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Reducing the late sodium current improves cardiac function during sodium pump inhibition by ouabain*

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d) List of nonstandard abbreviations

AIP	autocamide-2 related inhibitory peptide
ATX-II	sea anemone toxin-II
$[X]_i$	intracellular concentration of a metabolite or ion
CaMKII	Ca^{2+}-calmodulin-dependent kinase II
late I_{Na}	late sodium current (= persistent sodium current)
LVDevP	left ventricular developed pressure
LVEDP	left ventricular end diastolic pressure

LVSP	left ventricular systolic pressure
$Na_5Tm[DOTP]$	sodium [thulium (III) 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10-tetra (methylene phosphonate)]
NCX	sodium/calcium exchanger
NMR	nuclear magnetic resonance
PCr	phosphocreatine
pH_i	intracellular pH
Pi	intracellular inorganic phosphate
RPP	rate pressure product
sodium pump	Na^+, K^+-ATPase
TTX	tetrodotoxin

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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_{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 Na^+_i and high-energy phosphates using ^{23}Na and ^{31}P NMR, the amplitude of late I_{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 I_{Na}. Ouabain (1-1.3 μM) increased Na^+_i and late I_{Na} of guinea pig isolated hearts and myocytes by 3.7- and 4.2-fold, respectively. The late I_{Na} inhibitors ranolazine and tetrodotoxin significantly reduced ouabain-stimulated increases in Na^+_i and late I_{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_{Na} was also attenuated by the CaMKII inhibitors KN-93 and AIP, but not by KN-92. We conclude that ouabain-induced Na^+ and Ca^{2+} overload is ameliorated by inhibition of late I_{Na}.

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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_{Na}) (Ver Donck et al. 1993; Hale et al. 2008).

Late I_{Na} is caused by entry of Na^+ ions through myocyte Na^+ channels that fail to inactivate normally. These channels stay open or re-open during the action potential plateau, when "normal" Na^+ channels are inactivated, thereby contributing to intracellular Na^+ loading (Undrovinas and Maltsev, 2008; Makielski and Farley, 2006). An increase of late I_{Na} due to 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 (for review see Hale et al., 2008; Undrovinas and Maltsev, 2008). Although small in amplitude relative to peak I_{Na}, late I_{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 due to an enhanced late I_{Na} may lead to an increase of $[Na^+]_i$. Using a computational model, Noble (2008) found that reduction of late I_{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

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and leads to Ca^{2+} loading of myocardial cells (Bers, 2001; Imahashi et al., 2005). Thus, inhibition of late I_{Na} is cardioprotective (Hale et al., 2008; Makielski and Farley, 2006; Sossalla et al., 2008).

Reduction of late I_{Na} can be achieved using either ranolazine or tetrodotoxin (TTX). The antianginal drug ranolazine is a relatively selective late I_{Na} inhibitor (Antzelevitch et al., 2004; Hale et al., 2008). Ranolazine reduces late I_{Na} with an approximate IC_{50} value of 6.5 μM (vs. an IC_{50} value of 244 μM for inhibition of peak I_{Na}) and causes minimal or no inhibition of L-type Ca^{2+} channel current, NCX, or sodium proton exchange at therapeutic concentrations (≤ 10μM) (Antzelevitch et al., 2004; Hale et al., 2008). In recent studies it has been shown that ranolazine reduces sea anemone toxin-II (ATX-II)- and H_2O_2 -induced late I_{Na} in guinea pig and rabbit isolated ventricular myocytes, and suppresses early and delayed afterdepolarizations and arrhythmic activity (Song et al., 2008; Song et al., 2004). Ranolazine attenuates diastolic dysfunction in myocardium isolated from failing human hearts (Sossalla et al., 2008), in ATX-II-treated and ischemic/reperfused rat hearts (Fraser et al., 2006), and in 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_{Na} relative to hERG 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_{Na} than does ranolazine. Both inhibitors were therefore used in this study to test the hypothesis that a reduction of endogenous late I_{Na} will reduce effects of ouabain to cause cellular Na^+ loading, and metabolic and contractile dysfunction. It also has 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

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inactivation and enhance late I_{Na} (Hale et al., 2008; Maier and Bers, 2007; 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_{Na} and its attendant adverse functional consequences. The findings in this study were that ouabain itself led to an increase of late I_{Na} and that in the presence of inhibitors of late I_{Na} and CaMKII, Na^{+}_{i} accumulation in the presence of ouabain was reduced, energy loss was prevented, and mechanical function was improved.

Methods

Animals and Isolated Perfused Heart Preparation. Animal use protocols were approved by the Standing Committee on Animals of Harvard Medical Area and by 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 (NIH publication no. 85-23, revised 1996).

Guinea pigs (Duncan Hartley, 250-350 g, either sex) were anesthetized (180 mg/kg sodium pentobarbital, i.p.) and hearts were isolated and perfused in the isovolumic Langendorff mode at a constant pressure of 60 mmHg with a modified Krebs-Henseleit (KH) buffer (37° C, pH 7.4) containing (in mM) 118 NaCl, 4.8 KCl, 1.75 $CaCl_2$, 1.2 $MgSO_4$, 0.5 EDTA, 25 $NaHCO_3$, 1.2 KH_2PO_4, 5.5 glucose, 2 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 pressure transducer (Capto SP 844; Capto, Norway). Hearts were stimulated at a rate of 5 Hz during all experiments (SD9 Square Stimulator, West Warwick, USA). Data were collected and analyzed as previously described (Shen et al., 2001), using a PowerLab system (Bridge Amp, 8sp interface, Chart 5.Pro software; AD Instruments, USA). 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, Palo Alto, CA), and paced at a rate of 5 Hz.

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^{23}Na and ^{31}P Nuclear Magnetic Resonance (NMR) Spectroscopy for Measuring $[Na^+]_i$ and High-Energy Metabolites in Guinea Pig Isolated Hearts. For ^{23}Na NMR, 590 free induction decay (FIDs) 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, 3.5 mM of the shift reagent sodium (thulium (III) 1, 4, 7, 10-tetraazacyclododecane-1, 4, 7, 10- tetra (methylene phosphonate)) (Na_5Tm[DOTP]) was added to the KH buffer. To determine $[Na^+]_i$, the peak areas of ^{23}Na signals were compared to the peak area of a Na^+ internal reference standard (Jansen et al., 2003).

For ^{31}P-NMR, 125 FIDs 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 inorganic phosphate (Pi) were determined according to Shen et al. (2001).

Ouabain, Ranolazine, and Tetrodotoxin (TTX) Concentrations. Results of preliminary studies of guinea pig isolated perfused hearts indicated that concentrations of ouabain at 0.5 μM or above 1 μM were associated with either no dysfunction or rapid induction of arrhythmia, respectively. The ouabain concentration of 0.75 μM was therefore used in the majority of experiments. Ouabain induced two effects, first a positive inotropic effect and secondly a toxic effect (e.g. arrhythmia, elevated diastolic function).The shift reagent Na_5TmDOTP reduced the effect of ouabain, presumably secondary to Ca^{2+}-chelation. Therefore, in ^{23}Na NMR experiments the ouabain concentration was increased to 1.3 μM, to achieve an effect equivalent to that seen at 0.75 μM in the absence of Na_5TmDOTP. For measurements of contractile function in guinea pig papillary muscle the ouabain

concentration was increased to 2 μM, acknowledging that papillary muscle tissue is less ouabain-sensitive than either single cells or perfused hearts.

Ranolazine is a relatively selective late I_{Na} inhibitor. The ranolazine concentrations used in this study (3, 5, and 10 μM) are in the mid to high therapeutic range and are known to significantly reduce the late I_{Na} (Antzelevitch et al., 2004; Hale et al., 2008).

TTX reduces peak and late I_{Na} with IC_{50} values of 6.0 ± 0.2 μM and 0.5 ± 0.1 μM (Wu, L. et al., 2009), respectively. At the concentrations of 0.5 - 1 μM, TTX is a relatively selective late I_{Na} inhibitor.

Papillary Muscle Preparation and Tension Measurement. Guinea pigs were anesthetized and hearts were quickly removed and placed in an ice-cold Tyrode solution, containing (in mmol/L): NaCl 136, KCl 2.8, $CaCl_2$ 1, $MgCl_2$ 1.5, NaH_2PO_4 0.3, HEPES 10, glucose 10, and NaOH to adjust the pH to 7.4. The right ventricular papillary muscle was dissected free, mounted in a 36.5±0.5ºC tissue bath in Tyrode solution equilibrated with 100% 0_2, 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 developed tension was maximal. Contractile force was measured isometrically using a force displacement transducer (TRI 201, LSi Letica Scientific Instruments, Spain) and digitized using a PowerLab system (AD Instruments, USA).

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 (Axon Instruments, Union City, CA), using the whole-cell patch-clamp technique. The recording pipettes had a resistance of 2-3 MΩ when filled with a solution containing (in mM) 120 Cs-aspartate, 20 CsCl, 1 $MgSO_4$, 4 Na_2ATP, 0.1 Na_3GTP, and 10 HEPES, pH 7.2, and the series resistance was compensated by about 85%. Late I_{Na} 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 nano- or picocoulombs (nC or 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 ^oC) containing (in mM) 135 NaCl, 4.6 CsCl, 1.8 $CaCl_2$, 1.1 $MgSO_4$, 0.01 nitrendipine, 0.1 BaCl, 10 glucose and 10 HEPES, pH 7.4**.** Barium was present in the bath solution to reduce potential contamination of late I_{Na} by I_{K1}.

In selected experiments with isolated myocytes, KN-93, KN-92 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, Palo Alto, CA, and KN-92, KN-93, and myristoylated autocamtide-2 related inhibitory peptide (AIP) were obtained from Calbiochem (La Jolla, CA). The shift reagent $Na_5Tm[DOTP]$ was purchased from

Macrocyclics (Dallas, TX). All other drugs and reagents were obtained from Sigma-Aldrich (St. Louis, USA).

Statistics. Results are expressed as mean ± SEM. Data were analyzed by one-way analysis of variance (ANOVA; GraphPad Prism 5.01, San Diego, CA, USA) or ANOVA with repeated measures (Statistica 8.0, Stat Soft, Inc., Tulsa, OK, USA), 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 by 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 out of 13 hearts tested (Fig. 1A). A marked elevation of LV end diastolic pressure (LVEDP) and a decrease of developed pressure (LVDevP) were observed (Fig. 1A). The late I_{Na} 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.

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The responses of hearts that were exposed to ouabain in the presence of 0.5 μM TTX were comparable to 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 or biphasic contractions nor changes in LVEDP were observed. Ranolazine (Fig. 1B-D) and TTX (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 16-17 % (n = 6-8 each) during treatment with ranolazine alone (3, and 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 (RPP) in the presence of 0.75 μM ouabain with or without ranolazine or TTX increased significantly and were not different from each other (Table 1). 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 Table 1.

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

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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. 2A, 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. 2A, C). Diastolic tension of papillary muscle strips also increased significantly by 15% after a 1-hr ouabain treatment (Fig. 2E). Ranolazine attenuated the ouabain-induced contractile/electrical dysfunction in guinea pig papillary muscle preparations (Fig. 2B, D, E). Ranolazine (10 μM) alone had no significant effect on developed tension, and did not appear to decrease the effect of ouabain to increase developed tension (Fig. 2B, 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 tachyarrhythmias were not observed and diastolic force was not significantly increased compared to control.

Changes in $[Na^+]_i$ during Sodium Pump Inhibition in the Absence and Presence of Ranolazine or TTX. $[Na^+]_i$ 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 $Na_5TmDOTP$. After perfusion of the heart with ranolazine (10 μM) for 30 min, $[Na^+]_i$ was unchanged, 6.5 ± 0.4 mM (n = 5, $p>0.1$ vs. control; Fig. 3B). Upon exposure of the heart to 1.3μM ouabain, $[Na^+]_i$ increased rapidly by 3.7-fold at 60 min to reach a plateau level of 25.1 ± 1.2 mM (n = 9, $p<0.001$ vs. control; Fig. 3C). After washout of ouabain for 20 min, $[Na^+]_i$ was 10.9 ± 1.2 mM (n = 8, $p<0.001$ vs. plateau level, $p<0.05$ vs. control), indicating that the ouabain effect was at least partially reversible. The

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1.3 μM ouabain-induced increase of $[Na^+]_i$ could be attenuated by treatment of hearts with either ranolazine (10 μM) or TTX (1 μM) for 10 min prior to and during the exposure to ouabain (Fig. 3C,D). During treatment of hearts with 1.3 μM ouabain in the presence of either 10 μM ranolazine (n = 9) or 1 μM TTX (n = 5), values of $[Na^+]_i$ reached plateau concentrations of 15.6 ± 0.1 or 10.5 ± 0.1 mM, respectively (p<0.001 ranolazine or TTX vs. ouabain alone). The decrease in the ouabain-induced rise of $[Na^+]_i$ by ranolazine and TTX was concentration-dependent (Fig. 3D).

Changes in Energy-Related Phosphates during Sodium Pump Inhibition in the Absence and Presence of Ranolazine. One of the consequences of Na^+ and Ca^{2+} overload is a mismatch of energy supply and demand. Therefore, we measured changes of energy-related phosphates with ^{31}P 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 (pH_i) 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 pH_i. 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 pH_i declined to 7.07 ± 0.01 (Fig. 4A). Values of pH_i and [Pi] recovered fully or partially during a 20-min washout period; pH_i 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

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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 pH_i (7.13 ± 0.01 vs. 7.07 ± 0.01, p<0.05). In summary, inhibition of late I_{Na} effectively prevented or reduced the ouabain-induced decreases in high-energy phosphates and pH_i and the increase in [Pi].

Ouabain-Induced Late I_{Na}. To determine if ouabain has an effect on sodium channels we measured the amplitude of late I_{Na} in guinea pig isolated ventricular myocytes exposed to ouabain in the absence and presence of ranolazine. The amplitude of late I_{Na} was increased by exposure of cells to ouabain (1 μM). After a 3 to 5-min exposure of myocytes to ouabain, the integrated late I_{Na} was increased by 4.2-fold from 23.5 ± 4.9 to 99.6 ± 15.2 pC/pF (n = 8, p<0.001; Fig. 5A-C). Ranolazine (10 μM) applied to cells in the continuous presence of ouabain reduced late I_{Na} by 69 ± 9%, from 99.6 ± 15.2 to 50.6 ± 13.6 pC/pF (n = 8, p<0.001; Fig. 5A, C). In some experiments, after washout of ranolazine, cells were exposed to TTX (3 μM, n = 6, Fig. 2B). 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., $Na_V1.5$).

The ouabain-induced increase of intracellular Na^+ may lead to Ca^{2+} uptake and activation of CaMKII. To examine the hypothesis that a Ca^{2+}-dependent, CaMKII-mediated mechanism may underlie the effect of ouabain to increase late I_{Na}, cells were incubated with ouabain when either the CaMKII inhibitor KN-93 (10 μM) or the Ca^{2+} chelator EGTA (1 mM) was dialyzed into them by inclusion in the patch pipette solution. KN-92 (10 μM),

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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_{Na} by 318 ± 74% from 21 ± 2 to 84 ± 12 pC/pF (p=0.003) in 5-10 min (Fig. 6A-6C). In comparison, at the end of a 10-min exposure to ouabain in the presence of intracellular KN-93, late I_{Na} was increased by only 76 ± 35% (from 21 ± 2 to 33 ± 6 pC/pF; n = 7, p= 0.003 vs. ouabain alone). In contrast, in the presence of the inactive analog KN-92, late I_{Na} 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 vs. ouabain alone, and P<0.01 vs. KN-93). The intracellular application of 1 mM EGTA (a Ca^{2+}-chelating agent) via the patch pipette prior to a 10-min exposure of isolated myocytes to ouabain also attenuated the ouabain-induced increase of late I_{Na}: late I_{Na} increased by only 33 ± 28% (from 23 ± 3 to 31 ± 8 pC/pF; n = 6, p<0.001 vs. 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 intracellular Ca^{2+}, presumably by reduction 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_{Na} inhibitors but also CaMKII inhibitors reduced ouabain-induced late I_{Na} in myocytes suggested that CaMKII inhibitors may also improve function in the isolated guinea pig heart exposed to ouabain. KN-93 (1.8 μM) alone decreased LVSP by 24 ± 1.1 % (p<0.03, n = 5), whereas AIP (0.3 μM, n = 2) alone had no measurable effect

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on cardiac contractility. Exposure of hearts to ouabain in the presence of the CaMKII inhibitors KN-93 or AIP resulted in an increase of contractile amplitude (Table 1). In contrast to hearts treated with ouabain alone, however, hearts exposed to ouabain in combination with CaMKII inhibitors experienced neither episodes of cardiac standstill nor an elevation of LVEDP (Table 1). Thus, inhibition of either late I_{Na} or 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, [Pi], and pH_i. Exposure of hearts to KN-93 (1.8 μM, n = 3-6) or AIP (0.3 μM, n = 2) 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 [Pi] 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 to 7.14 ± 0.01 in hearts treated with ouabain in the presence of KN-93 or AIP, respectively (p<0.05 vs. ouabain alone).

Lastly, 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 mM 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

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(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, 5 μM, respectively) and 98 ± 10, 89 ± 12, and 106 ± 11% of control in the presence of AIP (0.03, 0.3, 0.8 μM, respectively).

Discussion

The results presented here suggest that a reduction of late I_{Na} 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 $[H^+]_i$ and decreased [ATP] and [PCr] in the heart. Ranolazine (10 μM) and TTX (1 μM) at concentrations reported to inhibit late I_{Na} (Song et al., 2008) significantly reduced the rise in $[Na^+]_i$ and attenuated the losses of [ATP] and

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[PCr] and the decrease of pH_i that were observed in the presence of ouabain alone. Ranolazine (5 and 10 μM), TTX (1 μM), KN-93 (1.8 μM), and AIP (0.3 μM) 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 4-8 mM (Bers et al., 2003). In this study using ^{23}Na NMR spectroscopy, $[Na^+]_i$ was found to be ~7 mM in guinea pig isolated hearts paced at 5 Hz, consistent with literature reports (Hotta et al., 1998; Jelicks and Siri, 1995). Treatment of hearts with 10 μM ranolazine for up to 30 min or with 1 μM TTX for 10 min did not significantly change $[Na^+]_i$ (Fig. 3B,D). This finding suggests that physiological late I_{Na} is either a small contributor to sodium entry in the beating isolated heart, or that a decrease of Na^+ influx via late I_{Na} 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 (Akera and Ng, 1991). In this study, ouabain (1.3 μM, in the presence of the NMR shift reagent and Ca^{2+} chelator Na_5TmDOTP) led to a 3.7-fold increase in $[Na^+]_i$ (Fig. 1D). Ranolazine (10 μM) and TTX (1 μM) as well as KN-93 (2 μM) significantly attenuated the increase of $[Na^+]_i$ caused by ouabain (Fig. 1D), suggesting that an enhancement of persistent Na^+ current (late I_{Na}) by ouabain (Fig. 5), was a factor contributing to the increase of $[Na^+]_i$.

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Ouabain-Induced Late I_{Na}. A novel finding in this study is that ouabain increased late I_{Na} 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} in the presence of ouabain (Fig. 5, 6) and attenuated the ouabain-induced increase of $[Na^+]_i$ (Fig. 3). These findings suggest that an increased late I_{Na} contributes to elevation of $[Na^+]_i$ in the whole heart (Makielski and Farley, 2006; Noble, 2008) and that elevation of $[Ca^{2+}]_i$, and/or activity of CaMKII are potential causes of the increase of late I_{Na} 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} (Aiba et al., 2010 and references therein; Bers and Grandi, 2009; Maltsev et al., 2008; Wagner et al., 2006). An increase of late I_{Na} 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}. Furthermore, it has been reported that ouabain can stimulate ROS production by the Na^+, K^+ ATPase (Liu et al., 2000; Xie et al., 1999), and an increase of ROS is reported to activate CaMKII by both Ca^{2+}-dependent and Ca^{2+}-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}, which leads to a further increase of Na^+ influx. In the present work we

sought to diminish the latter component in three ways: by use of a late I_{Na} inhibitor, by inhibition of CaMKII, and by reducing Ca^{2+} overload with EGTA. Each of these interventions (e.g., ranolazine, TTX, KN-93, and EGTA) reduced late I_{Na}. However, we are not able to distinguish how much of the increase in intracellular sodium comes from the late sodium current vs. the sodium pump inhibition because inhibition of the sodium pump and increase of late I_{Na} 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} plays a role in glycoside-induced cardiac dysfunction, and that either a direct (by TTX or ranolazine) or indirect inhibition of late I_{Na} 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} inhibitor R56865 is reported to reduce Na^+ and Ca^{2+} overload and to 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-R7943) 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 CPG-37157 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}, 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 LV systolic function, episodes of cardiac standstill (contracture, inexcitability), and a pronounced loss of about 50% of ATP and PCr. Sodium-induced Ca^{2+} overload is known to lead to a mismatch of energy demand and supply in the heart (Hotta et al., 1998; O'Rourke and Maack, 2007). Energy demand increases due to activation of myosin ATPase, sarcoplasmatic reticulum Ca^{2+} ATPase, the sarcolemmal Ca^{2+} ATPase, and the sodium pump. ATP synthesis may be reduced due to Na^+ and Ca^{2+} overload (Balaban, 2002; O'Rourke and Maack, 2007) as the activity of Ca^{2+}-dependent Krebs cycle dehydrogenases (pyruvate, isocitrate, and α-ketoglutarate) is reduced when the mitochondrial Ca^{2+} level falls in response to increased mitochondrial NCX driven by elevation of intracellular Na^+ (Maack et al., 2006; Kohlhaas et al., 2010). The mismatch of energy demand and supply results in decreases in [ATP] and [PCr], increases in [ADP], [Pi], and cellular acidosis, and ultimately in a decrease of the free energy available from hydrolysis of ATP.

Limitations of this study are that we have not measured the level of intracellular Ca^{2+}, and the CaMKII inhibitors KN-93 and AIP may not have been completely selective. Off-target effects of KN-93 include inhibition of L-type calcium and potassium channels (Xie et al., 2009 and references therein), which may reduce Ca^{2+} overload and arrhythmia. Furthermore, an effect of AIP to prolong the duration of the myocyte action potential

(presumably a nonspecific peptide effect; Xie et al., 2009) may counteract any potential shortening of action potential duration caused by ouabain (Lee and Klaus, 1971). Any of the above may potentially reduce ouabain toxicity independently of CaMKII inhibition.

In summary our results show that ouabain stimulated an increase of late I_{Na} in guinea pig myocytes, and direct and indirect inhibition of late I_{Na} attenuated the ouabain-induced Na^+ overload and metabolic, electrical, and mechanical dysfunction in the guinea pig isolated heart and papillary muscle. The effects of an enhanced late I_{Na} to cause Ca^{2+} overload and both electrical and mechanical dysfunction of the heart suggest that late I_{Na} has a key pathophysiological role in the heart. Therefore, inhibition of late I_{Na} in the diseased heart and during digitalis therapy may be of clinical relevance.

Authorship Contributions

Participated in research design: Hoyer, Song, Wang, Phan, Ingwall, Belardinelli, Shryock

Conducted experiments: Hoyer, Song, Wang, Phan, Balschi

Performed data analysis: Hoyer, Song, Wang

Wrote or contributed to the writing of the manuscript: Hoyer, Song, Wang, Ingwall, Belardinelli, Shryock

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Footnotes

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Figure Legends

Figure 1: Effect of ouabain (0.75 μM) on left ventricular (LV) pressure of the guinea pig isolated, electrically-paced heart, in the absence (**Panel A**) and presence of ranolazine (Ran, 3, 5, and 10 μM; **Panels B, C, D**, respectively) or 1.8 μM KN-93 (KN, **Panel 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.

Figure 2. Effect of ouabain (2 μM) on contractile function of guinea pig isolated papillary muscles in the absence and presence of ranolazine (Ran, 10 μM). **Panel A**, Representative recording (top) of contractile force development in the absence of drug (a, control) and during exposure to 2 μM ouabain (b-d) for 1 hr. Expanded portions of each record at times a-d are shown at the bottom of the panel. OUMax, OUArrhy, and OU1hr indicate the maximal inotropic response to ouabain, ouabain-induced arrhythmic activity, and the response to ouabain at 1 hr of treatment, respectively. **Panel B**, Representative recording (top) of contractile force development in the absence of drug (a, control), in the presence of 10 μM ranolazine (b, Ran), and during 1 hr exposure to both 2 μM ouabain and Ran (c,d). Expanded portions of each record at times a-d are shown at the bottom of the panel. OU+RanMax and OU+Ran1hr indicate the maximal inotropic response to ouabain+Ran and the response to ouabain+Ran at 1 hr of treatment, respectively. **Panels C-D**, Summary of data (mean ± SEM) for all experiments using the protocols shown in Panels A (n = 8) and B (n = 8), respectively. *$p<0.05$ and **$p<0.01$ for treatment vs. control (Ctrl). **Panel**

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E, Comparison of percentage changes (relative to control (set to 100%), mean ± SEM) in diastolic tension after 1 hour of exposure to either 2 μmol/L ouabain alone (Ou1hr) or 2 μmol/L ouabain + 10 μmol/L Ran (Ou+Ran 1hr). *p<0.05 vs. control.

Figure 3: Effects of ouabain, ranolazine (Ran), and TTX on intracellular Na^+-concentration ($[Na^+]_i$) measured by ^{23}Na NMR spectroscopy of the guinea pig isolated heart. **Panel A,** The extracellular Na resonance (Na_e) in this typical ^{23}Na spectrum was shifted to the left by 1.8 ppm in the presence of the shift reagent $Na_5TmDOTP$ (3.5 mM) compared to the intracellular Na resonance (Na_i). **Panel B**, Stacked plot of Na_i resonances obtained every 2 min during control perfusion (10 min) and during perfusion with 10 μM ranolazine (10 Ran, 30 min). **Panel C**, Effects of 1.3 μM ouabain on $[Na^+]_i$ in the absence (■, n = 9) and presence of either 10 μM Ran (●, n=9) or 1 μM TTX (▼, n=5). Timeline: 1-control, 2- vehicle, Ran or TTX pretreatment, 3- ouabain ± drug, 4 – washout. **Panel D**, Plateau values (mean ± SEM, n = 4-9) during exposure to 1.3 μM ouabain in absence and presence of either Ran (3 or 10 μM) or TTX (0.5 or 1 μM); *p<0.05 vs. 1.3 μM ouabain alone, $^{\dagger}p$<0.05 for 10 μM Ran vs. 3 μM Ran, and $^{\#}p$<0.05 for 1 μM TTX vs. 0.5 μM TTX or 3 μM Ran.

Figure 4: Ouabain-induced changes in concentrations of energy-related phosphates measured by ^{31}P NMR spectroscopy of guinea pig isolated hearts. **Panel A**, Values of ATP, phosphocreatine (PCr), inorganic phosphate (Pi), and intracellular pH (pH_i) during exposure to 0.75 μM ouabain (arrow, n = 5) in the absence or presence of 10 μM ranolazine (n = 5). Values of ATP and PCr are expressed relative to concentrations measured during

baseline (control). *p<0.05 ouabain alone vs. control; †p<0.05 during ouabain + inhibitor treatment vs. ouabain alone; symbols denote earliest time point when the values are significantly different during ouabain treatment. #p<0.05 for all ranolazine vs. all ouabain, Wilcoxon's rank sum test. **Panel B**, Representative ^{31}P NMR control spectrum. Peak assignments from left to right: phosphomonoesters (PME), extracellular inorganic phosphate ($_{ex}$Pi), intracellular Pi, PCr, and phosphorus atoms of γ-, α- and β- phosphates of ATP. **Panel C,** Representative stacks of sequential averaged spectra depicting Pi, PCr, and [γ-P]–ATP resonances during control (1), ± 10 μM ranolazine (2), 0.75 μM ouabain-treatment ± 10 μM ranolazine (3), and washout periods (4). Values are means ± SEM.

Figure 5. Ouabain increases late sodium current (late I_{Na}) in guinea pig isolated ventricular myocytes. **Panels A** and **B**, The effect of 1 μM ouabain (Ouab) to increase late I_{Na} in a patch-clamped myocyte is partially reversed by either ranolazine (Ran, 10 μM) or TTX (3 μM). Current traces a - e were successively recorded from a single myocyte. The effect of TTX was reversible upon washout (not shown). **Panel C**, Summary of effects of Ouab, Ran and TTX on late I_{Na} (n = 6-8 myocytes); * and ** p<0.001 vs. control and ouabain alone, respectively.

Figure 6. Intracellular applications (via the patch pipette) of either KN-93 (10 μM) or EGTA (1 mM), but not KN-92 (10 μM), attenuated the effect of ouabain (1 μM) to increase late I_{Na}. **Panel A**, Changes of late current amplitude (nC) in each of 4 individual myocytes during a 10-min treatment with ouabain in the absence (control) and presence of KN-92, KN-93, or EGTA. **Panel B**, Records of late I_{Na} recorded from the 4 cells shown in panel A,

at the beginning (0 min) and end (10 min) of an experiment. Dotted line indicates zero current. Calibration bars apply to all records. **Panel C**, Summary of effect of ouabain (bars represent mean ± SEM of data from 6-7 myocytes) on late I_{Na} (pC/pF) recorded at beginning (0 min) and end (10 min) of drug exposures as depicted in panel A. *p<0.01 vs. 0 min. NS, P>0.05 vs. 0 min. **Panel D**, Comparison of increases of late I_{Na} caused by 1 μM ouabain in the absence (Ctrl) and presence of either KN-92, KN-93, or EGTA, expressed as % of baseline (0 min) current. NS, p>0.05 vs. control; *p<0.01 vs. control and KN-92.

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Table 1: Contractile function of guinea pig isolated heart at [A] baseline and after 10 min pretreatment with late I_{Na} inhibitors, and [B] during 60 min exposure to ouabain ± drug. Maximal responses to ouabain are given. Data are presented as mean ± S.E.M.

A	**LVSP (mmHg)**	**RPP (mmHg/min)**	**LVDevP (mmHg)**	**LVEDP (mmHg)**
Baseline	96 ± 1	26,670 ± 440	90 ± 1	7 ± 0.3
3 μM ranolazine	93 ± 3	25,240 ± 1050	85 ± 4	7 ± 0.5
5 μM ranolazine	80 ± 7*	22,470 ± 2450	77 ± 8	8 ± 0.6
10μM ranolazine	82 ± 4*	22,300 ± 1390	75 ± 5	7 ± 0.8
0.5 μM TTX	85 ± 4*	23,230 ± 1500	76 ± 4	9 ± 0.8
1 μM TTX	79 ± 3*	21,270 ± 1190	71 ± 2	8 ± 1.0

B	Positive inotropic effect			Toxic effect
0.75 μM ouabain	142 ± 5	40,680 ± 1,160	139 ± 6	56 ± 6
+ 3 μM ranolazine	140 ± 6	40,030 ± 1,480	131 ± 5	38 ± 10
+ 5 μM ranolazine	147 ± 8	38,870 ± 2,900	140 ± 9	15 ± 3***
+ 10μM ranolazine	142 ± 4	41,080 ± 2,100	136 ± 5	10 ± 2***
+ 0.5 μM TTX	126 ± 7	35,750 ± 1,850	111 ± 7	36 ± 12
+ 1 μM TTX	137 ± 8	39,180 ± 2,220	129 ± 7	9 ± 1***
+ 1.8 μM KN-93	123 ± 5	34,880 ± 1,670	123 ± 9	6 ± 1***
+ 0.3 μM AIP[a]	123 ± 14	37,370 ± 4,160	123 ± 19	6 ± 1

[a]data are mean ± S.D., n=2; *p<0.05 vs. baseline; ***p < 0.001 vs. 0.75 μM ouabain

Figure 1

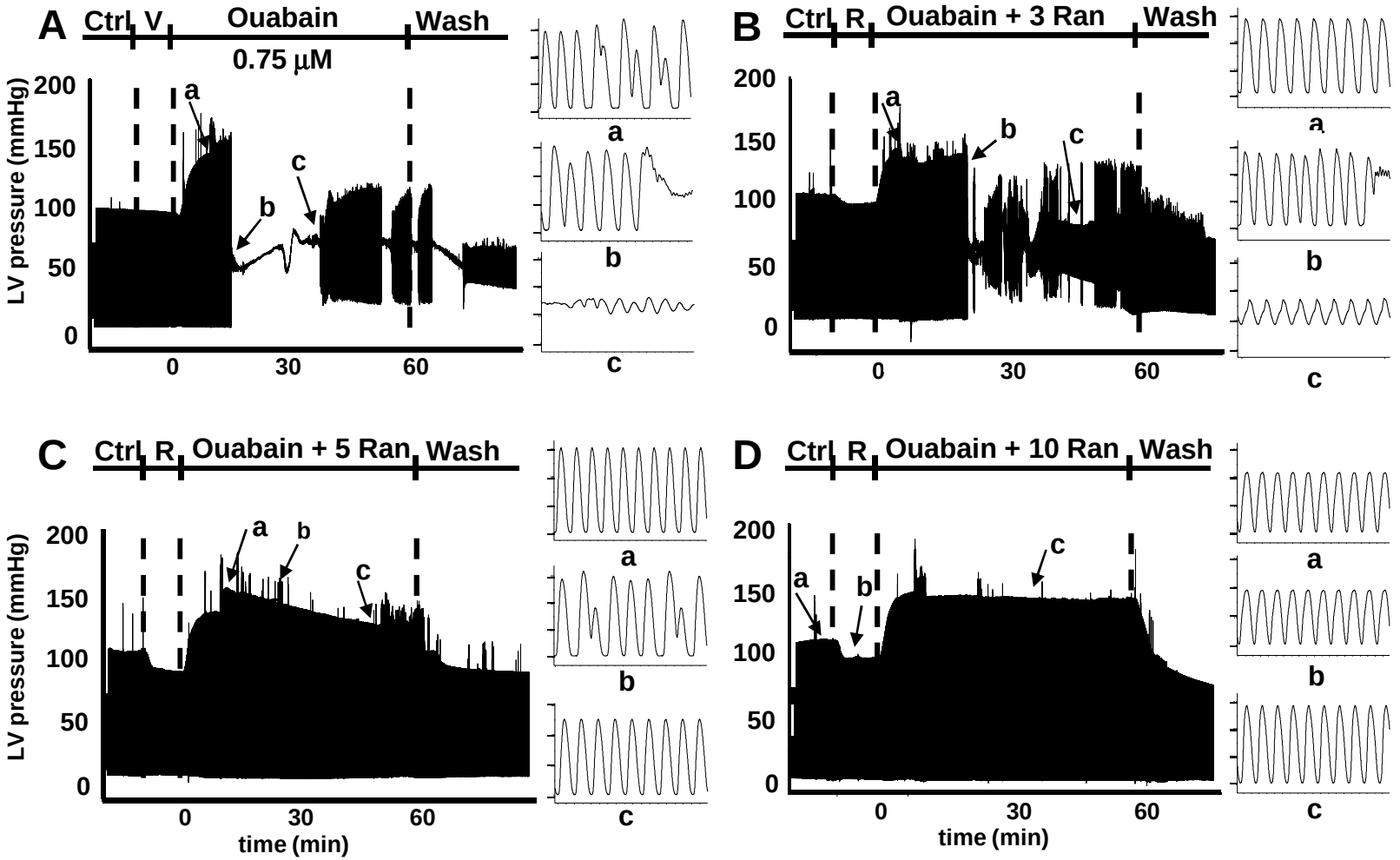

Figure 1

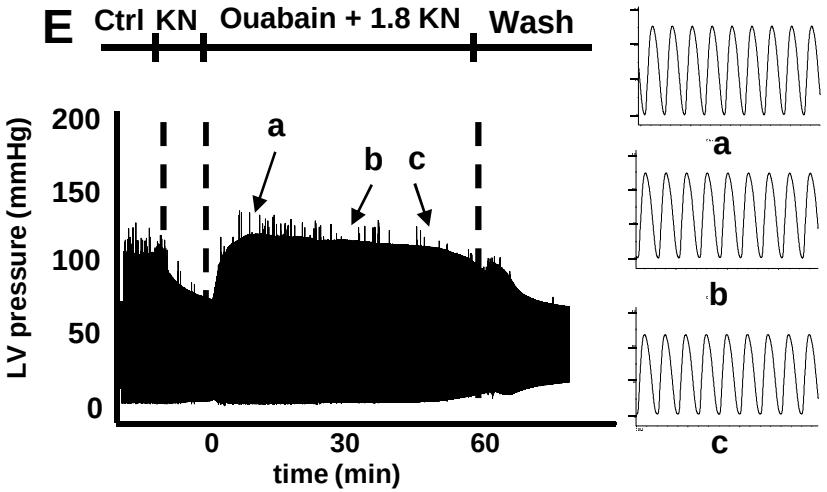

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Figure 2

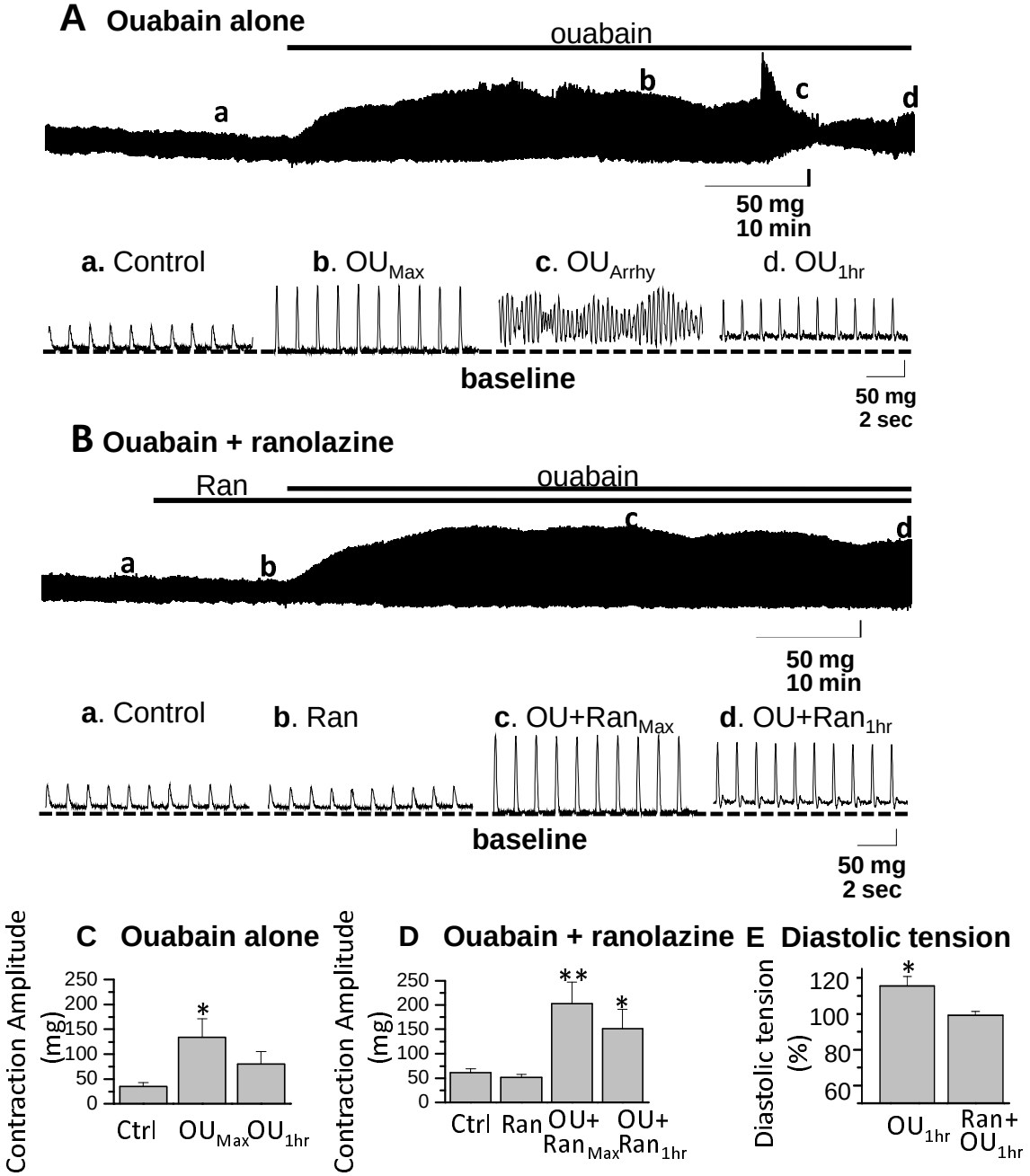
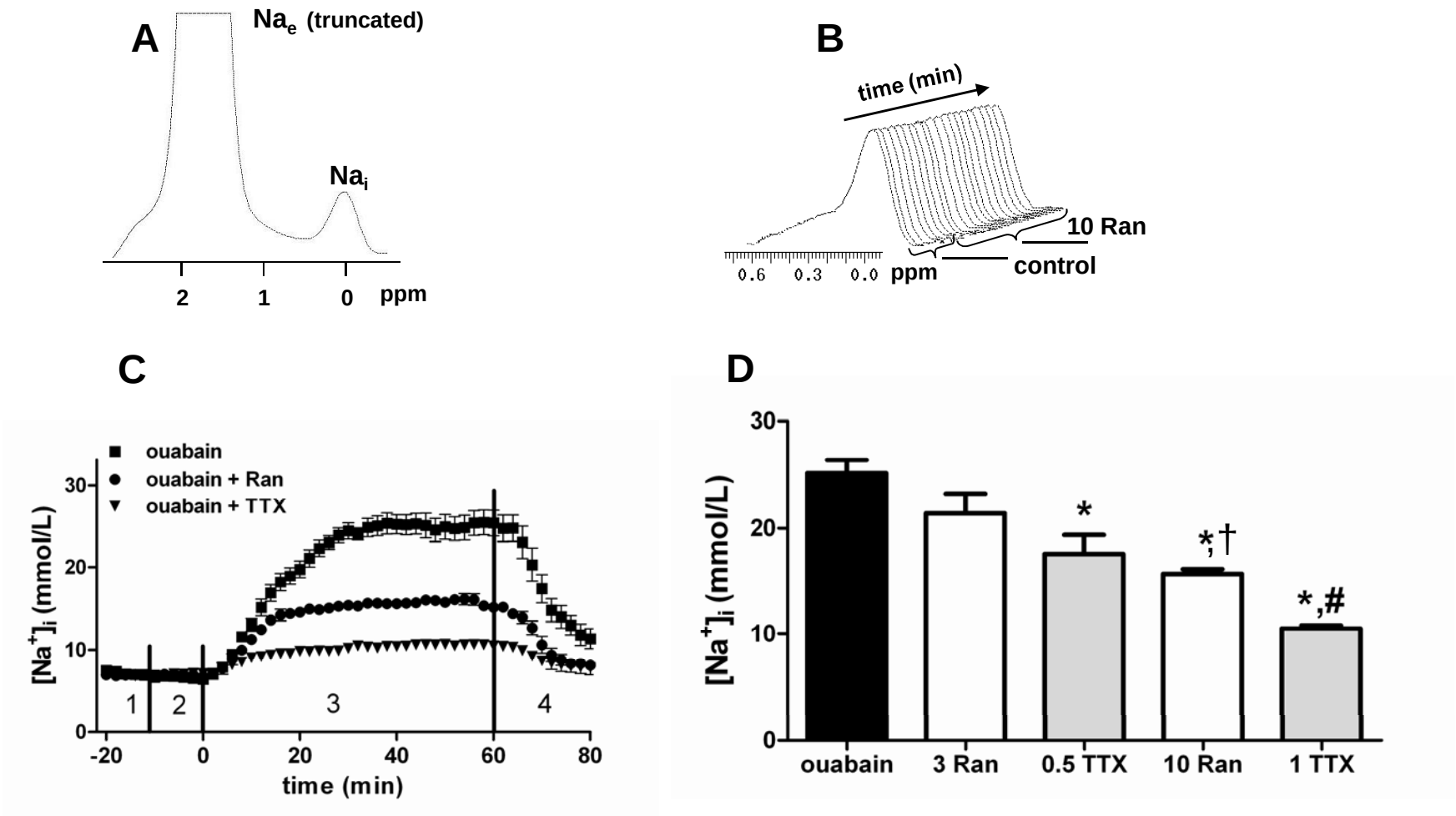


Figure 3



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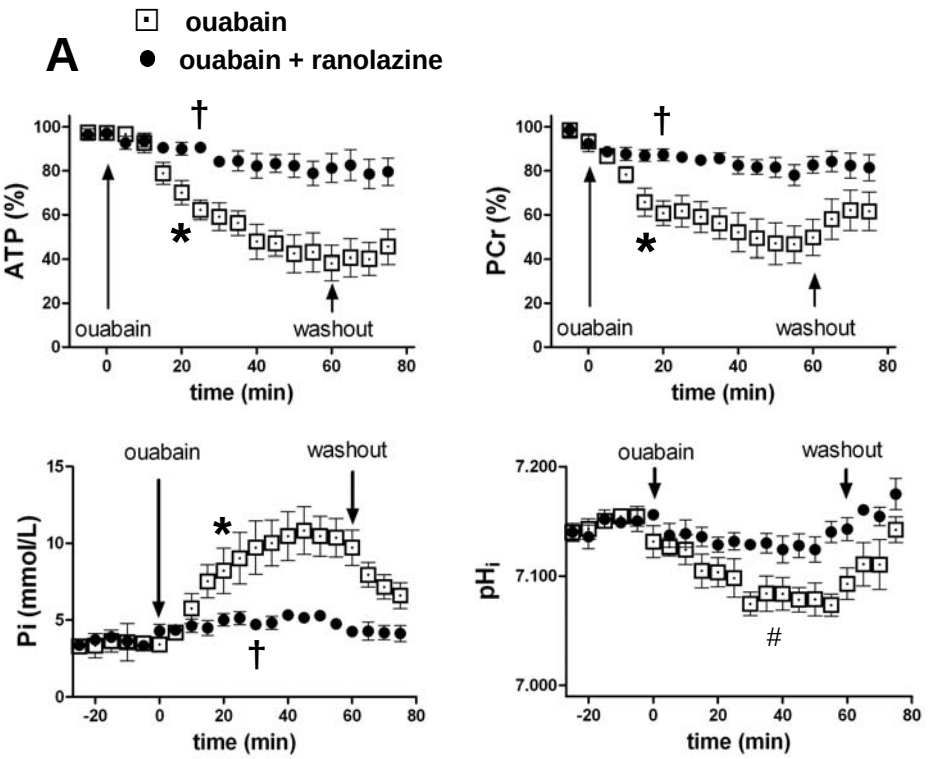

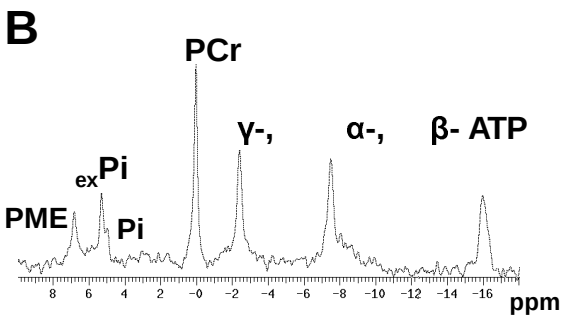

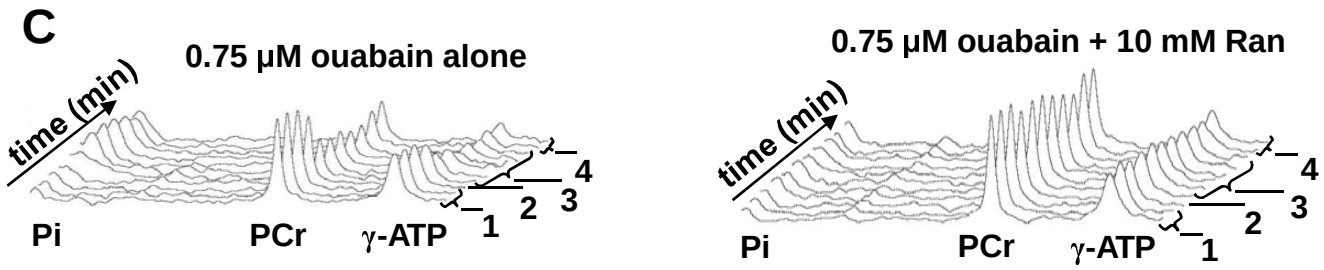

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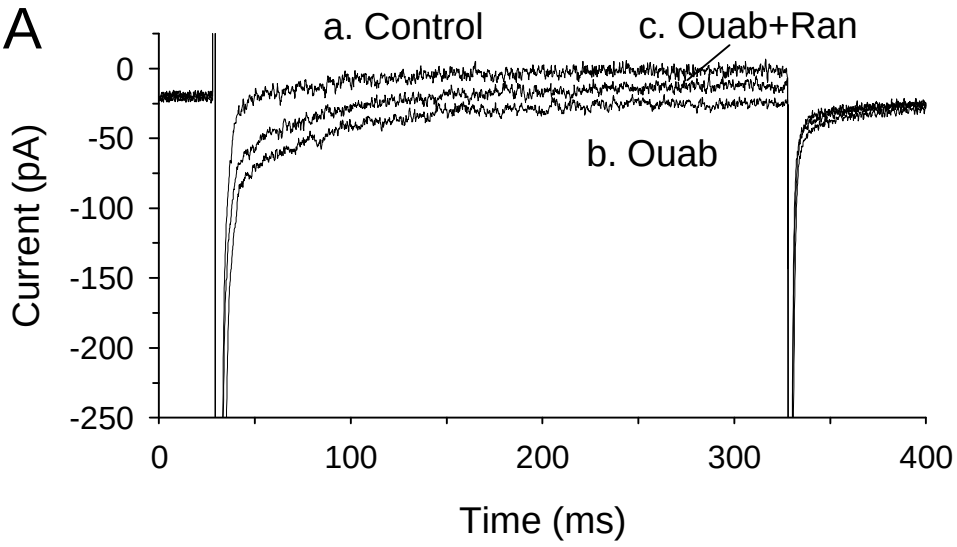

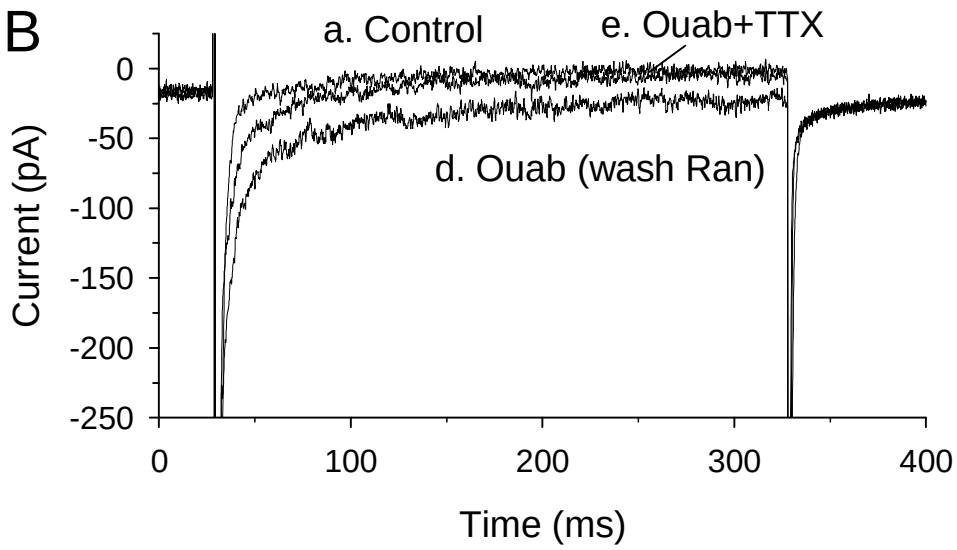

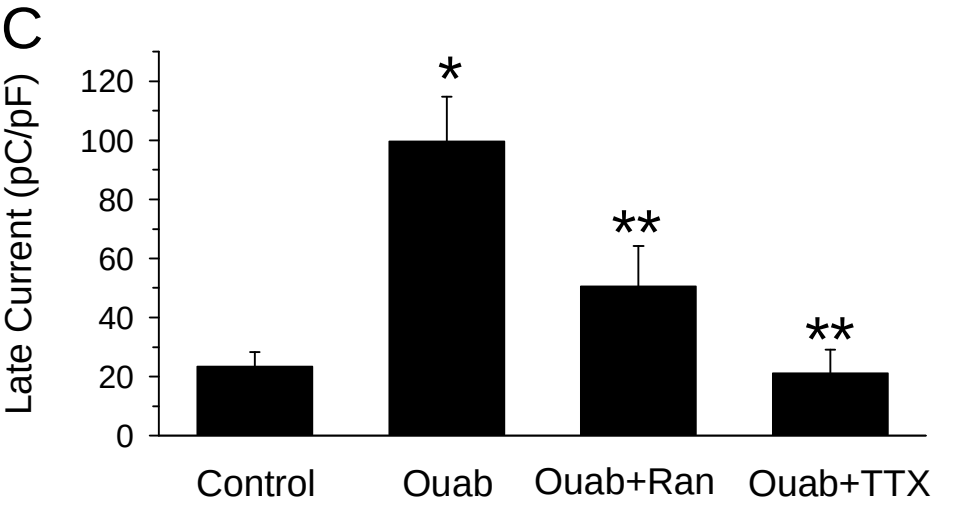

Figure 6