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The late $I_{\text{Na}}$ Inhibitor Ranolazine Attenuates Effects of Palmitoyl-L-Carnitine to Increase Late $I_{\text{Na}}$ and Cause Ventricular Diastolic Dysfunction

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ABBREVIATIONS:

CPP, coronary perfusion pressure; K-H, Krebs-Henseleit; LVEDP, left ventricular end-diastolic pressure; PC, palmitoyl-L-carnitine; Ran, ranolazine; ROS, reactive oxygen species; TTX, tetrodotoxin
Abstract

Palmitoyl-L-carnitine (PC), an ischemic metabolite, causes cellular Na\(^+\) and Ca\(^{2+}\) overload and cardiac dysfunction. This study determined if ranolazine attenuates PC-induced Na\(^+\) current and ventricular contractile dysfunction of the isolated heart. PC (4 \(\mu\)mol/L, 30 minutes) increased late Na\(^+\) current by 1,034+/\(-349\)% in guinea pig isolated ventricular myocytes; ranolazine (10 \(\mu\)mol/L) and tetrodotoxin (TTX, 3 \(\mu\)mol/L) significantly attenuated this effect of PC. PC increased left ventricular end-diastolic pressure (LVEDP), coronary perfusion pressure (CPP), wall stiffness, and cardiac lactate and adenosine release from the isolated heart. Ranolazine (10 \(\mu\)mol/L) significantly reduced the PC-induced increase in LVEDP by 72 ± 6 \% (n=6, p<0.001) reduced left ventricular wall stiffness, and attenuated the PC-induced increase of CPP by 53 ± 10\% (n=6-7, p<0.05). Ranolazine (10 \(\mu\)mol/L) reduced the PC-induced increases of lactate and adenosine release by 70 ± 8 and 81 ± 5 \%, respectively (n=6, p ≤ 0.05 for both). TTX (2 \(\mu\)mol/L) significantly (p<0.05) reduced PC-induced increases of CPP and LVEDP. Pretreatment of isolated myocytes or hearts with the free radical scavenger Tiron (1 mmol/L) significantly reduced the effects of PC to cause increases of late Na\(^+\) current and LVEDP, respectively, but unlike ranolazine or TTX, Tiron did not reverse increases of late Na\(^+\) current and LVEDP caused by PC. In summary, ranolazine and TTX — inhibitors of the late Na\(^+\) current — attenuated the PC-induced ventricular contractile dysfunction and increase of coronary resistance in the guinea pig isolated heart.
Introduction

Long chain acyl carnitines including palmitoyl-L-carnitine (PC) are intermediates of fatty acid metabolism that accumulate rapidly during brief ischemia (Sobel et al., 1978; DaTorre et al., 1991), secondary to a reduction of tissue oxygenation and mitochondrial lipid oxidation. Hypoxia of one hour duration was shown to increase the content of long-chain acylcarnitines in sarcolemmal membranes of cultured neonatal rat cardiac myocytes by 70-fold (Knabb et al., 1986). PC has been shown to cause metabolic, electrical, and mechanical cardiac malfunction (Corr et al., 1979; Sakata et al., 1989; Tanaka et al., 1992; Wu and Corr, 1992; Sato et al., 1992; Sato et al., 1993; Allely et al., 1993; Wu and Corr, 1994; Shen and Pappano, 1995; Wu and Corr, 1995; Hara et al., 1997; Netticadan et al., 1999; Maruyama et al., 2000). PC alters ion channel function and has been reported to decrease $I_{Ca,L}$ (Wu and Corr, 1992), to reduce the open probability of the inward rectifier $K^+$ current $I_{K1}$ (Sato et al., 1993), to inhibit $I_{KATP}$ (Haruna et al., 2000), and to reduce peak $I_{Na}$ (Sato et al., 1992). PC concentration- and time-dependently increased, then decreased the duration of the action potential of the guinea pig ventricular myocyte (Meszaros and Pappano, 1990; Shen and Pappano, 1995), induced $Ca^{2+}$ overload, $I_{Ti}$, and aftercontractions, and both early and late afterdepolarizations (Meszaros et al., 1990; Shen and Pappano, 1995; Wu and Corr, 1992). Palmitoyl-L-carnitine also induced a slowly-inactivating $Na^+$-sensitive current and increased $[Na^+]_i$ (Wu and Corr, 1995) in guinea pig ventricular myocytes. This latter finding suggests that palmitoyl-L-carnitine may increase the late (persistent) $Na^+$ current (late $I_{Na}$) in ventricular myocytes. An increase of late $I_{Na}$ in the heart is known to lead to $Na^+$ and $Ca^{2+}$ overload and both electrical and mechanical dysfunction (Ver Donck et al., 1993; Hale et al., 2008).
Several agents are reported to attenuate the toxic effects of PC on the isolated heart: the Na\(^+\) channel blockers tetrodotoxin (TTX) and dilazep (Hara et al., 1997), the Na\(^+\)/H\(^+\) exchange inhibitor 5-(N,N-dimethyl)-amiloride (Arakawa and Hara, 1999), and the local anesthetic, lidocaine (Arakawa et al., 1997). These findings suggest that inhibition of Na\(^+\) channels and reduction of sodium overload is beneficial to reduce PC-induced dysfunction. The antianginal and anti-ischemic drug ranolazine (Siddiqui and Keam, 2006; Hale et al., 2008) was also reported to attenuate PC-induced mechanical and metabolic derangement in the rat isolated heart (Allely et al., 1993; Maruyama et al., 2000). More recently it was discovered that ranolazine inhibits late I\(_{\text{Na}}\) and cellular Na\(^+\) and Ca\(^{2+}\) overload in the heart (Antzelevitch et al., 2004; Belardinelli et al., 2006). These findings suggest that enhancement of late I\(_{\text{Na}}\), which is normally a very small current in cardiac myocytes, may explain many of the effects of PC on the heart, and that inhibition of late I\(_{\text{Na}}\) is the mechanism by which ranolazine acts to attenuate PC-induced dysfunction. The present study was designed to test this hypothesis. We determined whether PC increases late I\(_{\text{Na}}\), if the effect of PC to increase late I\(_{\text{Na}}\) is reversed by ranolazine and by tetrodotoxin (TTX), and if ranolazine attenuates PC – induced diastolic dysfunction (increase wall stiffness, reduced vascular conductance and ischemia) of the heart. Furthermore, because ranolazine has been reported to be an antagonist of β-adrenergic receptors in rat heart (Letienne et al., 2001), to increase myocardial blood flow during exposure to PC (Maruyama et al., 2000), to attenuate the effects of reactive oxygen species (Song et al., 2006), and to inhibit hERG K\(^+\) current in the heart (Antzelevitch et al., 2004), we sought to clarify the potential roles of these actions as mechanisms of ranolazine’s effect to reduce the toxicity of PC. Guinea pig isolated hearts and single ventricular myocytes were used in these studies.
Methods

Isolation, perfusion, and recording of function of the guinea pig heart

Female Dunkin-Hartley guinea pigs, weighing between 300 and 400 g, were used for all experiments. Animal use was approved by the Institutional Animal Care and Use Committee of CV Therapeutics (isolated heart studies) or of the University of Florida (myocyte electrophysiology) and conformed to guidelines issued by the National Institutes of Health (NIH publication No. 85-25). Guinea pigs were anti-coagulated by injection of heparin (100 U/kg, intramuscular) and anesthetized with an intramuscular injection of ketamine/xylazine (48/6.4 mg/kg, respectively). The heart was excised and placed in oxygenated Krebs-Henseleit (K-H) buffer at room temperature. The aorta was rapidly cannulated and perfused in a retrograde manner to maintain a constant flow of 10 mL/min with K-H solution at 37 ± 2 °C using a Langendorff apparatus. Hearts were paced at a rate of 4 Hz. The contents of the K-H solution (in mmol/L) were: NaCl 118, KCl 4.8, KH₂PO₄ 1.2, CaCl₂ 2.5, MgSO₄ 1.2, pyruvic acid 2, glucose 5.5, Na₂EDTA 0.5, and NaHCO₃ 25. K-H solution was continuously bubbled with 95% O₂/5%CO₂ and pH was adjusted to 7.4 with 5 N NaOH. To measure coronary perfusion pressure (CPP), a pressure transducer was connected to the aortic cannula via a side port of the cannula positioned above the level of the heart. To measure left ventricular end-diastolic pressure (LVEDP), a water-filled custom-made balloon of latex film was attached to a small-diameter polyethylene catheter and inserted through an incision in the left atrium, across the mitral valve, and into the LV. The polyethylene catheter was connected to a pressure transducer (Biopac, Goleta, CA.) and the balloon was inflated to achieve an LVEDP of 5-8 mmHg. Balloon volume was kept constant through an experiment unless otherwise indicated. To measure left ventricular wall stiffness, LVEDP was measured in response to incremental increases in the volume of the
intraventricular balloon. Hearts were perfused for 20 min without intervention or until coronary perfusion pressure was stabilized before beginning an experiment.

**Drugs and solutions**

Palmitoyl-L-carnitine, sodium nitroprusside, adenosine, acetylcholine, the hERG current blocker E-4031, and DMSO were purchased from Sigma Chemical (St. Louis, MO). The β-adrenergic receptor blocker esmolol hydrochloride (Paddock Laboratories, Minneapolis, MN) was purchased from a local pharmacy. Ranolazine ((±)-1-piperazineacetamide, N-(2,6-dimethylphenyl)-4-[2-hydroxy-3-(2-methoxyphenoxy)propyl]-)(Siddiqui and Keam, 2006) and the A2A-adenosine receptor agonist CVT-3146 (regadenoson; 1-{9-[(4S, 2R, 3R, 5R)-3,4-dihydroxy-5-(hydroxymethyl)-oxolan-2-yl]-6-aminopurin-2-yl}-pyrazol-4-yl-N-methylcarboxamide) were synthesized by the Department of Medicinal and Bio-Organic Chemistry, CV Therapeutics, Inc. TTX was purchased from EMD Biosciences (San Diego, CA) and lactate assay kits were purchased from BioVision (Mountain View, CA). To prepare stock solutions, palmitoyl-L-carnitine, ranolazine, CVT-3146, adenosine, and acetylcholine were dissolved at a concentration of 10 mmol/L in DMSO. Sodium nitroprusside and the free-radical scavenger Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid, disodium salt) were dissolved in water to achieve concentrations of 10 mmol/L and 1 mol/L, respectively. All DMSO stock solutions were further diluted 1:1000 or greater in the cardiac perfusate on the day of an experiment.

**Experimental protocols using isolated hearts**

Control (drug-free) records of CPP and LVEDP were obtained after stabilization. To determine the effects of PC in the absence and presence of drugs, two protocols were used. In the first, hearts were perfused with PC (4 µmol/L) for 30 minutes, followed by a 30-min washout
of PC in the presence of either vehicle or drug. The effect of drug to reverse PC-induced
dysfunction was measured. In a second protocol, the effect of drug to prevent the actions of PC
was determined, and treatment drug was administered 10 min prior to exposure of the heart to
PC. In both protocols, measurements of CPP and LVEDP were recorded at intervals of 10 min.
In some experiments using both protocols, samples of the cardiac effluent were obtained at 10-
min intervals to determine lactate release by the heart. Using the first protocol, adenosine release
was determined by collection of effluent samples for 1 min at the single time point of 60 min
(i.e., following 30-min treatment with PC and 30-min washout of PC in the presence of either
vehicle or ranolazine).

**Determination of cardiac effluent concentrations of lactic acid and adenosine**

Samples (approx. 2 mL) of cardiac effluent were collected at selected times and stored at
–20 °C until they were thawed and analyzed. Lactic acid content of samples was determined
using a commercial kit (BioVision). Adenosine content of samples was determined by the
PreClinical Development Group at CV Therapeutics using liquid chromatography followed by
mass spectrometry.

**Electrophysiology of I_{Na,L} of guinea pig isolated ventricular myocytes**

**Cell isolation**

Hearts of adult guinea pigs of either sex were isolated and perfused via the aorta with
warm (35 °C) oxygenated solutions in the following sequence: 1) Tyrode solution containing (in
mmol/L) 135 NaCl, 4.6 KCl, 1.8 CaCl₂, 1.1 MgSO₄, 10 glucose and 10 HEPES, pH 7.4, for 5
min; 2) Ca²⁺-free solution containing (in mmol/L) 100 NaCl, 30 KCl, 2 MgSO₄, 10 glucose, 10
HEPES, 15 taurine, and 5 pyruvate, pH 7.4, for 5 min; and 3) Ca²⁺-free solution containing
collagenase (120 units/ml) and albumin (2 mg/ml), for 20 min. At the end of the perfusion, the
ventricles were minced and gently shaken for 10 min in solution #3 to release single myocytes for study.

Electrophysiological recording

Transmembrane 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. During an experiment, myocytes were superfused with a 36 °C bath solution containing (in mmol/L) 135 NaCl, 4.6 CsCl, 1.8 CaCl2, 1.1 MgSO4, 0.01 nitrendipine, 0.3 BaCl2, 10 glucose and 10 HEPES, pH 7.4. Nitrendipine, CsCl, and BaCl2 were used to block L-type Ca2+ and delayed-rectifier K+ channels, respectively. Ranolazine and TTX were applied to the bath solution. The recording pipettes were filled with a solution containing (in mmol/L) 120 Cs-aspartate, 20 CsCl, 1 MgSO4, 4 Na2ATP, 0.1 Na3GTP, and 10 HEPES, pH 7.2. To activate late INa, 300-ms voltage-clamp pulses from -90 to -50 mV at a frequency of 0.16 Hz were delivered to the cell through the patch pipette. Both the maximal amplitude (pA) and integrated current (nC) of late INa during the last 100 ms of a depolarizing pulse were calculated using pClamp software.

Data collection and statistical analysis

LVEDP, CPP, and the electrocardiogram were monitored continuously during each isolated heart experiment and recorded using a Biopac recording unit with either Biopac Acqknowledge V 3.8.1 software or Notocord V 4.2 software (Notocord, Cherry Hill, NJ). Coronary resistance (in mm Hg-1 • mL • min-1) was calculated as the ratio between CPP (in mm Hg) and coronary perfusion rate (which was fixed at 10 mL/min). Statistical analysis was performed using the software package Prism Version 5.0 (Graph Pad, San Diego, CA). The significance of differences among isolated heart treatment group values was determined using
two-way ANOVA with repeated measures of one variable, unless stated otherwise. For analysis of data from myocyte electrophysiology experiments, the one-way repeated measures ANOVA followed by Student-Newman-Keuls test for multiple comparisons was used. The percentage inhibition by ranolazine (Ran) of the effects of PC was calculated using the formula \[ \frac{\text{PC} - \text{Ran}}{\text{PC} - \text{control}} \times 100 \], where PC, Ran and control indicate measurements obtained in the presence of PC alone, PC plus Ran, and in the absence of drugs, respectively. Differences among measure values were considered to be significant when the probability of their chance occurrence was < 0.05. Values of n indicate the number of cells or hearts studied. Data are expressed as mean ± SEM.
**Results**

**PC increased late I_{Na}, and ranolazine attenuated the effect of PC**

The maximal amplitude of late I_{Na} and the total late I_{Na} during the last 100 msec of the clamp pulse (i.e., the integrated current) were significantly increased by PC (4 µmol/L, n = 7) from -8.5±1.8 to -27.8±3.5 pA (p < 0.001) and from -0.352±0.078 to -2.611±0.242 nC (p < 0.001), respectively (Figure 1). Ranolazine (10 µmol/L, n = 7) reduced late I_{Na} in the continuous presence of PC. The maximal amplitude of PC-induced late I_{Na} and the total integrated current were reduced by ranolazine from -27.8±3.5 to -15.5±3.2 pA (p < 0.001) and from -2.611±0.242 to -1.486±0.200 nC (p < 0.001), respectively (Figure 1). In some experiments, after washout of ranolazine, cells were exposed to TTX (3 µmol/L, n = 4) in the continued presence of PC. As shown in Figure 1B, the PC-induced late current was completely inhibited by 3 µmol/L TTX, indicating that it was a Na^+ current.

**Ranolazine partially reverses PC-induced cardiac dysfunction**

A 30-min treatment of the guinea pig isolated, perfused heart with 4 µmol/L PC led to increases of LVEDP, coronary resistance, lactate and adenosine release into the cardiac effluent, and left-ventricular wall stiffness, and a decrease of left-ventricular developed pressure. PC (4 µmol/L, 30 min) increased LVEDP from 7 ± 1.25 (control) to 30 ± 3.16 mmHg (n = 12 hearts, Figure 2). The PC-induced increase of LVEDP was not reversible during a 30-min washout period (Figure 2). Ranolazine (10 µmol/L) administration for 30 min significantly reversed the increase of LVEDP after PC treatment from 30 ± 1.91 to 14 ± 2.18 mmHg (n = 6, p < 0.001 compared to vehicle treatment)(Figure 2). Although much evidence indicates that the antianginal and cardioprotective effects of ranolazine are mediated by inhibition of late I_{Na} (Hale et al., 2008) ranolazine (10 µmol/L) has also been reported to reduce hERG K^+ (I_{K_r}) current in
the heart (Antzelevitch et al., 2004). Therefore, the effect of the selective, potent hERG blocker E-4031 to reverse a PC-induced increase of LVEDP was determined. Unlike ranolazine, E-4031 (10 nmol/L) did not reverse the effect of PC to increase LVEDP (Figure 2).

Because an increase of LVEDP reduces the perfusion gradient for coronary flow and compresses microvessels, the effects of PC and ranolazine on coronary resistance were measured. Typical values of CPP in hearts perfused with drug-free K-H solution were 55-60 mm Hg, whereas in hearts perfused with K-H solution containing palmitoyl-L-carnitine (4 μM), values of CPP were typically 90-95 mm Hg. A 30-min exposure to 4 μmol/L PC increased coronary resistance from 5.82 ± 0.21 to 9.24 ± 0.39 mmHg mL⁻¹ min⁻¹ (n = 13, Figure 3, top panel). The effect of PC to elevate coronary resistance was not reversible upon washout of PC with vehicle (i.e., K-H solution), but was partially and significantly reversed by ranolazine (10 μmol/L, 30 min), from a value of 9.02 ± 0.38 to 7.26 ± 0.53 mmHg mL⁻¹ min⁻¹ (n = 7, Figure 3).

The Na⁺ channel blocker TTX (2 μmol/L; Figure 3, top panel), but not acetylcholine (10 μmol/L), esmolol (10 μmol/L) or sodium nitroprusside (30 μmol/L), significantly reversed the increase of coronary resistance caused by PC (Figure 3, bottom panel). TTX reduced coronary resistance from 9.40 ± 0.43 (after 30 min treatment with PC) to 7.72 ± 0.50 mmHg/mL/min (n = 6, p < 0.05; Figure 3). The endothelium-dependent vasodilator acetylcholine had no effect on coronary resistance after treatment of the heart with PC (Figure 3), whereas esmolol and sodium nitroprusside reduced coronary resistance slightly but not significantly from 9.14 ± 0.27 to 8.64 ± 0.17 and 9.69 ± 0.68 to 8.32 ± 0.29, respectively (n = 5 each; p > 0.05 vs vehicle alone).

The release of lactate by the isolated perfused heart was also increased by PC. Lactate concentration in the cardiac effluent was increased from a control value of 0.028 ± 0.002 to 0.133 ± 0.006 mmol/L (n = 12, p < 0.001 vs no drug) after a 30-min exposure to 4 μmol/L PC.
(Figure 4). Ranolazine (10 μmol/L, 30 min) reduced the release of lactate by PC-treated hearts from 0.132 ± 0.007 to 0.053 ± 0.008 mmol/L (n = 6, p < 0.001 compared to vehicle alone; Figure 4). Similarly, the release of adenosine — another marker of an imbalance between oxygen supply and oxygen demand — from the perfused heart was increased by PC treatment, and the effect of PC was reversed by ranolazine (not shown). When hearts were treated for 30 min with 4 μmol/L PC then with either vehicle or ranolazine (10 μmol/L) for an additional 30 min, adenosine contents of the cardiac effluent were 26.3 ± 9.4 and 3.6 ± 1.5 ng/mL (n = 6, p < 0.01), respectively. Left ventricular wall stiffness (i.e., the increase of LVEDP per increment of increase of LV volume) was also increased by PC and this effect was significantly attenuated by ranolazine (Figure 5). Values of LVEDP at an intraventricular volume of 200 μL were 90 ± 7, 232 ± 17, and 132 ± 8 mmHg in control, 4 μmol/L PC-treated, and PC + ranolazine (10 μmol/L)-treated hearts, respectively (n = 3 hearts each, p < 0.05; Figure 5). The slope of the relationship between intraventricular volume and pressure was significantly steeper in hearts treated with palmitoyl-L-carnitine than in control hearts or hearts treated with both ranolazine and palmitoyl-L-carnitine (Figure 5). The slopes of the relationship between volume and LVEDP were 0.43 ± 0.01, 1.00 ± 0.03, and 0.49 ± 0.01 for control, PC and PC + Ran, respectively (P < 0.05 PC vs PC + Ran).

Treatment of hearts with ranolazine (10 μmol/L) or TTX (2 μmol/L) for 10 min prior to and during subsequent exposure to PC significantly but incompletely attenuated the effects of PC to increase LVEDP (Figure 6) and coronary resistance (not shown). The steady-state values of coronary resistance caused by a 30-min treatment with PC in the absence and presence of 10 μmol/L ranolazine were 9.47 ± 0.72 and 6.94 ± 0.16 mmHg mL⁻¹min⁻¹, respectively (n = 5-6, p <
whereas the value of coronary resistance prior to exposure to PC was 5.57 ± 0.38 mmHg mL⁻¹min⁻¹.

**Tiron pretreatment reduced PC-induced cardiac dysfunction**

The oxygen free-radical scavenger Tiron (1 mmol/L) partially prevented the effect of PC to increase LVEDP (Figure 7, top panel). The combination of Tiron (1 mmol/L) and ranolazine (10 μmol/L) did not have a significantly greater effect than either ranolazine or Tiron alone to reverse the action of PC on LVEDP (Figure 7, top panel). Unlike TTX and ranolazine, 1 mmol/L Tiron did not reverse the action of PC to increase LVEDP (Figure 7, bottom panel). This suggests that Tiron inhibits an early event (i.e., accumulation of reactive oxygen species) in a pathway leading to cardiac dysfunction, but is unable to reverse events after reactive oxygen species have modified a target (e.g., an enhanced late I_{Na}) proximal to the rise in diastolic tension (LVEDP). This interpretation was supported by the results of experiments using guinea pig single isolated ventricular myocytes. When Tiron (1 mmol/L) was applied to myocytes that were first treated for 5 min with PC to increase late I_{Na}, Tiron did not reverse the PC-induced increase of late Na⁺ current (Figure 8A,B). PC alone increased the maximal and late currents from -11.8 ± 0.03 to -23.8 ± 1.9 pA (p< 0.001; n = 4), and from -0.535 ± 0.055 to -1.810 ± 0.212 nC (p<0.001), respectively. After addition of Tiron for 5 min, the maximal and integrated late currents were -22.5 ± 2.9 pA (p>0.05 vs PC) and -1.715 ± 0.272 nC (p>0.05 vs PC), respectively. In contrast, when Tiron and PC were applied simultaneously for 5 min, the effect of PC to increase late I_{Na} was significantly less than in the presence of PC alone (compare Figure 8 panels C,D to panels A,B), and after 5-min washout of Tiron in the continued presence of PC, late current increased further (Figure 8C). The effect of PC alone to increase myocyte late I_{Na} was not readily reversed upon washout of PC (Figure 8E,F).
Discussion

Palmitoyl-L-carnitine (4 µmol/L) increased the magnitude of late $I_{Na}$ in guinea pig ventricular myocytes and caused increases of coronary resistance, LVEDP, and left ventricular wall stiffness in the guinea pig isolated, electrically-paced heart. Ranolazine reduced the PC-induced increase of late $I_{Na}$ in guinea pig myocytes (Figure 1), and reduced PC-induced mechanical and metabolic dysfunction in the isolated, saline-perfused heart (Figures 2-7). Because ranolazine, in addition to its action to reduce late $I_{Na}$, is also known to reduce hERG $K^+$ current (Antzelevitch et al., 2004) and is an antagonist of $\beta$-adrenergic receptors (Letienne et al., 2001), the effects of the selective sodium channel antagonist TTX, the selective hERG inhibitor E-4031, and the $\beta$-adrenergic receptor blocker esmolol were tested in hearts treated with PC. Tetrodotoxin (2 µM) significantly reversed the effects of PC to increase late $I_{Na}$ and coronary resistance. As the only known action of tetrodotoxin is to decrease sodium flux through the voltage-regulated Na$^+$ channel, the results are consistent with the view that many of the effects of PC are the result of its action to increase late $I_{Na}$, and that the protective effect of ranolazine is the result of its inhibition of late $I_{Na}$. Consistent with this hypothesis, an increase of late $I_{Na}$ has been previously reported to cause mechanical, electrical, and metabolic dysfunction in the heart, and ranolazine is reported to reduce dysfunction in the setting of enhanced late $I_{Na}$ (Belardinelli et al., 2006; Hale et al., 2008). Neither E-4031 nor esmolol reduced the effects of PC to increase LVEDP or coronary resistance, respectively (Figures 2, 3B).

The free radical scavenger Tiron also substantially prevented the effect of PC to increase LVEDP (Figure 7) and late $I_{Na}$ (Figure 8C,D), suggesting that an increased formation by PC of reactive oxygen species (ROS) may have played a significant role in the development of mechanical dysfunction in the PC-treated heart. However, unlike ranolazine, Tiron did not
reverse an elevation of LVEDP caused by PC (Figure 7), nor did it reduce the PC-enhanced late 
$I_{\text{Na}}$ (Figure 8A,B). One interpretation of this finding is that an elevation of late $I_{\text{Na}}$ induced by 
ROS is not readily reversed by subsequent reduction of ROS formation, but only by the direct 
inhibition of the ROS-mediated events, including an increase in Na$^+$ channel current (late $I_{\text{Na}}$). 
ROS are known to increase late $I_{\text{Na}}$ in heart (Ward and Giles, 1997; Ahern et al., 2000; Ma JH et 
al., 2005; Song et al., 2006; Gautier et al., 2008) and neurons (Meng and Nie, 2004). Thus, the 
mechanisms underlying PC-induced cardiac mechanical dysfunction may involve sequential 
increases of ROS and late $I_{\text{Na}}$. An increase of late $I_{\text{Na}}$ is a known cause of Na$^+$ and Ca$^{2+}$ overload 
in the heart, and is an explanation for the observed increases of LVEDP, coronary resistance, LV 
stiffness, and biochemical markers of ischemia, such as lactate and adenosine formation by 
isolated hearts in this study. This interpretation is consistent with the finding that lidocaine, 
another Na$^+$ channel blocker, attenuated cardiac dysfunction caused by palmitoyl-L-carnitine 
(Arakawa et al., 1997).

The effect of ranolazine to attenuate an increase of coronary resistance caused by PC 
could be explained by at least two mechanisms. First, ranolazine could reduce or reverse the 
effect of PC to cause Na$^+$-induced Ca$^{2+}$ overload in the myocardium. Ca$^{2+}$ overload of the 
myocardium can lead to partial contracture of myocardial cells, an increase of ventricular 
stiffness and extravascular fluid pressure, compression of the vascular space, and an increase of 
coronary resistance. PC increased late $I_{\text{Na}}$, LVEDP, LV stiffness (an indication of a failure of the 
heart to relax, and thus a sign of the development of Ca$^{2+}$ overload contracture), and coronary 
resistance. Because PC increased coronary resistance, it is not surprising that it also increased 
lactate and adenosine release from the perfused heart. The availability of oxygen to the isolated 
heart perfused with oxygenated K-H solution is expected to be reduced when coronary resistance
is increased, because the increase of coronary resistance indicates reduced vascular volume (and thus reduced delivery of oxygen to the muscle cells). Thus, the increase by PC of lactate and adenosine release suggests that PC reduced availability of oxygen to the myocardium. In this scenario, ranolazine (and TTX) would reduce the PC-induced increase of Na\(^+\) entry, and thereby the effects of PC on LVEDP, coronary resistance, and lactate release. Furthermore, vasodilators would fail to reduce the increase of coronary resistance caused by PC, because the increase of coronary resistance would be caused by extravascular compression of capillaries and not by a change in vascular tone per se.

Palmitoyl-L-carnitine and ranolazine could theoretically modulate coronary perfusion pressure by actions directly on the vasculature (endothelium and vascular smooth muscle) to increase and decrease, respectively, vascular tone. PC was reported to cause a loss of vascular responsiveness to the endothelial-dependent vasodilator acetylcholine (Inoue et al., 1994; Taki et al., 1999). We have confirmed this result. Hearts exposed to 4 µmol/L PC for 30 min were insensitive to 10 µmol/L acetylcholine (Figure 3). However, hearts treated with 4 µmol/L PC were somewhat responsive to sodium nitroprusside (Figure 3). This result suggests that it is not possible to disregard the possibility that PC has an action on the vessel wall to elevate coronary resistance, and that ranolazine reverses this action. However, evidence that ranolazine directly relaxes vascular smooth muscle is currently lacking, whereas ranolazine did reduce LVEDP and left ventricular wall stiffness in this study. Consistent with this result, ranolazine was shown to reduce diastolic tension in electrically-stimulated ventricular muscle strips from human failing hearts, and to improve intracellular Ca\(^{2+}\) handling in rabbit myocytes (Sossalla et al., 2008). Thus, the effects of ranolazine and TTX to reverse the increase of coronary resistance caused by treatment of the heart with PC are most likely the result of actions of the two drugs to decrease
sodium entry into myocardial cells and the resultant sequelae of Ca$^{2+}$ overload, impaired relaxation, increased extravascular pressure, and increased coronary resistance.

Ischemia is associated with an increase of late I$_{Na}$ (Verdonck et al., 1993; Haigney et al., 1994; LeGrand et al., 1995; Ju et al., 1996; Hale et al., 2008). The accumulation during ischemia of glycolytic metabolites (Kohlhardt et al., 1989) and the amphipathic lipids lysophosphatidylcholine (LPC) and long-chain acyl carnitines (Sobel et al., 1978; DeTorre et al., 1991) appears to be at least partly responsible for causing the increase of late I$_{Na}$ during ischemia. There are several studies (see Gauthier et al., 2008, and citations therein) demonstrating the effect of LPC to increase late I$_{Na}$ and cause cardiac electrical and mechanical dysfunction. The present report is the first to demonstrate that late I$_{Na}$ is increased by PC in cardiac myocytes, extending the results of two earlier studies suggesting the same (Wu and Corr, 1995; Shen and Pappano, 1995). Although the effects of glycolytic and amphipathic lipid metabolites to cause Na$^+$ channel, cellular and organ dysfunction have been shown primarily in studies of cardiac tissues, these metabolites are likely to be part of the ischemic milieu in all tissues with Na$^+$ channels. The content of long-chain acyl carnitines (including PC) increases in myocytes during hypoxia (DaTorre et al., 1991), and these molecules are reported to accumulate in sarcolemmal membranes (Knabb et al., 1986). Although the concentration of PC in ischemic myocardial cells has not been reported to our knowledge, and effects of endogenously-produced and exogenously-applied PC may be different, the concentration of 4 μmol/L PC used in this study is similar to concentrations used in previous studies (Arakawa et al., 1997; Arakawa and Hara, 1999; Maruyama et al., 2000; Meszaros and Pappano, 1990; Netticadan et al., 1999; Sato et al., 1992; Shen and Pappano, 1995; Wu and Corr, 1992), and the effects of an exogenous application of 4 μmol/L PC in this study are similar in magnitude to the effects of brief ischemia.
associated with a 4-5-fold elevation of long-chain acyl carnitine content in the heart (DaTorre et al., 1991). Thus, the results of the present study suggest that a pathologically-enhanced, PC-induced late $I_{Na}$ may be a contributor to ischemia-induced dysfunction.
References


Legends for Figures

Figure 1. Ranolazine (Ran) and tetrodotoxin (TTX) attenuated PC-induced late $I_{\text{Na}}$. Panels A and B, successive current records obtained from a single myocyte. Panel A, current traces recorded in the absence of drug (a), and in the presence of 4 µmol/L PC (b), or PC plus 10 µmol/L ranolazine (c). Panel B, currents recorded after a 10-min washout of ranolazine in the presence of PC (d), in the presence of PC plus 3 µmol/L TTX (e) and after washing out TTX in the continuous presence of TTX (f). Panel C, summary of the effects of PC and ranolazine on late $I_{\text{Na}}$. Current was integrated over the last 100 msec of the voltage clamp pulse. Each bar represents data collected from 7 myocytes. *** and † † †, p < 0.001 vs. control and PC alone, respectively. Ctrl, control.

Figure 2. Effect of ranolazine (10 µmol/L) to partially reverse the action of palmitoyl-L-carnitine (4 µmol/L) to increase left ventricular end-diastolic pressure (LVEDP) in the guinea pig isolated heart. After an equilibration period of 10 min, all hearts were treated with palmitoyl-L-carnitine (PC, 4 µmol/L) for 30 min, followed by 30 min treatment with either ranolazine (Ran, 10 µmol/L; open circles), the hERG K+ current blocker E-4031 (10 nmol/L; open diamonds), or vehicle (filled symbols). Each point represents a mean ± SEM of values from 5 (E-4031) or 6 Control, Ran) experiments. *** p < 0.001, Ran compared to vehicle.

Figure 3. Ranolazine and TTX, but not esmolol, acetylcholine, or sodium nitroprusside partially reversed the increase in coronary resistance caused by 4 µmol/L palmitoyl-L-carnitine in the guinea pig isolated heart. After an equilibration period of 10 min, hearts were treated with palmitoyl-L-carnitine (PC, 4 µmol/L) for 30 min, followed by 30 min treatment with either [top panel] ranolazine (Ran, 10 µmol/L, n = 7), TTX (2 µmol/L, n = 6), or saline vehicle (V, n = 6),
or [bottom panel] esmolol (10 μmol/L, n = 4), acetylcholine (ACh, 10 μmol/L, n = 4), or sodium nitroprusside (SNP, 30 μmol/L, n = 5). Each point represents a mean ± SEM. * p<0.05, TTX 40-60 min, compared to vehicle treatment; **p<0.01, Ran 40-60 min, compared to control.

Figure 4. Reversal by ranolazine of the effect of palmitoyl-L-carnitine to increase the release of lactic acid by the guinea pig isolated heart. After an equilibration period of 10 min, hearts were treated with palmitoyl-L-carnitine (PC, 4 μmol/L) for 30 min, followed by 30 min treatment with either ranolazine (Ran, 10 μmol/L, n = 6, open symbols) or vehicle (n = 6, filled symbols). Samples of cardiac effluent were collected at 10-min intervals and analyzed to determine the concentration of lactic acid. Each point represents a mean ± SEM. * p < 0.05, **p<0.01, Ran compared to vehicle.

Figure 5. Ranolazine (10 μmol/L) attenuated the action of palmitoyl-L-carnitine (4 μmol/L) to increase left ventricular wall stiffness (increase of LVEDP per increment of intraventricular balloon volume) in the guinea pig isolated heart. Hearts were treated with no drug (control), palmitoyl-L-carnitine (PC, 4 μmol/L), or palmitoyl-L-carnitine plus ranolazine (Ran, 10 μmol/L) for 30 min, followed by measurement of the incremental increases of LVEDP upon addition of fluid volume to the intraventricular balloon. Each point represents a mean ± SEM of values from three experiments.

Figure 6. Ranolazine or TTX administration before and during exposure of the guinea pig isolated heart to palmitoyl-L-carnitine (4 μmol/L) attenuated the effect of the latter to increase left ventricular end-diastolic pressure (LVEDP). After an equilibration period of 10 min, hearts were treated with vehicle (n = 6), TTX (2 μmol/L, n = 4), or ranolazine (Ran, 10 μmol/L, n = 4) for 10 min, then with palmitoyl-L-carnitine (PC, 4 μmol/L) for 30 min in the continued presence
of either vehicle, TTX, or ranolazine, and then with vehicle, TTX, ranolazine in the absence of PC. Each point represents a mean ± SEM. * p<0.05, ** p<0.01, compared to PC + vehicle.

Figure 7. Continuous exposure to Tiron (1 mmol/L) attenuated the effect of 4 µmol/L PC to increase left ventricular end-diastolic pressure (LVEDP) (top panel), but unlike ranolazine, Tiron administration after exposure of the heart to PC did not reverse the elevation of LVEDP caused by PC (bottom panel). Top Panel: After an equilibration period of 10 min, hearts were treated with vehicle (n = 6), Tiron (1 mmol/L, n = 4), or ranolazine plus Tiron (n = 7) for 10 min, then with palmitoyl-L-carnitine (PC, 4 µmol/L) for 30 min in the continued presence of either vehicle, Tiron, or Ran + Tiron. Bottom Panel: Hearts were treated with 4 µmol/L PC for 30 min, then with either vehicle (n = 7, filled squares) or Tiron (n = 5, open squares) in the absence of PC for an additional 30 min. Each point represents a mean ± SEM. *p<0.05, **p<0.01, ***p<0.001, compared to PC alone.

Figure 8. Palmitoyl-L-carnitine (PC, 4 µmol/L) increased late Na\(^+\) current in guinea pig isolated ventricular myocytes, and this effect was attenuated by simultaneous but not subsequent application of the free radical scavenger Tiron (1 mmol/L). Panels A,B: Representative experimental current traces (A) and summary data (B, 4 experiments) for (a) control (b) effect of PC (4 µmol/L, 5 min) and (c) PC + Tiron (5 min). ***p<0.001 vs control. Panels C,D: Representative experimental current traces (C) and summary data (D, 5 experiments) for (a) control (b) effect of PC (4 µmol/L, 5 min) plus Tiron (1 mmol/L) and (c) PC alone (5 min washout of Tiron). *, p<0.05 vs control and PC; ***p<0.001 vs control and PC + Tiron. Panels E,F: The stimulation by PC of late Na\(^+\) current persisted after 8-min washout of PC (n = 5 experiments). ***p<0.001 vs control.
Figure 1
Figure 2

LVEDP, mmHg

Time (min)

PC V, E-4031, or Ran

*** *** ***
Figure 3

**Graph 1:**
- **Y-axis:** Coronary Resistance (mmHg/ml/min)
- **X-axis:** Time (min)
- **Legend:**
  - Vehicle
  - TTX
  - Ran

**Graph 2:**
- **Y-axis:** Coronary Resistance (mmHg/ml/min)
- **X-axis:** Time (min)
- **Legend:**
  - Vehicle
  - ACh
  - Esmolol
  - SNP

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Figure 4
Figure 5
Figure 6
Figure 7
Figure 8

A. Control
B. PC
C. PC+T

E. Control
F. PC
G. Wash

Late Current [nC] vs. Time [ms]

B. 2.5
D. 3.0
F. 2.5

Control PC PC+Tiron Control PC+Tiron PC Control PC Wash

 Bars represent mean ± SEM; **p < 0.01, ***p < 0.001 vs. control; NS = non-significant.