Reducing the Late Sodium Current Improves Cardiac Function during Sodium Pump Inhibition by Ouabain

Kirsten Hoyer, Yejia Song, Desuo Wang, Dillon Phan, James Balschi, Joanne S. Ingwall, Luiz Belardinelli, and John C. Shryock

Department of Biology, Gilead Sciences, Inc., Palo Alto, California (K.H., D.P., L.B., J.C.S.); Division of Cardiovascular Medicine, University of Florida, Gainesville, Florida (Y.S.); Department of Basic Pharmaceutical Sciences, School of Pharmacy, Philadelphia College of Osteopathic Medicine, Suwanee, Georgia (D.W.); and NMR Laboratory for Physiological Chemistry, Department of Medicine, Brigham and Women’s Hospital, Harvard Medical School, Boston, Massachusetts (J.B., J.S.I.)

Received November 12, 2010; accepted February 14, 2011

ABSTRACT

Inhibition by cardiac glycosides of Na\(^{+}\), K\(^{+}\)-ATPase reduces sodium efflux from myocytes and may lead to Na\(^{+}\) and Ca\(^{2+}\) overload and detrimental effects on mechanical function, energy metabolism, and electrical activity. We hypothesized that inhibition of sodium persistent inward current (late I\(_{\text{Na}}\)) would reduce ouabain’s effect to cause cellular Na\(^{+}\) loading and its detrimental metabolic (decrease of ATP) and functional (arrhythmias, contracture) effects. Therefore, we determined effects of ouabain on concentrations of intracellular sodium (Na\(^{+}\)) and high-energy phosphates using \(^{23}\)Na and \(^{31}\)P NMR, the amplitude of late I\(_{\text{Na}}\) using the whole-cell patch-clamp technique, and contractility and electrical activity of guinea pig isolated hearts, papillary muscles, and ventricular myocytes in the absence and presence of inhibitors of late INa. Ouabain (1–1.3 μM) increased Na\(^{+}\), and late I\(_{\text{Na}}\) of guinea pig isolated hearts and myocytes by 3.7- and 4.2-fold, respectively. The late I\(_{\text{Na}}\) inhibitors ranolazine and tetrodotoxin significantly reduced ouabain-stimulated increases in Na\(^{+}\), and late I\(_{\text{Na}}\). Reductions of ATP and phosphocreatine contents and increased diastolic tension in ouabain-treated hearts were also markedly attenuated by ranolazine. Furthermore, the ouabain-induced increase of late I\(_{\text{Na}}\) was also attenuated by the Ca\(^{2+}\)-calmodulin-dependent kinase i inhibitors KN-93 [N-[2-[[3(4-chlorophenyl)-2-propenyl]methylamino]methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide] and autacamide-2 related inhibitory peptide, but not by KN-92 [2-[N-(4'-methoxybenzenesulfonyl)]amino-N-(4'-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate]. We conclude that ouabain-induced Na\(^{+}\) and Ca\(^{2+}\) overload is ameliorated by the inhibition of late I\(_{\text{Na}}\).

Introduction

Cardiac glycosides inhibit the sarcolemmal Na\(^{+}\), K\(^{+}\)-ATPase (sodium pump) and increase intracellular sodium concentration ([Na\(^{+}\)]\(_{i}\)). The effect of a glycoside to increase [Na\(^{+}\)]\(_{i}\), may lead to an increase of calcium influx via sodium/calcium exchange (NCX) and an increase in cardiac contractility. However, whereas a small increase of [Na\(^{+}\)]\(_{i}\), may lead to a positive inotropic effect (Bers et al., 2003), a larger increase may lead to arrhythmias and contractile dysfunction. We hypothesized that reduction of Na\(^{+}\) entry in the presence of the cardiac glycoside ouabain would reduce sodium overloading and its adverse mechanical, metabolic, and electrical consequences. A novel approach to reduce Na\(^{+}\) entry is by reduction of persistent Na\(^{+}\) inward current (late I\(_{\text{Na}}\)) (Ver Donck et al., 1993; Hale et al., 2008). Late I\(_{\text{Na}}\) is caused by entry of Na\(^{+}\) ions through myocyte Na\(^{+}\) channels that fail to inactivate normally. These channels stay open or reopen during the action potential plateau, when “normal” Na\(^{+}\) channels are inactivated, thereby con-
contributing to intracellular Na\(^+\) loading (Makielski and Farley, 2006; Undrovinas and Maltsev, 2008). An increase of late I\(_{\text{Na}}\) caused by impaired Na\(^+\) channel inactivation is common in inherited (e.g., SCN5A mutations; Ruan et al., 2009) and acquired (e.g., ischemia, heart failure, remodeling, and oxidative states) pathological conditions (Undrovinas and Maltsev, 2008; for review see Hale et al., 2008). Although small in amplitude relative to peak I\(_{\text{Na}}\), late I\(_{\text{Na}}\) persists throughout the duration of the cardiac action potential and contributes significantly to Na\(^+\) entry in myocytes (Makielski and Farley, 2006). The increase of Na\(^+\) entry caused by an enhanced late I\(_{\text{Na}}\) may lead to an increase of Na\(^+\) current ([Na\(^+\)]\(_{i}\)). Using a computational model, Noble (2008) found that reduction of late I\(_{\text{Na}}\) attenuated the rise of Na\(^+\)\(_{i}\) caused by repetitive 2-Hz stimulation of a ventricular myocyte. A rise of [Na\(^+\)]\(_{i}\) reduces the reversal potential of NCX and leads to Ca\(^2+\)-loading of myocardial cells (Bers, 2001; Imahashi et al., 2005). Thus, inhibition of late I\(_{\text{Na}}\) is cardioprotective (Makielski and Farley, 2006; Hale et al., 2008; Sossalla et al., 2008).

Reduction of late I\(_{\text{Na}}\) can be achieved using either ranolazine or tetrodotoxin (TTX). The antianginal drug ranolazine is a relatively selective late I\(_{\text{Na}}\) inhibitor (Antzelevitch et al., 2004; Hale et al., 2008). Ranolazine reduces late I\(_{\text{Na}}\) with an approximate IC\(_{50}\) value of 6.5 \(\mu\)M (versus an IC\(_{50}\) value of 244 \(\mu\)M for inhibition of peak I\(_{\text{Na}}\)) and causes minimal or no inhibition of L-type Ca\(^2+\)-channel current, NCX, or sodium proton exchange at therapeutic concentrations (\(= 10 \mu\)M) (Antzelevitch et al., 2004; Hale et al., 2008). In other studies it has been shown that ranolazine reduces sea anemone toxin-II- and H\_2O\_2-induced late I\(_{\text{Na}}\) in guinea pig and rabbit isolated ventricular myocytes and suppresses early and delayed afterdepolarizations and arrhythmic activity (Song et al., 2004, 2008). Ranolazine attenuates diastolic dysfunction in myocardium isolated from failing human hearts (Sossalla et al., 2008), sea anemone toxin-II-treated and ischemic/reperfused rat hearts (Fraser et al., 2006), and guinea pig hearts exposed to the ischemic metabolite palmitoyl-l-carnitine (Wu Y., et al., 2009). The limitation of ranolazine is that its selectivity for inhibition of late I\(_{\text{Na}}\) relative to human ether-a-go-go-related gene K\(^+\) current is only 2-fold (Hale et al., 2008). In contrast, TTX is very selective for Na\(^+\) channels relative to other ion channels, but has less selectivity for late relative to peak I\(_{\text{Na}}\) than does ranolazine. Both inhibitors were therefore used in this study to test the hypothesis that a reduction of endogenous late I\(_{\text{Na}}\) will reduce effects of ouabain to cause cellular Na\(^+\) loading and metabolic and contractile dysfunction. It has also been reported that a rise in intracellular Ca\(^2+\) and phosphorylation of the cardiac Na\(^+\) channel by Ca\(^2+\)-calmodulin-dependent kinase II (CaMKII) can alter Na\(^+\) channel inactivation and enhance late I\(_{\text{Na}}\) (Maier and Bers, 2007; Hale et al., 2008; Maltsev et al., 2008; Song et al., 2008; Undrovinas and Maltsev, 2008; Xie et al., 2009). Because ouabain is known to increase intracellular Ca\(^2+\), we also determined the effects of inhibition of NCX and CaMKII on late I\(_{\text{Na}}\) and its attendant adverse functional consequences. The findings in this study were that ouabain itself led to an increase of late I\(_{\text{Na}}\) and that in the presence of inhibitors of late I\(_{\text{Na}}\) and CaMKII Na\(^+\)\(_{i}\) accumulation in the presence of ouabain was reduced, energy loss was prevented, and mechanical function was improved.

### Animals and Isolated Perfused Heart Preparation

Animal use protocols were approved by the Standing Committee on Animals of Harvard Medical Area and the Institutional Animal Care and Use Committees of CV Therapeutics (now Gilead Sciences, Palo Alto, CA), the University of South Carolina (Columbia, SC), and the University of Florida (Gainesville, FL). Animal use conformed to National Institutes of Health guidelines (National Institutes of Health publication 85-23, revised 1996).

Guinea pigs (Duncan Hartley, 250–350 g, either sex) were anesthetized (180 mg/kg sodium pentobarbital, intraperitoneally), and hearts were isolated and perfused in the isovolumic Langendorff mode at a constant pressure of 60 mm Hg with a modified Krebs-Henseleit buffer (37°C, pH 7.4) containing 118 mM NaCl, 4.8 mM KCl, 1.75 mM CaCl\(_2\), 1.2 mM MgSO\(_4\), 0.5 mM EDTA, 25 mM NaHCO\(_3\), 1.2 mM KH\(_2\)PO\(_4\), 5.5 mM glucose, and 2 mM pyruvate, oxygenated with 95% O\(_2\)/5% CO\(_2\). For experiments in which contractile function was measured, a fluid-filled balloon was inserted into the left ventricle and connected to a physiological pressure transducer (AD Instruments, Colorado Springs, CO). Hearts were stimulated at a rate of 5 Hz during all experiments (SD9 Square Stimulator; Grass Technologies, West Warwick, RI). Data were collected and analyzed as described previously (Shen et al., 2001), using a PowerLab system (Bridge Amp, 8sp interface, Chart 5.Pro software; ADInstruments, Colorado Springs, CO). For experiments in which NMR signals were measured, hearts were isolated and perfused as described above, suspended in a Varian Inova wide-bore spectrometer (Varian Inc., Palo Alto, CA), and paced at a rate of 5 Hz.

### \(23\text{Na}\) and \(31\text{P}\) NMR Spectroscopy for Measuring [Na\(^+\)]\(_{i}\) and High-Energy Metabolites in Guinea Pig Isolated Hearts

For \(23\text{Na}\) NMR, 590 free induction decay signals obtained from the Varian Inova spectrometer were acquired at 105.5 MHz and averaged over 2 min (90° pulse, 0.2-s recycle time). To distinguish intracellular from extracellular sodium, the shift reagent sodium thulium (III)\(_{14},7,10\)-tetracazaacyclodecane-1,4,7,10-tetra(methylene phosphonate) (Na\(_5\)TmDOTP) (3.5 mM) was added to the Krebs-Henseleit buffer. To determine [Na\(^+\)]\(_{i}\), the peak areas of \(23\text{Na}\) signals were compared with the peak area of a Na\(^+\) internal reference standard (Jansen et al., 2003).

For \(31\text{P}\)-NMR, 125 free induction decay signals were acquired at 161.4 MHz and averaged over 5 min (60° pulse, 2.4-s recycle time). Cytosolic concentrations of ATP, phosphocreatine (PCr), and P\(_i\) were determined according to Shen et al. (2001).

### Ouabain, Ranolazine, and Tetrodotoxin Concentrations

Results of preliminary studies of guinea pig isolated perfused hearts indicated that concentrations of ouabain at 0.5 \(\mu\)M or above 1 \(\mu\)M were associated with either no dysfunction or rapid induction of arrhythmia, respectively. The ouabain concentration of 0.75 \(\mu\)M was therefore used in the majority of experiments. Ouabain induced two effects, a positive inotropic effect and a toxic effect (e.g., arrhythmia, elevated diastolic function). The shift reagent Na\(_5\)TmDOTP reduced the effect of ouabain, presumably secondary to Ca\(^2+\)-chelation. Therefore, in \(23\text{Na}\) NMR experiments the ouabain concentration was increased to 1.3 \(\mu\)M to achieve an effect equivalent to that seen at 0.75 \(\mu\)M in the absence of Na\(_5\)TmDOTP. For measurements of contractile function in guinea pig papillary muscle the ouabain concentration was increased to 2 \(\mu\)M, acknowledging that papillary muscle tissue is less ouabain-sensitive than either single cells or perfused hearts.

Ranolazine is a relatively selective late I\(_{\text{Na}}\) inhibitor. The ranolazine concentrations used in this study (3, 5, and 10 \(\mu\)M) are in the mild to high therapeutic range and are known to significantly reduce the late I\(_{\text{Na}}\) (Antzelevitch et al., 2004; Hale et al., 2008). TTX reduces peak and late I\(_{\text{Na}}\) with IC\(_{50}\) values of 6.0 \(\pm\) 0.2 and 0.5 \(\pm\) 0.1 \(\mu\)M (Wu L. et al., 2009), respectively. At concentrations of 0.5 to 1 \(\mu\)M, TTX is a relatively selective late I\(_{\text{Na}}\) inhibitor.

### Materials and Methods

#### Animal Use

*Animal use protocols were approved by the Standing Committee on Animals of Harvard Medical Area and the Institutional Animal Care and Use Committees of CV Therapeutics (now Gilead Sciences, Palo Alto, CA), the University of South Carolina (Columbia, SC), and the University of Florida (Gainesville, FL). Animal use conformed to National Institutes of Health guidelines (National Institutes of Health publication 85-23, revised 1996).*
Papillary Muscle Preparation and Tension Measurement. Guinea pigs were anesthetized, and hearts were quickly removed and placed in an ice-cold Tyrode’s solution containing 136 mM NaCl, 2.8 mM KCl, 1 mM CaCl₂, 1.5 mM MgCl₂, 0.3 mM Na₂HPO₄, 10 mM HEPES, 10 mM glucose, and NaOH to adjust the pH to 7.4. The right ventricular papillary muscle was dissected free, mounted in a 36.5 ± 0.5°C tissue bath in Tyrode’s solution equilibrated with 100% O₂ and electrically paced at a rate of 1 Hz. Muscle strips were equilibrated for 30 min with two changes of bathing solution and stretched stepwise with a micromanipulator to a rest length at which stimulated contractile tension was maximal. Contractile force was measured isometrically using a force displacement transducer (TRI 201; LSI Letica Scientific Instruments, Barcelona, Spain) and digitized using a PowerLab system (ADInstruments).

Isolation of Ventricular Myocytes and Electrophysiological Recordings. Single guinea pig ventricular myocytes were isolated using standard enzymatic procedures as described previously (Song et al., 2004). Transmembrane Na⁺ currents were measured with an Axopatch-200 amplifier, a Digidata-1440 digitizer, and pClamp-10 software (Molecular Devices, Sunnyvale, CA), using the whole-cell patch-clamp technique. The recording pipettes had a resistance of 2 to 3 MΩ when filled with a solution containing 120 mM Cs-aspartate, 20 mM CsCl, 1 mM MgSO₄, 4 mM Na₂ATP, 0.1 mM NaCl₂, and 10 mM HEPES, pH 7.2, and the series resistance was compensated by approximately 85%. Late İNa was activated using 300-ms voltage-clamp pulses from –90 to –50 mV at a frequency of 0.16 Hz. Transmembrane current during the last 100 ms of depolarizing pulse was integrated and expressed as nanocoulombs (nC) or picocoulombs (pC). Cell membrane capacitance was minimized using the amplifier, and values of capacitance compensation in picofarads (pF) were used to normalize the integrated current to the magnitude of the membrane capacitative current (pC/pF). During experiments, myocytes were superfused with a bath solution (36°C) containing 135 mM NaCl, 4.6 mM CsCl, 1.8 mM CaCl₂, 1.1 mM MgSO₄, 0.01 mM nitrendipine, 0.1 mM BaCl₂, 10 mM glucose, and 10 mM HEPES, pH 7.4. Barium was present in the bath solution to reduce potential contamination of late İNa by İKr.

In selected experiments with isolated myocytes, KN-93 [N-H-[3-(4-chlorophenyl)-2-propenyl]methylamino[methyl]phenyl]-N-(2-hydroxyethyl)-4-methoxybenzenesulphonamide, KN-92 [N-H-[4-methoxybenzenesulphonyl]amino-N’-(4’-chlorophenyl)-2-propenyl-N-methylbenzylamine phosphate], or EGTA were included in the recording pipette solution to avoid the CaMKII-independent effects of KN-93 that are reported to occur when the compound is applied extracellularly (Rezazadeh et al., 2006). Ouabain, TTX, and ranolazine were applied extracellularly via the bath solution.

Chemicals. Ranolazine was provided by CV Therapeutics, and KN-92, KN-93, and myristoylated autocamtide-2-related inhibitory peptide (AIP) were obtained from Calbiochem (La Jolla, CA). The shift reagent Na₃TmDOTP was purchased from Macrocycles (Dallas, TX). All other drugs and reagents were obtained from Sigma-Aldrich (St. Louis, MO).

Statistics. Results are expressed as mean ± S.E.M. Data were analyzed by one-way analysis of variance (Prism 5.01; GraphPad Software, Inc., San Diego, CA) or analysis of variance with repeated measures (Statistica 8.0; StatSoft, Tulsa, OK), followed by a post hoc test (e.g., Tukey’s test) when significant differences were observed. A p value < 0.05 was considered to indicate a significant difference.

Results

Changes in Contractile Function of the Isolated Heart during Ouabain-Induced Sodium Pump Inhibition in the Absence and Presence of Ranolazine or TTX. Exposure to 0.75 μM ouabain for 60 min led to a transient increase of left ventricular systolic pressure (LVSP) by 55 ± 5% (n = 13), followed by arrhythmic activity and episodes of cardiac standstill (i.e., absence of a contractile response during continuous electrical pacing at 5 Hz) alternating with periods of rhythmic contraction in 11 of 13 hearts tested (Fig. 1A). A marked elevation of left ventricular end diastolic pressure (LVEDP) and a decrease of left ventricular developed pressure (LVDevP) were observed (Fig. 1A). The late İNa inhibitors ranolazine and TTX reduced the occurrence of episodes of cardiac standstill and the rise of LVEDP caused by ouabain. Of eight hearts treated with 0.75 μM ouabain + 3 μM ranolazine, four hearts showed episodes of cardiac standstill including elevated LVEDP (Fig. 1B), whereas the remaining four hearts maintained enhanced but irregular contraction. The responses of hearts that were exposed to ouabain in the presence of 0.5 μM TTX were comparable with those exposed to ouabain in the presence of 3 μM ranolazine. When the concentration of ranolazine was increased to 5 μM, hearts treated with 0.75 μM ouabain (n = 6; Fig. 1C) did not have episodes of cardiac standstill, although biphasic contractions were occasionally observed and LVEDP was slightly, but not significantly, elevated. In hearts treated with either 10 μM ranolazine (n = 8; Fig. 1D) or 1 μM TTX (n = 6; not shown) throughout the 60-min duration of ouabain exposure, neither episodes of cardiac standstill nor biphasic contractions nor changes in LVEDP were observed. Ranolazine (Fig. 1, B–D) and TTX (data not shown) alone caused slight concentration-dependent decreases of LVSP but did not inhibit the positive inotropic response to ouabain (Fig. 1). LVSP decreased by 8 and 16% to 17% (n = 6–8 each) during treatment with ranolazine alone (3, 5, or 10 μM, respectively; p < 0.05) and by 9 and 18% (n = 5–6) during treatment with TTX alone (0.5 and 1 μM, respectively; p < 0.04). The values of rate pressure product in the presence of 0.75 μM ouabain with or without ranolazine or TTX increased significantly and were not different from each other (Tables 1 and 2). Hearts exposed to ouabain in the presence of 3, 5, or 10 μM ranolazine or 1 μM TTX also showed better recovery of contractile function (higher developed pressure) after drug washout than hearts treated with ouabain alone (Fig. 1). The effects of drug treatment on values of contractile parameters are summarized in Tables 1 and 2.

Changes in Contractile Function in Papillary Muscle Preparations during Ouabain-Induced Sodium Pump Inhibition in the Absence and Presence of Ranolazine. Contractile function of the guinea pig papillary muscle was measured to confirm results of experiments performed using the isolated heart. A concentration of 2 μM ouabain increased developed tension of papillary muscles by almost 4-fold after 20 min, from 35.4 ± 7.7 (n = 8) to 133.9 ± 37.0 mg (Fig. 2, A and C). The developed tension of papillary muscles declined with time during a 60-min exposure to ouabain, and episodes of tachyarrhythmia were often observed (Fig. 2, A and C). Diastolic tension of papillary muscle strips also increased significantly by 15% after a 1-h ouabain treatment (Fig. 2E). Ranolazine attenuated the ouabain-induced contractile/electrical dysfunction in guinea pig papillary muscle preparations (Fig. 2, B, D, and E). Ranolazine (10 μM) alone had no significant effect on developed tension and did not seem to decrease the effect of ouabain to increase developed tension (Fig. 2, B and D). In muscles pretreated with 10 μM ranolazine, the addition of 2 μM ouabain also caused a 4-fold increase in contractile force from 51.0 ± 6.7 to 202.9 ± 44.0 mg, but...
Fig. 1. Effect of ouabain (0.75 μM) on left ventricular pressure of the guinea pig isolated, electrically paced heart, in the absence (A) and presence of ranolazine (Ran) (3, 5, and 10 μM; B, C, and D, respectively) or 1.8 μM KN-93 (E). Records from five representative experiments are shown. Shown to the right of each record are expanded portions of the record at the points indicated by a, b, and c (arrows). The experimental treatment protocol is shown above each record. Ctrl, control (no drug); V, vehicle; R, ranolazine; KN, KN-93; Wash, drug washout.

TABLE 1
Contractile function of guinea pig isolated heart at baseline and after 10-min pretreatment with late \( I_{Na} \) inhibitors

<table>
<thead>
<tr>
<th></th>
<th>LVSP</th>
<th>RPP</th>
<th>LVDevP</th>
<th>LVEDP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mm Hg</td>
<td>mm Hg/min</td>
<td>mm Hg</td>
<td></td>
</tr>
<tr>
<td>Baseline</td>
<td>96 ± 1</td>
<td>26,670 ± 440</td>
<td>90 ± 1</td>
<td>7 ± 0.3</td>
</tr>
<tr>
<td>3 μM ranolazine</td>
<td>93 ± 3</td>
<td>25,240 ± 1050</td>
<td>85 ± 4</td>
<td>8 ± 0.5</td>
</tr>
<tr>
<td>5 μM ranolazine</td>
<td>80 ± 7*</td>
<td>22,470 ± 4250</td>
<td>77 ± 8</td>
<td>8 ± 0.6</td>
</tr>
<tr>
<td>10 μM ranolazine</td>
<td>82 ± 4*</td>
<td>22,300 ±1390</td>
<td>75 ± 5</td>
<td>7 ± 0.8</td>
</tr>
<tr>
<td>0.5 μM TTX</td>
<td>85 ± 4*</td>
<td>22,230 ± 1500</td>
<td>76 ± 4</td>
<td>9 ± 0.8</td>
</tr>
<tr>
<td>1 μM TTX</td>
<td>79 ± 3*</td>
<td>21,270 ± 1190</td>
<td>71 ± 2</td>
<td>8 ± 1.0</td>
</tr>
</tbody>
</table>

* \( p < 0.05 \) vs. baseline.
tachyarrhythmias were not observed and diastolic force was not significantly increased compared with control.

Changes in [Na\(^+\)]\textsubscript{i}, during Sodium Pump Inhibition in the Absence and Presence of Ranolazine or TTX.

[Na\(^+\)]\textsubscript{i} of the guinea pig isolated, perfused heart in the absence of drug was 6.9 ± 0.6 mM (n = 9; Fig. 3A), as determined by \(^{23}\)Na NMR spectroscopy in the presence of the shift reagent Na\(_5\)TmDOTP. After perfusion of the heart with ranolazine (10 \(\mu\)M) for 30 min, [Na\(^+\)]\textsubscript{i} during Sodium Pump Inhibition was unchanged, 6.5 ± 0.4 mM (n = 5, p > 0.1 versus control; Fig. 3B). Upon exposure of the heart to 1.3 \(\mu\)M ouabain, [Na\(^+\)]\textsubscript{i} increased rapidly by 3.7-fold at 60 min to reach a plateau level of 25.1 ± 1.2 mM (n = 9, p < 0.001 versus control; Fig. 3C). After washout of ouabain for 20 min, [Na\(^+\)]\textsubscript{i} was 10.9 ± 1.2 mM (n = 8, p < 0.001 versus plateau level, p < 0.05 versus control), indicating that the ouabain effect was at least partially reversible. The 1.3 \(\mu\)M ouabain-induced increase of [Na\(^+\)]\textsubscript{i} could be attenuated by treatment of hearts with either ranolazine (10 \(\mu\)M) or TTX (1 \(\mu\)M) for 10 min before and during the exposure to ouabain (Fig. 3, C and D). During treatment of hearts with 1.3 \(\mu\)M ouabain in the presence of either 10 \(\mu\)M ranolazine (n = 9) or 1 \(\mu\)M TTX (n = 5), values of [Na\(^+\)]\textsubscript{i} reached plateau concentrations of 15.6 ± 0.1 or 10.5 ± 0.1 mM, respectively (p < 0.001 ranolazine or TTX versus ouabain alone). The decrease in the ouabain-induced rise of [Na\(^+\)]\textsubscript{i} by ranolazine and TTX was concentration-dependent (Fig. 3D).

### Table 2

Contractile function of guinea pig isolated heart during 60-min exposure to ouabain ± drug

Maximal responses to ouabain are given. Data are presented as mean ± S.E.M.

<table>
<thead>
<tr>
<th>Positive Inotropic Effect</th>
<th>Toxic Effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>LVSP mm Hg</td>
<td>RPP mm Hg/min</td>
</tr>
<tr>
<td>0.75 (\mu)M ouabain</td>
<td>142 ± 5</td>
</tr>
<tr>
<td>+ 2 (\mu)M ranolazine</td>
<td>140 ± 6</td>
</tr>
<tr>
<td>+ 5 (\mu)M ranolazine</td>
<td>147 ± 8</td>
</tr>
<tr>
<td>+ 10 (\mu)M ranolazine</td>
<td>142 ± 4</td>
</tr>
<tr>
<td>+ 0.5 (\mu)T TTX</td>
<td>126 ± 7</td>
</tr>
<tr>
<td>+ 1 (\mu)T TTX</td>
<td>137 ± 8</td>
</tr>
<tr>
<td>+ 1.8 (\mu)M KN-93</td>
<td>123 ± 5</td>
</tr>
<tr>
<td>+ 0.3 (\mu)M AIP\textsuperscript{a}</td>
<td>123 ± 14</td>
</tr>
</tbody>
</table>

\textsuperscript{**} p < 0.001 vs. 0.75 \(\mu\)M ouabain.

\textsuperscript{a} Data are mean ± S.D., n = 2.

---

**Fig. 2.** Effect of ouabain (2 \(\mu\)M) on contractile function of guinea pig isolated papillary muscles in the absence and presence of ranolazine (Ran, 10 \(\mu\)M). A, representative recording (top) of contractile force development in the absence of drug (a, control) and during exposure to 2 \(\mu\)M ouabain (b–d) for 1 h. Expanded portions of each record at times a to d are shown at the bottom. OU\(_{\text{Max}}\), OU\(_{\text{Arrhy}}\), and OU\(_{\text{1hr}}\) indicate the maximal inotropic response to ouabain, ouabain-induced arrhythmic activity, and the response to ouabain at 1 h of treatment, respectively. B, representative recording (top) of contractile force development in the absence of drug (a, control), in the presence of 10 \(\mu\)M ranolazine (b, Ran), and during 1-h exposure to both 2 \(\mu\)M ouabain and Ran (c and d). Expanded portions of each record at times a to d are shown at the bottom. OU\(_{\text{Max}}\), OU\(_{\text{Arrhy}}\), and OU\(_{\text{1hr}}\) indicate the maximal inotropic response to ouabain, ouabain-induced arrhythmic activity, and the response to ouabain at 1 h of treatment, respectively. C and D, summary of data (mean ± S.E.M.) for all experiments using the protocols shown in A (n = 8) and B (n = 8), respectively. *, p < 0.05 and ***, p < 0.001 for treatment versus control (Ctrl). E, comparison of percentage changes [relative to control set to 100%], mean ± S.E.M.] in diastolic tension after 1 h of exposure to either 2 \(\mu\)M ouabain alone (OU\(_{\text{1hr}}\)) or 2 \(\mu\)M ouabain + 10 \(\mu\)M Ran (OU+Ran 1 h). *+, p < 0.05 versus control.
Changes in Energy-Related Phosphates during Sodium Pump Inhibition in the Absence and Presence of Ranolazine. One of the consequences of Na⁺ and Ca²⁺ overload is a mismatch of energy supply and demand. Therefore, we measured changes of energy-related phosphates with ³¹P NMR spectroscopy in ouabain-treated guinea pig isolated, perfused hearts in the absence and presence of ranolazine. Under control conditions [ATP], [PCr], and [Pi] were 10 ± 0.1, 18 ± 0.5, and 3 ± 0.2 mM, respectively (n = 18 each; Fig. 4), and intracellular pH (pHᵢ) was 7.15 ± 0.01 (n = 18). Exposure of hearts to ranolazine alone for 10 min (n = 5) did not alter either the concentrations of phosphates or pHᵢ. After exposure to 0.75 μM ouabain for 60 min, [ATP] and [PCr] declined by 53 ± 7 and 49 ± 5%, respectively, [Pi] increased by 3.6 ± 1-fold (from 3 ± 0.2 to 10.4 ± 1.3 mM; all n = 5), and pHᵢ declined to 7.07 ± 0.01 (Fig. 4A). Values of pHᵢ and [Pi] recovered fully or partially during a 20-min washout period; pHᵢ returned to 7.15 (control) and [Pi] decreased from 10.4 ± 1.3 to 6.6 ± 0.8 mM (p < 0.04; Fig. 4A). In hearts treated with 0.75 μM ouabain in the presence of 10 μM ranolazine, [ATP] and [PCr] did not change significantly after 60-min ouabain treatment (Fig. 4A). The value of [Pi] increased slightly, but not significantly, from 3.7 ± 0.3 to 4.8 ± 0.2 mM in hearts exposed to ouabain in the presence of ranolazine (p > 0.05 versus control). Ranolazine (10 μM; Fig. 4A) also attenuated the ouabain-induced decrease of pHᵢ (7.13 ± 0.01 versus 7.07 ± 0.01; p < 0.05). In summary, inhibition of late I₅Na effectively prevented or reduced the ouabain-induced decreases in high-energy phosphates and pHᵢ and the increase in [Pi].

Ouabain-Induced Late I₅Na. To determine whether ouabain has an effect on sodium channels we measured the amplitude of late I₅Na in guinea pig isolated ventricular myocytes exposed to ouabain in the absence and presence of ranolazine. The amplitude of late I₅Na was increased by the exposure of cells to ouabain (1 μM). After a 3- to 5-min exposure of myocytes to ouabain, the integrated late I₅Na was increased by 4.2-fold from 23.5 ± 4.9 to 99.6 ± 15.2 pC/pF (n = 8, p < 0.001; Fig. 5, A–C). Ranolazine (10 μM) applied to cells in the continuous presence of ouabain reduced late I₅Na by 69 ± 9%, from 99.6 ± 15.2 to 50.6 ± 13.6 pC/pF (n = 8, p < 0.001; Fig. 5, A and C). In some experiments, after washout of ranolazine, cells were exposed to TTX (3 μM, n = 6; Fig. 5B). Ouabain-induced late current was completely inhibited by 3 μM TTX, to 21.2 ± 7.9 pC/pF (p < 0.001), indicating that this current was a Na⁺ channel current (e.g., Naᵥ1.5).

The ouabain-induced increase of intracellular Na⁺ may lead to Ca²⁺ uptake and activation of CaMKII. To examine the hypothesis that a Ca²⁺-dependent, CaMKII-mediated mechanism may underlie the effect of ouabain to increase late I₅Na, cells were incubated with ouabain when either the CaMKII inhibitor KN-93 (10 μM) or the Ca²⁺ chelator EGTA (1 mM) was dialyzed into them by inclusion in the patch pipette solution. KN-93 (10 μM), an inactive analog of KN-93,
was used as a control. It has previously been shown that KN-93 applied intracellularly selectively blocks ion channels (Rezazadeh et al., 2006).

Ouabain alone (1 μM, n = 6) caused a time-dependent increase of late \(I_{\text{NaL}}\) by 318 ± 74% from 21 ± 2 to 84 ± 12 pC/pF (\(p = 0.003\)) in 5 to 10 min (Fig. 6, A–C). In comparison, at the end of a 10-min exposure to ouabain in the presence of intracellular KN-93 late \(I_{\text{NaL}}\) was increased by only 76 ± 35% (from 21 ± 2 to 33 ± 6 pC/pF; \(n = 7, p = 0.003\) versus ouabain alone). In contrast, in the presence of the inactive analog KN-92 late \(I_{\text{NaL}}\) at the end of a 10-min exposure to ouabain was increased by 273 ± 39% (from 20 ± 1 to 72 ± 7 pC/pF; \(n = 6, p > 0.05\) versus ouabain alone, and \(p < 0.01\) versus KN-93). The intracellular application of 1 mM EGTA (a \(Ca^{2+}\)-chelating agent) via the patch pipette before a 10-min exposure of isolated myocytes to ouabain also attenuated the ouabain-induced increase of late \(I_{\text{NaL}}\): late \(I_{\text{NaL}}\) increased by only 33 ± 28% (from 23 ± 3 to 31 ± 8 pC/pF; \(n = 6, p < 0.001\) versus ouabain alone; Fig. 6D). A similar intracellular application of 1 mM EGTA has been shown to reduce the effect of \(Ca^{2+}\) to induce delayed afterdepolarizations in myocytes (Song et al., 2008), indicating that this application of EGTA is effective to attenuate an action mediated by a rise of \(Ca^{2+}\)i itself.

Changes in Cardiac Contractility, High-Energy Phosphates, and \([Na^+]_i\) during Sodium Pump Inhibition in the Presence of CaMKII Inhibitors. The finding that not only late \(I_{\text{NaL}}\) inhibitors but also CaMKII inhibitors reduced ouabain-induced late \(I_{\text{NaL}}\) in myocytes also attenuated the ouabain-induced increase of late \(I_{\text{NaL}}\): late \(I_{\text{NaL}}\) increased by only 33 ± 28% (from 23 ± 3 to 31 ± 8 pC/pF; \(n = 6, p < 0.001\) versus ouabain alone; Fig. 6D). A similar intracellular application of 1 mM EGTA has been shown to reduce the effect of
CaMKII caused similar reductions of both late $I_{Na}$ and electrical/contractile dysfunction in the presence of ouabain.

The CaMKII inhibitors KN-93 and AIP also attenuated the effects of ouabain on high-energy phosphates, $[P_i]$, and pH$_i$. Exposure of hearts to KN-93 (1.8 μM, $n = 6–8$ cells) alone for 10 min did not alter the concentrations of phosphates or pH$_i$ (data not shown). During exposure to 0.75 μM ouabain in the presence of either 1.8 μM KN-93 or 0.3 μM AIP, [ATP] and [PCr] did not change significantly from baseline after 60 min (not shown). The values of $[P_i]$ increased slightly, but not significantly, from 2.5 ± 0.2 to 4.5 ± 0.8 mM and from 2.5 ± 0.2 mM to 3.9 ± 0.03 mM ($p > 0.05$ versus control), and the values of pH$_i$ decreased from 7.15 ± 0.01 to 7.13 ± 0.01 and 7.14 ± 0.01 in hearts treated with ouabain in the presence of KN-93 or AIP, respectively ($p < 0.05$ versus ouabain alone).

Finally, the effect of KN-93 on $[Na^+]_i$ in hearts exposed to ouabain was determined. KN-93 (2 μM) alone did not significantly alter $[Na^+]_i$. The concentrations of intracellular sodium in the absence and presence of KN-93 were 7.3 ± 0.4 and 6.9 ± 0.6 mM, respectively ($p > 0.05$, $n = 4$). The increase of $[Na^+]_i$ in hearts exposed to 1.3 μM ouabain was significantly reduced in the presence of KN-93 from 25.1 ± 1.2 mM in hearts treated with ouabain alone to 18.9 ± 1.8 mM in hearts treated with ouabain in the presence of 2 μM KN-93 ($p < 0.05$). In summary, the deleterious effects of ouabain on cardiac function (contractility, energy metabolism, intracellular sodium) were diminished by either CaMKII or late $I_{Na}$ inhibitors.

To exclude the possibility that either ranolazine or the CaMKII inhibitors KN-93 or AIP had a direct effect on the sodium pump, three different concentrations of each inhibitor were tested in a Na$^+$, K$^+$-ATPase activity assay (Chassande et al., 1988) by measuring the $^{86}$Rb$^+$ uptake of A7r5 cells in the presence of ouabain with or without ranolazine or CaMKII inhibitor. The activity of Na$^+$, K$^+$-ATPase was inhibited 77% by 1 mM ouabain in the absence (control) of either inhibitor. Values of $^{86}$Rb$^+$ uptake were 91 ± 11, 98 ± 2.5, and 93 ± 8.3% of control (activity in presence of ouabain) in the presence of 3, 10, and 30 μM ranolazine, respectively. Neither KN-93 nor myristoylated AIP had significant effects on Na$^+$, K$^+$-ATPase activity in this assay. Values of $^{86}$Rb$^+$ uptake were 90 ± 4, 92 ± 14, and 87 ± 20% of control in the presence of KN-93 (0.2, 2, and 5 μM, respectively) and 98 ±
The results presented here suggest that a reduction of late \(I_{Na}^L\) attenuates sodium accumulation and metabolic, contractile, and electrical dysfunction induced by the cardiac glycoside ouabain in the guinea pig isolated perfused heart and papillary muscle. Ouabain markedly increased \([Na^+]_i\) and \([H^+]\), and decreased [ATP] and [PCr] in the heart. Ranolazine (10 \(\mu\)M and TTX (1 \(\mu\)M) at concentrations reported to inhibit late \(I_{Na}^L\) (Song et al., 2008) significantly reduced the rise in \([Na^+]_i\), and attenuated the losses of [ATP] and [PCr] and the decrease of \(pH_i\) that were observed in the presence of ouabain alone. Ranolazine (5 and 10 \(\mu\)M), TTX (1 \(\mu\)M), KN-93 (1.8 \(\mu\)M), and AIP (0.5 \(\mu\)M) all prevented the rise of LVEDP and reduced occurrences of cardiac standstill caused by ouabain in the isolated perfused heart, and ranolazine attenuated the increase of diastolic tension of isolated guinea pig papillary muscles during ouabain treatment.

**Changes in \([Na^+]_i\) during Sodium Pump Inhibition in the Absence and Presence of Ranolazine, TTX, and KN-93.** The concentration of \(Na^+\) in resting heart cells of many mammals is in the range of 4 to 8 mM (Bers et al., 2003). In this study using \(^{23}\)Na NMR spectroscopy, \([Na^+]_i\) was found to be \(\sim 7\) mM in guinea pig isolated hearts paced at 5 \(Hz\), consistent with literature reports (Jelicks and Siri, 1995; Hotta et al., 1998). Treatment of hearts with 10 \(\mu\)M ranolazine for up to 30 min or 1 \(\mu\)M TTX for 10 min did not significantly change \([Na^+]_i\) (Fig. 3, B and C). This finding suggests that physiological late \(I_{Na}^L\) is either a small contributor to sodium entry in the beating isolated heart or a decrease of \(Na^+\) influx via late \(I_{Na}^L\) does not lead to reduction of \([Na^+]_i\), because the reserve capacity of the \(Na^+, K^+\)-ATPase to extrude \(Na^+\) from the cell is not normally exceeded (Akerá and Ng, 1991). In this study, ouabain (1.3 \(\mu\)M, in the presence of the NMR shift reagent and \(Ca^{2+}\)-chelator \(Na_2\)TmDTP) led to a 3.7-fold increase in \([Na^+]_i\) (Fig. 3C). Ranolazine (10 \(\mu\)M) and TTX (1 \(\mu\)M) as well as KN-93 (2 \(\mu\)M) significantly attenuated the increase of \([Na^+]_i\), caused by ouabain (Fig. 3D), suggesting that an enhancement of persistent \(Na^+\) current (late \(I_{Na}^L\)) by ouabain (Fig. 5), was a factor contributing to the increase of \([Na^+]_i\).

**Ouabain-Induced Late \(I_{Na}^L\).** A novel finding in this study is that ouabain increased late \(I_{Na}^L\) in guinea pig isolated ventricular myocytes. Ranolazine and TTX as well as intracellular applications of the CaMKII inhibitor KN-93 or the \(Ca^{2+}\)-chelator EGTA all reduced late \(I_{Na}^L\) in the presence of ouabain (Figs. 5 and 6) and attenuated the ouabain-induced increase of \([Na^+]_i\) (Fig. 3). These findings suggest that an increase late \(I_{Na}^L\) contributes to elevation of \([Na^+]_i\) in the whole heart (Makielksi and Farley, 2006; Noble, 2008) and elevation of \([Ca^{2+}]_i\), and/or activity of CaMKII are potential causes of the increase of late \(I_{Na}^L\) that occurs during exposure of cardiac myocytes to ouabain. This interpretation is supported by results of previous studies showing that glycosides increased both \([Na^+]_i\) and \([Ca^{2+}]_i\) in the heart and activated CaMKII (Sapia et al., 2010) and that \(Ca^{2+}/calmodulin/\)CaMKII may directly regulate the function of the cardiac \(Na^+\) channel to increase late \(I_{Na}^L\) (Wagner et al., 2006; Maltsev et al., 2008; Bers and Grandi, 2009; Aiba et al., 2010 and references therein). An increase of late \(I_{Na}^L\) itself leads to \(Ca^{2+}\) overload (Maier and Bers, 2007; Xie et al., 2009) to close a positive feedback loop between increases of \(Ca^{2+}\) and late \(I_{Na}^L\). Furthermore, it has been reported that ouabain can stimulate reactive oxygen species production by the \(Na^+, K^+\)-ATPase (Xie et al., 1999; Liu et al., 2000), and an increase of reactive oxygen species is reported to activate CaMKII by both \(Ca^{2+}\)-dependent and -independent pathways (Palomque et al., 2009; Xie et al., 2009). Thus, there are several potential mechanisms by which the ouabain-induced increase of intracellular \(Na^+\) may lead to \(Ca^{2+}\) dysregulation and altered cell function.

We suggest that the effect of ouabain on cardiac \(Na^+/Ca^{2+}\) homeostasis and cardiac function has at least two components: first, the rise of \([Na^+]_i\) caused by decreased \(Na^+\) efflux due to inhibition of \(Na^+, K^+\)-ATPase; second, the rise of \([Na^+]_i\) caused by an enhanced late \(I_{Na}^L\), which leads to a further increase of \(Na^+\) influx. In the present work we sought to diminish the latter component in three ways: use of a late \(I_{Na}^L\) inhibitor, inhibition of CaMKII, and reduction of \(Ca^{2+}\) overload with EGTA. Each of these interventions (e.g., ranolazine, TTX, KN-93, and EGTA) reduced late \(I_{Na}^L\). However, we are not able to distinguish how much of the increase in intracellular sodium comes from the late sodium current versus the sodium pump inhibition because inhibition of the sodium pump and increase of late \(I_{Na}^L\) probably act synergistically. More importantly, each of the interventions also reduced \(Na^+\) accumulation, loss of [ATP] and [PCr], and electrical and mechanical dysfunction caused by ouabain. These findings suggest that late \(I_{Na}^L\) plays a role in glycoside-induced cardiac dysfunction, and that either a direct (by TTX or ranolazine) or indirect inhibition of late \(I_{Na}^L\) is cardioprotective when \([Na^+]_i\) is elevated as a result of glycoside-induced inhibition of the \(Na^+, K^+\)-ATPase.

In addition to ranolazine and TTX, the putative late \(I_{Na}^L\) inhibitor R56865 \([N-[1-4-(4-fluorophenoxy)-butyl]-4-piperidinyl]-N-methyl-2-benzothiazolamine\) is reported to reduce \(Na^+\) and \(Ca^{2+}\) overload and improve electrical and mechanical function (e.g., reductions of arrhythmic activity and contracture) during exposure of cardiac tissues to cardiac glycosides (Ver Donck et al., 1993; Watano et al., 1999). Inhibition of NCX with KB-R7945 (2-[2-[4-(4-nitrobenzoxyl)phenyl]-ethyl]isothioura) in isolated guinea pig atria exposed to ouabain has also been shown to reduce \(Ca^{2+}\) overload pathology (Watano et al., 1999). The mitochondrial NCX inhibitor CGP-37157 \([7-chloro-5-(2-chlorophenyl)-1,5-dihydroxy-4,1-benzothiazepin-2(3H)-one]\) also improved electrical and mechanical function in isolated guinea pig hearts as well as energy metabolism in myocytes during concomitant exposure to ouabain and isoproterenol (Liu et al., 2010). Taken together, these results indicate that strategies to prevent a pathological increase in late \(I_{Na}^L\), and/or its downstream effects, may be cardioprotective.

**Changes in High-Energy-Related Phosphates and Contractile Performance during Sodium Pump Inhibition in the Absence and Presence of Ranolazine, KN-93, and AIP.** By inducing increases of \([Na^+]_i\) and \([Ca^{2+}]_i\), ouabain had a positive inotropic effect in the guinea pig isolated heart and papillary muscle preparations. This effect was transient and was followed by mechanical, electrical, and metabolic dysfunction, including a rise of LVEDP, a decrease in left ventricular systolic function, episodes of car-


Wu L, Rajamani S, Li H, January CT, Shryock JC, and Belardinelli L (2009) Regulation of repolarization reserve unmasks the proarrhythmic role of endoge...


Address correspondence to: Kirsten Hoyer, Gilead Sciences, Inc., 1651 Page Mill Rd., Palo Alto, CA 94304. E-mail: kirsten.hoyer@gilead.com