Uncoupling the mitochondria facilitates alternans formation in the isolated rabbit heart

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Submitted 12 December 2012; accepted in final form 26 April 2013

Smith RM, Visweswaran R, Talkachova I, Wothe JK, Tolkacheva EG. Uncoupling the mitochondria facilitates alternans formation in the isolated rabbit heart. Am J Physiol Heart Circ Physiol 305: H9–H18, 2013. First published May 3, 2013; doi:10.1152/ajpheart.00915.2012.—Alternans of action potential duration (APD) and intracellular calcium ([Ca2+]i) transients in the whole heart are thought to be markers of increased propensity to ventricular fibrillation during ischemia-reperfusion injuries. During ischemia, ATP production is affected and the mitochondria become uncoupled, which may affect alternans formation in the heart. The aim of our study was to investigate the role of mitochondria on the formation of APD and [Ca2+]i, alternans in the isolated rabbit heart. We performed dual voltage and [Ca2+]i optical mapping of isolated rabbit hearts under control conditions, global no-flow ischemia (n = 6), and after treatment with 50 nM of the mitochondrial uncoupler FCCP (n = 6). We investigated the formation of alternans of APD, [Ca2+]i, amplitude (CaA), and [Ca2+]i duration (CaD) under different rates of pacing. We found that treatment with FCCP leads to the early occurrence of APD, CaD, and CaA alternans; an increase of intraventricular APD but not CaD heterogeneity; and significant reduction in conduction velocity compared with that of control. Furthermore, we demonstrated that FCCP and global ischemia have similar effects on the prolongation of [Ca2+]i transients, whereas ischemia induces a significantly larger reduction of APD compared with that in FCCP treatment. In conclusion, our results demonstrate that uncoupling of mitochondria leads to an earlier occurrence of alternans in the heart. Thus, in conditions of mitochondrial stress, as seen during myocardial ischemia, uncoupled mitochondria may be responsible for the formation of both APD and [Ca2+]i alternans in the heart, which in turn creates a substrate for ventricular arrhythmias.

alternans; FCCP; calcium; action potential; mitochondria; ischemia

T-WAVE ALTERNANS and alternans of action potential duration (APD) in the whole heart are thought to be markers of increased propensity to ventricular arrhythmias during ischemia-reperfusion injuries (21, 29, 40). Repolarization T-wave alternans has been considered to be a stronger marker of electrical instability and a precursor for ventricular arrhythmias (25, 42, 46, 51, 53), which is a major cause of sudden cardiac death (3, 4, 63). At the whole heart level, T-wave alternans, seen on the electrocardiogram, has been shown to correspond to a beat-to-beat variation in the APD at the single-cell level, a phenom-
tion. Specifically, it is known that during myocardial ischemia, the electrons generated by the electron transport chain in the mitochondria can no longer be transferred to molecular oxygen, leading to a cessation of oxidative phosphorylation and inhibition of ATP synthesis (27, 59). Furthermore, inhibition of electron transport prevents pumping of protons across the inner membrane required to generate the mitochondrial membrane potential (20, 41, 6). Thus, to maintain the membrane potential, the mitochondria starts to consume ATP by running the F1F0-ATPase in reverse, rather than produce ATP. However, the exact role of the mitochondria in alternans formation is unknown.

Carbonyl cyanide p-trifluoromethoxyphenyldrazone (FCCP) is a potent mitochondrial uncoupler that has been used to study mitochondria and mitochondrial function in a variety of studies (9, 10, 45, 61). Different effects of FCCP on the mitochondrial network have been reported depending on the concentrations used. For instance, small concentrations of FCCP (<30 nM) lead to mitochondrial uncoupling caused by a detectable change in oxygen consumption within 5 min of FCCP treatment. It has been shown that treatment with FCCP at concentrations as low as 10 nM leads to uncoupling of the mitochondria (9) in isolated myocytes. On the other hand, FCCP at concentrations 30–100 nM has been shown to confer cardio-protection in the single cells while causing mitochondrial oxidation (9). However, similar concentrations of FCCP when used in the whole heart was shown to lead to ventricular arrhythmias because of development of interventricular heterogeneity (57). Larger concentrations of FCCP (>300 nM) was shown to cause mitochondrial depolarization (9). Recently, it has been shown in cat atrial myocytes that depolarizing the mitochondria, using FCCP, led to the formation of $\text{[Ca}^{2+}]_i$, alternans (24). However, the effect of FCCP and, hence, uncoupling the mitochondria on the formation of APD and $\text{[Ca}^{2+}]_i$ alternans in the whole heart has not been investigated.

In this study, we aim to determine the role of the mitochondria, through its uncoupling, on APD and $\text{[Ca}^{2+}]_i$ alternans formation in the isolated Langendorff-perfused rabbit heart. In this study, we will use a small concentration of FCCP (50 nM) since our previous study indicated that higher concentrations of FCCP (100 nM) lead to arrhythmias in the whole heart (57). In addition, we investigated the changes in various electrophysiological properties induced by the mitochondria uncoupling and global ischemia.

MATERIALS AND METHODS

The investigation conformed to the Guide for the Care and Use of Laboratory Animals (National Institutes of Health Publication No. 85-23, Revised 1996), and the protocol was approved by the Institutional Animal Care and Use Committee at the University of Minnesota. New Zealand White rabbits (1.3–2.0 kg, n = 12, Bakkom Rabbitry) were first heparinized (550 U 100 g) and then anesthetized with ketamine and xylazine (35 and 5 mg/kg, respectively). The heart was excised and placed in an ice-cold cardioplegia solution. The temperature of the bath solution was maintained at 37 ± 1°C at all times during the course of the experiment. All hearts were subjected to periodic pacing at different rates, as described below, during control, FCCP, and global ischemia to induce alternans.

Optical Mapping

Voltage and $\text{[Ca}^{2+}]_i$, were simultaneously recorded as previously described (14, 34). Briefly, the heart was stained with the voltage-sensitive dye RH-237 (10 μM) and the calcium-sensitive dye Rhod-2AM (1 nM) (52), and two 532-nm green lasers (1 W, Shanghai Dream Lasers) were used to illuminate the anterior surface of the heart. This fluorescent signal from the heart was first separated by a dichroic mirror and then filtered with 585 ± 20-nm band-pass (Rhod-2AM) and 720-nm long-pass (RH-237) filters. The cameras were precisely aligned using a grid pattern so that voltage and $\text{[Ca}^{2+}]_i$, signals were taken from the same pixel on the heart.

External stimuli (5-ms duration, twice the threshold) were applied to the base of the heart using a dynamic pacing protocol (39), which consisted of 100 stimuli applied at each basic cycle length (BCL) starting with BCL = 300 ms down to 200 ms in steps of 20 ms and then from 200 ms to 130 ms in steps of 10 ms. Optical movies of 6.7 s were acquired at 600 frames/s with 64 × 64 pixel resolution at the end of each BCL to record steady-state responses. Optical movies were recorded at 30 min of control; 5 and 10 min of FCCP treatment; and 5 and 10 min of no-flow global ischemia. The background fluorescence was subtracted from each frame, and spatial (3 × 3 pixels) and temporal (3 pixels) conical convolution filters were used.

Parameter Measurements

$\text{APD and } [\text{Ca}^{2+}]_i\text{ measurements.}$ Optical APD was measured at 80% repolarization, while the duration of $[\text{Ca}^{2+}]_i$, transients (CaD) was determined from the maximum first derivative of the $[\text{Ca}^{2+}]_i$, upstroke to the time point of 80% recovery of $[\text{Ca}^{2+}]_i$, to its original baseline, as previously described (14, 34). Two-dimensional (2-D) APD and CaD maps were constructed to reveal the spatial distribution of APD and CaD on the anterior surface of the heart. $[\text{Ca}^{2+}]_i$, amplitude (CaA) was measured by finding the local minima and maxima, which represent the diastolic and systolic values of $[\text{Ca}^{2+}]_i$. The mean and standard deviation for all APD, CaD, and CaA measurements were determined from the anterior surface at each BCL. To compare the electrophysiological changes caused by FCCP treatment and ischemia compared with those in control conditions, we calculated $\Delta$APD and $\Delta$CaD which quantified the relative change in APD and CaD according to the formula: $\Delta$X = (X - Xcontrol)/Xcontrol and $\Delta$Xischemia = (Xischemia - Xcontrol)/Xcontrol, where X is APD or CaD. All variations smaller than the temporal threshold of alternans (5 ms) were defined as 1:1 responses. Alternans maps (2-D) were constructed to reveal the spatial distribution of APD and CaD alternans for the anterior surface of the heart.

The degree of CaA alternans was quantified at each pixel as the alternans ratio (60): $\Delta$CaA = $I - B/A$, where B is the net amplitude of the smaller transient and A is the net amplitude of the larger transient. The threshold for the presence of alternans was set at 0.15, and all variations smaller than 0.15 were defined as 1:1 responses. The
phase of alternans was not taken into account, so no distinction between spatially concordant and discordant alternans was made. The local spatial onset of alternans was defined as the BCL (B_{onset}) at which at least 10% of the anterior surface exhibited alternans (17).

**Conduction velocity measurements.** Local conduction velocity (CV) was calculated as previously described (6, 39). Specifically, the distributions of activation times [measured at (dV/dr)_{max}] for the spatial regions of 3 × 3 pixels were fitted with the plane, and gradients of activation times g_x and g_y were calculated for each plane along the x- and y-axes, respectively. The magnitude of the local CV was calculated for each pixel as (g_x^2 + g_y^2)^{-1/2}. Mean values for CV were calculated for the anterior surface. The relative change in CV compared with that of control was calculated as ΔCV = (CV_{FCCP} − CV_{control})/CV_{control}.

**Intraventricular APD and CaD heterogeneity.** The spatial dispersion of APD and CaD at the anterior surface of the heart was estimated based on the heterogeneity index, μ_X = (X^{95} − X^{50})/X^{50} (39), where X is APD or CaD; X^{95} and X^{50} represent the 95th and 5th percentiles of either APD or CaD distribution, respectively; and X^{50} is the median of either APD or CaD distribution. The μ value for both APD and CaD was calculated as the average of the heterogeneity index for the odd and even beats.

**Statistical analysis.** All data are presented as means ± SE. Statistical comparisons between control and FCCP and between control and ischemia conditions in the same heart were performed using a paired Student’s t-test. Statistical comparisons between FCCP and ischemia in different hearts were performed using ANOVA. Values of P < 0.05 were considered statistically significant.

**RESULTS**

We first investigated the effects of uncoupling the mitochondria on the formation of APD alternans in the rabbit heart. Figure 1A, left, shows representative 2-D APD maps of two consecutive beats for control and 5 and 10 min of FCCP treatment at different BCLs: 220, 190, 170, 150, and 130 ms. Corresponding action potential traces from a single pixel marked as a star in Fig. 1A, left, are shown in Fig. 1A, right, for BCL = 170 ms. Note the presence of APD alternans after 10 min of FCCP treatment. Figure 1B shows the corresponding spatial distribution of APD alternans at different BCLs for control and 5 and 10 min of FCCP treatment. The red and blue color indicates the amplitude of APD alternans, whereas the white color indicates the absence of alternans. Although no APD alternans is present in any regions of the heart in control, after 5 min of FCCP treatment, APD alternans occurs locally at B_{onset} = 150 ms. Further treatment with FCCP (10 min) causes APD alternans to occur earlier, at B_{onset} = 190 ms. In our experiments (n = 6), APD alternans always formed at earlier B_{onset} after treatment with FCCP than during control, as indicated in Fig. 1C. Note that B_{onset} increases for both 5 min (B_{onset} = 174 ± 22 ms, P = not significant (NS)) and 10 min (B_{onset} = 192 ± 18 ms, P < 0.05) of FCCP treatment compared with control (B_{onset} = 136 ± 6 ms). Therefore, uncoupling the mitochondria promotes APD alternans formation in isolated rabbit heart.

We then examined the effect of uncoupling the mitochondria on [Ca^{2+}]_i dynamics and formation of [Ca^{2+}]_i alternans. Figure 2 illustrates how uncoupling the mitochondria affect CaD. Figure 2A, left, shows representative 2-D CaD maps for two consecutive beats of control and 5 and 10 min after FCCP treatment at different BCLs. [Ca^{2+}]_i traces from a single pixel marked as a star in Fig. 2A, left, are shown in Fig. 2A, right, for BCL = 170 ms. In this specific example, there is no CaD alternans formed during control at BCL = 170 ms. However, note the presence of CaD alternans after 5 and 10 min of FCCP treatment. ΔCaD maps (2-D), which illustrate the spatial development of CaD alternans at different BCLs are shown in Fig. 2B. Note that in this specific example, CaD alternans formed at B_{onset} = 200 ms, both for 5 and 10 min of treatment.
with FCCP, whereas no alternans is present in control at the same BCL. Mean values of B_{Onset} for CaD alternans at different conditions are shown in Fig. 2C for all experiments (n = 6). Note that treatment with FCCP for both 5 min (B_{Onset} = 172 ± 11 ms, P < 0.05) and 10 min (B_{Onset} = 182 ± 20 ms, P < 0.05) facilitates CaD alternans formation compared with that of control (B_{Onset} = 133 ± 6 ms). Therefore, uncoupling the mitochondria also promotes CaD alternans formation in isolated rabbit heart.

Since CaD was affected by treatment with FCCP, CaA was then examined. Representative 2-D CaA maps and corresponding [Ca^{2+}] traces taken from a single pixel at BCL = 170 ms are shown in Fig. 3A, left and right, respectively. In this example, at BCL = 170 ms, no CaA alternans is seen during control and 5 min of treatment of FCCP. However, longer FCCP treatment (10 min) induced CaA alternans. Figure 3B shows corresponding spatial distribution of CaA alternans at different BCLs for control and 5 and 10 min of FCCP treatment. Note that the local onset of CaA alternans occurred earlier after 5 min (B_{Onset} = 150 ms) and 10 min (B_{Onset} = 190 ms) of FCCP treatment than during control (B_{Onset} = 130 ms). Figure 3C shows the mean values of B_{Onset} for CaA alternans at different conditions from all of our experiments (n = 6). B_{Onset} of CaA occurs earlier after 5 min (B_{Onset} = 162 ± 5 ms, P < 0.05) and 10 min (B_{Onset} = 152 ± 5 ms, P < 0.05) than during control (B_{Onset} = 141 ± 3 ms). Therefore, uncoupling the mitochondria promotes CaA alternans formation in isolated rabbit heart.

We also investigated how uncoupling the mitochondrial network affects other important electrophysiological characteristics, such as spatial heterogeneity and CV of impulse propagation in the heart. To examine the spatial dispersion of APD and CaD, the corresponding heterogeneity indexes, μ_{APD} and μ_{CaD}, were calculated for control and 5 and 10 min of FCCP treatment at different BCLs and shown in Fig. 4. Treatment with FCCP for 5 or 10 min significantly increased μ_{APD} at all BCLs compared with control (Fig. 4A). On the other hand, the effect of FCCP on μ_{CaD} was not so pronounced and was not significant at any BCL (Fig. 4B). Therefore, our data suggest that uncoupling the mitochondria affects the spatial dispersion of APD more than [Ca^{2+}].

We next investigated changes in CV caused by treatment with FCCP. Figure 4C shows a representative example of an activation maps illustrating action potential propagation during control and 5 and 10 min of FCCP treatment at BCL = 130 ms. Color represents activation times across the epicardial surface, and isochrones are shown 5 ms apart. Note the slowing of propagation during FCCP treatment. From all our experiments, CV calculated at BCL = 130 ms was significantly reduced both at 5 min (CV = 0.53 ± 0.03 m/s, P < 0.05) and 10 min (CV = 0.54 ± 0.03 m/s, P < 0.05) compared with that in control conditions (CV = 0.62 ± 0.03 m/s). Figure 4D illustrates the relative change in CV, ΔCV, at 5 and 10 min of FCCP treatment compared with control conditions at different BCLs across all experiments. Treatment with FCCP significantly decreases CV at both 5 and 10 min, although the effect is not consistent over all BCLs at 5 min. Analysis of beat-to-beat differences in CV revealed no evidence of CV alternans during treatment with FCCP.

Disruption of ATP production through mitochondrial uncoupling and oxidation is one of the main consequences of ischemia. To understand whether uncoupling of mitochondria with FCCP produces similar electrophysiological changes in the APD and [Ca^{2+}], as in no-flow global ischemia, we performed additional experiments in which no-flow global ischemia was induced in the isolated rabbit heart. Similar to FCCP, ischemia
induced alternans in APD, CaD, and CaA. However, we were not able to directly calculate B_{Onset} since all alternans were present at the largest BCL (300 ms). Figure 5 compares changes in APD and CaD at different BCLs caused by 10 min of FCCP treatment (Fig. 5, A and B) and 10 min of global no-flow ischemia (Fig. 5, C and D). We observed that both FCCP (Fig. 5A) and ischemia (Fig. 5C) significantly increase CaD only at large BCLs and have no effect on CaD at faster pacing rates. On the other hand, Fig. 5, B and D, illustrates a significant reduction of APD at all BCLs caused by FCCP and ischemia, respectively. Note that the significant reduction of APD and increase of APD heterogeneity by global no-flow ischemia was previously demonstrated (34, 49). We observed that the effect of FCCP treatment of APD reduction is qualitatively similar to global ischemia.

These data are further quantified in Fig. 6, where the relative change in CaD (ΔCaD) and APD (ΔAPD) compared with those in control conditions are shown at different values of BCL. Figure 6A shows that FCCP and global ischemia cause very similar increases in CaD across all BCLs. For instance, at BCL = 220 ms, the relative increase in CaD caused by FCCP is similar to that caused by ischemia (ΔCaD_{FCCP} = 4.5 ± 0.7 vs. ΔCaD_{ischemia} = 4 ± 1.3, P = NS). In contrast, Figure 6B shows that even though FCCP and ischemia both significantly reduced the APD across all BCLs, the relative reduction during ischemia (ΔAPD_{ischemia}) was significantly larger than the one during FCCP (ΔAPD_{FCCP}). For instance, at BCL = 220 ms, the reduction in APD was much larger during ischemia than during FCCP treatment (ΔAPD_{ischemia} = −25 ± 2.3 vs. ΔAPD_{FCCP} = −6.6 ± 1.5, P < 0.05). In addition, we investigated the change in CV caused by ischemia. At the lowest BCL = 150 ms, CV is significantly reduced during 5 min (CV = 0.48 ± 0.06 m/s, P < 0.05) and 10 min (CV = 0.4 ± 0.03 m/s, P < 0.05) of ischemia, compared with that control conditions (CV = 0.77 ± 0.02 m/s). Therefore, CV is significantly reduced during ischemic conditions, as has been previously reported (13, 32).

**DISCUSSION**

In this article, we examined the role of FCCP in the formation of APD and [Ca^{2+}], alternans in the whole rabbit heart.
The main findings of this study are the following: 1) treatment with FCCP facilitates early onset of both APD and \([\text{Ca}^{2+}]_i\) alternans in the heart; 2) FCCP significantly increases intraventricular heterogeneity in APD but not in CaD and significantly reduces the CV of electrical propagation in the heart; 3) uncoupling the mitochondrial network increases CaD at higher BCLs, similar to global ischemia; and 4) uncoupling the mitochondrial network significantly reduces APD at all BCLs; however, this effect is significantly smaller than the one caused by global ischemia. Therefore, uncoupling the mitochondrial network, similar to global ischemia, facilitates APD and \([\text{Ca}^{2+}]_i\) alternans formation in the heart, which in turn creates a substrate conducive to formation of ventricular arrhythmias.

FCCP uncouples the mitochondria at low concentrations and increases oxygen consumption by disrupting the proton gradient that is required for electron transport (2, 9, 61). This is similar to what occurs during myocardial ischemia. During the early phase of ischemia, electron transport and the ejection of \(\text{H}^+\) in the mitochondria is ceased (5). Moreover, the damaged mitochondria cannot efficiently transfer electrons through the electron chain, which, in the long run, damages mitochondrial proteins (5). As an immediate consequence, the electrochemical gradient necessary for ATP synthesis to occur is insufficient to maintain the energy demands of the cell, resulting in depolarization of the mitochondrial membrane, which causes a significant drop in ATP and an increase in \([\text{Ca}^{2+}]_i\).

Fig. 4. Effects of 50 nM FCCP on intraventricular heterogeneity and conduction velocity (CV). A: intraventricular APD heterogeneity (\(\mu_{\text{APD}}\)). B: intraventricular CaD heterogeneity (\(\mu_{\text{CaD}}\)). C: representative example of action potential activation maps for different conditions at BCL = 130 ms. D: relative change of CV (\(\Delta\text{CV}\)) for different BCLs during 5 and 10 min of treatment with FCCP. Isochrones for D are shown 5 ms apart. \(*P < 0.05\), statistical significance with control.

Fig. 5. Effects of 10 min of treatment with 50 nM FCCP and 10 min of no-flow global ischemia on APD and CaD. A: mean CaD for control and FCCP at different BCLs. B: mean APD for control and FCCP at different BCLs. C: mean APD for control and ischemia at different BCLs. D: mean APD for control and ischemia at different BCLs. \(*P < 0.05\), statistical significance with control.
Any factor that diminishes Ca\textsuperscript{2+} sequestrations generates favorable conditions for calcium alternans to occur (24). Ca\textsuperscript{2+} sequestration normally occurs against an electrochemical gradient, thereby requiring the consumption of ATP. Therefore, a reduction in the production of ATP by the mitochondria is usually followed by impairment of Ca\textsuperscript{2+} removal from the cytosol. Indeed, it has been shown that mitochondria accumulate Ca\textsuperscript{2+} when exposed to higher frequencies of Ca\textsuperscript{2+} transients (19, 28, 44). However, this elevated Ca\textsuperscript{2+} in the mitochondrial matrix will eventually reduce the electrochemical gradient for mitochondrial Ca\textsuperscript{2+} uptake.

Previous studies have investigated the effects of mitochondrial stress and depolarization in the isolated myocytes and whole hearts. For instance, Brown et al. (11) reported that collapse of the mitochondrial potential mediated by diamate treatment caused increased occurrences of arrhythmias and initiated mechanical dysfunction caused by marked increase in glutathione disulfide (35, 37). They also reported that prevention of the mitochondrial potential collapse afforded protection from electromechanical dysfunction in the guinea pig hearts. Similar results were reported by Akar et al. (1), who demonstrated that preventing mitochondrial stress and depolarization using MBzR prevents the occurrences of spontaneous arrhythmias upon reperfusion in the heart. These reports support the hypothesis that ischemia-reperfusion-related electrophysiological alterations and arrhythmias in intact hearts are in part a consequence of the failure of the cellular mitochondrial network to maintain the mitochondrial membrane potential. Although these studies show that mitochondrial stress is an important factor, they have not looked at the development of abnormal rhythms like alternans during ischemia.

In recent study, Florea and Blatter (24) investigated the role of mitochondria in alternans formation in isolated cat atrial myocytes and demonstrated that any intervention that interferes with mitochondrial ATP production or mitochondrial Ca\textsuperscript{2+} buffering, using various pharmaceutical agents, enhanced Ca\textsuperscript{2+} alternans. Similar to our experiments (Figs. 2 and 3), after treatment with FCCP, there was an increase of CaD and CaA alternans. However, they did not examine what occurs in voltage with each intervention. To our knowledge, our study is the first to show that uncoupling the mitochondria, similar to what occurs during ischemia, facilitates the formation of APD and [Ca\textsuperscript{2+}]i alternans in the whole heart. Therefore, it is plausible that during ischemia, uncoupling of the mitochondria alone contributes to proarrhythmic alternans. It is important to note that although FCCP places mitochondria under a lot of stress and causes mitochondrial uncoupling at the concentrations used in this study, another consequence of FCCP involves the accumulation of protons, thereby creating acidic conditions in the heart. Since acidosis is present very early during ischemia and it facilitates calcium alternans in the heart, as has been previously described (31), acidosis may be an alternative mechanism through which FCCP promotes calcium and APD alternans in the heart.

It has been previously shown that at the single cell level, small concentrations (30–100 nM) of FCCP are cardioprotective (9, 10). However, our study in the whole rabbit heart (57) suggested that pretreatment of the heart with FCCP at these concentrations before no-flow global ischemia is arrhythmogenic. The mechanism of arrhythmogenesis is suggested to be interventricular heterogeneity, which cannot be evaluated at the single-cell level. In our study, we used 50 nM FCCP to uncouple the mitochondrial network, but it is feasible that different concentrations of FCCP may provide a different effect on both voltage and calcium and needs to be further investigated.

The effect of no-flow global ischemia on the heart has been studied in detail, whereas the effect of uncoupling the mitochondria has not received a lot of attention. Previous studies have shown that ischemia reduces APD and increases spatial dispersion of refractoriness by increasing heterogeneity of APD (34, 38), and it has been suggested that these significant changes can occur within the first 5–10 min of interruption of perfusion (1). These studies have also shown that ischemia also affects calcium dynamics by increasing the Ca\textsuperscript{2+} in a more homogenous way. Our results show that uncoupling the mitochondria has similar effects to the one from no-flow global ischemia in isolated hearts. FCCP treatment led to a lengthening of the [Ca\textsuperscript{2+}]i transients without any increase in its 2-D dispersion and reduction of CV of impulse propagation. Furthermore, our results also show that FCCP treatment increased the heterogeneity of APD at both 5 and 10 min, although the APD shortening effect is much milder than that caused by ischemia at the same point. The direct comparison of B\textsubscript{Onset} between FCCP and ischemia was not possible due to the fact that during ischemia, APD, CaD, and CaA alternans occurred at the beginning of the pacing, at BCL = 300 ms. The discrepancy between the onset of alternans caused by FCCP and ischemia can be attributed to the concentration and time course of FCCP treatment. It is possible that a higher concentration or longer time of mitochondria uncoupling could produce similar effects as 10 min of no-flow global ischemia. In this study, we used 10 min of 50 nM of FCCP in accordance with previous studies also using FCCP (9, 10). Another pos-
possible explanation is that other electrophysiological changes during ischemia, such as hyperkalemia, acidosis, etc. (54, 55), may cause a larger effect on the membrane voltage than on [Ca$^{2+}$], alternans.

In our previous study (57), we found that FCCP pretreatment increased interventricular heterogeneity during no-flow global ischemia, which we suggested caused ventricular fibrillation. It is possible that ventricular fibrillation occurred because of the formation of alternans during treatment with FCCP, although we did not look at this phenomenon in detail. In our current study, we compared electrophysiological parameters between 10 min of treatment with FCCP and 10 min of no-flow global ischemia (Figs. 5 and 6). We found similarities in the electrophysiological effects of both conditions, which suggest that uncoupling the mitochondria may lead to alternans formation during ischemia. Our data also suggest that even though voltage is affected more than calcium when the mitochondria is uncoupled, the effect of FCCP treatment on membrane voltage is milder than that of ischemia. However, more studies need to be done to understand the exact cellular mechanism of APD and [Ca$^{2+}$], alternans formation.

**Conclusion**

FCCP, at a concentration of 50 nM, facilitates APD and [Ca$^{2+}$], alternans in the whole rabbit heart. We suggest that during myocardial ischemia, uncoupling the mitochondria might be one of the important mechanisms capable of creating a proarrhythmic substrate more conducive to development of fatal or severe ventricular arrhythmias.

**Limitations**

In this study, we investigate the effect of mitochondrial stress on the alternans formation in the heart using FCCP. Although it is known that FCCP causes mitochondrial stress and uncoupling at low concentrations by affecting proton conductance across the mitochondrial membrane, it is still debatable and unclear whether they have similar effects on the plasma membrane. Indeed, plasma membrane changes were reported in other types of cell such as endothelial cell (45), astrocytes, and neurons (30). Hence, there remains the possibility that electrophysiological changes in the FCCP-treated heart that facilitates alternans formation may be partly caused by FCCP-induced changes in the plasma membrane.

In this study, we assume that within a single downsweep pacing protocol (2.5 min), the conditions of no-flow ischemia and FCCP treatment remain the same. However, it is important to note that since no-flow ischemia is such a dynamic process, it is difficult to attain true steady state. While we believe we have overcome this by implementing protocols at different time points, we would like to address this as a potential limitation. In addition, we would like to address some limitations associated with the global ischemic model used in our experiments, although it has been well accepted for optical mapping studies so far (34, 49, 50, 60, 62). Interruption of coronary flow creates ischemic conditions in the intramural muscle layers; however, superfusion with deoxygenated Tyrode solution might reduce the degree of ischemia on the epicardium relative to intramural layers. In addition, the diffusion through the epicardial surface layer might reduce potassium accumulation and acidosis, and therefore not all hallmark of ischemia might be fully present at the superficial epicardial surface. However, this diffusion is most likely limited, so that the relevant ion concentrations of K$^+$, H$^+$, lactate, etc., remain elevated.

Moreover, it is important to note that Rhod-2 may accumulate in organelles that are capable of storing Ca$^{2+}$ (52), such as the mitochondria. However, this effect is negligible at the small concentration of the dye that was used in this study, and therefore it is likely the change of fluorescence we observe is due to cytoplasmic Ca$^{2+}$ and not the Ca$^{2+}$ in the matrix of the mitochondria or any other organelle.

**ACKNOWLEDGMENTS**

We thank Dr. Xueyi Xie, Joseph Ippolito, and Stephen McIntyre for the careful reading of the manuscript.

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