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Involvement of Mitochondrial Permeability Transition Pore (mPTP) in Cardiac Arrhythmias: Evidence from Cyclophilin D Knockout Mice

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Abstract

In the present study, we have used a genetic mouse model that lacks cyclophilin D (CypD KO) to assess the cardioprotective effect of mitochondrial permeability transition pore (mPTP) inhibition on Ca^{2+} waves and Ca^{2+} alternans at the single cell level, and cardiac arrhythmias in whole-heart preparations. The protonophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) caused mitochondrial membrane potential depolarization to the same extent in cardiomyocytes from both WT and CypD KO mice, however, cardiomyocytes from CypD KO mice exhibited significantly less mPTP opening than cardiomyocytes from WT mice ($p < 0.05$). Consistent with these results, FCCP caused significant increases in CaW rate in WT cardiomyocytes ($p < 0.05$) but not in CypD KO cardiomyocytes. Furthermore, the incidence of Ca^{2+} alternans after treatment with FCCP and programmed stimulation was significantly higher in WT cardiomyocytes (11 of 13), than in WT cardiomyocytes treated with CsA (2 of 8; $p < 0.05$) or CypD KO cardiomyocytes (2 of 10; $p < 0.01$). (Pseudo-)Lead II ECGs were recorded from *ex vivo* hearts. We observed ST-T-wave alternans (a precursor of lethal arrhythmias) in 5 of 7 WT hearts. ST-T-wave alternans was not seen in CypD KO hearts ($n=5$) and in only 1 of 6 WT hearts treated with CsA. Consistent with these results, WT hearts exhibited a significantly higher average arrhythmia score than CypD KO ($p < 0.01$) hearts subjected to FCCP treatment or chemical ischemia-reperfusion ($p < 0.01$). In conclusion, CypD deficiency-induced mPTP inhibition attenuates CaWs and Ca^{2+} alternans during mitochondrial depolarization, and thereby protects against arrhythmogenesis in the heart.

Graphical Abstract

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6. Author Contributions:

Conceived and designed the experiments: LHX, RG, JKG. Performed the experiments: RG, NF. Analyzed the data: RG, NF, LHX. Wrote the manuscript: RG, LHX, JKG.

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Keywords

Cyclophilin D; mPTP; Calcium; Arrhythmias; Mitochondria; Heart

1. Introductions

Mitochondria have been shown to play a vital role in the regulation of intracellular Ca^{2+} (Ca_i^{2+}) homeostasis in cardiomyocytes [1–3]. Mitochondrial Ca^{2+} influx is primarily mediated by the mitochondrial calcium uniporter (mCU), a low-affinity, high-capacity ion channel [4, 5]. Mitochondrial Ca^{2+} efflux is dependent on two channels: the Na^+ - Ca^{2+} exchanger (mNCX), the primary channel in physiologic conditions, and the mitochondrial permeability transition pore (mPTP), which opens during times of pathophysiologic stress [6]. These Ca^{2+} channels and transporters derive their driving forces from the Ca^{2+} gradient and mitochondrial membrane potential (Ψ_m) established by a proton gradient generated by the mitochondrial electron transport chain [1, 7–9].

Previous studies have shown that mitochondria are physically associated with the sarcoplasmic reticulum (SR) through electro-dense tethering structures opposite the location of ryanodine receptors [10]. Mitofusin 2, a protein necessary for mitochondrial membrane fusion, creates a tether between the mitochondrial outer membrane and the SR [11] [12]. This link is approximately 10 to 50 nm wide, and likely creates a micro-domain in which Ca_i^{2+} levels can be readily manipulated to induce a variety of ionic flux responses by either the mitochondrial or the SR Ca^{2+} handling pathways.

Our previous work has demonstrated that mitochondrial Ca^{2+} fluxes likely alter Ca_i^{2+} levels within this micro-domain, and thus modulate Ca_i^{2+} handling properties, including the behavior of the SR ryanodine receptors (RyR)[13]. We found that mitochondrial uncoupling by the protonophore carbonyl cyanide *p*-(trifluoromethoxy) phenylhydrazone (FCCP) results in Ψ_m depolarization, which subsequently causes mitochondrial Ca^{2+} release via the mPTP. This mitochondrial Ca^{2+} release promoted spontaneous Ca^{2+} release from the SR, and exacerbated Ca^{2+} waves (CaWs), which can be potentially arrhythmogenic. However, the following issues remained to be elucidated: 1) the less than ideal selectivity of cyclosporin A (CsA) as a mPTP blocker; 2) the relevance of the mPTP to arrhythmogenesis at the whole heart level.

To further delve into mechanistic research, we have employed the *Ppif*^{-/-}, CypD knockout mouse model (CypD KO). CypD has been demonstrated to be a necessary component and regulator of the mPTP. As a result, the mPTP opening should be severely reduced in CypD KO cardiomyocytes. It has been shown that CypD KO mice are generally protected from ischemia/reperfusion (I/R) injury *in vivo*, and mitochondrial swelling, Ca²⁺ overload, and ROS-induced cell death *in vitro* [14, 15]. These cardioprotective effects are thought to occur as a result of the inability of the mPTP to open in a high-conductance mode during instances of mitochondrial matrix Ca²⁺ overload or other severe mitochondrial stress, such as membrane potential dissipation via superoxide flashes [16]. This ultimately prevents mitochondrial inner membrane permeabilization and apoptosis. On the other hand, CypD KO may also have deleterious consequences under different stress conditions. For example, CypD KO mice demonstrate increased hypertrophy, fibrosis, and accelerated development of congestive heart failure in response to transaortic constriction pressure-overload models [17]. This may be mediated by increased mitochondrial matrix Ca²⁺ overload and/or lack of dynamic cardiac metabolic range. Our previous study has shown that suppression of mPTP by CsA attenuates CaWs, suggesting a potential antiarrhythmic effect. However, it still remains to be elucidated if a selective genetic approach to knock out CypD ameliorates arrhythmogenesis.

In the present study, we have found that inhibition of the mPTP by CypD KO reduces mPTP-mediated pathological mitochondrial Ca²⁺ effluxes, attenuates spontaneous CaWs, and prevents Ca²⁺ alternans development. Furthermore, CypD KO prevents arrhythmogenesis at the whole heart level in an *ex-vivo* model. A preliminary report has been communicated to the American Heart Association annual meeting [18].

2. Materials and Methods

2.1. Animal Models

WT and CypD KO mice (2–4 months, either gender) were purchased from Jackson Laboratories (stock #: 009071; Donating Investigator: Jeffery Molkentin). All animal experimental protocols were reviewed by the Institutional Animal Care and Use Committee at Rutgers-New Jersey Medical School, and were in accordance with the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health (Revised 1996). Briefly, cyclophilin D, a vital component to the mPTP, was encoded by the *Ppif* gene. *Ppif*^{-/-} mice were generated by replacing the first three coding exons of the *Ppif* gene, and were eventually used to generate chimeric, then full-null mice.

2.2. Cell Isolation

Ventricular myocytes were enzymatically isolated from mouse hearts as described previously [19]. Briefly, mice were anesthetized with pentobarbital (0.07 mg/g, i.p.), the hearts were removed and were retrogradely perfused at 37°C in Langendorff fashion with nominally Ca²⁺-free Tyrode's solution containing 0.5 mg/ml collagenase (Type II; Worthington) and 0.1 mg/ml protease (type XIV; Sigma) for 10 minutes. The enzyme solution was then washed out and the hearts were removed from the perfusion apparatus. The left ventricles were placed in petri dishes, and were gently teased apart with forceps.

Finally, the cardiomyocytes were filtered through nylon mesh. The Ca^{2+} concentration was gradually increased to 1.0 mM, and the cells were stored at room temperature and used within 8 hours. Only cells from the left ventricular wall were used.

2.3. Measurement of Mitochondrial Membrane Potential (Ψ_m)

Isolated cardiomyocytes were loaded with 50 nM TMRM (Molecular Probes) at 37 °C for 90 minutes. Loaded cardiomyocytes were then washed free of extraneous TMRM and excited at 548 nm. Emitted fluorescence was acquired at 570 nm continuously. The fluorescence was monitored using a Nikon Eclipse TE200 inverted microscope and recorded using an Andor Ixon CCD camera, as described in our previous studies [20, 21]. Cells were stimulated at 2 Hz. The decrease in fluorescence rate was used as an index of mitochondrial membrane depolarization [13].

2.4. Measurement of mPTP Opening with Calcein AM

Isolated cardiomyocytes were co-loaded with 1 μM calcein AM (Molecular Probes) and 1 mM CoCl_2 at room temperature for 30 minutes. After de-esterification, loaded cardiomyocytes were washed with Tyrode's solution. The cells were then allowed to incubate for 20 minutes in fresh Tyrode's containing CoCl_2 , but no calcein. Fluorescence was excited at 484 nm and acquired at 520 nm. The results were obtained by averaging 3 consecutive 200 ms exposures per minute continuously. The baseline value (before perfusion of FCCP) was normalized to 1. It is known that intact membranes are permeable to esterified calcein AM, but not for unesterified calcein. Since mitochondrial calcein is always unesterified, the exit of calcein only reflects the degree of mPTP opening. When cells were co-loaded with calcein AM and CoCl_2 , calcein fluorescence was quenched by Co^{2+} in the cytosolic compartment. The rate of calcein leaving the mitochondria was estimated from the rate of decrease in fluorescence during a 10 minute period and was used as an index of mPTP opening [13].

2.5. Measurements of Ca_i^{2+} Fluorescence

Cardiomyocytes were loaded with Fluo-4 AM (Molecular Probes) for 40 minutes at room temperature, followed by ~20 min of additional incubation with fresh Tyrode's solution containing no Fluo-4 AM to allow intracellular de-esterification. The fluorescence (E_X/E_M : 485/530) was monitored using a Nikon Eclipse TE200 microscope and recorded using an Andor Ixon CCD camera, as described in our previous studies [20, 21]. Ca^{2+} fluorescence intensity was recorded as the ratio F/F_0 of the fluorescence (F) over the basal diastolic fluorescence (F_0) [14].

2.6. Single Cell Ca^{2+} Alternans Testing

Single cardiomyocytes were stimulated by a Grass stimulator that was triggered by a custom-designed computer program via a sound file. During the pacing protocol, the initial pacing cycle length (PCL) was set at 300 ms and decremented every 8 beats by 20 ms (from 300 to 200 ms), 10 ms (from 200 to 150 ms), and 5 ms (from 150 to 80 ms).

2.7. ECG Recording and Arrhythmia Induction Testing

(Pseudo-)Lead II ECGs were recorded in isolated Langendorff-perfused hearts. A pair of Ag-AgCl electrodes were placed close to the apex of the left ventricle as well as at the right atrial appendage to obtain a (Pseudo-)Lead II ECG at a sampling rate of 1 kHz. Two additional platinum electrodes were placed on the free wall of the right ventricle for stimulation using a stimulator (Grass) triggered by a custom-designed computer program. To induce ventricular arrhythmias, a standard S_1 - S_2 arrhythmia induction protocol at twice the pacing threshold intensity was adapted from Jeron *et al* [22]. Following a 20-beat train with a basic cycle length of 100 ms (S_1), 3 extra stimuli (S_2) with a coupling cycle length of 50, 40, or 30 ms, respectively, were introduced. Each of these 3 sets of stimulation were repeated 3 times. It should be noted that due to the nature of Langendorff ECG recordings, the position of the Ag-AgCl electrodes in the bath, the volume of the conducting solution in the bath, and the orientation of the heart in the recording chamber may generate different ECG waveform morphologies from heart to heart. We focused on the generation of ventricular arrhythmias and beat-to-beat alternans after FCCP or chemical I/R treatment.

2.8. Chemical I/R procedure

A chemical I/R protocol was adapted from Ruiz-Meana *et al* and Seidlmayer *et al* [23, 24]. Briefly, ischemia was mimicked by: 1) adjusting the pH to 6.4 to induce acidosis, 2) replacing 10 mM glucose with 20 mM deoxyglucose to inhibit glycolysis, 3) adding 2 mM NaCN to inhibit mitochondrial respiration, and 4) deoxygenate the ischemia solution by restricting oxygen flow [23, 24]. The ischemic solution contained (in mM): 20 deoxyglucose, 2 NaCN, 135 NaCl, 4 KCl, 1 $MgCl_2$, 2 $CaCl_2$, 10 HEPES, and the pH adjusted to 6.4[24]. The hearts were exposed to simulated chemical ischemia for 10 minutes, followed by a 50 minute reperfusion period in normal Tyrode's solution. The same standard S_1 - S_2 arrhythmia induction protocol was used. For the CsA treated group, hearts were perfused with CsA (1 μM) for 5 minutes prior to the chemical ischemia treatment, then continuously perfused thereafter. (Pseudo-)Lead II ECGs were recorded to monitor arrhythmic events as previously described.

2.9. Arrhythmia Scoring System

The definitions and point values of various arrhythmic events are as follows [25–27]: no arrhythmia: 0 points; PVC (one to three): 1 point; non-sustained VT (up to 10 beats): 2 points; sustained ventricular tachycardia: 3 points; ventricular fibrillation: 4 points. The highest scoring arrhythmogenic event from each heart was responsible for its score.

2.10. Chemicals and Reagents

Chemicals and reagents were purchased from Sigma-Aldrich or Molecular Probes, as indicated in the text. The following reagents were dissolved in DMSO as stock solutions and then further diluted to final working concentrations in normal Tyrode's: FCCP, CsA, thapsigargin (Tha), Fluo-4 AM, calcein AM, TMRM. The maximum DMSO concentration was < 0.2% by volume.

2.11. Statistics

Data were expressed as mean $\pm$ SE. Statistical significance was assessed by using Student's *t*-test or Fischer's Exact test, as indicated in the text. Results were considered statistically significant if the *P* value was less than 0.05.

3. Results

3.1. FCCP Induced the Same Ψ_m , But Less mPTP Opening in CypD KO Compared to WT Cardiomyocytes

In order to determine the relationship between mPTP opening and its effect on mitochondrial Ca^{2+} efflux, we compared cardiomyocytes isolated from CypD KO mice to their WT counterparts. CypD deficiency has been confirmed in CypD KO mouse heart in Baines' original paper [14, 15]. To determine if any secondary alterations of major handling proteins occur in CypD KO cardiomyocytes, we carried out Western blotting analyses in heart tissue. As shown in Supplemental Figure 1, the expression levels of mCU, RyR, SERCA2a, DHPR (L-type Ca^{2+} channel), total and phosphorylated phospholamban (PLN) remained the same in CypD KO as compared to WT.

First, we assessed the extent to which FCCP depolarized the Ψ_m in cardiomyocytes from both groups. As shown in Figure 1 A, B, and C we observed that 100 nM, 1 μM , and 20 μM FCCP significantly depolarized the Ψ_m (appearing as a decrease in TMRM fluorescence) in both WT and CypD KO cells (Figure 1 A-b, B-b, C-b, ** $p < 0.01$ compared to the baseline value). However, there was no significant difference in the Ψ_m depolarization between the two strains at all three concentrations of FCCP (Figure 1 A-b, B-b, C-b), suggesting FCCP has the same ability to depolarize the mitochondrial membrane potential by directly disrupting the proton gradient.

Although FCCP caused Ψ_m depolarization to the same degree in cardiomyocytes from CypD KO mice compared to WT, our following experiments revealed that the mPTP opening in CypD KO cells was more resistant to FCCP compared to WT. The extent of mPTP opening was visualized by monitoring calcein fluorescence decline in calcein and Co^{2+} co-loaded cells. As shown in Figure 2A, we observed less opening of mPTP in response to FCCP-induced Ψ_m depolarization in CypD KO than in WT cardiomyocytes. For example, perfusing 1 μM FCCP for 6 minutes caused a smaller decrease (28%) in calcein fluorescence (F/F_0) in CypD KO cells, compared to 61% in WT cardiomyocytes ($p < 0.05$ between WT and CypD KO). This result is consistent with the low levels of mPTP function previously reported in CypD KO cells [14]. It should be noted that mPTPs were still available to be opened in CypD KO cardiomyocytes (Figure 2A, * $p < 0.05$, ** $p < 0.01$, compared to the baseline value in each group), indicating that partial, albeit decreased mPTP function is still present. This was further confirmed by exposure to a higher level of FCCP (20 μM), which resulted in similar mPTP opening in both CypD KO and WT cardiomyocytes (Figure 2A-b, # $p < 0.05$, ## $p < 0.01$ between the WT and CypD KO; NS: no significant difference noted between WT and CypD KO).

3.2. CypD KO Cardiomyocytes Release Less Mitochondrial Ca^{2+} than WT Myocytes in Response to mPTP Opening

Our previous study has revealed that FCCP-induced mitochondrial depolarization leads to mPTP opening and Ca^{2+} release from mitochondria [13]. Using the same experimental strategy, we compared FCCP-induced mitochondrial Ca^{2+} release between WT and CypD KO cardiomyocytes. As described previously [13], the elevation in basal intracellular Ca^{2+} was taken to represent mitochondrial Ca^{2+} release after both extracellular calcium (Ca^{2+} -free Tyrode's solution) and SR sources (with 10 mM caffeine plus 1 μM thapsigargin) were eliminated. As shown in Figure 3A, we observed that treatment with 1 μM FCCP raised basal Ca^{2+} levels from 1 to 1.13 ± 0.02 in WT cells ($p < 0.01$, paired Student's t -test), while the same FCCP treatment induced no significant increase in the basal Ca^{2+} level of CypD KO cells (1 compared to 1.00 ± 0.02). A significant difference in mitochondrial Ca^{2+} release was found in the presence of 1 μM FCCP between the two groups (Figure 3C, unpaired Student's t -test, $** p < 0.01$). To exclude the possibility that mitochondria in cardiomyocytes from CypD KO hearts might possess lower mitochondrial matrix Ca^{2+} concentrations than the WT, we compared mitochondrial Ca^{2+} release induced by high-dose FCCP (20 μM), which we have shown to cause maximum Ψ_m dissipation and mPTP opening (Figure 1B). As shown in Figure 3B and 3C, both WT and CypD KO cells showed similar levels of mitochondrial Ca^{2+} release after high dose FCCP treatment, raising their basal Ca^{2+} levels from 1 to 1.29 ± 0.08 (WT) and 1.29 ± 0.02 (CypD KO), respectively, indicating that mitochondria from WT and CypD KO cardiac myocytes retain the same amount of Ca^{2+} . In order to avoid overestimation of mitochondrial Ca^{2+} due to buffering the Ca^{2+} released by the SR after treatment with caffeine, these results were confirmed using another protocol: inhibiting both SR Ca^{2+} release via pretreatment with 5 μM ryanodine and SR Ca^{2+} reuptake via pretreatment with 1 μM Tha (Supplemental Figure 2). These results suggest that CypD KO cells do not have proper mPTP function resulting in less Ca^{2+} release when their Ψ_m is depolarized.

3.3. CypD KO Cardiomyocytes Are Resistant to CaW Acceleration in Response to FCCP Challenge

Next, we aimed to compare the intracellular Ca^{2+} behavior, specifically CaWs, in response to Ψ_m resulting from FCCP treatment. While WT cardiomyocytes exhibited significant increases in the rate of CaW generation (82% and 180% increase in 50 nM and 100nM FCCP, respectively; Figure 4A, C), CypD KO cardiomyocytes maintained a consistent CaW rate when exposed to both 50 nM and 100 nM FCCP concentrations (Figure 4B, C). These results suggest that mitochondrial Ca^{2+} release via the mPTP promotes CaWs. CypD KO cardiomyocytes were significantly resistant to Ψ_m depolarization-promoted CaWs in comparison to WT cardiomyocytes (Figure 4C; $## p < 0.01$ between WT and CypD KO).

3.4. Generation of Ca^{2+} Alternans in Single Cells and ECG ST-T Wave Alternans in Ex-Vivo Hearts Treated with FCCP

In addition to CaWs, Ca^{2+} alternans is another proarrhythmic factor [28, 29]. It has been shown that metabolic inhibition promotes Ca^{2+} alternans and arrhythmias [30, 31]. We further examined whether CypD KO cells may be protected from cardiac Ca^{2+} alternans

induced by FCCP treatment in the following experiments. A custom-designed pacing protocol with progressively shorter PCL (see Methods) was employed in isolated cardiomyocytes (Figure 5 B). As seen in Figure 5, Ca^{2+} alternans was observed in 11 out of 13 WT cardiomyocytes studied in the presence of 50 nM FCCP, while a significantly lower incidence rate was detected when mPTP opening was inhibited either pharmacologically (2 out of 8 cardiomyocytes treated with 1 μM CsA) or after genetic manipulation in CypD KO cardiomyocytes (2 out of 10; $p < 0.05$ Fischer's Exact test). Representative traces in the absence or presence of 50 nM FCCP are shown in each group (Figure 5 A–C).

To examine this effect at the whole-heart level, a *post-hoc* analysis of *ex-vivo*, Langendorff perfused WT and CypD KO hearts was carried out to evaluate the incidence of ST-T wave alternans, a precursor of lethal arrhythmias [32]. As seen in Figure 6, we observed that FCCP (30 nM) perfusion induced a significantly higher instance of ST-T wave alternans in WT compared to CypD KO hearts. ST-T wave alternans was seen in 5 of 7 WT hearts perfused with FCCP, however, they were not seen in CypD KO hearts ($n = 5$; Figure 6C, $p < 0.05$, Fischer's test), and were only seen in 1 of 6 WT hearts pretreated with CsA 1 μM .

3.5. Relevance of mPTP to Arrhythmias Induced by FCCP or IR in ex-Vivo Hearts

Having demonstrated the effect of V_m depolarization on CaWs and alternans in isolated cardiomyocytes, our next aim was to examine the potential arrhythmogenic consequences at the whole-heart level. FCCP (30 nM) was perfused in *ex-vivo* WT and CypD KO hearts via a Langendorff perfusion apparatus for up to one hour. Programmed stimulation protocols (as described in Methods section 2.9) were used to assess the propensity to develop arrhythmias in each group. As shown in Figure 7, WT hearts were particularly susceptible to various types of arrhythmias in the presence of 30 nM FCCP and had an average arrhythmia score of 3.25 ± 0.40 ($n = 8$), as indicated in Figure 7D. Interestingly, CypD KO hearts were resistant to programmed stimulation-induced arrhythmias and had a significantly lower arrhythmia score (1.40 ± 0.25 , $n = 5$) compared to the WT group (Figure C & D, $p < 0.01$). Additionally, mPTP inhibition by CsA (1 μM) protected the WT hearts and significantly lowered average arrhythmia scores to 1.40 ± 0.38 , which was similar to the score obtained in CypD KO mice (Figure 7 B & D, $** p < 0.01$).

In order to demonstrate pathological relevancy of mPTP opening in arrhythmogenesis, a chemical I/R model was then used in conjunction with the CypD KO mouse model. Briefly, Langendorff-perfused hearts were treated with a chemical ischemia solution for 10 minutes, followed by up to 50 minutes of reperfusion with normal Tyrode's solution, as described in the methods (section 2.10). The susceptibility to programmed S_1 - S_2 stimulation-induced arrhythmias was evaluated after 10 minutes of reperfusion, then every ten minutes thereafter. Compared to WT hearts (Figure 7E), which exhibited an average arrhythmia score of 3.78 ± 0.28 , CypD KO hearts had significantly lower arrhythmia scores, averaging 1.83 ± 0.60 (Figure 7G $** p < 0.01$). WT hearts treated with CsA (1 μM) also showed the tendency to prevent I/R-induced arrhythmias, although the difference did not reach statistical significance (arrhythmia score: 2.29 ± 0.35 ; $p = 0.09$). Taken together, these results suggest that mPTP inhibition, either through genetic knockout or pharmacological intervention,

significantly lowers the arrhythmia susceptibility in mouse hearts under conditions with dysfunctional mitochondria, such as depolarization caused by FCCP treatment, or I/R.

4. Discussion

Mitochondria are located in close proximity to the SR and SR-mitochondrial interactions mediated by Ca^{2+} may contribute to the regulation of heart function [33–35]. For example, the automaticity of rabbit pacemaker (sinoatrial-node) cells [36] or the mouse HL-1 cell line [37] is regulated by mitochondria-SR Ca^{2+} crosstalk. A very recent study by Santulli *et al* [38] has also demonstrated that altered Ca^{2+} -mediated SR-mitochondrial interactions may mediate heart failure. Our previous study has also provided insights into how a similar mechanism accounts for the arrhythmogenesis in mouse ventricular myocytes under Ca^{2+} -overload conditions. Specifically, Ψ_m depolarization leads to mitochondrial Ca^{2+} efflux via the mitochondrial permeability transition pore (mPTP) consequently promoting spontaneous CaWs [13].

In the present study, we have used the CypD KO mouse model [14, 17, 39]. The goal was to define the protection that genetic or pharmacological ablation of the mPTP might confer in the context that mitochondrial Ca^{2+} flux plays a key role in the modulation of CaWs and arrhythmogenesis. The major findings of this study were: 1) Ψ_m depolarization results in the opening of the mPTP, which in turn causes Ca^{2+} efflux from mitochondria and into the mitochondrial-SR micro domain, thus promoting CaWs and Ca^{2+} alternans; 2) The adverse effects of the mitochondrial depolarization on CaWs and Ca^{2+} alternans can be attenuated by inhibition of the mPTP, either by inhibition with CsA or genetic ablation of CypD (a protein necessary for mPTP complex opening); 3) Metabolic block and Ψ_m depolarization result in increased susceptibility to induced arrhythmias in *ex-vivo* whole heart settings, mimicking ischemic conditions. CypD deficiency-induced mPTP inhibition also attenuates CaWs and Ca^{2+} alternans during mitochondrial depolarization, and thereby protects against arrhythmogenesis in the heart.

4.1. Lower Sensitivity of mPTP Opening in Response to Ψ_m Depolarization in CypD KO Myocytes

Our earlier study [13] demonstrated that mPTP opening is involved in mitochondrial dysfunction-promoted CaWs and is potentially proarrhythmic. For example, the mPTP blocker CsA effectively attenuated the CaW frequency and amplitude enhanced by FCCP treatment (Ψ_m depolarization). It should be noted that CsA is not completely selective and also inhibits calcineurin. However, in our current experimental setting, CsA alone did not affect CaW frequency suggesting its effect is mediated via mitochondrial function. To further exclude any off-target effects of CsA, we employed the unique CypD KO mouse model in the present study. The deletion of the *Ppif* gene in CypD KO mice causes the absence of cyclophilin D proteins, without a change in the protein levels of the voltage-dependent anion channel (VDAC1) or the adenine nucleotide translocator (ANT1/2) [14]. We have also confirmed that no alteration in mCU, RyR, SERCA2a, or DHPR protein levels occur (Supplemental Figure 1).

It is known that mPTPs are Ca^{2+} , redox, Ψ_m , ADP, and pH-sensitive [40–42]. FCCP-induced mPTP opening can occur in the absence of ROS and therefore the mitochondrial membrane depolarization primarily and directly increases mPTP opening probability. Thus, FCCP has been frequently used as a mPTP promoter [1, 41, 43]. As shown in Figure 1, FCCP depolarized the Ψ_m in CypD KO cells to the same degree as that in WT, since the proton ionophore has the same ability to depolarize Ψ_m via direct disruption of the proton gradient. However, consistent with the role of CypD in regulating mPTP activity, FCCP induced mPTP opening in the CypD KO myocytes to a lesser extent compared to WT myocytes, as evidenced by the decline in calcein fluorescence (Figure 2). These results suggest that mPTPs are still partially functional in CypD KO myocytes, albeit with less sensitivity to Ψ_m depolarization. In accordance with the calcein assay indicating a difference in mPTP opening level, we observed lower Ca^{2+} release from mitochondria in CypD KO myocytes than in WT (Figure 3 Aa & B). It should be noted that the mitochondrial Ca^{2+} content (*i.e.* baseline mitochondrial Ca^{2+} level) appeared to be at the same level in both CypD and WT myocytes, since the same mitochondrial Ca^{2+} releases were observed at high concentrations of FCCP (20 μM ; Figure 3Ab & B, Supplemental Figure 2C). It is of note that the same phenomenon was also observed in mCU KO mouse cardiac mitochondria, suggesting that baseline mitochondrial Ca^{2+} levels could be regulated by other additional Ca efflux/influx pathways [44].

4.2. Reduction in mPTP Opening Attenuates CaWs, Ca^{2+} Alternans, and ST-T Wave Alternans

Our earlier [13] and present studies have provided evidence that opening of the mPTP serves as a mode of mitochondrial Ca^{2+} efflux when Ψ_m is depolarized, which may occur under ischemic conditions [17]. Furthermore, suppression of mPTP opening via either pharmacological inhibition (CsA) or deletion of the *Ppif* gene (CypD KO) attenuated Ca^{2+} release via the mPTP. We next demonstrated how these manipulations may affect cellular Ca^{2+} handling behaviors such as CaWs and Ca^{2+} alternans.

In accordance with our previous data using CsA [13], reduction of mPTP opening by a specific genetic approach (CypD KO) was also resistant to Ψ_m -promoted CaWs (Figure 4). Since CaWs activate sarcolemmal Na^+ - Ca^{2+} exchange current (or inward transient current, I_{ti}) and subsequently induces EADs, DADs, and triggered activities [45], which serve as triggers for arrhythmias, we speculated that CypD KO mouse hearts should be more protected from arrhythmias.

Additionally, cardiac ST-T wave alternans, a significant precursor for lethal arrhythmias, can be generated from action potential duration (APD) alternans [46]. APD, itself, is also influenced by Ca^{2+} cycling, as many of the key Ca^{2+} cycling-related currents (L-type calcium current, sodium-calcium exchange current, Cl^-) directly shape APD. As a result of this coupling, Ca^{2+} alternans can lead to APD alternans which can ultimately manifest as ST-T-wave repolarization alternans at the whole heart level. In the present study, we have also found that inhibition of mPTP opening via CsA or genetic knockout of CypD provided protection from developing Ca^{2+} alternans in single cells (Figure 5). This protection ameliorated the generation of ST-T-wave alternans in the whole-heart setting. The ST-T-

wave alternans was observed in the majority of WT hearts, but not in the hearts whose mPTPs were blocked by either CsA or CypD KO (Figure 6), indicating that Ca^{2+} release via the mPTP may contribute to this phenomena, and ultimately to its role in arrhythmogenesis. These results are also indicative of a potential role for reducing mPTP activity to prevent arrhythmias induced by metabolic inhibition.

4.3. Attenuation of mPTP Opening Protects Against Arrhythmias in the Whole Heart Setting

It has been well accepted that mPTPs open when there is elevation of matrix $[\text{Ca}^{2+}]$, an increase in oxidative stress, or mitochondrial depolarization occurs. Transient or sustained opening of mPTPs can lead to apoptotic or necrotic cell death [47]. Inhibition of mPTP by CypD inhibitors sanglifehrin A or CsA has been shown to reduce ischemic death and to improve functional recovery during cardiac I/R injury [16]. When subjected to cardiac I/R, CypD KO hearts showed a 40% reduction in myocardial infarction compared to WT [14]. Furthermore, CypD KO mice subjected to prolonged myocardial infarction also displayed reduced mortality and decreased infarct size [16, 48]. These studies suggest that CypD elimination and consequent reduction of mPTP activity generates significant protection in hearts subjected to I/R or myocardial infarction. On the contrary, it is interesting to note that in contrast to the WT, CypD-deficient mice displayed accelerated cardiac disease in response to a surgical model of chronic pressure overload (transverse aortic constriction) as well as increased hypertrophy and mortality in response to forced exercise [17] which are likely due to metabolic deficiencies in the mitochondria lacking CypD.

While the role of mPTP opening in cell death and the protective effect of CypD inhibition under ischemia have been well established, there is less evidence how mPTP activity influences the generation of cardiac arrhythmias. It remains unknown if inhibition of mPTP opening prevents arrhythmias. Some previous studies, using the non-selective CypD inhibitor CsA, have failed to show antiarrhythmic, protective effects of suppressing mPTP activities in animals or humans [49]. However, the potential antiarrhythmic property in the CypD KO mice has not been evaluated. In the present study, we have presented evidence, for the first time, that selectively suppressing mPTP via CypD KO is protective against the occurrence of cardiac arrhythmias induced by metabolic inhibition (FCCP) in the whole heart setting, or by a chemical I/R procedure. We have observed a significantly lower susceptibility to arrhythmias in *ex-vivo* hearts of CypD KO mice compared to hearts of WT mice in both models. These results suggest that the resistance to arrhythmias under I/R may be conferred by attenuation of mitochondrial Ca^{2+} release via mPTP opening. These results are consistent with previous data that CypD KO or inhibition protects the heart from injury induced by acute I/R models [14] [50].

4.4. Pathophysiological and Clinical Relevance

It has been well established that the mPTP can open in response to mitochondrial stress, including a buildup of reactive oxygen species [51, 52] and I/R injury [53]. The mitochondrial uncoupler FCCP has been used as a tool to mimic ischemic conditions [54]. In our present study, we have demonstrated that suppression of the mPTP via CypD KO

attenuates CaWs during mitochondrial depolarization and subsequently protects the heart from arrhythmias at the whole heart level.

We conclude that certain types of arrhythmias resulting from mitochondrial dysfunction or metabolic inhibition (most likely during I/R), are likely to be alleviated or prevented by genetic or pharmacological deficiency of the mPTP opening, thus potentially providing a novel therapeutic strategy for prevention/treatment of arrhythmias.

4.5. Limitations

CsA is not a completely selective inhibitor of mPTP, as it also inhibits calcineurin, which is a serine/threonine phosphatase that controls phosphorylation of PLN [55]. One would assume that calcineurin (also known as phosphatase 2B) inhibition may enhance SERCA activity to compensate for the loss of mitochondrial Ca^{2+} uptake in the presence of FCCP, providing protection independently of mPTP block. However, our results revealed no differences in the expression of either total or phosphorylated PLN (Supplemental Figure 1), which likely excludes potential remodeling of the calcineurin-PLN-SERCA pathway in CypD KO mice.

Additionally, we have also excluded any potential secondary alterations of major handling proteins, such as mCU, RyR, SERCA2a, and DHPR, in CypD KO cardiomyocytes (Supplemental Figure 1). Although we have not shown Western blotting data for mNCX due to lack of effective and available mNCX-specific antibodies, our functional data did not show any significant effect of the mNCX inhibitor CGP37157 on CaW frequency (Supplemental Figure 3), suggesting mNCX contributes less to CaW regulation under our experimental condition. Further experiments are warranted to clarify potential remodeling of other mitochondrial Ca^{2+} influx/efflux pathways in CypD KO cardiomyocytes.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

CaWs	Ca^{2+} waves
FCCP	carbonyl cyanide <i>p</i> -(trifluoromethoxy) phenylhydrazine
CsA	cyclosporin A
CypD KO	CypD knockout mouse model
DHPR	α_1 subunit of L-type Ca channel, dihydropyridine receptor
Ca_i^{2+}	Intracellular Ca^{2+}

I/R	ischemia/reperfusion
mCU	mitochondrial calcium uniporter
Ψ_m	mitochondrial membrane potential
mPTP	mitochondrial permeability transition pore
mNCX	Na^+ - Ca^{2+} exchanger
PCL	pacing cycle length
PLN	phospholamban
RyR	ryanodine receptors
SR	sarcoplasmic reticulum
Tha	thapsigargin

References

1. Bernardi P. Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization. *J Biol Chem.* 1992; 267:8834–8839. [PubMed: 1374381]
2. Dedkova EN, Blatter LA. Mitochondrial Ca^{2+} and the heart. *Cell Calcium.* 2008; 44:77–91. [PubMed: 18178248]
3. Bers DM. Calcium cycling and signaling in cardiac myocytes. *Annu Rev Physiol.* 2008; 70:23–49. [PubMed: 17988210]
4. De Stefani D, Raffaello A, Teardo E, Szabo I, Rizzuto R. A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter. *Nature.* 2011; 476:336–340. [PubMed: 21685888]
5. Baughman JM, Perocchi F, Girgis HS, Plovanich M, Belcher-Timme CA, Sancak Y, Bao XR, Strittmatter L, Goldberger O, Bogorad RL, Kotliansky V, Mootha VK. Integrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter. *Nature.* 2011; 476:341–345. [PubMed: 21685886]
6. Halestrap AP, Pasdois P. The role of the mitochondrial permeability transition pore in heart disease. *Biochim Biophys Acta.* 2009; 1787:1402–1415. [PubMed: 19168026]
7. Griffiths EJ. Mitochondrial calcium transport in the heart: physiological and pathological roles. *J Mol Cell Cardiol.* 2009; 46:789–803. [PubMed: 19285504]
8. Gunter TE, Buntinas L, Sparagna G, Eliseev R, Gunter K. Mitochondrial calcium transport: mechanisms and functions. *Cell Calcium.* 2000; 28:285–296. [PubMed: 11115368]
9. Hoppe UC. Mitochondrial calcium channels. *FEBS Lett.* 2010; 584:1975–1981. [PubMed: 20388514]
10. Ruiz-Meana M, Fernandez-Sanz C, Garcia-Dorado D. The SR-mitochondria interaction: a new player in cardiac pathophysiology. *Cardiovasc Res.* 2010; 88:30–39. [PubMed: 20615915]
11. Chen Y, Csordas G, Jowdy C, Schneider TG, Csordas N, Wang W, Liu Y, Kohlhaas M, Meiser M, Bergem S, Nerbonne JM, Dorn GW 2nd, Maack C. Mitofusin 2-containing mitochondrial-reticular microdomains direct rapid cardiomyocyte bioenergetic responses via interorganelle Ca^{2+} crosstalk. *Circulation research.* 2012; 111:863–875. [PubMed: 22777004]
12. Dorn GW 2nd, Song M, Walsh K. Functional implications of mitofusin 2-mediated mitochondrial-SR tethering. *J Mol Cell Cardiol.* 2015; 78:123–128. [PubMed: 25252175]
13. Zhao Z, Gordan R, Wen H, Fefelova N, Zang WJ, Xie LH. Modulation of intracellular calcium waves and triggered activities by mitochondrial Ca^{2+} flux in mouse cardiomyocytes. *PloS one.* 2013; 8:e80574. [PubMed: 24348912]

14. Baines CP, Kaiser RA, Purcell NH, Blair NS, Osinska H, Hambleton MA, Brunskill EW, Sayen MR, Gottlieb RA, Dorn GW, Robbins J, Molkentin JD. Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death. *Nature*. 2005; 434:658–662. [PubMed: 15800627]
15. Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. *Nature*. 2005; 434:652–658. [PubMed: 15800626]
16. Kwong JQ, Molkentin JD. Physiological and pathological roles of the mitochondrial permeability transition pore in the heart. *Cell metabolism*. 2015; 21:206–214. [PubMed: 25651175]
17. Elrod JW, Wong R, Mishra S, Vagnozzi RJ, Sakthivel B, Goonasekera SA, Karch J, Gabel S, Farber J, Force T, Brown JH, Murphy E, Molkentin JD. Cyclophilin D controls mitochondrial pore-dependent Ca(2+) exchange, metabolic flexibility, and propensity for heart failure in mice. *J Clin Invest*. 2010; 120:3680–3687. [PubMed: 20890047]
18. Gordan, R.; Fefelova, N.; Zhao, Z.; Xie, L. Involvement of Mitochondrial Permeability Transition Pore (mPTP) in Cardiac Arrhythmias: Evidence From Cyclophilin D Knockout Mice. *American Heart Association Scientific Sessions*; Orlando, Florida: 2015.
19. Kabaeva Z, Zhao M, Michele DE. Blebbistatin extends culture life of adult mouse cardiac myocytes and allows efficient and stable transgene expression. *Am J Physiol Heart Circ Physiol*. 2008; 294:H1667–1674. [PubMed: 18296569]
20. Xie LH, Weiss JN. Arrhythmogenic consequences of intracellular calcium waves. *Am J Physiol Heart Circ Physiol*. 2009; 297:H997–H1002. [PubMed: 19561309]
21. Xie LH, Chen F, Karagueuzian HS, Weiss JN. Oxidative-stress-induced afterdepolarizations and calmodulin kinase II signaling. *Circulation research*. 2009; 104:79–86. [PubMed: 19038865]
22. Jeron A, Mitchell GF, Zhou J, Murata M, London B, Buckett P, Wiviott SD, Koren G. Inducible polymorphic ventricular tachyarrhythmias in a transgenic mouse model with a long Q-T phenotype. *Am J Physiol Heart Circ Physiol*. 2000; 278:H1891–1898. [PubMed: 10843886]
23. Ruiz-Meana M, Abellan A, Miro-Casas E, Agullo E, Garcia-Dorado D. Role of sarcoplasmic reticulum in mitochondrial permeability transition and cardiomyocyte death during reperfusion. *Am J Physiol Heart Circ Physiol*. 2009; 297:H1281–1289. [PubMed: 19684187]
24. Seidlmayer LK, Juettner VV, Kettlewell S, Pavlov EV, Blatter LA, Dedkova EN. Distinct mPTP activation mechanisms in ischaemia-reperfusion: contributions of Ca²⁺, ROS, pH, and inorganic polyphosphate. *Cardiovasc Res*. 2015; 106:237–248. [PubMed: 25742913]
25. Wu Y, Temple J, Zhang R, Dzhura I, Zhang W, Trimble R, Roden DM, Passier R, Olson EN, Colbran RJ, Anderson ME. Calmodulin kinase II and arrhythmias in a mouse model of cardiac hypertrophy. *Circulation*. 2002; 106:1288–1293. [PubMed: 12208807]
26. Curtis MJ, Walker MJ. Quantification of arrhythmias using scoring systems: an examination of seven scores in an in vivo model of regional myocardial ischaemia. *Cardiovasc Res*. 1988; 22:656–665. [PubMed: 3242835]
27. Gao J, Zhang L, Wang Y, Lu B, Cui H, Fu W, Wang H, Yu Y, Yu X. Antiarrhythmic effect of acupuncture pretreatment in rats subjected to simulative global ischemia and reperfusion--involvement of adenylate cyclase, protein kinase A, and L-type Ca²⁺ channel. *J Physiol Sci*. 2008; 58:389–396. [PubMed: 18845056]
28. Weiss JN, Karma A, Shiferaw Y, Chen PS, Garfinkel A, Qu Z. From pulsus to pulseless: the saga of cardiac alternans. *Circ Res*. 2006; 98:1244–1253. [PubMed: 16728670]
29. Xie LH, Sato D, Garfinkel A, Qu Z, Weiss JN. Intracellular Ca alternans: coordinated regulation by sarcoplasmic reticulum release, uptake, and leak. *Biophys J*. 2008; 95:3100–3110. [PubMed: 18539635]
30. Chen F, De Diego C, Xie LH, Yang JH, Klitzner TS, Weiss JN. Effects of metabolic inhibition on conduction, Ca transients, and arrhythmia vulnerability in embryonic mouse hearts. *Am J Physiol Heart Circ Physiol*. 2007; 293:H2472–2478. [PubMed: 17660398]
31. Florea SM, Blatter LA. The role of mitochondria for the regulation of cardiac alternans. *Front Physiol*. 2010; 1:141. [PubMed: 21423381]
32. Qu Z, Xie Y, Garfinkel A, Weiss JN. T-wave Alternans and Arrhythmogenesis in Cardiac Diseases. *Front Physiol*. 2010; 1:1–15. [PubMed: 21522484]

33. Lukyanenko V, Chikando A, Lederer WJ. Mitochondria in cardiomyocyte Ca²⁺ signaling. *Int J Biochem Cell Biol.* 2009; 41:1957–1971. [PubMed: 19703657]
34. Williams GS, Boyman L, Chikando AC, Khairallah RJ, Lederer WJ. Mitochondrial calcium uptake. *Proc Natl Acad Sci U S A.* 2013
35. Williams GS, Boyman L, Lederer WJ. Mitochondrial calcium and the regulation of metabolism in the heart. *J Mol Cell Cardiol.* 2015; 78:35–45. [PubMed: 25450609]
36. Yaniv Y, Spurgeon HA, Lyashkov AE, Yang D, Ziman BD, Maltsev VA, Lakatta EG. Crosstalk between mitochondrial and sarcoplasmic reticulum Ca²⁺ cycling modulates cardiac pacemaker cell automaticity. *PloS one.* 2012; 7:e37582. [PubMed: 22666369]
37. Takeuchi A, Kim B, Matsuoka S. The mitochondrial Na⁺-Ca²⁺ exchanger, NCLX, regulates automaticity of HL-1 cardiomyocytes. *Scientific reports.* 2013; 3:2766. [PubMed: 24067497]
38. Santulli G, Xie W, Reiken SR, Marks AR. Mitochondrial calcium overload is a key determinant in heart failure. *Proc Natl Acad Sci U S A.* 2015; 112:11389–11394. [PubMed: 26217001]
39. Baines CP. The mitochondrial permeability transition pore as a target of cardioprotective signaling. *Am J Physiol Heart Circ Physiol.* 2007; 293:H903–904. [PubMed: 17513492]
40. Honda HM, Korge P, Weiss JN. Mitochondria and ischemia/reperfusion injury. *Annals of the New York Academy of Sciences.* 2005; 1047:248–258. [PubMed: 16093501]
41. Saotome M, Katoh H, Satoh H, Nagasaka S, Yoshihara S, Terada H, Hayashi H. Mitochondrial membrane potential modulates regulation of mitochondrial Ca²⁺ in rat ventricular myocytes. *Am J Physiol Heart Circ Physiol.* 2005; 288:H1820–1828. [PubMed: 15563537]
42. Sokolova N, Pan S, Provazza S, Beutner G, Vendelin M, Birkedal R, Sheu SS. ADP protects cardiac mitochondria under severe oxidative stress. *PloS one.* 2013; 8:e83214. [PubMed: 24349464]
43. Scorrano L, Petronilli V, Bernardi P. On the voltage dependence of the mitochondrial permeability transition pore. A critical appraisal. *J Biol Chem.* 1997; 272:12295–12299. [PubMed: 9139672]
44. Kwong JQ, Lu X, Correll RN, Schwanekamp JA, Vagnozzi RJ, Sargent MA, York AJ, Zhang J, Bers DM, Molkentin JD. The Mitochondrial Calcium Uniporter Selectively Matches Metabolic Output to Acute Contractile Stress in the Heart. *Cell Rep.* 2015; 12:15–22. [PubMed: 26119742]
45. Ter Keurs HE, Boyden PA. Calcium and arrhythmogenesis. *Physiol Rev.* 2007; 87:457–506. [PubMed: 17429038]
46. Goldhaber JJ, Xie LH, Duong T, Motter C, Khuu K, Weiss JN. Action potential duration restitution and alternans in rabbit ventricular myocytes: the key role of intracellular calcium cycling. *Circulation research.* 2005; 96:459–466. [PubMed: 15662034]
47. Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. *Physiol Rev.* 2007; 87:99–163. [PubMed: 17237344]
48. Lim WY, Messow CM, Berry C. Cyclosporin variably and inconsistently reduces infarct size in experimental models of reperfused myocardial infarction: a systematic review and meta-analysis. *Br J Pharmacol.* 2012; 165:2034–2043. [PubMed: 21950961]
49. Brown DA, O'Rourke B. Cardiac mitochondria and arrhythmias. *Cardiovasc Res.* 2010; 88:241–249. [PubMed: 20621924]
50. Wang K, An T, Zhou LY, Liu CY, Zhang XJ, Feng C, Li PF. E2F1-regulated miR-30b suppresses Cyclophilin D and protects heart from ischemia/reperfusion injury and necrotic cell death. *Cell death and differentiation.* 2015; 22:743–754. [PubMed: 25301066]
51. Wang YH, Shi CX, Dong F, Sheng JW, Xu YF. Inhibition of the rapid component of the delayed rectifier potassium current in ventricular myocytes by angiotensin II via the AT1 receptor. *British journal of pharmacology.* 2008; 154:429–439. [PubMed: 18414380]
52. Saotome M, Katoh H, Yaguchi Y, Tanaka T, Urushida T, Satoh H, Hayashi H. Transient opening of mitochondrial permeability transition pore by reactive oxygen species protects myocardium from ischemia-reperfusion injury. *Am J Physiol Heart Circ Physiol.* 2009; 296:H1125–1132. [PubMed: 19202002]
53. Halestrap AP, Clarke SJ, Javadov SA. Mitochondrial permeability transition pore opening during myocardial reperfusion - a target for cardioprotection. *Cardiovascular Research.* 2004; 61:372–385. [PubMed: 14962470]

54. Suh SH, Droogmans G, Nilius B. Effects of cyanide and deoxyglucose on Ca²⁺ signalling in macrovascular endothelial cells. *Endothelium*. 2000; 7:155–168. [PubMed: 10912910]
55. Chu G, Carr AN, Young KB, Lester JW, Yatani A, Sanbe A, Colbert MC, Schwartz SM, Frank KF, Lampe PD, Robbins J, Molkentin JD, Kranias EG. Enhanced myocyte contractility and Ca²⁺ handling in a calcineurin transgenic model of heart failure. *Cardiovasc Res*. 2002; 54:105–116. [PubMed: 12062367]

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Highlights

- Mitochondrial Ca^{2+} efflux via mPTP opening increases Ca^{2+} wave generation.
- Mitochondrial Ca^{2+} efflux promotes Ca^{2+} alternans in single cells.
- mPTP inhibition reduces the Ca^{2+} wave rate and incidence of Ca^{2+} alternans.
- Ca^{2+} efflux via mPTP is likely involved in FCCP- and I/R-induced arrhythmias.
- mPTP inhibition reduces susceptibility to FCCP and I/R-induced arrhythmias.

Figure 1. FCCP Depolarized the Ψ_m to the Same Extent in WT and CypD KO Myocytes
 FCCP (A, 100 nM; B, 1 μM; C, 20 μM) was used to depolarize ψ_m . The fluorescence in the presence of 30 μM FCCP was set as 100% dissipation (not shown). (A-a, B-a, C-a) Snapshots of TMRM fluorescence at baseline (0), 6, and 10 minutes in the presence of FCCP are shown. (A-b, B-b, C-b) Summary data showing a decrease in TMRM fluorescence, * $p < 0.05$, ** $p < 0.01$ compared to the respective baseline value.

Figure 2. FCCP-Induced mPTP Opening was Attenuated in CypD KO Myocytes

(**A-a**, **B-a**) Snapshots of calcein fluorescence at baseline (0 min), 6 min, and 10 min after the treatment with FCCP at 1 and 20 μ M, respectively. (**A-b**, **B-b**) Summary time course data showing the decrease in calcein fluorescence in the presence of FCCP, * $p < 0.05$, ** $p < 0.01$ compared to baseline within each group; # $p < 0.05$, ## $p < 0.01$ compared to WT.

Figure 3. FCCP-Induced Mitochondrial Ca²⁺ Release was Reduced in CypD KO Myocytes FCCP-Induced mitochondrial Ca²⁺ release was evaluated by the elevation of basal Ca²⁺ levels in Ca_o-free Tyrode's solution after SR Ca²⁺ was depleted by 10 mM caffeine (Caff) and 1 μM thapsigargin (Tha). **(A)** Less mitochondrial Ca²⁺ release by 1 μM FCCP was observed in CypD KO myocytes compared to WT. **(B)** FCCP at high concentration (20 μM) induced same mitochondrial Ca²⁺ release in both CypD KO and WT myocytes, indicating equal mitochondrial Ca²⁺ content. **(C)** Summarized data, ** p < 0.01.

Figure 4. FCCP-Induced CaW Rate Increase was Attenuated in CypD KO Myocytes
(A) A representative Ca^{2+} fluorescence trace showing the effect of FCCP (50–100 nM) on spontaneous CaWs in a WT ventricular myocyte. **(B)** The same as A, except in a CypD KO ventricular myocyte. **(C)** Data summary, * $p < 0.05$, compared to control within each group; ## $p < 0.01$ between CypD KO and WT.

Figure 5. Lower Incidence of FCCP-Induced Ca^{2+} Alternans was Observed in CypD KO Myocytes

(A–C) Representative traces of control (top), treated with FCCP 50 nM (middle), and expanded area of interest (bottom), for each group: WT (A), CypD KO (B), and WT pretreated with CsA 1 μM (C). Cells were field stimulated using a programmed protocol as indicated in B. The pacing cycle length decreased progressively from 300 to 80 ms (B). (D) Summarized Data. * $p < 0.05$, ** $p < 0.01$ compared to the WT group.

Figure 6. Lower Incidence of FCCP-Induced ST-T Wave Alternans was Observed in CypD KO Mouse *ex-vivo* Hearts

(A) Pseudo-Lead II ECG signals recorded from Langendorff-perfused WT or CypD KO hearts in the absence (Ctl) and presence of 30 nM FCCP (FCCP). Arrows indicated alternating ST level alternans. (B) Overlapped ECG waveforms; **a** and **b** represent individual complexes appearing in the ECG traces as indicated in A. (C) Summarized Data showing incidences of ST alternans in WT and CypD KO hearts. * $p < 0.05$ by Fisher's exact test.

Figure 7. FCCP and Chemical I/R Induced Arrhythmias in *ex-Vivo* Hearts and Relevance to mPTP

(A–C) Pseudo-Lead II ECG signals recorded from WT mice treated with FCCP (A), or FCCP + CsA (B), and CypD KO mice treated with FCCP (C). Programmed stimulations were applied to evaluate the vulnerability to arrhythmias in each case. (E–G) Pseudo-Lead II ECG signals recorded from WT mice treated with a chemical ischemia solution (E), or a chemical ischemia solution + CsA (F), and CypD KO mice treated with a chemical ischemia solution (G). Programmed stimulations were applied 10 minutes after reperfusion, and every 10 minutes thereafter to evaluate the vulnerability to arrhythmias in each case. The top panels show baseline ECG before treatment. A representative arrhythmia event is shown in each case. (D, H) Summarized arrhythmia scores for each group, * $p < 0.05$, ** $p < 0.01$, compared to the WT.