Palmitoyl-carnitine increases RyR2 oxidation and sarcoplasmic reticulum Ca2+ leak in cardiomyocytes: Role of adenine nucleotide translocase

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ARTICLE INFO

Keywords:
Long chain acyl carnitine
Reactive oxygen species
ANT
RyR2

ABSTRACT

Long chain fatty acids bind to carnitine and form long chain acyl carnitine (LCAC), to enter into the mitochondria. They are oxidized in the mitochondrial matrix. LCAC accumulates rapidly under metabolic disorders, such as acute cardiac ischemia, chronic heart failure or diabetic cardiomyopathy. LCAC accumulation is associated with severe cardiac arrhythmia including ventricular tachycardia or fibrillation. We thus hypothesized that palmitoyl-carnitine (PC), alters mitochondrial function leading to Ca^{2+} dependent-arrhythmia. In isolated cardiac mitochondria from C57Bl/6 mice, application of 10 μM PC decreased adenine nucleotide translocase (ANT) activity without affecting mitochondrial permeability transition pore (mPTP) opening. Mitochondrial reactive oxygen species (ROS) production, measured with MitoSOX Red dye in isolated ventricular cardiomyocytes, increased significantly under PC application. Inhibition of ANT by bongkrekic acid (20 μM) prevented PC-induced mitochondrial ROS production. In addition, PC increased type 2 ryanodine receptor (RyR2) oxidation, S-nitrosylation and dissociation of FKBP12.6 from RyR2, and therefore increased sarcoplasmic reticulum (SR) Ca^{2+} leak. ANT inhibition or anti-oxidant strategy (N-acetylcysteine) prevented SR Ca^{2+} leak, FKBP12.6 depletion and RyR2 oxidation/S-nitrosylation induced by PC. Finally, both bongkrekic acid and NAC significantly reduced spontaneous Ca^{2+} wave occurrences under PC. Altogether, these results suggest that an elevation of PC disturbs ANT activity and alters Ca^{2+} handling in a ROS-dependent pathway, demonstrating a new pathway whereby altered FA metabolism may contribute to the development of ventricular arrhythmia in pathophysiological conditions.

1. Introduction

In physiological conditions, the high-energy demand required for cardiac function is mainly provided by long-chain fatty acids (FAs). The rate of FAs uptake and oxidation is directly related to the level of circulating FA [1]. As soon as the FAs supply increases, the cardiac efficiency, defined as the ratio between the cardiac power and the oxygen consumption drops [2]. In skeletal muscle, under high acute FAs supply, uncoupling protein 3 (UCP3) may export fatty anions from the mitochondrial matrix leading to a reduction of the mitochondrial proton motive force and so of ATP synthesis [3]. In normal heart, UCP3 expression level is low, and FAs-mediating uncoupling has been attributed to the adenine nucleotide translocase (ANT) [4–6]. Before entering into the mitochondria, FAs are first esterified to give fatty-acyl-CoA and then the acyl group is transferred to carnitine to form acylcarnitine. Long chain acyl carnitine (LCAC) such as palmitoyl-carnitine (PC) is shuttled in the mitochondria and converted again in fatty-acyl-CoA to be β-oxidyzed [7]. Changes in FA metabolism affect the LCAC levels and the concentration increases from 2–6 μM to 10–30 μM during diabetic cardiomyopathy, genetic disorders or ischemic heart failure [8–11]. LCAC accumulation contributes to the ventricular dysfunctions as ventricular extrasystole, tachycardia and ventricular fibrillation [12–14]. Similarly, cardiac ANT deficiency in human is associated with ventricular arrhythmia [15]. Although a decrease in LCAC accumulation is known to reduce ventricular arrhythmias, the mechanisms whereby LCAC induces ventricular arrhythmia remain elusive.

Abbreviations: AK2, adenylate kinase 2; ANT, adenine nucleotide translocase; Ap5A, P1P5-diadenosine-5′-pentaphosphate; BA, bongkrekic acid; C, carnitine; CsA, cyclosporine A; DPI, diphenyleneiodonium; FA, fatty acid; FKBP12.6, FK506 binding protein 12.6 kDa; I_{K1}, inward rectifying potassium current; IV, intravenous; LCAC, long chain acyl carnitine; mPTP, mitochondrial permeability transition pore; NAC, N-acetylcysteine; NOX, NADPH oxidase; NT, non-treated; OC, octanoyl-L-carnitine; OlC, oleoyl-L-carnitine; PC, palmitoyl-carnitine; ROS, reactive oxygen species; RyR2, type 2 ryanodine receptor; SR, sarcoplasmic reticulum; UCP3, uncoupling protein 3; WT, wild type

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In addition to energy expenditure, acute application of the long-chain free FA palmitate disturbs Ca^{2+} handling in healthy cardiomyocytes via a mechanism involving markedly increased reactive oxygen species (ROS) production [16]. Similarly, electro-mechanical dysfunction induced by PC has been linked to an early increase in cellular ROS production [17]. More generally, increased ROS production impairs cellular Ca^{2+} handling by interfering with a wide range of proteins implicated in excitation–contraction coupling, e.g. the sarcoplasmic reticulum (SR) Ca^{2+} release channels (type 2 ryanodine receptors, RyR2) [18,19]. RyR2 contains several thiols groups that are highly sensitive to redox modification and, RyR2 oxidation may dissociate the regulatory protein, FKBP12.6, from the channel to promote SR Ca^{2+} leak [20,21]. Although PC accumulation induces Ca^{2+} overload and initiates transient inward current [22], whether LCAC affects RyR2 function through redox modification is still unknown.

In the present study we aimed to determine (1) whether acute application of PC on wild type (WT) cardiomyocytes affects Ca^{2+} handling through redox modification of RyR2 and (2) whether regulation of ANT activity may account for these changes. We found that PC-altered ANT activity leading to an increased mitochondrial ROS production, RyR2 oxidation and SR Ca^{2+} leak that results in the triggering of cellular Ca^{2+} waves and ventricular extrasystoles.

2. Material and methods

2.1. Chemicals

Fluo-4 AM, and MitoSOX Red were from Molecular Probes/Invitrogen. Carnitine (C), octanoyl-L-carnitine (OC), palmitoyl-L-carnitine (PC), oleoyl-L-carnitine (OlC), N-acetylcysteine (NAC), bongkrekic acid (BA), and isoprenaline hydrochloride were purchased from Sigma-Aldrich (France). Ap5A was from VWR (France). All compounds were prepared as stock solutions in appropriate solvents. On the day of the experiment, stock solutions were diluted to the desired final concentration in the bath solution. Control solutions contain the same solution of solvent when required (1% dilution of stock solution).

2.2. Isolated mitochondria

Mice hearts were excised and homogenized with the Dounce homogenizer. Then, mitochondria were isolated by differential centrifugation [23]. Mitochondrial protein contents were determined using micro BCA assay (BCA Protein Assay Kit, Thermo Fisher Scientific).

In order to measure mitochondrial permeability transition pore (mPTP) opening, isolated heart mitochondria were diluted in 200 μl of hypo-osmotic buffer composed of (in mM): 200 saccharose, 5 succinate, 10 MOPS, 0.01 EGTA, 1 H_3PO_4 (pH adjusted at 7.4), and incubated with different PC concentrations. mPTP opening was estimated by spectrophotometry where the decrease of optical density at 540 nm reflects the mitochondrial swelling. Different Ca^{2+} concentrations (6, 25, 50 μM) were used as positive control [24]. The effects of different treatments were normalized according to the following equation (NT − X) / (NT − Ca^{2+} 50 μM) ∗ 100, where NT is the value obtained in non-treated mitochondria, X is the value obtained with the test condition, and "Ca^{2+} 50 μM" is the value obtained with 50 μM of Ca^{2+}.

A non-radioactivity assay was used to estimate ANT activity [25]. Briefly, the ADP/ATP exchange rate was evaluated by following NADPH fluorescence (λexc: 360 nm, λem: 465 nm) in the presence of 6.5 μM external ADP and 2.5 mM glucose, 1 E.U. hexokinase, 0.2 mM NADPH, 0.5 E.U. glucose-6-phosphate dehydrogenase. An increase of NADPH fluorescence reflects an increase of ANT activity as previously described [26,27]. 10 μM of P1P5-diadenosine-5′-pentaphosphate (Ap5A) was used to determine the influence of adenylate kinase-dependent ATP synthesis.

2.3. Cell isolation

All procedures conformed to European Parliament Directive 2010/63/EU and the 22 September 2010 Council on the protection of animals, and were approved by the institutional Ethics Committee for Animal Experiments, Languedoc Roussillon (N CEEA-LR-12080).

7 weeks-old C57Bl/6 male mice (Centre d'élevage Janvier, Le Genest Saint Isle, France) were killed by rapid cervical dislocation. Hearts were excised, mounted on the Langendorff apparatus and retrogradely perfused with dissociation buffer contained (in mM): 113 NaCl, 4.7 KCl, 0.6 KH_2PO_4, 0.6 Na_2HPO_4, 1.2 $MgSO_4$, 12 $NaHCO_3$, 10 $KHCO_3$, 10 Hepes, 30 Taurine (pH 7.4 adjusted with NaOH) and 0.1 mg/ml Liberase TM Research Grade (Roche Diagnostic, Germany). After enzymatic dissociation, hearts were mechanically dissociated in the same solution without enzyme. Cardiomyocytes were then filtered and resuspended in the dissociation buffer where Ca^{2+} was reintroduced gradually to reach a final concentration of 1 mM Ca^{2+} [16].

2.4. Confocal imaging

Cells were placed in a bath chamber perfused with a Tyrode solution (in mM): 135 NaCl, 4 KCl, 1.8 $CaCl_2$, 1 $MgCl_2$, and 2 HEPES (pH 7.4 adjusted with NaOH) supplemented or not with 10 μM of palmitoyl-L-carnitine (PC10). The bath chamber was placed on the stage of a Zeiss LSM 510 inverted confocal microscope (Zeiss, LePecq France) equipped with a 63× lens (oil immersion, numerical aperture, N.A. = 1.2) allowing the measurement of Fluo4-AM and MitoSOX Red fluorescence.

To measure cytoplasmic Ca^{2+} transients and spontaneous RyR2 activities (i.e. Ca^{2+} sparks), isolated cardiomyocytes were loaded for 15 min with the permeant Ca^{2+} indicator Fluo4-AM (3 μM, Molecular Probes), at room temperature. All measurements were performed in line-scan mode (1.5 ms/line), and scanning was carried out along the long axis of the cell. An excitation wavelength of 488 nm was used, and emitted light was collected through a 505 nm long-pass filter. The laser intensity used (3%–6% of the maximum) had no noticeable deleterious effect on the fluorescence signal or cell function over the course of the experiment. Ca^{2+} transient were recorded under field stimulation (10 V at 1 Hz). Analyses were performed using ImageJ software. To enable comparisons between cells, fluorescence signals were divided by the minimal fluorescent (F_0) obtained immediately before the 1 Hz stimulation pulse. The SR Ca^{2+} content was assessed by measuring the amplitude of cytosolic Ca^{2+} transients induced by the rapid application of caffeine (10 mM). Spontaneous Ca^{2+} sparks were recorded in quiescent cells following 5 min stimulations in order to reach steady state SR-Ca^{2+} content. Ca^{2+} sparks frequency was analyzed using ImageJ software with the Sparksmaster plugin [28].

TMRM was used to measure mitochondrial membrane potential ($\Delta\Psi_m$) [29]. Isolated cardiomyocytes were loaded with TMRM (10 nM) for 20 min at room temperature. Confocal images of TMRM fluorescence were obtained by excitation at 568 nm while measuring the emitted light at 585 nm. TMRM fluorescence was measured in five different areas in each cell to minimize the subcellular variability in $\Delta\Psi_m$. Images were taken every minute and fluorescence signals were normalized to the fluorescence measured in each cell at the start of the experiment, which was set to 100%. At the end of each experiment, cells were exposed to the mitochondrial uncoupler FCCP (10 μM) to determine the dynamic range of the dye.

Mitochondrial ROS production was measured using MitoSOX Red dye. Cardiomyocytes were incubated 45 min at 37 °C with MitoSOX Red [16]. Cells were perfused with the Tyrode solution and field stimulated during 5 min to reach a steady state. Then the PC solution was perfused during 15 min. x-y confocal images of the emitted light at 585 nm were recorded every 2 min intervals by excitation at 488 nm. The first acquisition was done after 1 min of Tyrode perfusion. Fluorescence changes, measured after 10 min of PC application, were

normalized to the steady state values recorded after 5 min of Tyrode perfusion (Fig. 3B).

2.5. Cellular arrhythmia

Cellular arrhythmias, such as abnormal Ca^{2+} waves, were quantified with the IonOptix® system (Hilton, USA) [30,31]. Cardiomyocytes were loaded with the ratiometric dye 30 min at RT with 10 μM indo-1 AM (Invitrogen) and perfused with a Tyrode solution containing 1 nM isoprenaline. Cardiomyocytes were field-stimulated at 3 Hz (20 V, 1 ms), and simultaneously illuminated at 305 nm using a xenon arc bulb light. Indo-1 AM fluorescence emitted at 405 nm and 480 nm was recorded simultaneously using IonOptix® acquisition software (Hilton). To record spontaneous Ca^{2+} waves in the resting condition, stimulation was stopped for 30 s once Ca^{2+} transient steady state was reached.

2.6. RyR2 biochemistry

Using Langendorff technique, the whole heart was perfused 10 min with Tyrode's solution containing or not PC10 and/or BA. Atria and right ventricles were then excised and the left ventricles were quickly frozen. Left ventricle was lysed in 1 ml of a buffer containing (in mM) 10 Tris-maleate (pH 6.8), 35 NaF, 1% Triton and a cocktail of protease inhibitors (Roche 11873580001). An anti-RyR antibody was used to immunoprecipitate RyR2 from 500 μg of left ventricular homogenate. Samples were incubated with an anti-RyR antibody in 0.5 ml of a modified RIPA buffer (10 mM Tris–HCl, pH 7.4; 150 mM NaCl; 1% Triton; 5 mM NaF and protease inhibitor cocktail) for 2 h at 4 °C. The immune complex was incubated with protein A/G magnetic beads (Pierce 88802) at 4 °C for 2 h, after which the beads were washed out three times with RIPA buffer. To detect RyR2 protein oxidation, the immune complex was treated with 2.4 μM dinitro-phenylhydrazine (DNPH) and the DNP-derivatized carbonyls were detected using on Oxyblot Protein oxidation detection Kit (Millipore S7150). Proteins were separated on SDS/PAGE gels and transferred onto nitrocellulose membranes for 1 h at 100 V. The immunoblots were prepared using antibodies against RyR (1:1000), anti Cys-NO antibody (Sigma-Aldrich N5411, 1:1000), anti-DNPH (1:300) and anti-FKBP12.6 (RD System AF 4174, 1:1000). All immunoblots were developed and quantified using the Odyssey infrared imaging system (LICOR Biosystems) and infrared-labeled secondary antibodies.

2.7. Electrocardiogram recording

Mice were equipped of subcutaneous implantable devices (PhysiothelTAE-F10 model, Data Sciences International, USA). Surgical implantation of ECG devices was realized under general gaseous anesthesia (2.5% isoflurane/O_2, Iso-vet, Piramal Healthcare UK) and monitoring, on retro-controlled heating pad. Lidocaïne was solely disposed on the wound after surgery during 2 days of recuperation

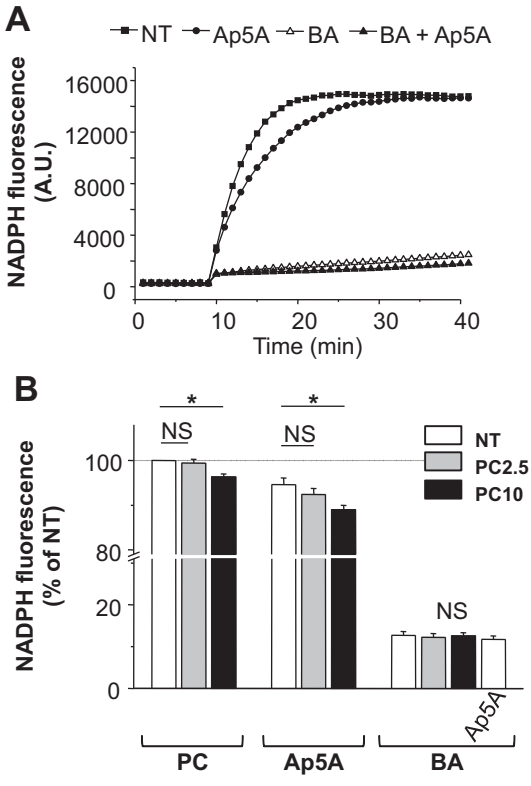

Fig. 1. PC10 affects ADP/ATP translocase activity at a non-saturating ADP concentration (6.5 μM). (A) Typical example of changes of NADPH fluorescence that reflects ANT activity (λ_{ex} 360 nm, λ_{em} 465 nm). The test was performed in different conditions: non-treated mitochondria (NT, square), mitochondria incubated with Ap5A (circle), BA (open triangle), or Ap5A + BA (filled triangle). (B) The ANT activity is estimated using the area under the curve in the different conditions normalized to the activity of non-treated mitochondria. (C) The application of PC10, or PC10 + Ap5A decreases ANT activity. Experiments were performed in triplicate. Statistical differences between NT and other groups *$p < 0.05$; ($n = 6$).

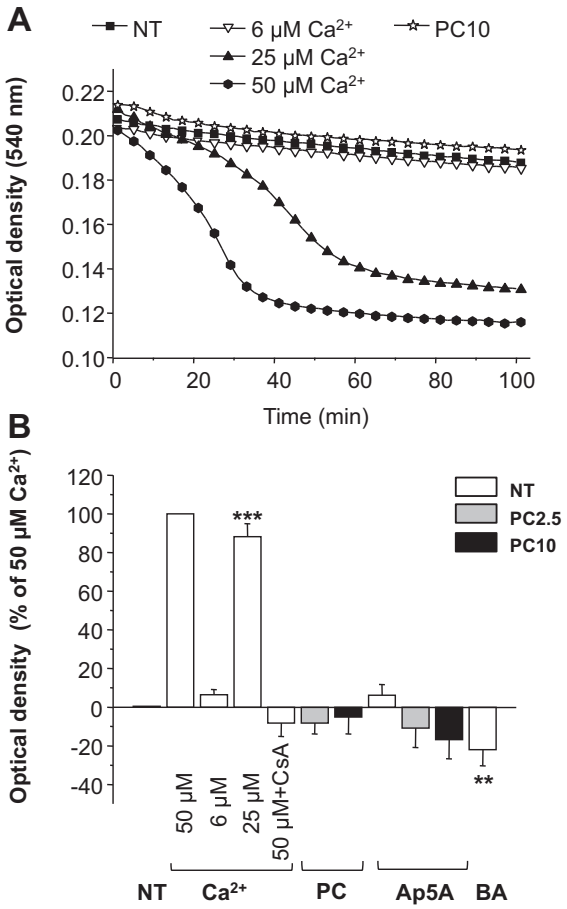

Fig. 2. PC10 does not modify the mPTP opening. (A) Typical example of an experiment showing the decrease in optical density reflecting the mPTP opening. The optical density measured at 543 nm of mitochondria was followed in non-treated mitochondria (square), in presence of PC10 (open star) or in the presence of different concentrations of Ca^{2+}, 6 μM (open triangle), 25 μM (filled triangle) or 50 μM (filled circle). (B) Summarizes the mean results from 5 different experiments performed in duplicate. Different PC concentrations were applied with Ap5A. As for PC, BA alone did not induce mPTP opening. Statistical differences between NT and other groups; **$p < 0.01$; and ***$p < 0.001$ ($n = 6$).

and experiments began one week after surgery. ECGs were recorded using IOX and analyzed with ECG auto software (EMKA technologies, France). Heart rate, PR, QRS, and QT intervals were measured during the 6 h after 100 μL intravenous (IV) injection of a physiological serum solution (control) or after PC10. Experiments were performed in the same mice after one day of clearance. QT interval was defined between the first deviations from an isoelectric PR interval until the return of the ventricular repolarisation to the isoelectric TP baseline. This method included in the measure the low-amplitude portion of the T-wave and allows a complete ventricular repolarization of ventricles. The QT correction was performed with the adapted Bazett's formula of Mitchell. The triggering of spontaneous arrhythmia as single or in salvos ventricular extrasystoles was counted after control or PC10 challenge. The housing, the recording and the analyses of ECG respected the Lambeth convention.

2.8. Statistical analysis

Data are presented as mean ± SEM. Statistical significance was defined as $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ using Student's t test (paired or unpaired) or analysis of variance (one- or two-way), followed by a Bonferroni selected-comparison test. The n represents the number of cells and N the numbers of animals studied.

3. Results

3.1. Effects of palmitoyl-carnitine on ANT activity

Depending on the FAs nature, ANT activity is regulated differentially. For instance, free FAs activates ANT whereas palmitoyl-CoA inhibits it [5]. Here we first determined whether PC form would affect ANT activity. We used an ADP/ATP translocase assay on ventricular isolated mitochondria as previously described [23]. This assay is based on

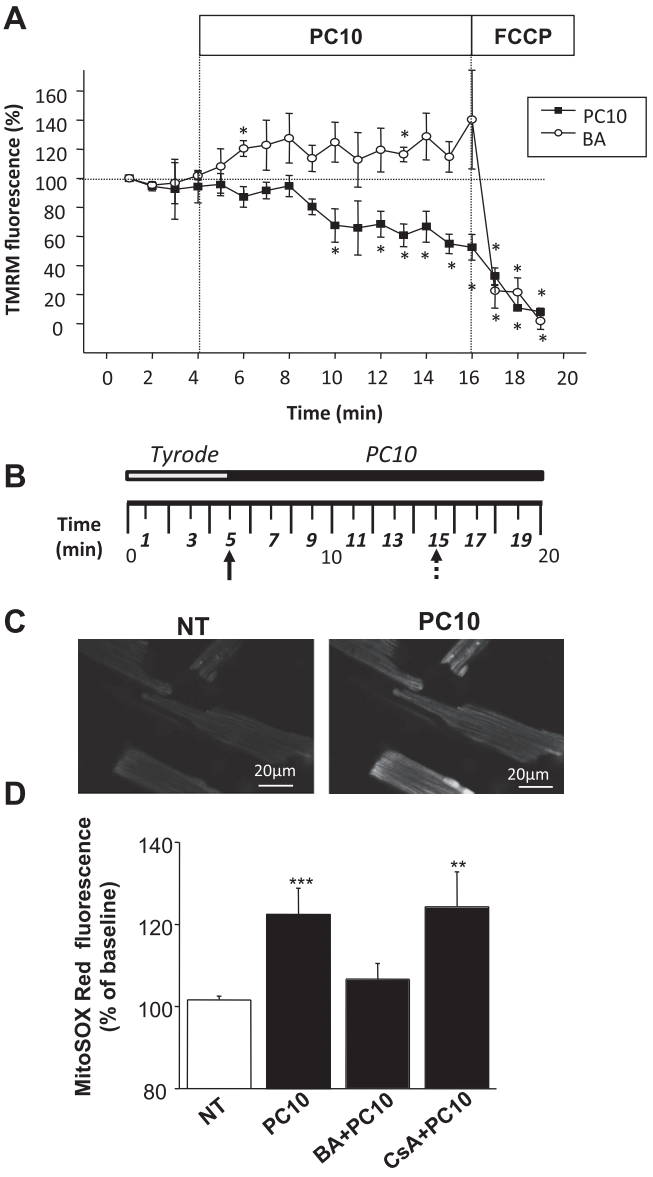

Fig. 3. PC10 decreases ΔΨm and increases mitochondrial ROS production. (A) ΔΨm was measured using TMRM dye, and TMRM fluorescence was normalized to the fluorescence at the start of the experiments. PC10 was applied on cardiomyocytes in absence (filled square) or in presence of 20 μM BA (open circle). At the end of the experiments 10 μM FCCP was applied to fully dissipate ΔΨm. (B) Mitochondrial ROS production was measured using MitoSOX Red dye under confocal. The first acquisition was done after 1 min of Tyrode perfusion every 2 min. Fluorescence changes, measured after 10 min of PC application (dash arrow), were normalized to the steady state values recorded after 5 min of Tyrode perfusion (filled arrow). (C) Typical images of cells in absence of PC (NT) or after 10 min with PC10. (D) Mean values of MitoSOX fluorescence normalized to basal values obtained prior to 10 min incubation of PC10, PC10 in presence of 20 μM BA or 0.1 μM CsA. $^{*}p < 0.05$, $^{**}p < 0.01$; and $^{***}p < 0.001$ compared to the same conditions prior to application of PC10 (n = 9–12 cardiomyocytes in each condition).

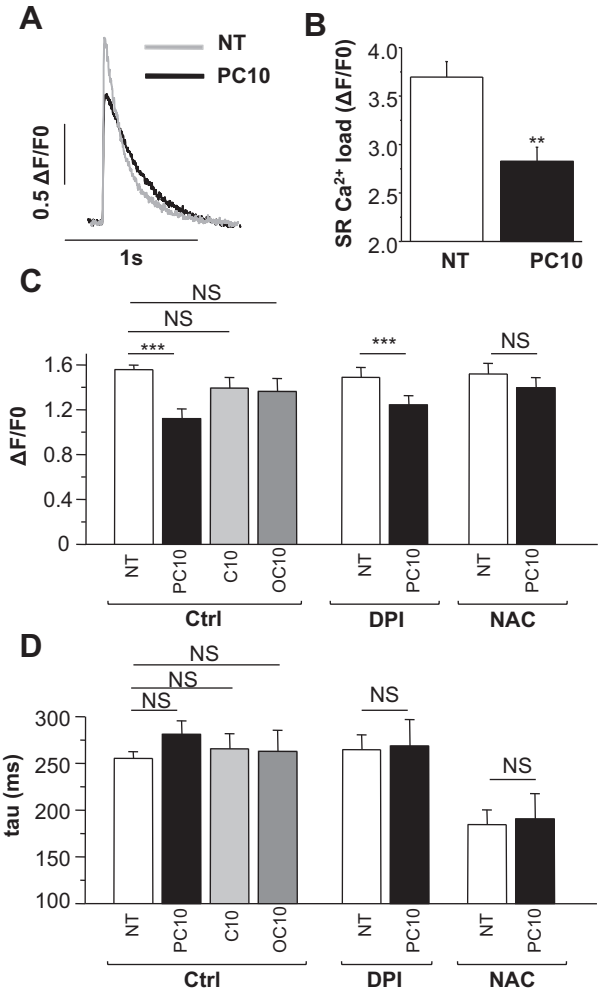

Fig. 4. PC10 decreases SR Ca^{2+} content and Ca^{2+} transient amplitude. (A) Representative ΔF/F0 signal recorded in non-treated (NT) or incubated with PC10. (B) Mean value of SR Ca^{2+} content, (C) Ca^{2+} transient amplitude or (D) decay time constant (Tau) of Ca^{2+} transient signal obtained in isolated ventricular cardiomyocytes treated or not with PC10, 10 μM carnitine (C10), and 10 μM octanoylcarnitine (OC10). Cardiomyocytes were pre-incubated 15 min with NAC or DPI. $^{**}p < 0.01$; and $^{***}p < 0.001$ compared to non-treated conditions.

NADPH fluorescence measurement upon ADP application (6.5 μM). The subsequent NADPH formation depends on both the adenylate kinase 2 (AK2) and the ANT activities. We thus used diadenosine-pentaphosphate (Ap5A, 10 μM) and bongkrekic acid (BA, 20 μM), specific inhibitors of AK2 and ANT respectively in order to differentiate both activities (Fig. 1A) [23]. In the presence of 2.5 μM PC (PC2.5) NADPH fluorescence, with or without Ap5A, was unchanged whereas 10 μM PC (PC10) decreased NADPH formation in presence or in absence of Ap5A (Fig. 1B). In the presence of BA, PC2.5 and PC10 were ineffective, indicating that PC10 specifically decreased the ATP/ADP translocase activity without affecting AK2.

Under stress conditions, ANT associated with other mitochondrial proteins, such as cyclophilin D, forms the mitochondrial permeability transition pore (mPTP) [32]. To determine whether PC affects mPTP formation, we performed a mitochondrial-swelling test [24] (Fig. 2). In the presence of PC2.5 or PC10, the absorbance was unchanged whereas application of Ca^{2+} (50 μM) as a positive control induced a drop of optical density. Altogether these results show that PC10 modulates ANT activity without affecting mPTP opening.

3.2. Effects of palmitoyl-carnitine on mitochondrial ΔΨm and ROS production

To estimate the uncoupling effects of PC10, we measured mitochondrial membrane potential (ΔΨm) using TMRM on intact ventricular cardiomyocytes. FCCP (10 μM) was applied at the end of each experiments to fully uncouple and dissipate ΔΨm. (Fig. 3A) PC10 application decreases TMRM fluorescence by ~20% whereas in the presence of BA, PC10 was ineffective (Fig. 3A), indicating that BA prevents uncoupling mediated by PC10 [4,6]. We next measured mitochondrial ROS production using MitoSOX Red on intact ventricular cardiomyocytes [16]. Application of PC10 significantly increased mitochondrial ROS production by ~20% (122.4 ± 6.4 vs. 101.6 ± 0.9; Fig. 3C, D). In presence of the ANT inhibitor, BA (20 μM), PC10 was unable to increase mitochondrial ROS production (Fig. 3D). When applied with the mPTP inhibitor, cyclosporine A (CsA, 0.1 μM), PC10 significantly increased MitoSOX Red fluorescence to the same extent than in the absence of CsA (Fig. 3D). These results indicate that PC increases mitochondrial ROS production through its effects on ANT and independently of mPTP.

3.3. Effects of palmitoyl-carnitine on Ca^{2+} transients

PC has been reported to increase intracellular Ca^{2+} level on quiescent cardiomyocytes, however the effects of PC on triggered Ca^{2+} transients is unknown [33]. In intact isolated ventricular cardiomyocytes, application of PC10 significantly decreased Ca^{2+} transient amplitude (Fig. 4A, C) as well as SR Ca^{2+} content (Fig. 4B). The use of carnitine alone (10 μM) or a middle chain fatty acid, octanoyl-carnitine (10 μM) did not affect Ca^{2+} transient amplitude. This result suggests that the decrease of Ca^{2+} transient amplitude is specific to PC. NAC (20 mM), a broad-spectrum anti-oxidant, prevented PC10-induced Ca^{2+} transients decrease, whereas DPI (diphenyleneiodonium), an inhibitor of NADPH oxidase (NOX) was ineffective (Fig. 4B). Although, PC10 decreased Ca^{2+} transients' amplitude, the Ca^{2+} transients' decay was not affected (Fig. 4D). To summarize, these results indicate that LCAC affect SR release of Ca^{2+} through an increased ROS production independently of NOX activity.

3.4. Effects of palmitoyl-carnitine on SR Ca^{2+} leak

We next determined whether PC10 affects RyR2 activities by measuring the SR spontaneous Ca^{2+} release events (Ca^{2+} sparks) using confocal microscopy. In intact isolated ventricular cardiomyocytes, application of PC10 significantly increased Ca^{2+} sparks frequency reflecting a SR Ca^{2+} leak (Fig. 5A, B) whereas in the presence of BA (20 μM), PC10 was ineffective (Fig. 5B). Similarly, when cardiomyocytes were incubated with the non-specific antioxidant, NAC (20 mM), PC10 did not affect Ca^{2+} sparks frequency (Fig. 5A, B). It is to note that BA and NAC alone did not affect calcium sparks frequency. In addition sparks frequency was not affected by carnitine or octanoyl-carnitine (Fig. 5C). These results indicate that LCAC only induce SR Ca^{2+} leak via ANT activity and ROS production.

Increased oxidation state of RyR2 may account for SR Ca^{2+} leak [18, 21]. Therefore, we assessed RyR2 post-translational modification in left ventricle from heart perfused for 10 min with either PC10 alone or

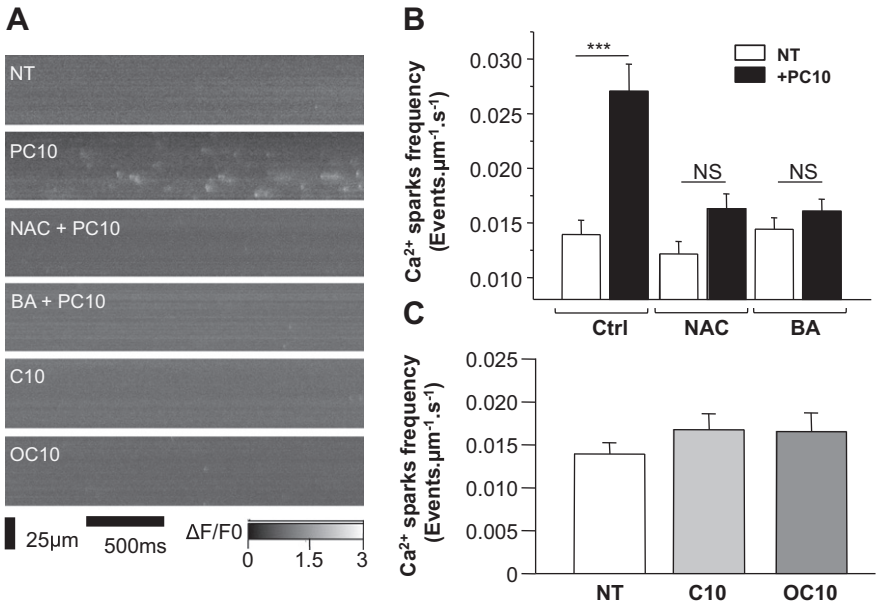

Fig. 5. PC10 increases Ca^{2+} sparks frequency. (A) Representative ΔF/F0 line-scan images recorded in non-treated or incubated with PC10, NAC + PC10, and BA + PC10, carnitine (C10) or 10 μM octanoylcarnitine (OC10) cells. (B) Mean values of sparks frequency obtained in isolated ventricular cardiomyocytes treated or not with PC10. Cardiomyocytes were pre-treated 15 min with NAC or BA prior to 10 min with PC10 incubation. (C) Mean values of sparks frequency obtained in isolated ventricular cardiomyocytes treated or not with 10 μM carnitine (C10) or 10 μM octanoylcarnitine (OC10). $p > 0.05$ (not significant, NS), and $^{***}p < 0.001$ compared to the same condition without PC10 ($n > 15$ cells in each condition).

PC10 + BA (20 μM), PC10 + NAC (20 mM) (Fig. 6). We observed that PC10 increased both RyR2 oxidation and S-nitrosylation (Fig. 6C, D), and a dissociation of FKBP12.6/RyR2 complex (Fig. 6E). BA or NAC alone did not affect the basal RyR2 oxidation and S-nitrosylation state and the FKBP12.6/RyR2 interaction. However, they both partially decreased the RyR2 oxydation and S-nitrosylation induced by PC10 (Fig. 6). Similarly, they reduced the dissociation of FKBP12.6 from RyR2 macromolecular complex induced by PC10 application (Fig. 6E). To sum up, PC10 increases SR-mediated Ca^{2+} leak through RyR2 oxidation, S-nitrosylation and FKBP12.6 dissociation from RyR2. PC10-induced RyR2 post-translational modification results from reduced ANT activity and mitochondrial ROS production.

3.5. Effects of palmitoyl-carnitine on spontaneous Ca^{2+} waves and ventricular arrhythmia

In pathological conditions, an increased SR Ca^{2+} leak is known to initiate Ca^{2+} waves and to trigger arrhythmic events, originating from delayed after depolarization [34]. To investigate the propensity of intact ventricular cardiomyocytes to generate spontaneous Ca^{2+} waves in the presence of PC10, we field-stimulated cardiomyocytes, loaded with the Ca^{2+} indicator indo-1-AM, during 30 s at 3 Hz frequency. Spontaneous Ca^{2+} waves were detected during a subsequent resting period (30 s). While in control conditions, the diastolic Ca^{2+} remained stable during the rest period, after PC10 application, about 70% of the cardiomyocytes triggered Ca^{2+} oscillations and/or waves, indicative of an arrhythmic behavior (69.3 ± 3.6%, Fig. 7B). Confocal microscopy experiments show that PC10-induced Ca^{2+} waves originate from an increase in Ca^{2+} sparks events (Fig. 7C). Again, when cardiomyocytes were incubated with BA or NAC, PC10 failed to trigger spontaneous Ca^{2+} events (Fig. 7B). PC10 propensity to induce arrhythmic events was further studied in vivo by telemetric approach (Fig. 8). Although IV injection of PC10 did not significantly impact basal ECG parameters (Fig. 8A–E), PC10 increased the occurrence of ventricular extrasystoles (Fig. 8F–G). In addition we also observed in 2 out of 6 PC10 treated mice the triggering of non-sustained ventricular tachycardia as defined by the Lambeth conventions [35], whereas untreated mice did not (Chi2 = 0.12; Fig. 8H). None of the untreated and treated PC10 mice developed sustained ventricular tachycardia.

4. Discussion

LCAC level varies depending on the metabolic state and may increase under pathophysiological conditions. In the present study, we

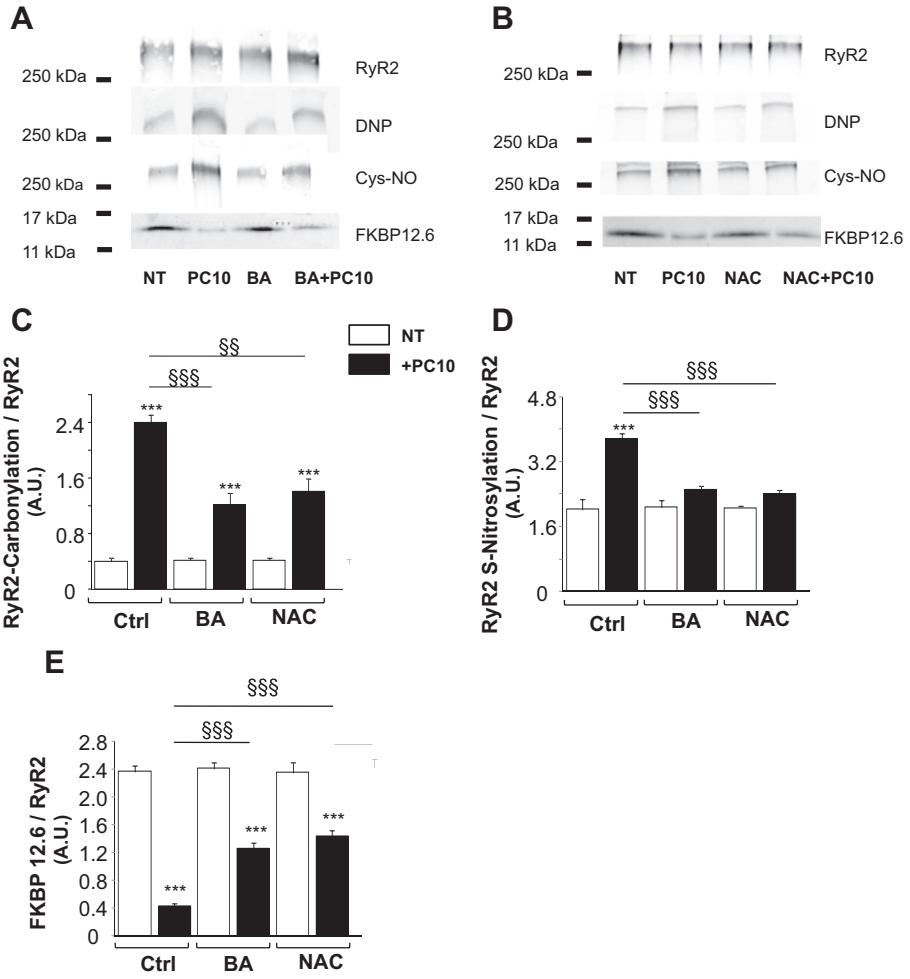

Fig. 6. PC10 increases RyR2 oxidation and S-nitrosylation and dissociates FKBP12.6. RyR2 was coimmuno-precipitated from beating heart homogenate perfused with standard Tyrode solution or with PC10, BA or BA + PC10. (A) Representative immunoblots for S-nitrosylation (Cys-NO) and S-carbonylation (DNP) of cysteine residue of RyR2 and FKBP12.6 bound to RyR2, obtained in three independent experiments in presence or not of BA or (B) NAC. (C) Mean values of the relative amount of RyR2 S-nitrosylation for each group, determined by dividing the Cys-NO signals by the total amount of RyR2 coimmuno-precipitated. (D) Mean values of the relative amount of RyR2 carbonylation for each group, determined by dividing the DNP signals by the total amount of RyR2 coimmuno-precipitated. (E). Mean values of relative amount of FKBP12.6 associated with the channel complex for each group, determined by dividing the FKBP12.6 signals by the total amount of RyR2 coimmuno-precipitated. Western-blot performed on 3 hearts in each condition in triplicates. Statistical significance $^{**}p < 0.01$; and $^{***}p < 0.001$ compared to the same condition without PC10. §§p < 0.01, and §§§p < 0.001 compared to PC10 conditions.

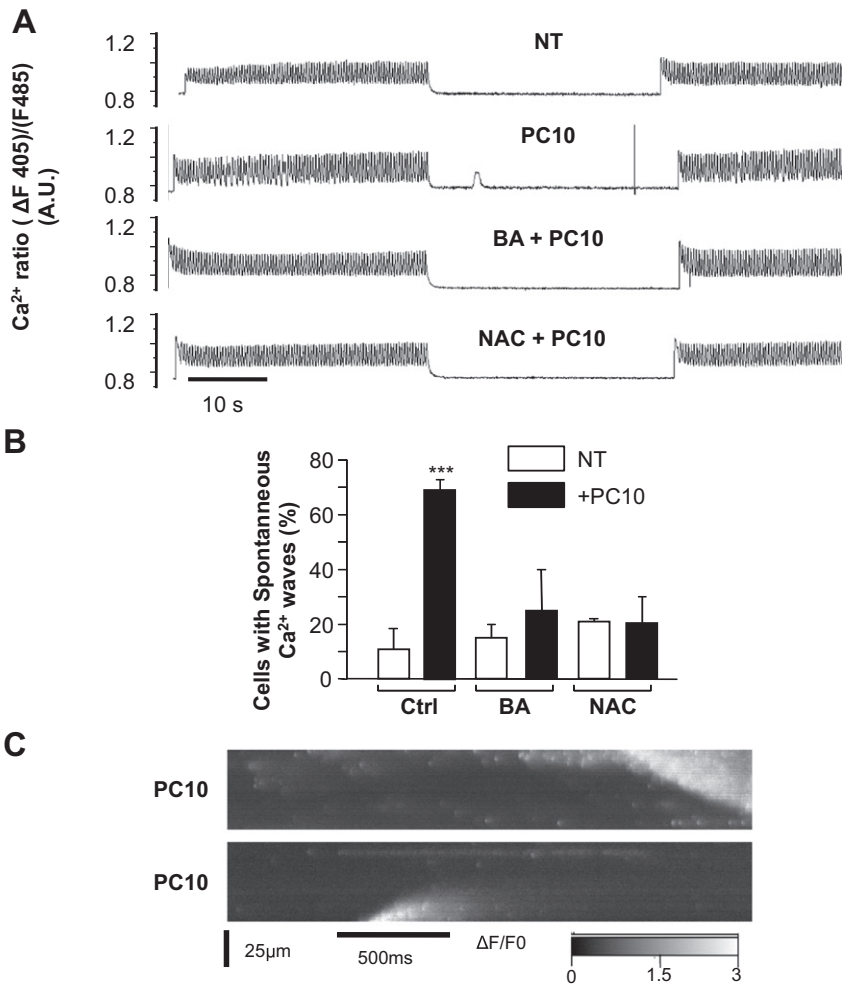

Fig. 7. PC10 increases the occurrence of cellular Ca^{2+} waves. Variation of cytosolic Ca^{2+} of isolated cardiomyocytes was followed during the field stimulation and rest periods. (A) Representative trace obtained in control (NT) and in the presence of PC10, NAC + PC10 and BA + PC10 perfusion. (B) Results are presented as the percentage of cells having arrhythmic events during the rest period. (C) Representative images of spontaneous Ca^{2+} waves observed, under confocal microscopy, in presence of PC10. Each cell is its own control. NT (n = 28), PC10 (n = 28), NAC + PC10 (n = 19), BA + PC10 (n = 20) ***$p < 0.001$ compared to non-treated conditions in each group.

demonstrate that PC-induced decrease in ANT activity causes an increased mitochondrial ROS production and consequently RyR2 oxidation and S-nitrosylation, and FKBP12.6 dissociation. These RyR2 post-translational modifications trigger SR Ca^{2+} leak and promotes the occurrences of diastolic Ca^{2+} waves and arrhythmic events.

In physiological conditions, the heart is preferentially fuelled with FAs. However, compared to glucose, an increase in FAs consumption reduces cardiac efficiency. Among the mechanisms involved, a modulation of ANT has been suggested [2]. Indeed, increased palmitoyl-CoA content inhibits ANT both from the extra-mitochondrial side and the mitochondrial matrix [36]. Altered ANT activity would reduce the ATP/ADP turnover and subsequently the ATP synthase activity. Consequently, it would increase mitochondrial ROS production from the electron transport chain [37]. Accordingly, PC-induced ROS production has been ascribed to an incomplete blockade of the respiratory chain [38]. In the present study the decrease in ANT activity induced by PC is presumably due to its conversion into palmitoyl-CoA rather than a direct effect, as LCAC does not inhibit ANT on submitochondrial particles [5,39,40]. In addition, free fatty acids have been proposed to uncouple mitochondria via the ANT either through an allosterical stimulation of H^+ transfer or through the translocation of fatty acid under their anionic form. Alternatively, fatty acid anions could also increase negative surface charges facilitating H^+ transfer trough the ANT [2]. Consequently, an increased uncoupling through the ANT may compete with the ATP^{4-}/ADP^{3-} translocation capacity of the ANT as reported in Fig. 1, associated with $\Delta\Psi m$ dissipation (Fig. 3A and [4,6]) and mitochondrial ROS production (Fig. 3D). Conversely, bongkrekic acid, which inhibits both the uncoupling and the ATP^{4-}/ADP^{3-} transport (Fig. 1; [4,6]), prevents $\Delta\Psi m$ and mitochondrial ROS production.

A cellular elevation of ROS leads to the oxidation of numerous proteins resulting in myocardial dysfunction [41]. Among ROS potential targets, calcium-handling proteins are particularly sensitive to redox modulation [18]. RyR2 is highly sensitive to redox state due to the large number of cysteines that composed the homo-tetramer. The nature and the number of oxidized and/or S-nitrosylated cysteine residues directly impact on the channel properties. Indeed, a RyR2 monomer contains 89 cysteine residues and 2 per FKBP12.6 protein, and it was estimated that around 20 cysteine residues per subunit are free (80 per tetrameric RyR2). It was also estimated that channel activation requires a poly-S-nitrosylation where 3 sites per subunit (~11 per tetramer) induced a maximal RyR2 activation, whereas ~2 sites per RyR2 did not significantly affect RyR2 open probability. Meaning that a low level of RyR2 S-nitrosylation per se does not affect significantly RyR2 function. On the other hand, oxidation of >7 thiols per subunit induces an irreversible activation of the channel through disulfide bonds formation between RyR2 subunits [42–44]. Irreversible RyR2 oxidation unambiguously increases RyR2 open probability and SR Ca^{2+} leak, however the level of RyR2 S-nitrosylation, which is reversible, has been proposed to increase or decrease RyR2 open probability (for review see [45,46]). Indeed, more than S-nitrosylation

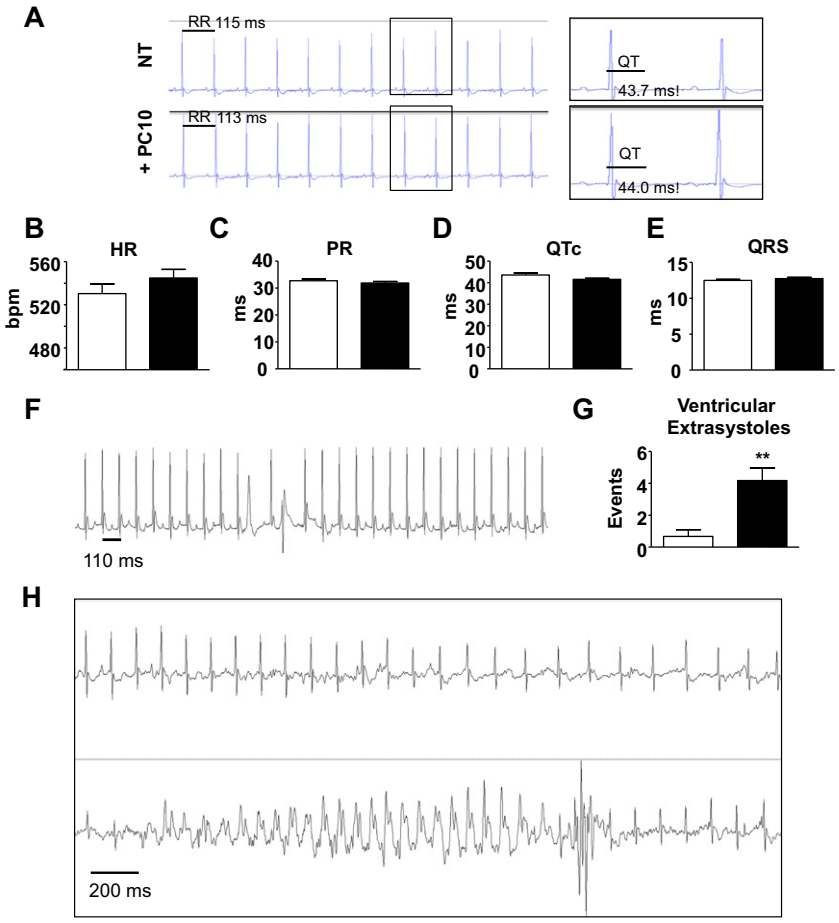

Fig. 8. PC10 increases the occurrence of ventricular extrasystoles. (A) Typical examples of ECG recorded in vigil animals during 6 h after intravenous PC10 injection or saline solution (NT). Mean ECG parameters, heart rate H(R) (B), PR (C), QRS (D), and QT (E) calculated after intravenous saline injection (NT) and after intravenous PC10 injection (PC10). (H) Typical example of a ventricular extra systole after PC10 injection. (G) Mean ventricular extra systoles events counted over 2 h after PC10 injection. (H) Example of a continuous ECG recording presenting a non-sustained ventricular tachycardia. Such arrhythmic events were triggered in 2 out of 6 mice injected with PC10. N = 6. Statistical significance *p < 0.05; **p < 0.01; and ***P < 0.001.

or oxidation itself, the nitroso/redox balance is critical for the modulation of RyR2 function. In physiological conditions, RyR2 is endogenously S-nitrosylated at a low level, which does not affect the RyR2 open probability. Nevertheless, a decrease in the basal S-nitrosylation level increases the number of free cysteines available for oxidation. Consequently, the RyR2 oxidation may increase which leads to SR Ca^{2+} leak [47,48]. In this context RyR2 S-nitrosylation competes with the RyR2 oxidation level and is considered as a protective reaction to counteract an irreversible thiol oxidation of the RyR2. On the other hand, in pathophysiological conditions where NO homeostasis is altered and the number of S-nitrosylated cysteine is substantially increased, SR Ca^{2+} leak is enhanced [31,46]. However, in pathological conditions, the nitroso/redox balance was not systematically measured and a concomitant increase in oxidation may also contribute to SR Ca^{2+} leak [31]. In the present study, increase S-nitrosylation might be a compensatory mechanism to limit the extent of thiol oxidation or might contribute, in synergy with oxidation, to altered Ca^{2+} release. One challenging perspective of the present work would be to determine the specific S-nitrosylated- and/or oxidized cysteines that are modified under PC treatments compared to control. However, in the present study, the use of both anti-SNO and anti-DNPH antibodies does not allow us to identify the number and the specific location of the S-nitrosylated and/or oxidized cysteine. A recent study identified 21 cysteine residues sensitive to oxidation in RyR1 isoform that are conserved in RyR2 isoforms [49]. Most importantly several cysteine residues are located in a regulated domain of the cytoplasmic part of the channel such as the FKBP12 interacted site [49]. Although involvement of these specific cysteine residues in the displacement of the FKBP12/FKBP12.6 under oxidation and/or poly-S-nitrosylation is still speculative, these results reinforce the hypothesis that RyRs/FKBPs interactions in heart and skeletal muscle might be directly sensitive to oxidation and/or poly-S-nitrosylation [20,21].

Normal closing of RyR2 in diastole is a key parameter, which prevents Ca^{2+} leak and subsequent SR Ca^{2+} depletion, thereby preserving appropriate conditions for an optimal Ca^{2+} release during systole. Increased RyR2 oxidation and/or S-nitrosylation are associated with both a decrease in FKBP12.6 binding affinity to the channel and an increase RyR2 Ca^{2+} sensitivity [20,21]. Therefore, disruption of the RyR2–FKBP12.6 interaction increases diastolic SR Ca^{2+} leak through increased RyR2 activity [50]. As a matter of fact, PC-induced RyR2 oxidation and S-nitrosylation disturb diastolic Ca^{2+} homeostasis with decreased peak Ca^{2+} transients and triggering of ectopic Ca^{2+} waves. Increased diastolic SR Ca^{2+} leak and Ca^{2+} waves destabilize secondary resting membrane potential and triggered ectopic action potential [51]. When SR Ca^{2+} leak exceed the SR Ca^{2+} uptake capacity, Ca^{2+} extrusion from the Na^{+}/Ca^{2+} exchanger is increased, generating an inward current [51]. In the mean time, the increased SR Ca^{2+} leak decreases the outward component of the inward rectifying potassium current (I_{K1}) [52,53]. The increased inward Na^{+}/Ca^{2+} exchanger current and reduced outward I_{K1} synergistically contribute to the genesis of delayed afterdepolarizations [51–53]. Accordingly PC has been reported to trigger transient inward current and delayed afterdepolarization in adult ventricular cardiomyocytes [22]. Moreover, NAC treatment reduces RyR2 oxidation, S-nitrosylation and prevents RyR2 SR Ca^{2+}

leak as previously reported [31]. This confirms the deleterious role of ROS on Ca^{2+} handling and subsequent arrhythmic events [29,47,54]. In addition, we showed that the blockade of ANT activity by BA reduces PC10 effects on RyR2. These results reveal a direct link between ANT dysfunction, ROS production and RyR2 function. Although in the present study PC10 application did not significantly affect Ca^{2+} transients decay, cumulative deleterious effects of PC on other Ca^{2+} handling proteins could not be excluded [55].

The uptake of cardiac FAs is directly correlated to the plasmatic level of non-esterified FAs [56]. Under metabolic stress, plasmatic FA concentration increases dramatically as well as FAs covalently bound to coenzyme A or carnitine [13,51,57]. For instance a rapid increase of plasmatic FA concentration in diabetic cardiomyopathy rats is correlated with a four-fold increase of LCAC in myocardium [57] and mitochondrial dysfunction during metabolic syndrome is associated with arrhythmogenic events [58]. Moreover, increased levels of long chain FAs have also been suspected to be involved in unexplained sudden death in infants with conduction defects or ventricular fibrillation and tachycardia [59]. In addition to LCAC accumulation, acute ischemia is also associated with a decreased ANT activity, independently of any change in oxidative phosphorylation, [60–62] and an increased RyR2 oxidation leading to reperfusion injury [63]. Similarly, PC level was shown to correlate with NYHA classification and high plasma levels of PC were significantly associated with serious adverse events and poor prognostic of heart failure patients [64]. Finally, decreased ANT function per se triggers a progressive cardiomyopathy and elicits arrhythmic events [15,65–67] associated with mitochondrial ROS production [65,68].

To conclude growing evidences demonstrate a link between altered FAs acids metabolism and arrhythmia in various pathological and stress conditions in both animal model and human. The present study proposes a novel model whereby mitochondrial ANT-induced ROS production is central for FA-induced arrhythmias. Strategies based on a re-equilibration of mitochondrial function could be promising to limit PC accumulation and subsequent fatal arrhythmias in a wide range of pathology [12,69].

Disclosures

None.

Transparency Document

The Transparency document associated with this article can be found, in the online version.

Acknowledgments

J.R. was supported by the “Groupe de Reflexion sur la Recherche Cardiovasculaire (GRRC)”. A.L., N.S. and J.F. were supported by the ANR Cydiacor and FRM (Mitochondrial Physiopathology, 2012). The authors would like to thanks Claire Nicolas for technical assistance.

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