricular myocytes. Furthermore, ranolazine, inhibited RM NCX1.1 currents (IC\(_{50}\) = 1.7 µM), without affecting forward mode currents, revealing that ranolazine has novel RM NCX1.1 inhibitory actions. However, as lidocaine provides similar protection to ranolazine in whole heart models but does not inhibit RM NCX1.1, we conclude that induction of late I\(_{Na}\) is upstream of RM NCX1.1 activity and selective inhibition of late I\(_{Na}\) alone is sufficient to reduce Ca\(^{2+}\)\(_i\) overload and contractile dysfunction in IR injury and cardiac glycoside toxicity.
Introduction

Intracellular calcium (Ca\textsuperscript{2+}\textsubscript{i}) overload is a key contributor to the mechanical and electrical dysfunction observed in cardiac ischemia-reperfusion (IR) injury and cardiac glycoside toxicity (Barry, 1987; Ferrari et al., 1993; Piper et al., 2003; Ruch et al., 2003; Silverman et al., 1994; Tani, 1990). Excessive Ca\textsuperscript{2+} influx mediated by reverse-mode (RM) operation of the cardiac Na\textsuperscript{+}-Ca\textsuperscript{2+} exchanger (NCX1.1) is thought be the major mediator of the observed Ca\textsuperscript{2+}\textsubscript{i} overload. Deleterious RM NCX1.1 activity occurs as a consequence of intracellular sodium (Na\textsuperscript{+}) loading in response to 1) increased Na\textsuperscript{+} influx by the Na\textsuperscript{+}-H\textsuperscript{+} exchanger NHE-1 (Eigel et al., 1999; Fliegel, 2009), 2) impaired ATP availability to support Na\textsuperscript{+}/K\textsuperscript{+} ATPase-mediated Na\textsuperscript{+} extrusion (Ju et al., 1996; Noble et al., 2006) in ischemia or direct inhibition by cardiac glycosides and more recently, 3) augmentation of a late and persistent Na\textsuperscript{+} current mediated by voltage-gated Na\textsuperscript{+} channels (late I\textsubscript{Na}). Therefore, RM NCX1.1, NHE-1 and late I\textsubscript{Na} have been investigated extensively as therapeutic targets to limit Ca\textsuperscript{2+} overload and elicit anti-ischemic actions (Karmazyn et al., 2001; Lee et al., 2005; Moreno et al., 2012; Saint, 2008). However, the relative importance of late I\textsubscript{Na} and RM NCX1.1 inhibition in reducing IR injury and cardiac glycoside toxicity remains to be clearly delineated.

With respect to late I\textsubscript{Na}, ranolazine (Ranexa\textsuperscript{TM}) is an approved drug for the treatment of angina pectoris. At therapeutic plasma concentrations (1 to 10 \textmu M), ranolazine’s anti-ischemic action is thought to arise by inhibition of late I\textsubscript{Na} (Antzelevitch et al., 2004a,b) reducing Na\textsuperscript{+} loading and thus attenuating RM NCX1.1-mediated Ca\textsuperscript{2+} overload to decrease contractile and electrical dysfunction caused by ischemia or by selective augmentation of late I\textsubscript{Na} by Sea Anemone Toxin-II (ATX-II) (Fraser et al., 2006; Sossalla et al., 2008; Wasserstrom et al., 2009). Furthermore, ranolazine has recently been shown to reduce Ca\textsuperscript{2+}\textsubscript{i} overload and contractile
dysfunction in a model of cardiac glycoside-induced toxicity, presumably by inhibition of late $I_{\text{Na}}$ (Hoyer et al., 2011). However, it is not known whether inhibition of $\text{Na}^+/\text{K}^+$ ATPase with cardiac glycosides involves augmentation of late $I_{\text{Na}}$. Although NCX1.1 has been previously excluded as a target, as 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. As RM NCX1.1 activity is the key mediator of $\text{Ca}^{2+}$ overload caused either by ischemia or cardiac glycoside toxicity, the possibility remains that the beneficial effects of ranolazine may be due, in part, to direct inhibition of RM NCX1.1 activity.

Accordingly, we examined the effects of ranolazine and lidocaine, a known late $I_{\text{Na}}$ inhibitor (Fedida et al. 2006), in 1) whole-heart models of IR injury and cardiac glycoside toxicity 2) RM NCX1.1-mediated cellular $\text{Ca}^{2+}$ overload independent of late $I_{\text{Na}}$ 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 $I_{\text{Na}}$ alone with lidocaine and suggests that induction of late $I_{\text{Na}}$ is upstream of deleterious RM NCX1.1 activity in both IR injury and cardiac glycoside toxicity.

**Methods**

*Heart perfusions and measurements of LV mechanical function in working mode*

Adult male Sprague-Dawley rats (300-400 g) were anesthetized with pentobarbital (150 mg/kg i.p.) in accordance with the Canadian Council on Animal Care guidelines and the US National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by
the University of Alberta Animal Policy and Welfare Committee (protocols #027 and #152). Hearts were excised, the aorta cannulated and a non-working (Langendorff) perfusion was initiated within 30 sec with Krebs-Henseleit solution (Fraser et al., 2006; Ju et al., 1996; Karmazyn et al., 2001). After 10 min, working-mode perfusion was initiated with re-circulating perfusate (volume 100 ml, 37 °C, pH 7.4, gassed with a 95 % O₂-CO₂ mixture) consisting of a modified Krebs-Henseleit solution containing the following (in mM): 118 NaCl, 4.7 KCl, 1.2 KH₂PO₄, 1.2 MgSO₄, 2.5 CaCl₂, 25 NaHCO₃, 11 glucose, 1.2 palmitate and 100 mU/L insulin. Palmitate was pre-bound to bovine serum albumin (final concentration of 3% wt/vol). Heart perfusions were conducted at a constant workload (preload: 11.5 mmHg, afterload: 80 mmHg, pacing rate of 5 Hz).

Systolic and diastolic aortic pressures (mmHg) were measured with a pressure transducer (AD Instruments, Colorado Springs, CO) attached to the aortic outflow line. Cardiac output (mL/min) and aortic flow (mL/min) was measured using ultrasonic flow probes (Transonic T206, Transonic Systems Inc., Ithica, NY) placed in the left atrial inflow line and the aortic outflow line, respectively. All parameters were acquired using Chart 5.0 software (AD Instruments, Colorado Springs, CO). LV minute work (LV work, L/min-mmHg) was used as a continuous index of LV mechanical function and was calculated as cardiac output x 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 (CVC, mL/min/mmHg) was measured as coronary flow divided by aortic diastolic perfusion pressure (Fraser et al., 2006).

Heart perfusions and measurements of LV intracellular Ca²⁺

Hearts were loaded with the fluorescent Ca²⁺ indicator indo-1AM (5 μM) and fluorescence was measured in a ~0.3 cm² area of the epicardial surface of the LV free wall using
a bifurcated fiber optic cable and spectrofluorometer (Photon Technology International, London, ON) (Wang et al., 2001). Fluorescent signals were sampled at 500 Hz and the ratio of indo-1 fluorescence emitted at 405 nm and 485 nm was calculated to give an index of beat-to-beat Ca$^{2+}_i$ (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$^{2+}_i$, systolic Ca$^{2+}_i$, and LV work. Following 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 μM), lidocaine (10 μM) or vehicle (control) were added 5 min before ischemia and remained present throughout ischemia and reperfusion, or 2) exposed to ouabain (80 μM) during normal aerobic perfusion for 30 min in the absence (control) or presence of either ranolazine (10 μM) or lidocaine (10 μM) that were 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 previously described (Kovacic et al., 2003). After 48 to 72 hours of isolation, NRVMs were loaded with calcium green-1AM (2 μM) for 30 min at room temperature (22±1 ºC) and for 30 min at 37 ºC, and then subjected to the protocol below, as previously detailed (Eigel et al., 2001; Soliman et al., 2009). Cells were excited with 488±10 nm wavelength light and fluorescent signals emitted at 520±10 nm were measured using a photomultiplier detection system (PTI, Photon Technology International, London, ON). Data were analyzed using Felix32 (PTI) and Clampfit 10.1 software (Axon Instruments, Foster City, CA).
To assess $\text{Ca}^{2+}$ influx via RM NCX1.1 activity, NRVMs were superfused for 2 min with a solution containing (in mM): 140 NaCl, 4 KCl, 10 HEPES, 2.5 CaCl$_2$, 1 MgCl$_2$ and 10 dextrose. Cells were then superfused with a K$^+$-free solution (144 NaCl, 10 HEPES, 2.5 CaCl$_2$, 1 MgCl$_2$, and 10 dextrose) for 5 min, followed by 5 min superfusion with Na$^+$-free solution (140 LiCl, 4 KCl, 10 HEPES, 2.5 CaCl$_2$, 1 MgCl$_2$, and 10 dextrose). Tetrodotoxin (TTX, 10 μM) was added to both K$^+$-free and Na$^+$-free solutions to inhibit cardiac Na$^+$ channels. Thapsigargin (2 μM) was added to the Na$^+$-free solution to reduce Ca$^{2+}$ sequestration by the sarcoplasmic reticulum Ca$^{2+}$-ATPase (SERCA2a). KB-R7943 (5 μM), ranolazine (10 μM), lidocaine (10 μM) or NiCl$_2$ (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 hrs prior to experiments as described previously (Soliman et al., 2009). The AdshRNA-NCX1 construct was generously provided by Dr. Grant Pierce, University of Manitoba (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 using a similar procedure to that previously described (Su et al., 1999), with the exception that pipettes were pulled from borosilicate glass to yield resistances of 2-4 MΩ when filled with pipette solution. The pipette solution contained (in mM) NaCl 20, CsCl 130, HEPES 10, glucose 5.5, MgCl$_2$ 3.3, CaCl$_2$ 1 and EGTA 15. pH was adjusted to 7.4 with CsOH. Outward currents were elicited by rapidly switching from Na$^+$-containing extracellular solution (in mM) NaCl 138, HEPES 12, glucose 11, MgCl$_2$ 1 and CaCl$_2$ 1 to a Na$^+$-free solution containing (in mM) NaCl 0,
CsCl 138, HEPES 12, glucose 11, MgCl₂ 1 and CaCl₂ 1. Holding potential was -40mV and outward RM NCX1.1 currents were measured and analyzed using an Axopatch 200B amplifier and Clampex 8.1 software (Axon Instruments, Foster City, CA). Experiments were performed at room temperature (22±1 ºC).

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 the following (in mM): CsCl 140, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4. pH was adjusted to 7.4 with CsOH. Outward RM currents were elicited by rapidly switching from an intracellular cesium-based solution containing (in mM) CsCl 120, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4.28 to an intracellular sodium-based solution containing (in mM) CsCl 120, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4.28. 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 (in mM) CsCl 30, NaCl 90, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4.28. 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-calcium solution containing (in mM) CsCl 120, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4.28 and EGTA 5 to a cesium-based high-calcium solution containing (in mM) CsCl 140, TEA 20, HEPES 5, MgATP 2, glucose 10, MgCl₂ 1.4 and CaCl₂ 4. The pH of all intracellular solutions was adjusted to 7.2 with CsOH. NCX1 currents were activated for a period of 60 sec. Excised patches were held at 0 mV and NCX1.1 currents were measured using an Axopatch 200B amplifier and Clampex 10.1.
software (Axon Instruments, Foster City, CA). All experiments were performed at room temperature (22±1 °C).

**Experimental compounds**

Ouabain, lidocaine, thapsigargin and NiCl₂ were purchased from Sigma Aldrich (Oakville, ON). KB-R7943 and ranolazine were purchased from Tocris Biosciences (Ellisisville, MO). Tetrodotoxin citrate was obtained from Alomone Laboratories (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 ± SEM. Late NCX1.1 current amplitudes were measured and normalized to peak current. The time constant of RM NCX1.1 current inactivation (Tau, 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 when p<0.05.

**Results**

**Attenuation of IR-induced LV dysfunction and Ca²⁺ overload by ranolazine or lidocaine**

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

In order to further investigate the underlying mechanisms for cardioprotection afforded by ranolazine or lidocaine, the Na$^{+}$/K$^{+}$-ATPase inhibitor ouabain was used to increase Na$^{+}$i and thereby enhance RM NCX1.1-mediated Ca$^{2+}$i accumulation by mechanisms independent of IR injury. While 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. 3A,B), ouabain-induced depression of LV work was significantly attenuated by ranolazine or lidocaine and LV work was only depressed by 23% (n=5, p<0.01) or 31% (n=6, p<0.01 (Fig. 3A,B). Ouabain (80 μM) significantly increased diastolic Ca$^{2+}$i (Fig. 1C) and after exposure for 30 min values were 33% higher than baseline (p<0.001, n=6 Fig. 3C,D). Application of ranolazine (10 μM) or lidocaine (10 μM) 10 min after ouabain significantly reduced ouabain-induced diastolic Ca$^{2+}$i accumulation relative to vehicle-treated hearts to 14% (n=5, p<0.001) or 16% (n=6, p<0.001), respectively (Fig. 3C,D). However, systolic Ca$^{2+}$i was not affected by either ranolazine or lidocaine (Fig. 3E,F), despite the reduction in diastolic Ca$^{2+}$i.

Ranolazine inhibits activity in a cellular model of RM NCX1.1-induced Ca$^{2+}$i overload.
As 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 activity in an intact cellular model (Fig. 4). We utilized neonatal rat ventricular myocytes (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 using Ca\(^{2+}\) imaging. The Na\(^+\) channel toxin TTX (10 μM) was used throughout experiments to eliminate any involvement of I\(_{Na}\). 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±1.7 % reduction, \(p<0.01\), Fig 4A,C). Similarly, molecular knockdown of NCX1.1 by adenoviral delivery of NCX1 shRNA (AdshRNA-NCX1) also resulted in a marked decrease in the observed Ca\(^{2+}\) signal (73.9±13.6% reduction, \(p<0.01\), Fig 4A,C). These data confirm that the induced Ca\(^{2+}\) overload occurring during Na\(^+\)-free conditions is predominately mediated by RM NCX1.1 activity.

Using this validated cellular model of evoked RM NCX1.1 activity, the effects of ranolozine, the Na\(^+\) channel inhibitor lidocaine and the NCX inhibitor KB-R7943, were compared. Ranolazine (10 μM) decreased Ca\(^{2+}\) overload by 23.6±2.6 % (\(p<0.001\)) (Fig. 4B,C). In direct contrast, lidocaine (10 μM) had no significant effect on reducing Ca\(^{2+}\) overload (Fig. 4B,C). KB-R7943 (5 μM) had an effect similar to ranolazine and decreased Ca\(^{2+}\) overload by 20.7±3.9% (\(p<0.01\)). These data indicate that ranolazine, but not lidocaine inhibits RM NCX1.1-dependent Ca\(^{2+}\) overload independently of peak or late I\(_{Na}\) 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 utilized to measure Ni^{2+}-sensitive RM currents (Fig. 5A,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 ~80% occurring at a concentration of 100 μM (IC_{50} = 1.7 μM) (Fig. 5B-D). The inhibitory effect of ranolazine (10 μM) was readily reversible after 1 min of drug washout (99.1±2.3% vs control). In direct contrast, lidocaine (10 μM) did not significantly inhibit RM NCX1.1 currents (Fig. 5E,F). Upon re-introduction of extracellular Na^+, NCX1.1 switches to FM operation to extrude 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. 5B-E).

As many NCX1.1 inhibitors have been demonstrated to display a selectivity for RM NCX1.1 currents displaying Na^+-dependent I_f inactivation (Iwamoto, 2004; Iwamoto et al., 2004), the excised 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 μM) reversibly inhibited the inactivating RM NCX1.1 currents, even a high concentration of ranolazine (100 μM) failed to inhibit the non-inactivating FM NCX1.1 currents (Fig. 6A,B) 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_f inactivation and ranolazine was unable to inhibit these mutant non-inactivating RM currents (Fig. 7A). A similar lack of effect was observed in the non-
inactivating FM NCX1.1-WT currents (Fig. 7B). Conversely, NCX1.1-F255E RM currents exhibit a faster inactivation process ($\tau = 519 \pm 42$ msec) and this was significantly accelerated by ranolazine (10 $\mu$M, $\tau = 404 \pm 29$ msec, $p < 0.05$, Fig. 7C,D).

**Discussion**

**Late $I_{Na}$, IR injury and cardiac glycoside toxicity.**

Late $I_{Na}$ inhibition has been suggested to be a useful approach to reduce myocardial dysfunction associated with Na$^+$ and Ca$^{2+}$ overload during IR injury (Maier, 2009; Saint, 2008; Shryock et al., 2008; Stone, 2008). Furthermore, previous studies have demonstrated that the late $I_{Na}$ inhibitors ranolazine and lidocaine attenuate ATP depletion during ischemia and reduce intracellular acidosis and Na$^+$ accumulation (Belardinelli et al., 2006; Butwell et al., 1993). Indeed, our results using intact hearts confirm that ranolazine and lidocaine are equally effective in enhancing recovery of post-ischemic LV mechanical function (Fig. 2A,B) and returning post-ischemic diastolic Ca$^{2+}$ levels to near pre-ischemic values (Fig. 2C,D), presumably via inhibition of late $I_{Na}$.

Cardiac glycosides such as ouabain are useful clinically to increase inotropy of the failing heart (Kjeldsen et al., 2003; Schoner et al., 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 et al., 2007). In contrast, higher concentrations of ouabain are cardiotoxic and impair LV mechanical function due to excessive Na$^+$ accumulation that promotes NCX1.1-mediated Ca$^{2+}$ overload. Recent studies suggest that ouabain may also induce late $I_{Na}$ through voltage-gated Na$^+$ channel phosphorylation by Ca$^{2+}$/calmodulin-dependent kinase II (CaMKII) (Hoyer et al., 2011; Maltsev et al., 2008), 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 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 as follows: 1) that 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) that 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 employed 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, 2004; Iwamoto et al., 2004) inhibited evoked RM NCX1.1 activity by ~21 to 24 %, respectively (Fig. 4). Interestingly, 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\(_{Kr}\), I\(_{to}\), L-type Ca\(^{2+}\) channels, and NCX1.1
(Antzelevitch et al., 2004a; Antzelevitch et al., 2004b). At therapeutic concentrations of 1 to 10 μM, 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 μM) (Antzelevitch et al., 2004b). However, in that study, NCX currents were elicited by FM NCX1.1 exchange activity and 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 (IC_{50} = 1.7 μM, Fig. 5B-D). Furthermore, ranolazine is a selective RM inhibitor as 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 would likely be a desirable therapeutic property as it reduces the pathophysiological Ca^{2+} influx that leads to Ca^{2+} overload, while sparing Ca^{2+} efflux resulting from FM activity.

RM-selectivity of NCX1.1 inhibitors is thought to be due to the drug binding to exchangers undergoing the I_{1}-inactivation process, a characteristic of RM, but not FM NCX1.1 currents (Bouchard et al., 2004; Elias et al., 2001; Hamming et al., 2008). To assess whether the RM selectivity of ranolazine is similarly dependent on the I_{1}-inactivation process, we measured the effects of ranolazine on RM currents arising from NCX1.1 exchangers with altered I_{1}-inactivation properties (Matsuoka et al., 1997). While ranolazine did not inhibit the non-inactivating 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 that it 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 Ca\textsuperscript{2+} loading in whole-heart models of IR injury and cardiac glycoside 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 I\textsubscript{Na} is involved in both IR injury and cardiac glycoside toxicity and that the observed RM NCX1.1 inhibitory effects of ranolazine do not confer any additional protection in these models. Therefore, induction of late I\textsubscript{Na} is likely upstream of RM NCX1.1-mediated Ca\textsuperscript{2+} overload and that the observed inhibition of RM NCX1.1 activity with ranolazine does not confer any additional protection over late I\textsubscript{Na} inhibition alone in models of IR injury and cardiac glycoside toxicity.

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Authorship Contributions

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References


Footnotes

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Figure Legends

**Fig. 1.** Representative calcium transient recordings. (A) aerobic baseline, (B) during global ischemia and (C) after treatment with ouabain (80 μM). Grouped diastolic and systolic calcium levels shown in Figs 2 and 3 (B-F) were generated 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.

**Fig. 2.** Effects of ranolazine and lidocaine on IR-induced alterations in LV work and diastolic Ca\(^{2+}\). LV work (A, B), diastolic Ca\(^{2+}\) (C, D) and Ca\(^{2+}\) transient amplitude (E,F) were measured during aerobic perfusion (0 to 15 min), during global, no-flow ischemia (15 to 35 min, indicated by bar), as well as during reperfusion (35 to 65 min). Hearts were treated with vehicle (DMSO, n=14, ■), ranolazine (10 μM, n=8, □) or lidocaine (10 μM, n=5, □) that was applied 5 min before onset of ischemia. Significant differences between values in absence and presence of ranolazine or lidocaine are denoted by *, **, *** for p<0.05, 0.01 and 0.001 respectively. Analysis of the time-dependent changes were performed by repeated measures ANOVA. Ranolazine results were reproduced from Fraser et al 2006.

**Fig. 3.** Effects of ranolazine and lidocaine on ouabain-induced alterations in LV work and diastolic Ca\(^{2+}\). LV work (A,B), diastolic Ca\(^{2+}\) (C,D) and Ca\(^{2+}\) transient amplitude (E,F) were measured during aerobic perfusion in the absence of presence of ouabain (80 μM). Hearts were perfused initially under aerobic conditions (0-15min), and then exposed to ouabain (15-45 min). After 10 min, hearts were treated during the remainder of the perfusion period (25 to 45 min) with either vehicle (DMSO, n=5, ■), ranolazine (10 μM, n=5) or lidocaine (10μM, n=6). Significant differences between values in absence and presence of ranolazine or lidocaine are
denoted by *, **, *** for $p<0.05$, $p<0.01$ and $p<0.001$ respectively. Analysis of the time-dependent changes were performed by repeated measures ANOVA.

**Fig. 4.** Effect of ranolazine on evoked RM NCX1.1 activity in neonatal rat cardiac myocytes. Sample traces (A) of evoked RM NCX1.1 activity as assessed by Ca$^{2+}$ imaging in the presence of thapsigargin (TG, 2 $\mu$M), NiCl$_2$ (5 mM) or after NCX1.1 expression was suppressed by treatment of NCRMs with Ad-shRNA-NCX1 for 72 hrs. Representative recordings (B) of Ca$^{2+}$ imaging of evoked RM NCX1.1 activity in the absence or presence of ranolazine (10 $\mu$M), lidocaine (10 $\mu$M) or KB-R7943 (5 $\mu$M). Quantification of $\Delta F$-AUC data (C) for changes in RM NCX1.1 activity in response to ranolazine (n=9), lidocaine (n=5), KB-R7943 (n=8), Ni$^{2+}$ (n=3) and shRNA-NCX1 knockdown of NCX1.1 expression (n=6). **$p<0.01$, ***$p<0.001$ when compared with untreated group; paired Student’s t-test.

**Fig. 5.** The effects of ranolazine and lidocaine on recombinant human reverse-mode (RM) and forward-mode (FM) NCX1.1 currents recorded using the whole-cell patch-clamp technique. Representative recordings of inactivating RM currents showing inhibition by 5 mM Ni$^{2+}$ (A) and concentration-dependent inhibition by 1 and 10 $\mu$M ranolazine (B,C). (D) Grouped data concentration-inhibition curve (reverse-mode) for ranolazine ($IC_{50} = 1.7$ $\mu$M, n=5-9 patches per concentration, open circles, AUC = total current area under curve, Hill co-efficient = 0.85). Filled circle = lack of FM NCX1.1 inhibition (10 $\mu$M ranolazine, n=7 patches). (E) Representative recording of inactivating RM currents showing lack of inhibition by lidocaine (10 $\mu$M, n=11 patches). (F) Grouped data showing either a lack of lidocaine inhibitory effect (NS = no significant difference) or significant Ni$^{2+}$ inhibition on peak or late RM currents and total
current (area under curve, AUC). *** denotes p<0.001 vs control. Dashed line denotes zero current level.

**Fig 6.** Representative traces showing the effect of ranolazine (10 μM) on (A) outward reverse-mode (RM) inactivating currents and (B) inward forward-mode (FM) non-inactivating currents from excised inside-out (I-O) patches from tsA201 cells transiently expressing human NCX1.1. The inhibitory effect of ranolazine on RM currents is fully reversible (washout). Dashed line denotes zero current level.

**Fig. 7.** The effects of ranolazine on RM NCX1.1 currents containing mutations that alter inactivation properties. Representative recordings of (A) non-inactivating RM currents from NCX1.1-K264Q mutant exchangers in the absence or presence of ranolazine (10 μM). Analysis of relative peak current (B) showing that ranolazine does not alter NCX1.1-K264Q currents, in which the I₁-dependent inactivation process is removed, in comparison to wild type (WT) FM NCX1.1 current (n=4 patches, NS = not significant). Representative trace (C) of the effect of ranolazine on RM NCX1.1-F255E mutant currents where the I₁-dependent inactivation process is accelerated. Grouped τ data (D) (n=6) indicating that ranolazine (10 μM) decreases time-to-inactivation. *p<0.05, ranolazine compared with control, paired Student’s t-test. Dashed line denotes zero current level.
Figure 1

A. Aerobic baseline

- Systolic $Ca^{2+}$
- Diastolic $Ca^{2+}$

B. Global ischemia

C. Ouabain

$\Delta F_{405/F485}$
Figure 3

A) LV Work

B) LV Work

C) Diastolic [Ca\(^{2+}\)]

D) Diastolic [Ca\(^{2+}\)]

E) Ca\(^{2+}\) Transient

F) Ca\(^{2+}\) Transient

Ouabain ± Ranolazine

Ouabain ± Lidocaine

Time of Perfusion (min)
Figure 4.
Figure 5
Figure 6

(A) Reverse-mode

(B) Forward-mode

excised I-O patch

washout

control

ranolazine

[Na⁺], (mM)

0 90

3pA

15s

[Ca²⁺], (mM)

0 3.2

4pA

15s

control

ranolazine
Figure 7