Slow Ca^{2+} Efflux by Ca^{2+}/H$^+$ Exchange in Cardiac Mitochondria Is Modulated by Ca^{2+} Re-uptake via MCU, Extra-Mitochondrial pH, and H$^+$ Pumping by F$_O$F$_1$-ATPase

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Mitochondrial (m) Ca^{2+} influx is largely dependent on membrane potential ($\Delta\Psi_m$), whereas mCa^{2+} efflux occurs primarily via Ca^{2+} ion exchangers. We probed the kinetics of Ca^{2+}/H$^+$ exchange (CHE$_m$) in guinea pig cardiac muscle mitochondria. We tested if net mCa^{2+} flux is altered during a matrix inward H$^+$ leak that is dependent on matrix H$^+$ pumping by ATP$_m$ hydrolysis at complex V (F$_O$F$_1$-ATPase). We measured [Ca^{2+}]$_m$, extra-mitochondrial (e) [Ca^{2+}]$_e$, $\Delta\Psi_m$, pH$_m$, pH$_e$, NADH, respiration, ADP/ATP ratios, and total [ATP]$_m$ in the presence or absence of protonophore dinitrophenol (DNP), mitochondrial uniporter (MCU) blocker Ru360, and complex V blocker oligomycin (OMN). We proposed that net slow influx/efflux of Ca^{2+} after adding DNP and CaCl$_2$ is dependent on whether the ΔpH$_m$ gradient is/is not maintained by reciprocal outward H$^+$ pumping by complex V. We found that adding CaCl$_2$ enhanced DNP-induced increases in respiration and decreases in $\Delta\Psi_m$ while [ATP]$_m$ decreased, ΔpH$_m$ gradient was maintained, and [Ca^{2+}]$_m$ continued to increase slowly, indicating net mCa^{2+} influx via MCU. In contrast, with complex V blocked by OMN, adding DNP and CaCl$_2$ caused larger declines in $\Delta\Psi_m$ as well as a slow fall in pH$_m$ to near pH$_e$ while [Ca^{2+}]$_m$ continued to decrease slowly, indicating net mCa^{2+} efflux in exchange for H$^+$ influx (CHE$_m$) until the ΔpH$_m$ gradient was abolished. The kinetics of slow mCa^{2+} efflux with slow H$^+$ influx via CHE$_m$ was also observed at pH$_e$ 6.9 vs. 7.6 by the slow fall in pH$_m$ until ΔpH$_m$ was abolished; if Ca^{2+} reuptake via the MCU was also blocked, mCa^{2+} efflux via CHE$_m$ became more evident. Of the two components of the proton electrochemical gradient, our results indicate that CHE$_m$ activity is driven largely by the ΔpH$_m$ chemical gradient with H$^+$ leak, while mCa^{2+} entry via MCU depends largely on the charge gradient $\Delta\Psi_m$. A fall in $\Delta\Psi_m$ with excess mCa^{2+} loading can occur during cardiac cell stress.

Cardiac cell injury due to mCa^{2+} overload may be reduced by temporarily inhibiting F$_O$F$_1$-ATPase from pumping H$^+$ due to $\Delta\Psi_m$ depolarization. This action would prevent additional slow mCa^{2+} loading via MCU and permit activation of CHE$_m$ to mediate efflux of mCa^{2+}.

HIGHLIGHTS

- We examined how slow mitochondrial (m) Ca^{2+} efflux via Ca^{2+}/H$^+$ exchange (CHE$_m$) is triggered by matrix acidity after a rapid increase in [Ca^{2+}]$_m$ by adding CaCl$_2$ in the presence of dinitrophenol (DNP) to permit H$^+$ influx, and oligomycin (OMN) to block H$^+$ pumping via F$_O$F$_1$-ATP synthase/ase (complex V).
- Declines in $\Delta\Psi_m$ and pH$_m$ after DNP and added CaCl$_2$ were larger when complex V was blocked.
- [Ca^{2+}]$_m$ slowly increased despite a fall in $\Delta\Psi_m$ but maintained pH$_m$ when H$^+$ pumping by complex V was permitted.
- [Ca^{2+}]$_m$ slowly decreased and external [Ca^{2+}]$_e$ increased with declines in both $\Delta\Psi_m$ and pH$_m$ when complex V was blocked.
- ATP$_m$ hydrolysis supports a falling pH$_m$ and redox state and promotes a slow increase in [Ca^{2+}]$_m$.
- After rapid Ca^{2+} influx due to a bolus of CaCl$_2$, slow mCa^{2+} efflux by CHE$_m$ occurs directly if pH$_e$ is low.

Keywords: cardiac mitochondria, Ca^{2+} uptake/release, mitochondrial Ca^{2+} uniporter, Ca^{2+}/H$^+$ exchange, H$^+$ leak and pumping, complex V

INTRODUCTION

Mitochondrial (m) Ca^{2+} overload is a damaging consequence of cardiac ischemia-reperfusion (IR) injury and hence is an important subject for potential therapy (Brookes et al., 2004; O'Rourke et al., 2005; Stowe and Camara, 2009; Camara et al., 2010). During IR, mitochondria can consume rather than generate ATP (Chinopoulos and Adam-Vizi, 2010; Chinopoulos et al., 2010), which consequently can augment mCa^{2+} overload (Riess et al., 2002) sufficient to induce cell apoptosis and necrosis (Murphy and Steenbergen, 2008). [Ca^{2+}]$_m$ is regulated in part by electrochemical dependent cation flux via Ca^{2+} transporters and by cation exchangers within the inner mitochondrial membrane (IMM) (Gunter and Pfeiffer, 1990; Gunter et al., 1994; Bernardi, 1999; Brookes et al., 2004). The major route for mCa^{2+} uptake is via the ruthenium red (RR) sensitive mitochondrial Ca^{2+} uniporter (MCU), now considered a macromolecular complex composed of two pore components, MCU and MCUb, and MCU regulators MCU1, 2, 3, and EMRE (essential MCU regulator), and other components (De Stefani et al., 2015). Ca^{2+} influx via the MCU is reduced by competition with cytosolic Mg^{2+} (Boelens et al., 2013; Tewari et al., 2014). Additional modes of mCa^{2+} uptake are proposed to occur via a ryanodine type channel (RTC) in the IMM (Ryu et al., 2011; O-Uchi et al., 2013; Tewari et al., 2014) and at the sarcoplasmic reticular-MCU interface where functional Ca^{2+} signaling between the cytoplasmic and mitochondrial compartments is believed to occur (Csordas et al., 2010).

A primary mCa^{2+} efflux pathway is the Na$^+$/Ca^{2+} exchanger (NCE$_m$) (Boyman et al., 2013). In unicellular organisms and in some non-cardiac tissues there is firm evidence (Azzone et al., 1977; Pozzan et al., 1977; Wingrove et al., 1984; Brand, 1985; Rottenberg and Marbach, 1990; Gunter et al., 1991, 1994; Bernardi, 1999; Demaurex et al., 2009; Nishizawa et al., 2013) for slow homeostatic mCa^{2+} efflux through a Na$^+$-independent Ca^{2+} exchanger (NICE), i.e., a non-electrogenic Ca^{2+}/H$^+$ exchanger (CHE) that might be activated when the ΔpH$_m$ gradient across the IMM is altered. The amount of free (ionized) [Ca^{2+}]$_m$ available for exchange depends on the extent of dynamic mCa^{2+} buffering (Bazil et al., 2013; Blomeyer et al., 2013; Tewari et al., 2014). mCa^{2+} influx via the MCU and efflux via the NCE$_m$ are largely voltage ($\Delta\Psi_m$) dependent, whereas Ca^{2+} transport via the CHE$_m$, while pH-dependent, may be electrogenic (1 H$^+$ for 1 Ca^{2+}) or non-electrogenic (2 H$^+$ for 1 Ca^{2+}). However, CHE$_m$ can be indirectly dependent on the full IMM electrochemical gradient if there is a decrease in the IMM ΔpH$_m$ gradient (Rottenberg and Marbach, 1990; Dash and Beard, 2008; Dash et al., 2009).

There is a well-known direct correlation between $\Delta\Psi_m$ and mCa^{2+} uptake based on the Nernst equation; a more polarized $\Delta\Psi_m$ permits greater mCa^{2+} uptake (Wingrove et al., 1984; Gunter et al., 1994). mCa^{2+} uptake via the MCU depends both on the electrical (charge) gradient, $\Delta\Psi_m$, and on the concentration gradient for [Ca^{2+}] across the IMM. ATP$_m$ hydrolysis with H$^+$ pumping can occur at complex V (F$_O$F$_1$-ATPsynthase/ase) during cardiac ischemia (Jennings et al., 1991) in an attempt to maintain the ΔpH$_m$ gradient, and along with the $\Delta\Psi_m$

gradient (Chinopoulos and Adam-Vizi, 2010; Chinopoulos, 2011), equals the proton motive force, *pmf*. However, it is not known how the magnitude, rate, and route of mCa^{2+} uptake or release in cardiac muscle cell mitochondria is affected by manipulating the IMM $\Delta[H^+]_m$ gradient by allowing mATP hydrolysis, which would result in H$^+$ pumping and better maintain the $\Delta[H^+]$ gradient when $\Delta\Psi_m$ is low, vs. blocking mATP hydrolysis (no H$^+$ pumping with collapsing $\Delta[H^+]$) and lower $\Delta\Psi_m$.

Exposure of mitochondria to external (e) CaCl$_2$ when the IMM is fully charged (high $\Delta\Psi_m$), defined here by the presence of substrate in state 2 conditions without an induced inward H$^+$ leak, promotes rapid voltage-dependent mCa^{2+} uptake via MCU (Hoppe, 2010). In contrast, decreased net mCa^{2+} uptake might be expected during a protonophore-induced inward H$^+$ leak if H$^+$ influx leads to Ca^{2+} efflux. However, an inward H$^+$ flux that slowly decreases $\Delta\Psi_m$ can still result in a slow, continued uptake of mCa^{2+} via the MCU if there remains sufficient $\Delta\Psi_m$ and Ca^{2+} chemical gradient ($[Ca^{2+}]_e > [Ca^{2+}]_m$) across the IMM. mCa^{2+} influx via the MCU can partially depolarize $\Delta\Psi_m$ (Delcamp et al., 1998; Di Lisa and Bernardi, 1998) due to the influx of positive charges without an effect on the $\Delta[H^+]_m$, and more so with a fall in $\Delta[H^+]_m$ gradient from the added influx of H$^+$ in the presence of a protonophore.

Our aim was to mechanistically examine the slow mode kinetics of mCa^{2+} influx/efflux in cardiac cell mitochondria. The conditions under which CHE$_m$ may occur in cardiac mitochondria are unknown. We proposed that an induced, net influx of H$^+$ is coupled to net mCa^{2+} efflux by activation of CHE$_m$ in the face of continued mCa^{2+} uptake via the MCU in partially depolarized $\Delta\Psi_m$ mitochondria. In addition, if the extra-mitochondrial milieu is acidic, pH$_m$ would slowly decrease as mH$^+$ entry by mCHE$_m$ is exchanged for mCa^{2+} efflux in Ca^{2+} overloaded mitochondria. We postulated that CHE$_m$ is activated under conditions of slow a H$^+$ influx and a high m[Ca^{2+}], and especially when H$^+$ pumping by complex V, stimulated by the lowered $\Delta\Psi_m$, is prevented. To carry out our aim, we examined the time dependent changes in $\Delta\Psi_m$, [Ca^{2+}]$_m$ and pH$_m$, and extra-mitochondrial [Ca^{2+}]$_e$ and pH$_e$, after a bolus of CaCl$_2$ either by inducing an inward H$^+$ leak that causes an outward pumping of H$^+$ by complex V, or by altering the extra-mitochondrial pH$_e$.

In one set of experiments, we challenged isolated energized mitochondria with a bolus of CaCl$_2$ in the absence or presence of increasing concentrations of the protonophore 2,4-dinitrophenol (DNP) in the absence or presence of the complex V inhibitor oligomycin (OMN) to block ATP hydrolysis-induced H$^+$ pumping, and or Ru360 to block the reuptake of Ca^{2+} via the MCU. To understand how DNP, OMN, and Ru360 dynamically alter [Ca^{2+}]$_m$ or [Ca^{2+}]$_e$ after a bolus of CaCl$_2$, we considered it crucial to also dynamically measure $\Delta\Psi_m$, pH$_m$, and NADH, as well as mitochondrial respiration (extent of uncoupling), total [ATP]$_m$, and ATP$_m$/ADP$_m$ ratio. In another set of isolated mitochondrial experiments, we directly induced

mCa^{2+} efflux via CHE$_m$ after CaCl$_2$ loading by altering the Na$^+$-free medium from a control pH$_e$ of 7.15 to either pH 7.6 or 6.9. We show that secondary Ca^{2+} influx vs. efflux is $\Delta[H^+]_m$ dependent.

MATERIALS AND METHODS

Isolated Mitochondrial Experiments

All experiments conformed to the Guide for the Care and Use of Laboratory Animal and were approved by the Medical College of Wisconsin Biomedical Resource Center animal studies committee. Detailed methods for mitochondrial isolation and measurements of $\Delta\Psi_m$, [Ca^{2+}]$_m$, NADH redox state, pH$_m$, [ATP]$_m$, ADP$_m$/ATP$_m$ ratio, respiration, and the number of animals per group, are furnished (see section **"Supplementary Materials S.1.1–S.1.12"**). Briefly, mitochondria were isolated from guinea pig heart ventricles in iced buffer and were suspended in experimental buffer containing in mM: KCl 130, K$_2$HPO$_4$ 5, MOPS 20, bovine serum albumin 0.016 and EGTA ∼0.036–0.040 at pH 7.15 (adjusted with KOH) at room temperature (21°C). The experimental buffer had a final protein concentration of 0.5 mg/mL. Specific fluorescent probes and spectrophotometry (Qm-8, Photon Technology International, Birmingham, NJ, United States) were used to measure [Ca^{2+}]$_m$ (indo-1AM) and buffer [Ca^{2+}]$_e$ (indo-1 or Fura 4 F penta-K$^+$ salt), NADH, an indicator of mitochondrial redox state (autofluorescence), pH$_m$ (BCECF-AM), and mitochondrial membrane potential ($\Delta\Psi_m$) assessed by rodamine-123 or TMRM (Heinen et al., 2007; Huang et al., 2007; Aldakkak et al., 2010; Haumann et al., 2010) (all fluorescence probes from Invitrogen^{TM} – Thermo Fisher Scientific). Respiration (Clark electrode) and ATP$_m$ (bioluminescence) and ATP$_m$/ADP$_m$ ratio (HPLC, luminometry) were also measured. The experimental buffer, mitochondrial substrates, and drugs were Na$^+$-free to prevent activation of NCE$_m$ by extra-mitochondrial Na$^+$. The inactivity of the NCE was verified by comparing data from these experiments to data from experiments with added CGP-37157, a known mitochondrial NCE$_m$ inhibitor (data not shown).

Experimental Protocols
Medium pH$_e$-Induced Changes in pH$_m$
The experimental buffer was identical to that described above except that in addition to the pH 7.15 buffer, buffers at pH 6.9 and 7.6 were prepared by titration with HCl and KOH, respectively. The residual EGTA carried over from the isolation buffer to the experimental buffer resulted in an ionized extra-mitochondrial [Ca^{2+}]$_e$ of <200 nM (**Figure 1**). To measure changes in [Ca^{2+}]$_e$ after adding a bolus of 40 μM CaCl$_2$, each pH buffer contained Fura 4 F penta-K$^+$ salt. The K$_D$'s for Ca^{2+} were calculated and corrected for each buffer pH because pH affects the binding of Ca^{2+} to the fluorescence dye (see section **"Supplementary Materials S.1.4, S.1.8"**). In other experiments, pH$_m$ and $\Delta\Psi_m$ were measured using BCECF-AM and TMRM fluorescent dyes, respectively. Experiments were initiated at $t = 30$ s when mitochondria were added to the
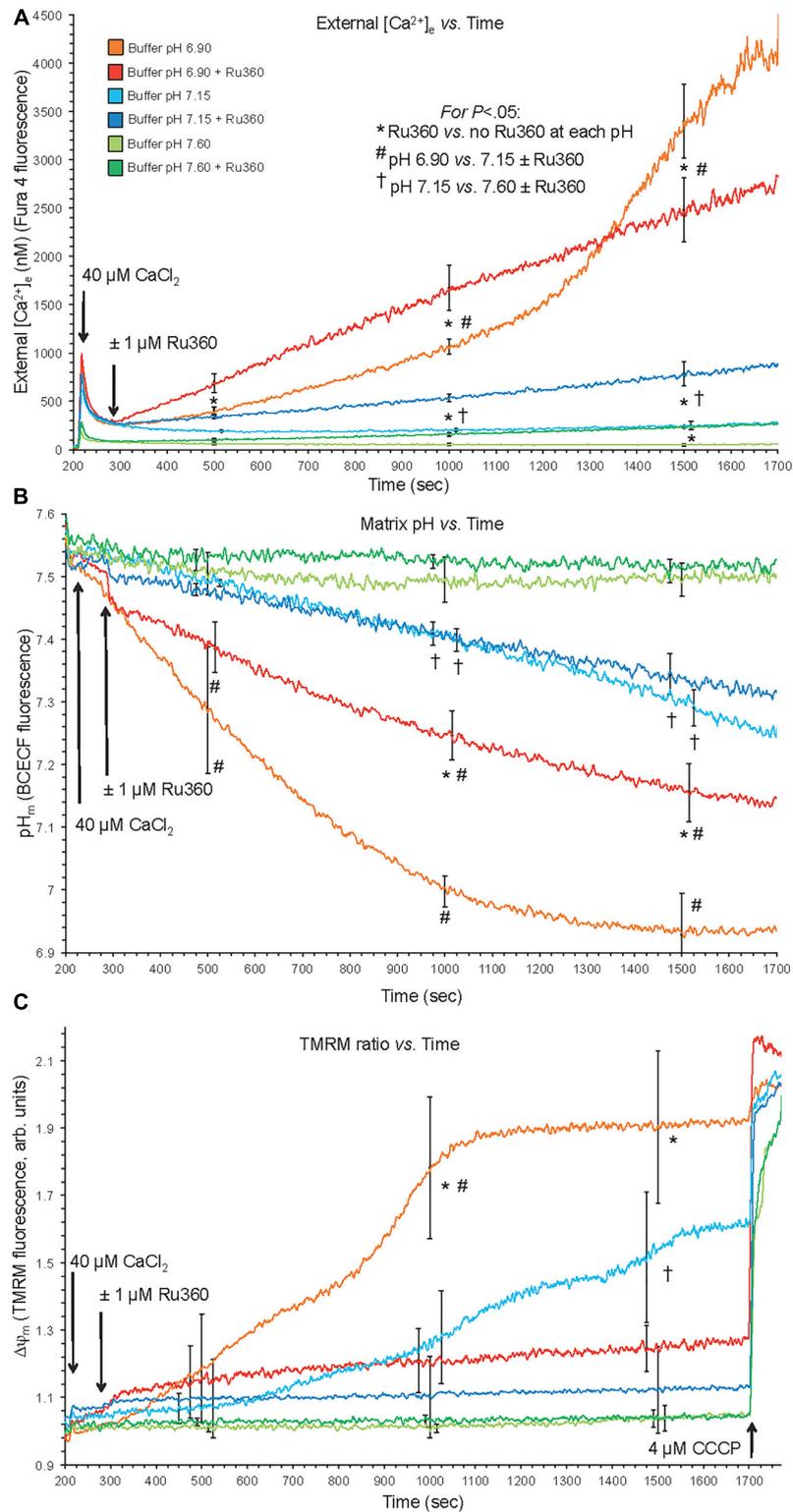
FIGURE 1 | Changes in buffer $[Ca^{2+}]_e$ **(A)**, matrix pH_m **(B)**, and $\Delta\Psi_m$ **(C)** over time after adding 40 μM CaCl₂ (210 s) at extra-mitochondrial pH_e 7.6, 7.15, and 6.9 with or without 1 μM Ru360 (300 s) to inhibit additional mCa^{2+} uptake via MCU. Note the rapid fall in $[Ca^{2+}]_e$ due to fast mCa^{2+} uptake via the MCU and the following slow rise in $[Ca^{2+}]_e$ (Ca^{2+} efflux) **(A)**, slow decline in pH_m **(B)**, and slow depolarization of $\Delta\Psi_m$ **(C)** at pH 6.9 (each line = mean of 3–4 replicates from 12 guinea pig hearts for each fluorescence measurement). Note in the pH 6.9 medium the faster rate of mCa^{2+} efflux **(A)** over time when MCU was blocked, and the faster declines in pH_m **(B)** and $\Delta\Psi_m$ **(C)** over time when MCU was not blocked.

FIGURE 2 | Plots of extra-mitochondrial [Ca^{2+}]$_e$ as a function of [H$^+$]$_m$ from the mean data of **Figures 1A,B**. Arrows denote the difference in net mCa^{2+} efflux at a given [H$^+$]$_m$ when MCU was or was not blocked with Ru360 given 90 s after the CaCl$_2$ bolus. While mCa^{2+} was extruded by CHE$_m$, much of it re-entered via the MCU when it was not blocked. At trans-membrane pH equilibrium (pH 6.9) Ca^{2+} uptake via MCU stopped as the $\Delta\Psi_m$ was nearly depolarized having resulted in a large net extrusion of Ca^{2+}.

buffer; at $t = 90$ s pyruvic acid (PA, 0.5 mM) was added, followed by a bolus of 40 µM CaCl$_2$ at $t = 210$ s to initiate rapid mCa^{2+} uptake via MCU. Note that in gui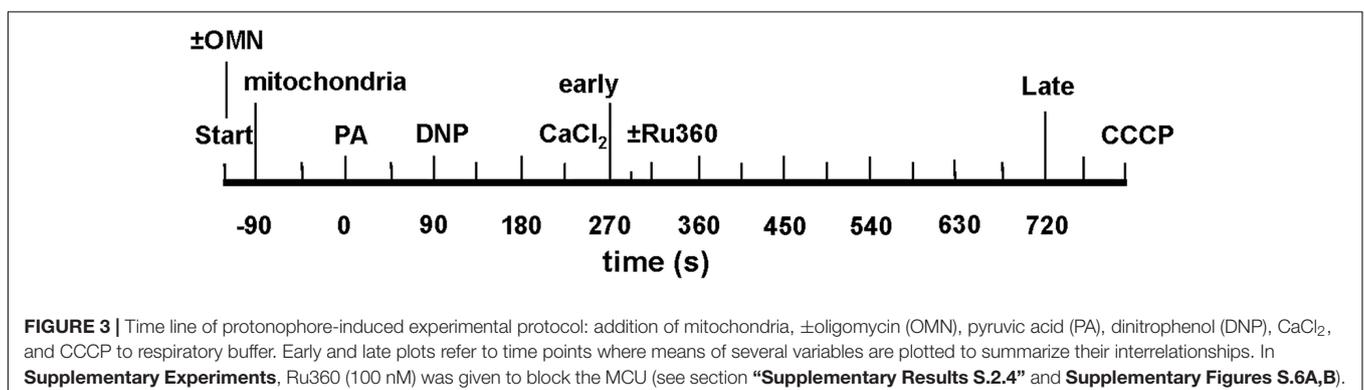nea pig cardiac mitochondria, the respiratory control index (RCI) is higher in the presence of pyruvate alone (Heinen et al., 2007; Blomeyer et al., 2013; Boelens et al., 2013) than with pyruvate plus malate (Riess et al., 2008). For some experiments, 1 µM Ru360 (or vehicle, 0.1% DMSO) was added at $t = 300$ s shortly after adding CaCl$_2$ to block Ca^{2+} reuptake into mitochondria via MCU after the Ca^{2+} was extruded from mitochondria. At the end (1700 s) of each experiment, the potent protonophore, carbonyl cyanide m-chlorophenyl hydrazone (CCCP, 4 µM) was given to completely abolish the ΔpH gradient and depolarize $\Delta\Psi_m$. Data for each pH group were collected in mitochondrial suspensions from the same heart; approximately 8–10 hearts were used for each fluorescent probe. At pH 7.15, adding 40 µM CaCl$_2$, which increased extra-mitochondrial [Ca^{2+}]$_e$ into the

1 µM range and increased the initial [Ca^{2+}]$_m$ to approximately 500 nM (**Figures 1**, **2**), is unlikely to induce membrane permeability transition pore (mPTP) opening. However, to test the possibility of mPTP opening, 500 nM cyclosporine A (CsA), a modulator of cyclophilin D required to open mPTP, was given before adding CaCl$_2$ in several experiments at pH$_e$ 6.9 and 7.15.

Protonophore-Induced Changes in pH$_m$

Experiments were initiated at $t = -120$ s; at $t = -90$ s, mitochondria were added to the experimental buffer (time line, **Figure 3**); external pH$_e$ was 7.15. At $t = 0$ s, pyruvic acid (PA, 0.5 mM) was added to the mitochondria suspended in the experimental buffer, followed by 0, 10, 20, 30, or 100 µM DNP, a mild protonophore, at $t = 90$ s, followed by the addition of de-ionized H$_2$O, 10, or 25 µM CaCl$_2$ at $t = 225$ s. The 90 s period allowed for full $\Delta\Psi_m$ polarization

FIGURE 3 | Time line of protonophore-induced experimental protocol: addition of mitochondria, ±oligomycin (OMN), pyruvic acid (PA), dinitrophenol (DNP), CaCl$_2$, and CCCP to respiratory buffer. Early and late plots refer to time points where means of several variables are plotted to summarize their interrelationships. In **Supplementary Experiments**, Ru360 (100 nM) was given to block the MCU (see section **"Supplementary Results S.2.4"** and **Supplementary Figures S.6A,B**).
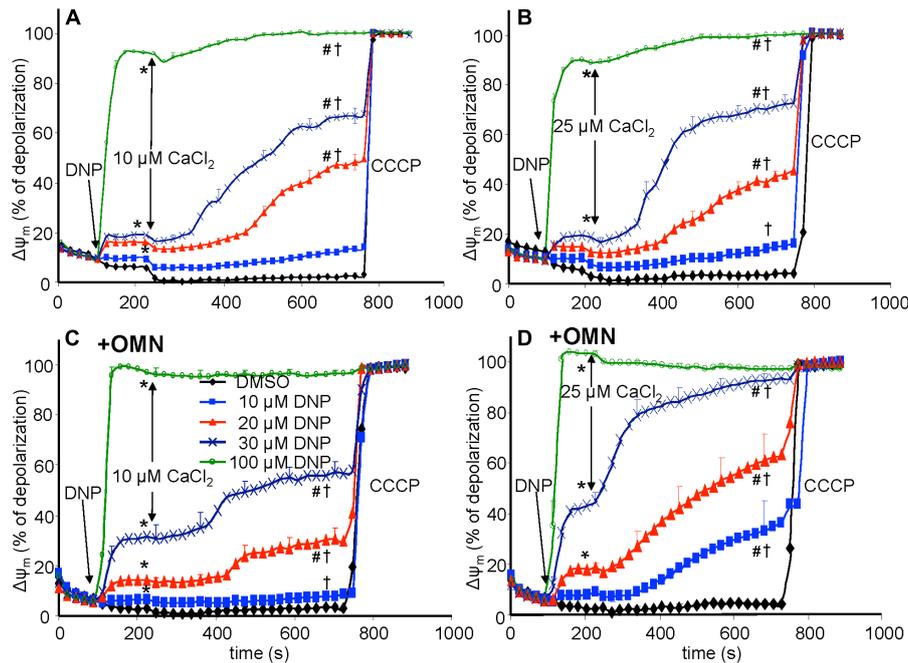
FIGURE 4 | Change in mitochondrial membrane potential ($\Delta\Psi_m$), assessed with rhodamine-123 as % of maximal depolarization, as a function of time after adding dinitrophenol (DNP) and CaCl₂ in the absence **(A,B)** and presence **(C,D)** of oligomycin (OMN). Note that adding DNP caused a concentration-dependent fall in $\Delta\Psi_m$ that was more pronounced in the presence of OMN. Adding CaCl₂ caused a small polarization, while increasing depolarization occurred over time. Adding 25 μM CaCl₂ **(B,D)** resulted in a more pronounced fall in $\Delta\Psi_m$ compared to 10 μM CaCl₂ **(A,C)**. Buffer pH = 7.15. Data obtained from 10 hearts with 4–5 replicates per heart. For $P < 0.05$: *after DNP vs. before DNP; #after CaCl₂ vs. before CaCl₂; †late (700 s) vs. early (215 s) after CaCl₂.

and stabilization of pH$_m$ and NADH. In some experiments (see section "**Supplementary Results S.2.4**" and **Supplementary Figure S.6**), 100 nM Ru360 was added at t = 300 s, after the addition of CaCl₂, to block any reuptake of mCa²⁺ by the MCU that was extruded by CHE$_m$. For the OMN treated groups, 10 μM OMN was added to the experimental buffer at the start of the experimental protocol (**Figure 3**). At the end of each experiment CCCP was added at t = 760 s to maximally depolarize $\Delta\Psi_m$. DNP, Ru360, OMN, and CCCP were each dissolved initially in DMSO and then in buffer to yield a final buffer concentration for DMSO of 0.1 to 0.4% (wt/vol). Each drug or DMSO alone was added to a final volume of 10 μL. To test for mPTP opening, CsA was given before adding 20 or 30 μM DNP and 25 μM CaCl₂ in several experiments conducted at pH$_e$ 7.15.

Statistical Analyses

Data were summarized at 500, 1000, and 1500 s (for **Figures 1**, **2**) for external buffer-induced changes in pH$_m$ on [Ca²⁺]$_e$. Data were summarized for protonophore-induced changes in pH$_m$ on [Ca²⁺]$_m$ at 80 s (after adding PA), 215 s (after adding DNP), 275 s (early after adding CaCl₂), and 700 s (late after adding CaCl₂) (e.g., **Figure 4**). All data points were presented and expressed as average ± SEM. Repeated measure ANOVAs followed by a *post hoc* analyses using Student-Newman-Keuls' test was performed to determine statistically significant differences among groups. A *P*-value < 0.05 (two-tailed)

was considered significant. See Figure legends for statistical notations.

RESULTS

CHE$_m$ Activation Was Exposed by Efflux of Ca²⁺ With Influx of H⁺ and Was Greater If MCU Was Inhibited

Direct evidence for CHE$_m$ activation was observed by acidifying the extra-mitochondrial buffer (low pH$_e$), which subsequently decreased the matrix pH$_m$ slowly over time (**Figure 1**). With NCE$_m$ and Na⁺/H⁺ (NHE$_m$) inactivated by using Na⁺-free solutions and substrates, fast mCa²⁺ influx via the MCU, induced after adding 40 μM CaCl₂ at pH 6.9, was followed by a slow mCa²⁺ efflux over time ∼(300–1700 s) as shown by the increase in extra-mitochondrial [Ca²⁺]$_e$ from <200 nM to nearly 4500 nM in the absence of Ru360 (**Figure 1A**). When Ru360 was added 90 s after adding CaCl₂, [Ca²⁺]$_e$ rose even more over the first 1000 s, indicating blockade of Ca²⁺ recycling via the MCU and revealing the total mCa²⁺ effluxed via CHE$_m$. In the pH 6.9 plus Ru360 group the mean rate (slope) of increase in [Ca²⁺]$_e$ (mCa²⁺ efflux) over time (300–1700 s) was 1.5 ± 0.1 nM/s, ΔpH 0.4 units). This was greater than in the pH 6.9 minus Ru360 group (1.0 ± 0.2 nM/s over 300–1000 s), suggesting that approximately 1/3 of the mCa²⁺ extruded was retaken up across the IMM

via the MCU. In contrast, mCa^{2+} efflux was not observed in the pH 7.6 medium without Ru360, and minimally at 1500 s at pH 7.6 with Ru360. There was less mCa^{2+} efflux at pH 7.15 $\pm$ Ru360 compared to pH 6.9 $\pm$ Ru360. However, even at pH 7.15 $\pm$ Ru360, there were similar steady declines in pH$_e$ while net slow Ca^{2+} efflux was noted only in the plus Ru360 groups, indicating Ca^{2+} re-uptake via MCU. Therefore, in the acidic extra-mitochondrial medium, slow decreases in pH$_m$ (H$^+$ influx) were accompanied by slow increases in mCa^{2+} efflux, indicating CHE$_m$ activity. Eventually, matrix acidification was more pronounced in the pH 6.9 medium (ΔpH 0.62 units) in the absence of Ru360 than in all other groups so that over time as H$^+$ influx was exchanged for Ca^{2+} efflux the IMM ΔpH gradient was eventually obliterated, halting Ca^{2+} efflux (**Figure 1B**). Eventually, because of mCa^{2+} influx, near complete depolarization of $\Delta\Psi_m$ occurred in the pH 6.9 medium (**Figure 1C**), as shown by little change after adding CCCP, and by the complete depolarization of $\Delta\Psi_m$ when Ca^{2+} recycling via the MCU was permitted (minus Ru360 group). Although adding CaCl$_2$ at an external pH$_e$ of 6.9 led eventually to near complete dissipation of $\Delta\Psi_m$, when CsA was first added to the buffer, CsA prevented the gradual, slow extrusion of mCa^{2+} and declines in pH$_m$ and $\Delta\Psi_m$ induced by adding CaCl$_2$ at pH$_e$ 6.9 indicating a complete lack of CHE$_m$ activity (see section "**Supplementary Results S.2.1**" and **Supplementary Figures S.1A–C**).

Increasing Matrix Acidification Led to Ca^{2+} Efflux Until Loss of the ΔpH$_m$ Gradient and a Lack of Ca^{2+} Re-uptake via MCU on Full Depolarization of $\Delta\Psi_m$

A plot of extra-mitochondrial [Ca^{2+}]$_e$ as a function of matrix [H$^+$]$_m$ at each extra-mitochondrial pH (**Figure 2**) indicates maximal mCa^{2+} efflux occurred in the pH$_e$ 6.9 medium (largest IMM (ΔH$^+$] gradient), much less so in the pH 7.15 medium, and not at all in the pH 7.6 medium. Ca^{2+} efflux was accentuated in the presence of Ru360 given just after the added CaCl$_2$ bolus (**Figure 2**). The difference (arrow) between the absence and presence of Ru360 indicates the rapid reuptake (recycling) of Ca^{2+} via MCU on extrusion via CHE$_m$. Thus total Ca^{2+} efflux was greater in the pH 6.9 group when MCU was not blocked because [H$^+$]$_m$ rose higher than when MCU was blocked. The steep, vertical increase in mCa^{2+} efflux at the highest [H$^+$]$_m$ in the pH 6.9 group resulted from cessation of mCa^{2+} reuptake via MCU due to depolarization of $\Delta\Psi_m$ (**Figure 1C**). The net amount of H$^+$ entering mitochondria per Ca^{2+} exiting mitochondria may be indeterminate because much of the H$^+$ entering is pumped out via the respiratory enzyme complexes.
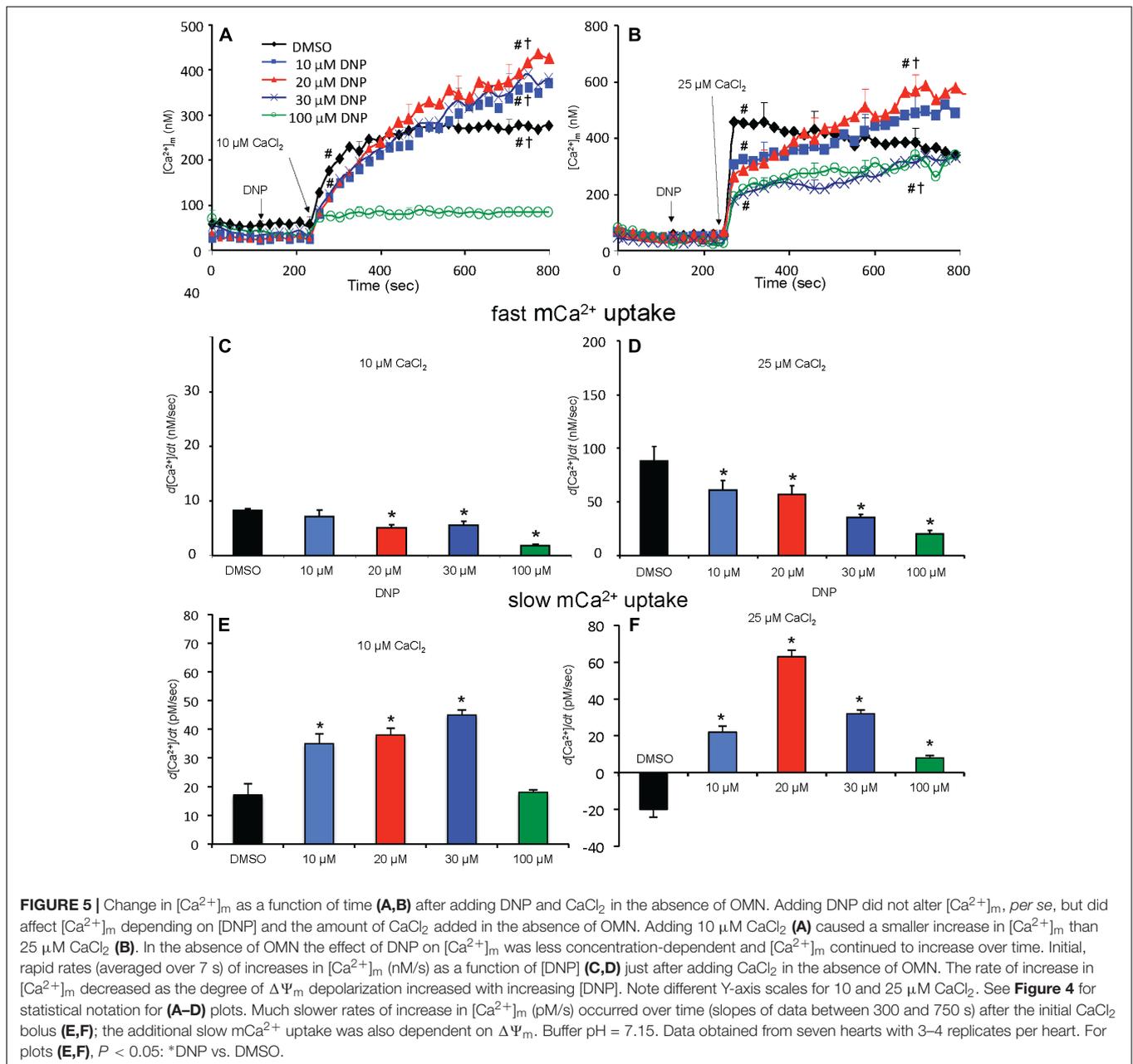
Mitochondrial Membrane Potential ($\Delta\Psi_m$) Was Depressed by DNP After Adding CaCl$_2$

In the protonophore series of experiments (time line, **Figure 3**), DNP alone decreased $\Delta\Psi_m$ slightly as assessed by rodamine-123

(R123) (Huang et al., 2007) (**Figure 4**), in a concentration-dependent manner, except at 100 µM DNP, which alone fully (+OMN) or nearly (−OMN) depolarized $\Delta\Psi_m$. $\Delta\Psi_m$ was estimated as % of maximal depolarization, where the baseline after adding substrate with OMN signifies full polarization (0%) and addition of CCCP denotes complete depolarization (100%). Adding 10 µL of 0.1% DMSO (DNP vehicle) or 10 µM DNP had no significant effect when given before CaCl$_2$, whereas adding 20, 30, or 100 µM DNP before 10 µM CaCl$_2$ reduced the R123 $\Delta\Psi_m$ signals by 12.7, 18.7, and 92.4% vs. DMSO (**Figure 4A**), respectively. In the presence of OMN (**Figure 4C**), adding 20, 30, or 100 µM DNP before 10 µM CaCl$_2$ increased the fluorescence signal intensities (i.e., depolarized $\Delta\Psi_m$) by 16.2, 33.0, and 99.0%, respectively, vs. DMSO (0%). Overall, before adding either 10 or 25 µM CaCl$_2$, 20 and 30 µM DNP moderately decreased $\Delta\Psi_m$ in the absence of OMN but greatly decreased $\Delta\Psi_m$ in the presence of OMN, suggesting blocked proton pumping from complex V (**Figures 4C,D** vs. **Figures 4A,B**). If no CaCl$_2$ was given after DNP, the moderate decrease in $\Delta\Psi_m$, which was unaffected by CsA, persisted for up to 25 min (see section "**Supplementary Results S.2.5**" and **Supplementary Figure S.7A**). After adding 10 and 30 µM DNP, and then CaCl$_2$, there were large decreases in $\Delta\Psi_m$ resulting from entry of Ca^{2+}. Although $\Delta\Psi_m$ depolarization by DNP alone was unaffected by CsA, the subsequent slow $\Delta\Psi_m$ depolarization induced by 25 µM CaCl$_2$ was delayed by CsA (**Supplementary Figure S.7B**). **Supplementary Results S.2.3** and **Supplementary Figures S.3A–D** shows statistics on mean $\pm$ SEM data for $\Delta\Psi_m$ replotted from **Figure 4** at time points 215, 275, and 700 s.

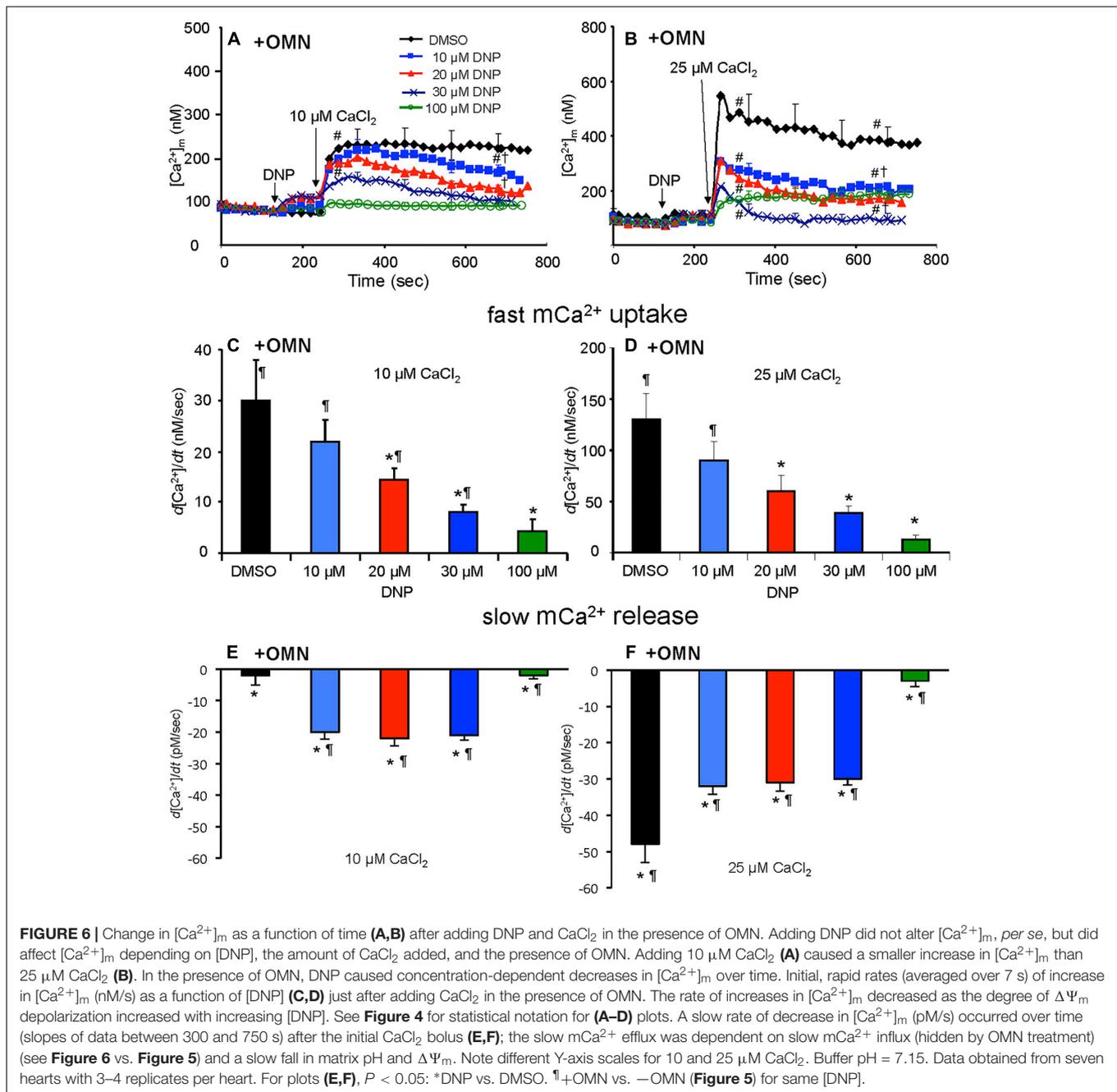## Matrix Free [Ca^{2+}]$_m$ Rose or Fell Slowly Depending on Block of Complex V

Adding 10 µM CaCl$_2$ without DNP ($\Delta\Psi_m$ fully polarized) caused [Ca^{2+}]$_m$ to increase rapidly from 80 nM (no added CaCl$_2$) initially to 235 nM at 300 s, whereas after adding 25 µM CaCl$_2$, [Ca^{2+}]$_m$ rose more rapidly to 450 nM (**Figures 5A,B**); [Ca^{2+}]$_m$ remained unchanged over time (300–750 s) after adding 10 µM CaCl$_2$ but fell slightly and gradually (non-significantly) over time after adding 25 µM CaCl$_2$ (DMSO group, **Figures 5A,B**). After adding 10–30 µM DNP, adding 10 µM CaCl$_2$ promoted a slow, secondary rise in [Ca^{2+}]$_m$ (**Figure 5A**). The secondary, slow increase in [Ca^{2+}]$_m$ beginning 300 s after adding 10 µM CaCl$_2$ plus DNP was accompanied by a slow decrease in extra-mitochondrial [Ca^{2+}]$_e$ (see **Supplementary Figure S.6A**). When $\Delta\Psi_m$ was nearly or totally depolarized by 100 µM DNP in the absence of OMN, and after adding 10 µM CaCl$_2$, there was no change in [Ca^{2+}]$_m$ over 300–750 s and thus no mCa^{2+} uptake over time (**Figure 5A**). [Ca^{2+}]$_m$ slowly increased over 300–750 s after first adding 10 and 20 µM DNP and then 25 µM CaCl$_2$ (**Figure 5B**), which caused the slow declines in $\Delta\Psi_m$ (**Figure 4B**). In the 100 µM DNP group [Ca^{2+}]$_m$ increased moderately immediately after adding 25 µM CaCl$_2$, but did not change further over time. **Supplementary Results S.2.6** and **Supplementary Figures S.4A,B** display statistics on

FIGURE 5 | Change in $[Ca^{2+}]_m$ as a function of time **(A,B)** after adding DNP and $CaCl_2$ in the absence of OMN. Adding DNP did not alter $[Ca^{2+}]_m$, *per se*, but did affect $[Ca^{2+}]_m$ depending on [DNP] and the amount of $CaCl_2$ added in the absence of OMN. Adding 10 μM $CaCl_2$ **(A)** caused a smaller increase in $[Ca^{2+}]_m$ than 25 μM $CaCl_2$ **(B)**. In the absence of OMN the effect of DNP on $[Ca^{2+}]_m$ was less concentration-dependent and $[Ca^{2+}]_m$ continued to increase over time. Initial, rapid rates (averaged over 7 s) of increases in $[Ca^{2+}]_m$ (nM/s) as a function of [DNP] **(C,D)** just after adding $CaCl_2$ in the absence of OMN. The rate of increase in $[Ca^{2+}]_m$ decreased as the degree of $\Delta\Psi_m$ depolarization increased with increasing [DNP]. Note different Y-axis scales for 10 and 25 μM $CaCl_2$. See **Figure 4** for statistical notation for **(A–D)** plots. Much slower rates of increase in $[Ca^{2+}]_m$ (pM/s) occurred over time (slopes of data between 300 and 750 s) after the initial $CaCl_2$ bolus **(E,F)**; the additional slow mCa^{2+} uptake was also dependent on $\Delta\Psi_m$. Buffer pH = 7.15. Data obtained from seven hearts with 3–4 replicates per heart. For plots **(E,F)**, $P < 0.05$: *DNP vs. DMSO.

mean ± SEM data for $[Ca^{2+}]_m$ replotted from **Figure 5** (−OMN) at time points 215, 275, and 700 s.

In marked contrast, when complex V was blocked by OMN, adding 10 μM $CaCl_2$ (**Figure 6A**) after adding 10–30 μM DNP caused a marked decrease in $[Ca^{2+}]_m$ over time (300–750 s); after adding 25 μM $CaCl_2$ in the absence of DNP (**Figure 6B**), $[Ca^{2+}]_m$ rose higher initially, whereas 10–30 μM DNP caused a slow decrease in $[Ca^{2+}]_m$ over this period, indicating net mCa^{2+} efflux. **Supplementary Results S.2.6** and **Supplementary Figures S.4C,D** shows statistics on mean ± SEM data for $[Ca^{2+}]_m$ replotted from **Figure 6** (+OMN) at time points 215, 275, and 700 s. The secondary, slow decrease in $[Ca^{2+}]_m$ after adding 20 μM DNP plus 25 μM $CaCl_2$ was accompanied by
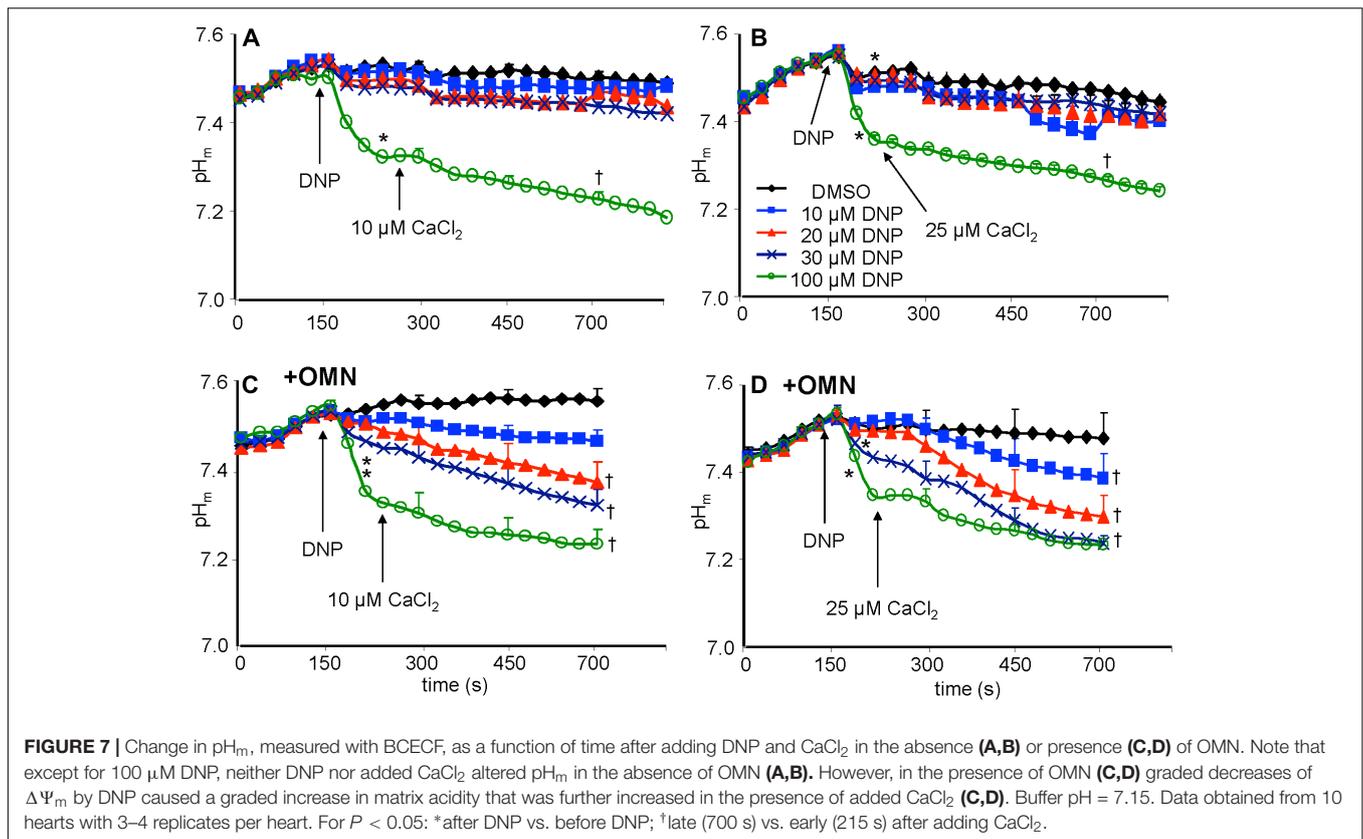
an increase in extra-mitochondrial $[Ca^{2+}]_e$ (see **Supplementary Figure S.6B**). Note that additional mCa^{2+} uptake after giving 25 μM $CaCl_2$ was halted after adding Ru360, 90 s later (at $t = 325$ s) and converted to mCa^{2+} efflux in the presence of OMN as shown by the increase in $[Ca^{2+}]_e$ (see **Supplementary Figures S.6B vs. S.6A**). A summary of slope data collected over the first 7 s (1 sample/s) after adding 10 or 25 μM $CaCl_2$ in the absence (**Figures 5C,D**) or presence (**Figures 6C,D**) of OMN shows that the average initial, rapid increase in $[Ca^{2+}]_m$ via the MCU was much faster after adding 25 μM $CaCl_2$ than after 10 μM $CaCl_2$ in the ± OMN groups; this initial rate of mCa^{2+} uptake decreased as $\Delta\Psi_m$ fell with added DNP. The initial rate of increase in $[Ca^{2+}]_m$ during the first 7 s after

FIGURE 6 | Change in $[Ca^{2+}]_m$ as a function of time **(A,B)** after adding DNP and $CaCl_2$ in the presence of OMN. Adding DNP did not alter $[Ca^{2+}]_m$, *per se*, but did affect $[Ca^{2+}]_m$ depending on [DNP], the amount of $CaCl_2$ added, and the presence of OMN. Adding 10 μM $CaCl_2$ **(A)** caused a smaller increase in $[Ca^{2+}]_m$ than 25 μM $CaCl_2$ **(B)**. In the presence of OMN, DNP caused concentration-dependent decreases in $[Ca^{2+}]_m$ over time. Initial, rapid rates (averaged over 7 s) of increase in $[Ca^{2+}]_m$ (nM/s) as a function of [DNP] **(C,D)** just after adding $CaCl_2$ in the presence of OMN. The rate of increases in $[Ca^{2+}]_m$ decreased as the degree of $\Delta\Psi_m$ depolarization increased with increasing [DNP]. See **Figure 4** for statistical notation for **(A–D)** plots. A slow rate of decrease in $[Ca^{2+}]_m$ (pM/s) occurred over time (slopes of data between 300 and 750 s) after the initial $CaCl_2$ bolus **(E,F)**; the slow mCa^{2+} efflux was dependent on slow mCa^{2+} influx (hidden by OMN treatment) (see **Figure 6** vs. **Figure 5**) and a slow fall in matrix pH and $\Delta\Psi_m$. Note different Y-axis scales for 10 and 25 μM $CaCl_2$. Buffer pH = 7.15. Data obtained from seven hearts with 3–4 replicates per heart. For plots **(E,F)**, $P < 0.05$: *DNP vs. DMSO. ¶+OMN vs. −OMN (**Figure 5**) for same [DNP].

adding 10 μM $CaCl_2$ (**Figure 5C**) decreased from 8 to 2 nM/s (DNP 0–100 μM). After adding 25 μM $CaCl_2$ (**Figure 5D**), the rate decreased from 88 to 20 nM/s. In the presence of OMN (**Figures 6C,D**), the initial increases in $[Ca^{2+}]_m$ in fully coupled mitochondria (no DNP) were larger than those in the absence of OMN (**Figures 6C,D** vs. **Figures 5C,D**). With OMN present, the initial increases in $[Ca^{2+}]_m$ decreased from 30 to 4 nM/s after adding 10 μM $CaCl_2$ and from 130 to 13 nM/s after adding 25 μM $CaCl_2$, Thus the initial rates of increase in $[Ca^{2+}]_m$ with 10 μM $CaCl_2$ were consistently faster in the presence of OMN (**Figure 6C** vs. **Figure 5C**), and at

25 μM $CaCl_2$, with or without 10 μM DNP (**Figure 6D** vs. **Figure 5D**).

A summary of slope data collected between 300 and 750 s, i.e., after the initial, rapid increase in $[Ca^{2+}]_m$ via the MCU with added 10 μM $CaCl_2$, demonstrates a much slower and smaller (pM/s) gradual increase in $[Ca^{2+}]_m$ over time in the absence of OMN with a threefold greater slope after 30 μM DNP vs. DMSO (**Figure 5E**). After adding 25 μM $CaCl_2$, the slow increase in $[Ca^{2+}]_m$ was about fourfold higher after 20 μM DNP vs. DMSO (**Figure 5F**). The secondary slow rise in $[Ca^{2+}]_m$ was about 1000 times slower than the initial fast phase and roughly dependent on

FIGURE 7 | Change in pH$_m$, measured with BCECF, as a function of time after adding DNP and CaCl$_2$ in the absence **(A,B)** or presence **(C,D)** of OMN. Note that except for 100 µM DNP, neither DNP nor added CaCl$_2$ altered pH$_m$ in the absence of OMN **(A,B)**. However, in the presence of OMN **(C,D)** graded decreases of ΔΨ$_m$ by DNP caused a graded increase in matrix acidity that was further increased in the presence of added CaCl$_2$ **(C,D)**. Buffer pH = 7.15. Data obtained from 10 hearts with 3–4 replicates per heart. For $P < 0.05$: *after DNP vs. before DNP; †late (700 s) vs. early (215 s) after adding CaCl$_2$.

both the amount of mCa^{2+} that was taken up initially just after adding CaCl$_2$ and the extent of ΔΨ$_m$ depolarization. In contrast, in the presence of OMN under the same conditions of added CaCl$_2$ and DNP, the slope data showed slow and small declines (rather than increases) in [Ca^{2+}]$_m$ over time (**Figures 6E,F**). The slow rate of extrusion of mCa^{2+} by CHE$_m$ when complex V was blocked with OMN (**Figures 6E,F**) became greater when mCa^{2+} entry via the MCU was greater (**Figures 6 A,B**).

Matrix pH Remained Steady Without OMN but Fell With OMN-Induced Block of Complex V

Baseline matrix pH$_m$ was approximately 7.55 in each group after adding PA and before adding DNP (**Figures 7A–D**). In the absence of OMN, adding 10–30 µM DNP did not result in a significant net decrease in pH$_m$; however, 100 µM DNP markedly decreased pH$_m$ (**Figures 7A,B**). This effect to collapse the ΔpH$_m$ gradient was proportional to the collapse of the ΔΨ$_m$ gradient (**Figure 4**). In the absence of OMN, adding CaCl$_2$ had no appreciable effect on pH$_m$ (ΔΨ$_m$ partially depolarized) even in the presence of DNP, except for 100 µM DNP, when pH$_m$ fell markedly (ΔΨ$_m$ fully depolarized) (**Figures 7A,B**). In the absence of OMN, H$^+$ influx was matched by H$^+$ pumping as pH$_m$ did not change appreciably. In contrast, in the presence of OMN there was a strong DNP concentration-dependent fall in matrix pH$_m$ (**Figures 7C,D**) after adding CaCl$_2$. This fall in pH$_m$ was likely due to blocked H$^+$ pumping by

complex V in the presence of OMN (see below). **Supplementary Figures S.5A–D** shows statistics on mean ± SEM data on pH$_m$ replotted from **Figure 7** (main text) at time points 215, 275, and 700 s. **Supplementary Figure S.8** displays plots of pH$_m$ as a function of [Ca^{2+}]$_m$ at 700 s after adding DNP and CaCl$_2$; these correlations show how [Ca^{2+}]$_m$ decreases while pH$_m$ decreases in the presence, but not in the absence of OMN.

Mitochondrial Redox State Remained Steady Without OMN but Fell With OMN-Induced Block of Complex V

A reduced redox state is associated with maintenance of pH$_m$. Adding the substrate PA increased the redox state (more reduced) as determined by high NADH autofluorescence (**Figure 8**). In the absence of OMN, adding 10 to 30 µM DNP ± 10 or 25 µM CaCl$_2$ (**Figures 8A,B**) did not cause a significant change in NADH. NADH was unchanged despite up to 60% decrease in ΔΨ$_m$ fluorescence (**Figures 4A,B**) after adding DNP and CaCl$_2$. However, when complex V was blocked by OMN (**Figures 8C,D**), there was significant oxidation (low NADH) by DNP in a concentration dependent manner. In contrast to the condition without OMN, with OMN present as little as a 20% fall in ΔΨ$_m$ fluorescence (**Figures 4C,D**) led to a more oxidized NADH state. Moreover, NADH was fully oxidized at 20 µM DNP with OMN present (**Figures 8C,D**), and the oxidized state was not altered significantly by adding CaCl$_2$ after DNP. In the absence or presence of CaCl$_2$, NADH

FIGURE 8 | Change in mitochondrial redox state, measured by NADH autofluorescence, as a function of time after adding DNP and CaCl$_2$ in the absence **(A,B)** or presence **(C,D)** of OMN. Note that the redox state was maintained after adding 10–30 μM DNP and CaCl$_2$ in the absence of OMN **(A,B)** but that in the presence of OMN **(C,D)** there was a concentration-dependent decrease in NADH autofluorescence. Adding CaCl$_2$ did not alter DNP-induced changes in redox state in the presence of OMN. Buffer pH = 7.15. Data obtained from eight hearts with 3–4 replicates per heart. See **Figure 4** for statistical notation.

was completely oxidized after adding 100 μM DNP (data not shown).

ATP Concentration Fell Without OMN but Remained Steady With OMN-Induced Block of Complex V

Total medium [ATP] was measured and mitochondrial [ATP]$_m$ was estimated (see section **"Supplementary Materials S.1.10"**). Basal [ATP]$_m$ was measured after adding mitochondria to the experimental buffer in the absence of OMN (**Figures 9A,B**). There was no change in basal [ATP]$_m$ after adding PA. DNP, at 10 μM, did not significantly change [ATP] before or after adding CaCl$_2$ (**Figures 9A,B**). Basal [ATP]$_m$ was unchanged if CaCl$_2$ was not added (data not displayed). Adding 20 or 30 μM DNP alone had no significant effect on [ATP]$_m$, but adding CaCl$_2$ resulted in a decrease in [ATP]$_m$ (**Figures 9A,B**). In the presence of OMN (**Figures 9C,D**), adding mitochondria to the buffer did not change [ATP]$_m$, indicating inhibited complex V activity. [ATP]$_m$ remained at a very low level and was unaffected by DNP or CaCl$_2$ in the presence of OMN. With OMN present, ATP$_m$/ADP$_m$ ratios (see section **"Supplementary Materials S.1.11, S.1.12** and **Supplemental Results S.2.9"**) also decreased with added DNP and CaCl$_2$, along with the progressive declines in $\Delta\Psi_m$.
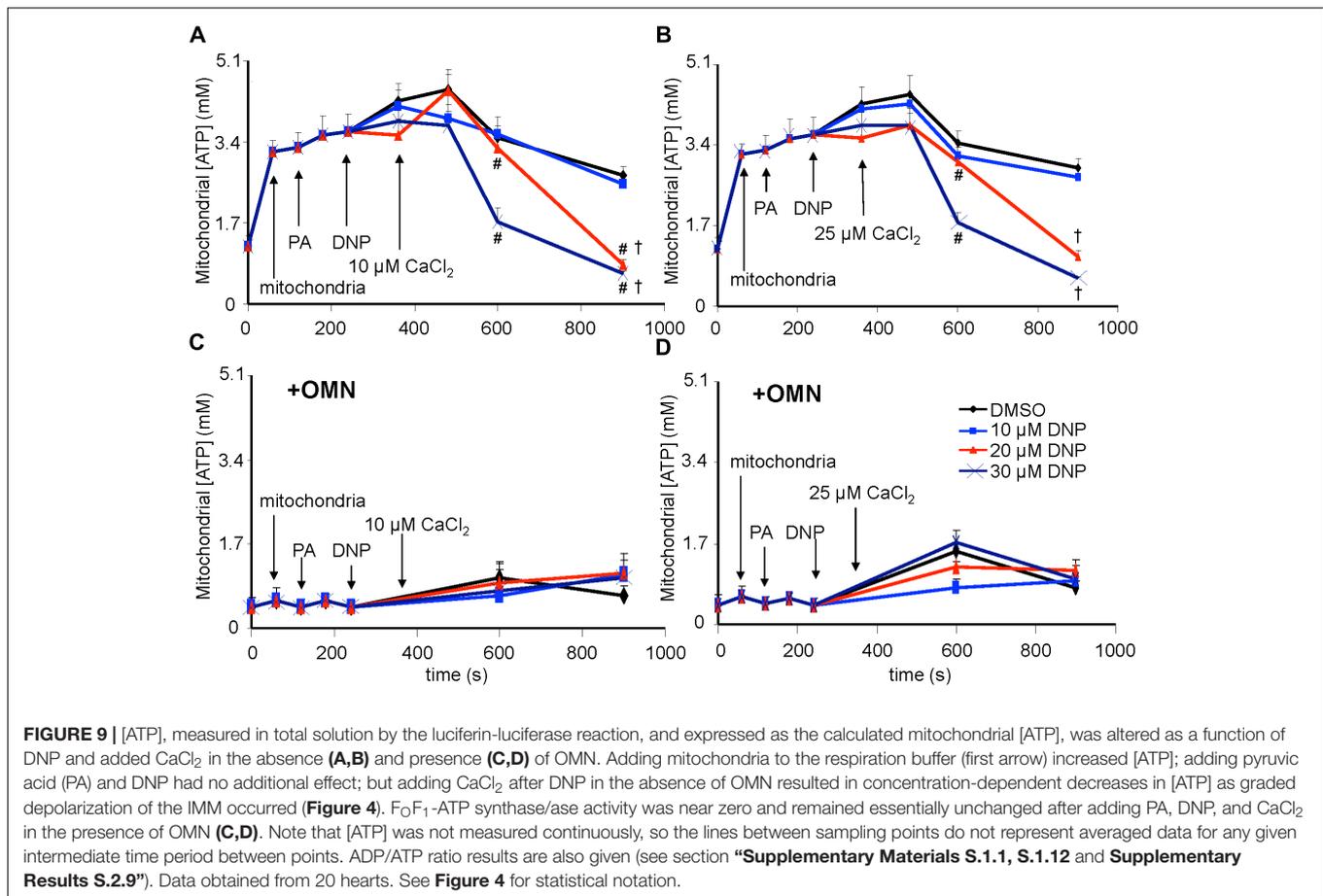
Additional Supplemental Comparisons and Calculations

Supplementary Results S.2.2 and **Supplementary Figure S.2** demonstrate the effect of adding DNP and CaCl$_2$ on respiration. **Supplementary Results S.2.7** and **Supplementary Figure S.9** furnish values for $\Delta\Psi_m$, [Ca^{2+}]$_m$, and pH$_m$ at 700 s, replotted from **Figures 4–7**, to compare these results in the presence or absence of OMN. The **Supplementary Table** shows DNP concentrations that produced 50% inhibitions (IC$_{50}$) of $\Delta\Psi_m$, [Ca^{2+}]$_m$, fast (initial) d[Ca^{2+}]$_m$/dt, and pH$_m$ as a linear function of 0–30 μM DNP ± OMN at the 700 s time point. **Supplementary Figure S.10** displays calculated mCa^{2+} flux rates (J_{CHE}) for CHE$_m$ (see section **"Supplementary Results S.2.8"**) in the absence and presence of OMN.

DISCUSSION

Ca^{2+}/H$^+$ Exchange Activity Is Identified by Manipulating IMM Δ[H$^+$] and Δ[Ca^{2+}] Gradients

We provide firm support for a role of CHE$_m$ in maintaining homeostasis of Ca^{2+} against H$^+$ under certain conditions in cardiac cell mitochondria that may mimic some sequelae of

FIGURE 9 | [ATP], measured in total solution by the luciferin-luciferase reaction, and expressed as the calculated mitochondrial [ATP], was altered as a function of DNP and added $CaCl_2$ in the absence **(A,B)** and presence **(C,D)** of OMN. Adding mitochondria to the respiration buffer (first arrow) increased [ATP]; adding pyruvic acid (PA) and DNP had no additional effect; but adding $CaCl_2$ after DNP in the absence of OMN resulted in concentration-dependent decreases in [ATP] as graded depolarization of the IMM occurred (**Figure 4**). F_OF_1-ATP synthase/ase activity was near zero and remained essentially unchanged after adding PA, DNP, and $CaCl_2$ in the presence of OMN **(C,D)**. Note that [ATP] was not measured continuously, so the lines between sampling points do not represent averaged data for any given intermediate time period between points. ADP/ATP ratio results are also given (see section **"Supplementary Materials S.1.1, S.1.12** and **Supplementary Results S.2.9"**). Data obtained from 20 hearts. See **Figure 4** for statistical notation.

cardiac IR injury. Our results: (1) furnish direct evidence for CHE_m activity by the secondary, slow increases in matrix Ca^{2+} efflux coupled to slow increases in matrix H^+ influx, when both NCE and NHE activities are blocked, and particularly, when MCU-dependent mCa^{2+} re-uptake is blocked with Ru360; (2) demonstrate that respiration increases while $\Delta\Psi_m$ decreases mildly, whereas pH_m and redox state are relatively maintained when inducing a matrix inward H^+ leak with DNP before adding $CaCl_2$; adding $CaCl_2$ results in a secondary, slow increase in $[Ca^{2+}]_m$ that slowly depolarizes $\Delta\Psi_m$; (3) show that with permissive H^+ influx, but inhibited outward H^+ pumping at complex V, adding $CaCl_2$ causes larger decreases in $\Delta\Psi_m$, pH_m, and NADH and results in a slow decrease in $[Ca^{2+}]_m$; (4) indicate that blocking complex V with OMN to prevent H^+ pumping causes $\Delta\Psi_m$ to further decrease after adding $CaCl_2$ because the influx of mCa^{2+} via the MCU is not opposed by H^+ pumping at complex V; (5) suggest that the lack of a slow fall or rise in $[Ca^{2+}]_m$ in the presence of 100 μM DNP is due to the loss of $\Delta\Psi_m$-dependent mCa^{2+} uptake by MCU; (6) point out that only in partially depolarized mitochondria does added $CaCl_2$ result in a pH_m-independent gradual increase in $[Ca^{2+}]_m$ that is reciprocated by H^+ pumping to maintain pH_m; preventing matrix acidification is associated with a maintained redox state; and (7) show that the decrease in [ATP] in the absence of

OMN supports ATP hydrolysis with H^+ pumping. These two scenarios, ±OMN, are depicted graphically in **Figure 10A** vs. **Figure 10B**.

Net Mitochondrial Ca²⁺ Influx Occurs via MCU and Net Ca²⁺ Efflux Can Occur via Ca²⁺/H⁺ Exchange

The dependence of rapid MCU-mediated mCa^{2+} uptake on $\Delta\Psi_m$ has been examined extensively (Gunter and Pfeiffer, 1990; Gunter et al., 1994; Dash et al., 2009; Haumann et al., 2010). But our study demonstrates that net $m[Ca^{2+}]$ can additionally increase slowly via the MCU, and that this happens when pH_m is relatively maintained despite a decline in $\Delta\Psi_m$ resulting from the DNP-mediated inward H^+ flux and after the initial rapid Ca^{2+} influx via MCU. A gradual increase in $[Ca^{2+}]_m$ at the expense of maintaining the ΔpH_m may be deleterious to mitochondrial function. We propose that this secondary rise in net $[Ca^{2+}]_m$ results from an adequate $\Delta\Psi_m$ with Ru360-dependent slow mCa^{2+} influx, which eventually leads to a slow, continued fall in $\Delta\Psi_m$. Because H^+ pumping at complex V maintains the $\Delta[H^+]_m$ gradient, mCa^{2+} efflux via CHE_m in exchange for mH^+ influx due to the H^+ leak is likely masked by mCa^{2+} re-uptake. Thus, the DNP-induced H^+ leak and the concomitant dissipation of the IMM $\Delta[H^+]$ gradient, when
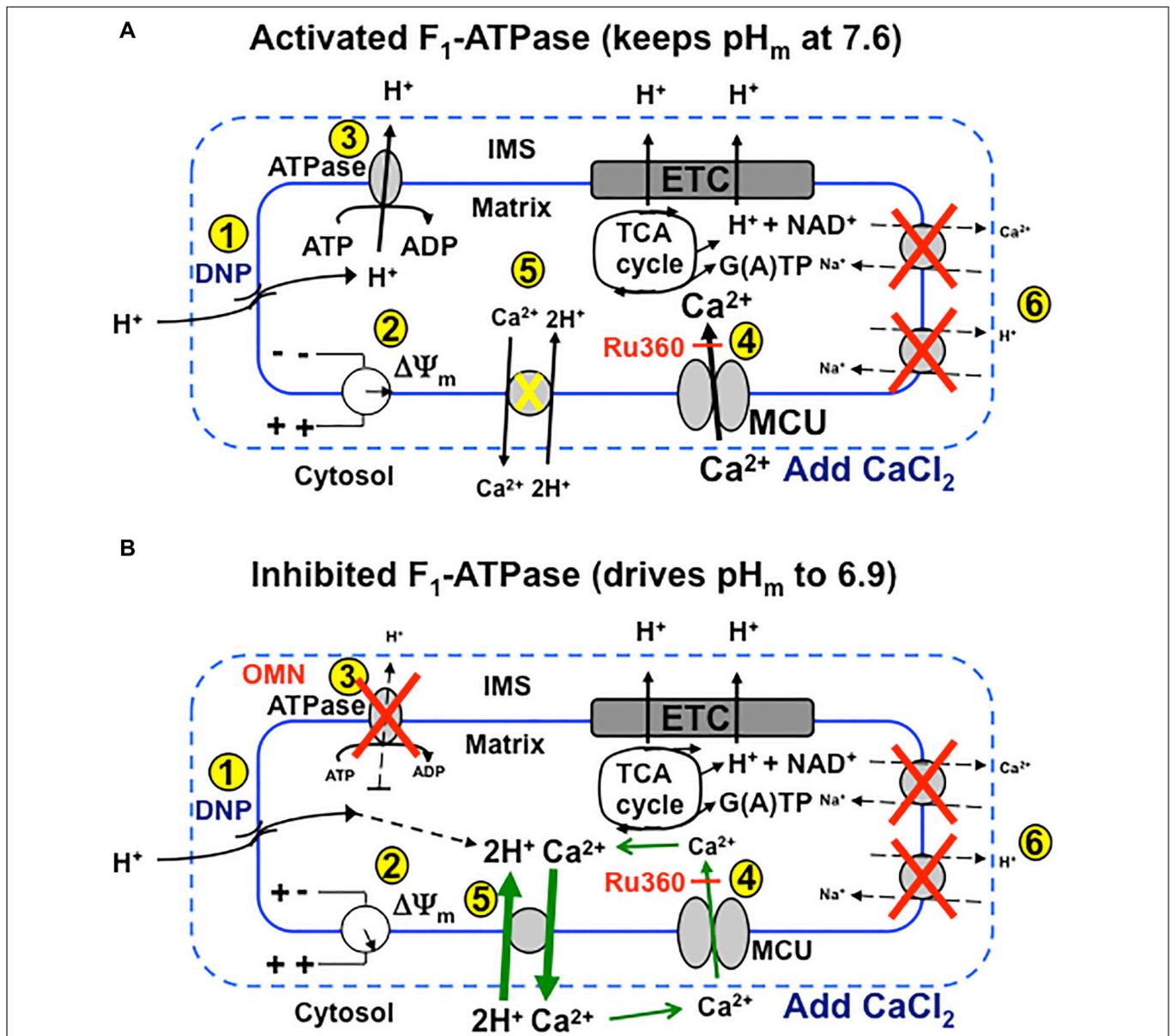
FIGURE 10 | Schema depicting putative role of MCU and CHE$_m$ on slow Ca^{2+} influx and efflux, respectively, during stepwise depolarization with DNP with un-inhibited (i.e., minus OMN) **(A)** vs. inhibited **(B)** F$_O$F$_1$-ATPsynthase(ase) (i.e., plus OMN) after a bolus addition of CaCl$_2$ to the mitochondrial medium. **(A)** (1) DNP permits H$^+$ entry that tends to (2) decrease $\Delta\Psi_m$, which enhances H$^+$ pumping by respiratory complexes, including (3) F$_O$F$_1$-ATPase, so that pH$_m$ does not decrease appreciably and $\Delta\Psi_m$ is partially supported (2). Adding CaCl$_2$ further depolarizes $\Delta\Psi_m$ by allowing more cationic (Ca^{2+}) charges into the matrix via the MCU (4). Over time, in the range of a 20–60% decline in $\Delta\Psi_m$, pH$_m$ remains unchanged as (5) H$^+$ is pumped out (3) in exchange for permissive H$^+$ entry (1) in triggering additional slow mCa^{2+} uptake by MCU and causing $\Delta\Psi_m$ to decrease further. CHE$_m$ is inhibited by the lack (pH 7.6) of matrix acidity (5) and NCE$_m$ and NHE$_m$ are inactivated by the lack of substrate and buffer Na$^+$ (6). **(B)** Alternatively, when F$_O$F$_1$-ATPase is inhibited (3), matrix acidity gradually increases (pH 6.9), $\Delta\Psi_m$ is less supported (2) and Ca^{2+} slowly exits (CHE$_m$) in exchange for slow H$^+$ entry due to DNP (5). This sequence triggers a net loss of mCa^{2+} even though uptake of Ca^{2+} via the MCU continues, as shown by a greater efflux of mCa^{2+} by CHE$_m$ when additional mCa^{2+} uptake via MCU is blocked by Ru360 (4). DNP, dinitrophenol; ETC, electron transport chain; IMS, inner membrane space; MCU, mitochondrial Ca^{2+} uniporter; OMN, oligomycin; TCA, tricarboxylic acid.

countered by H$^+$ pumping at complex V (in addition to other complexes), can maintain the ΔpH$_m$ and support the *pmf* ($\Delta\Psi_m$ + RT/FΔpH$_m$) (Dzbek and Korzeniewski, 2008). This view is especially supported by the smaller decline in extra-mitochondrial [Ca^{2+}]$_e$ in the presence of 20 µM DNP, 25 µM CaCl$_2$, and OMN, as well as in the presence of Ru360, by

the gradual increase in [Ca^{2+}]$_e$ due to CHE$_m$ mediated Ca^{2+} efflux. These results are reinforced by the exaggerated effect of added CaCl$_2$ to enhance the decline in $\Delta\Psi_m$ over time and by the slow decreases in [Ca^{2+}]$_m$ linked to slow decreases in pH$_m$. Blocking outward H$^+$ pumping by complex V prevented compensation for DNP-mediated H$^+$ influx. Consistent with

our observations, it was reported that matrix acidification may reduce Ca^{2+} uptake in cardiac mitochondria by its effect on decreasing $\Delta\Psi_m$ (Gursahani and Schaefer, 2004). In contrast, when ATP$_m$ hydrolysis is prevented, pH$_m$ slowly decreases toward pH$_e$ with a greater fall in $\Delta\Psi_m$; the slow H$^+$ influx is accompanied by a slow net fall in [Ca^{2+}]$_m$ mediated by CHE$_m$ even though the extruded Ca^{2+} is recycled via the MCU. Since H$^+$ influx (DNP-induced leak) is not countered by reciprocal H$^+$ pumping to restore ΔpH$_m$, the slow influx of H$^+$ is exchanged for slow Ca^{2+} efflux via CHE$_m$ until the ΔpH gradient is dissipated.

Ca^{2+} and H$^+$ gradients across the IMM are largely dependent on $\Delta\Psi_m$ and ΔpH gradients resulting from H$^+$ pumping by respiratory complexes. Ionic homeostasis requires one cation efflux pathway to oppose another cation influx pathway and *vice versa*. Cation exchangers fulfill this need. Unlike mCa^{2+} uptake via MCU, which is dependent on $\Delta\Psi_m$ and on the chemical gradient, exchange of Ca^{2+} and H$^+$ via CHE$_m$ may or may not be dependent on $\Delta\Psi_m$ (Rottenberg and Marbach, 1990; Gunter et al., 1991). But the direction of Ca^{2+} and H$^+$ flux mediated solely by CHE$_m$ is dependent on a large IMM [H$^+$] or [Ca^{2+}] gradient to shuttle Ca^{2+} or H$^+$ across the IMM. This can be expressed by an electroneutral J_{CHE} flux equation (Tewari et al., 2014), calculated here in the presence and absence of OMN (see section **"Supplementary Results S.2.8"** and **Supplementary Figure S.10**). J_{CHE} flux analysis of our data suggests that slow mCa^{2+} influx could have occurred via CHE$_m$ in the absence of OMN, whereas mCa^{2+} efflux could have occurred in the presence of OMN. Indeed, we have provided strong support for slow net mCa^{2+} efflux mediated by CHE$_m$ (despite slow mCa^{2+} uptake by MCU) when complex V cannot pump H$^+$ in the presence of OMN.

Although CHE$_m$ likely occurs both in the absence or presence of OMN, our results suggest that the observed secondary, slow influx of mCa^{2+} influx (minus OMN) is due primarily to re-uptake by a Ru360 sensitive mechanism, presumably MCU, that may overwhelm any CHE$_m$ activity. This is because Ru360 blocked the slow rise in [Ca^{2+}]$_m$ and the slow fall in [Ca^{2+}]$_e$, thus supporting MCU as the mediator of the slow mCa^{2+} influx. The J_{CHE} flux equation only monitors differences in [H$^+$] and [Ca^{2+}] on either side of the IMM and does not rely on effects of the ΔpH$_m$ gradient on H$^+$ pumping or the $\Delta\Psi_m$ gradient on mCa^{2+} uptake via MCU. Thus the secondary, slow mCa^{2+} uptake after the initial CaCl$_2$ bolus (**Figures 5A,B,E,F**) appears to be a direct effect of H$^+$ pumping by complex V (minus OMN) to maintain the ΔpH$_m$ charge gradient and support the *pmf* although $\Delta\Psi_m$ continues to fall due to the continued mCa^{2+} influx. On the other hand, inhibiting ATP$_m$ hydrolysis (**Figures 9C,D**) to prevent H$^+$ pumping not only enhances the fall in $\Delta\Psi_m$ (**Figures 4C,D**) to retard further mCa^{2+} loading by the MCU, but also permits slow CHE$_m$-mediated mCa^{2+} efflux (**Figures 6A,B,E,F**) in exchange for mH$^+$ influx until the diminishing ΔpH$_m$ gradient is abolished (**Figures 7C,D**).

Alternatively, we demonstrated CHE$_m$ activity by acidifying the external medium before adding CaCl$_2$, while blocking NCE$_m$ and NHE$_m$ activities by using Na$^+$ free buffer and substrates. We observed a slowly increasing [Ca^{2+}]$_e$ coupled to a slowly

increasing [H$^+$]$_m$. We used Ru360 to expose the net amount of mCa^{2+} efflux via CHE$_m$ by blocking the effluxed Ca^{2+} from re-entering via MCU (**Figures 1**, **2**). It is unlikely that 0.1–1 μM Ru360 inhibits CHE$_m$ because Ru360 did not block mCa^{2+} efflux (**Figures 1**, **2**), only mCa^{2+} influx. Of course, Ru360 might block another mode of non-MCU Ca^{2+} uptake. Our proposed mechanism is described schematically in **Figures 10A,B**. We postulate that CHE$_m$ activity is completely inhibited if the matrix remains alkaline (large ΔpH$_m$ gradient), thus exposing net Ca^{2+} uptake via MCU. The slow increases in [Ca^{2+}]$_m$ that we observed previously (Haumann et al., 2010) likely represent net slow mCa^{2+} via MCU (reference **Figure 5**).

A leucine zipper EF hand-containing trans-membrane protein (LETM1) found in non-mammalian cells is thought to be a molecular component of CHE$_m$ (Jiang et al., 2009; Shao et al., 2016). Knockdown and expression of LETM1 in a number of cell lines support its role in Ca^{2+}/H$^+$ exchange, particularly in mitochondria (Jiang et al., 2013; Doonan et al., 2014). Alternatively, other studies (Nowikovsky et al., 2004, 2012; Froschauer et al., 2005; Malli and Graier, 2010; Austin et al., 2017) support that LETM1 either does not mediate Ca^{2+} efflux (De Marchi et al., 2014) or that it mediates K$^+$/H$^+$ and/or Na$^+$/H$^+$ exchange, so conclusive genetic evidence for CHE requires more study. It is important to note that the elusive CHE protein appears to be insensitive to MCU inhibitors, i.e., ruthenium red (RR) compounds (Bernardi et al., 1984), and to CGP-37157, the NCE inhibitor (Tsai et al., 2014). The present study explores for the first time the kinetics of CHE$_m$ activity in relation to MCU activity in cardiac cell mitochondria.

$\Delta\Psi_m < E_{REV-ATPase}$ Promotes ATP Hydrolysis

F$_O$F$_1$-ATPsynthase/ase directionality is governed by $\Delta\Psi_m$ and its "reversal potential" $E_{REV-ATPase}$, which in turn is dependent on the concentration of the reactants ATP/ADP, and H$^+$ (Metelkin et al., 2009; Chinopoulos and Adam-Vizi, 2010; Chinopoulos et al., 2010). Additional factors of E_{REV} that affect the direction and rate of ATP synthesis/hydrolysis are the free [P$_i$] and the H^+_m/ATP$_m$ coupling ratio, *n* (Cross and Muller, 2004). When $\Delta\Psi_m$ becomes less negative than E_{REV}, which depends on a high [ATP]$_m$ and ΔpH$_m$, but a low [ADP]$_m$, H$^+$ ejection by complex V becomes thermodynamically favorable (Metelkin et al., 2009; Chinopoulos and Adam-Vizi, 2010; Chinopoulos et al., 2010; Chinopoulos, 2011). $E_{REV-ATPase}$ can occur when $\Delta\Psi_m$ falls between −130 and −100 mV, depending on matrix [ATP]$_m$/[ADP]$_m$, [P$_i$]$_m$, ΔpH$_m$, and the coupling ratio (Chinopoulos et al., 2010; Chinopoulos, 2011). Others (Leyssens et al., 1996; Bains et al., 2006; Chinopoulos and Adam-Vizi, 2010) have observed that a fall in $\Delta\Psi_m$ caused by a protonophore, such as DNP or CCCP, can induce ATP hydrolysis through reversal of F$_O$F$_1$-ATPsynthase. The consequent H$^+$ pumping by complex V would tend to partially restore $\Delta\Psi_m$ to offset the protonophore-induced decreases in pH$_m$ and $\Delta\Psi_m$ as discussed above. The electrical gradient $\Delta\Psi_m$ and the H$^+$ chemical gradient Δ[H$^+$]$_m$ together contribute to the total *pmf* that powers the synthesis of ATP; when *pmf* is not maintained, hydrolysis of matrix ATP

occurs. Previous studies have also furnished indirect evidence for reversal of F_OF_1-ATPsynthase under conditions of reduced mCa^{2+} uptake and a fully depolarized $\Delta\Psi_m$ with CCCP (Leyssens et al., 1996; Bains et al., 2006). ATP_m hydrolysis has been reported to occur *in vivo* during cardiac ischemia (Grover et al., 2004), but the *in vivo* $\Delta\Psi_m$ at which this occurs is not known. Here we show how a DNP-induced fall in $\Delta\Psi_m$ induces ATP hydrolysis.

In the absence of OMN, the lack of a fall in ATP levels after adding 10 μM DNP indicated that ATP_m hydrolysis (**Figure 9**) did not occur because $\Delta\Psi_m$ remained relatively stable before adding $CaCl_2$. However, adding $CaCl_2$ resulted in a gradual, but large, fall in $\Delta\Psi_m$ over time. In the presence of 20 μM DNP and 25 μM $CaCl_2$, ATP hydrolysis occurred (20–25% of maximum) with a decrease in $\Delta\Psi_m$ at an IMM gradient of approximately 0.35 ΔpH_m units (**Figures 7A,B**). A faster rate of ATP hydrolysis was indicated by the additional fall in $[ATP]_m$ over time after adding 30 μM DNP and $CaCl_2$. The DNP-induced falls in $\Delta\Psi_m$ were accompanied by reduced ATP_m/ADP_m ratios (see section **"Supplementary Materials S.1.11, S1.12** and **Supplementary Results S.2.9"**) indicating consumption of ATP, as also shown by the lower $[ATP]_m$ (**Figures 9A,B**). A calculation of available matrix ATP is given (see section **"Supplementary Results S.2.10"**). In the presence of 100 μM DNP and added $CaCl_2$, $\Delta\Psi_m$ was maximally depolarized (**Figures 4A,B**), the ΔpH_m gradient was abolished (**Figures 7A,B**), and NADH was oxidized (**Figures 8A,B**), indicating that ATP_m hydrolysis was insufficient to maintain the *pmf*. This contrasts to the situation with 10–30 μM DNP where *pmf* was supported largely by the ΔpH_m gradient, as also reflected by the maintained NADH redox state.

$\Delta\Psi_m$ is normally fully polarized when complex V is blocked by OMN (Valdez et al., 2006; Brand and Nicholls, 2011); however, the effect of DNP to slightly decrease $\Delta\Psi_m$ was intensified when OMN was present, particularly after adding 25 μM $CaCl_2$ that intensifies the depolarization of $\Delta\Psi_m$ in the presence of DNP. This effect of DNP in the absence of OMN indicates that ATP hydrolysis indeed supported the ΔpH_m via H^+ pumping even at a relatively small decline in $\Delta\Psi_m$ with DNP. With OMN present, ATP hydrolysis cannot occur (**Figures 9C,D**) and so complex V cannot contribute to maintaining pH_m; therefore, the low pH_m accompanied by a high $[Ca^{2+}]_m$ must have activated CHE_m.

Changes in pH_m, $[Ca^{2+}]_m$, and NADH Are Larger With OMN Than Without OMN

An interesting observation of our study is the contribution of complex V to maintain the ΔpH_m gradient (and thus supporting the *pmf*) whereby the H^+ leak is compensated by augmented H^+ pumping by complex V; this resulted in slow mCa^{2+} influx ("Ca^{2+} leak") that could be blocked by Ru360, which indicates the influx likely occurred via MCU. But if compensatory H^+ pumping is blocked by OMN, the matrix becomes acidic, the ΔpH_m gradient falls lower, and slow mCa^{2+} efflux occurs via CHE_m thus masking the slow mCa^{2+} influx (**Figure 10B**). Evidence for H^+ pumping during ATP hydrolysis during DNP-mediated H^+ influx was provided by the maintenance of an alkaline pH_m; moreover, pH_m indeed

fell when H^+ pumping was blocked by OMN. Similarly, if mitochondria reside in an acidic environment (**Figures 1, 2**), $[H^+]_m$ falls as $[Ca^{2+}]_e$ rises, indicating CHE_m. Indeed, in a previous study it was reported that adding lactic acid to a Na^+ free mitochondrial suspension increased buffer Ca^{2+} by 43% (Gambassi et al., 1993); it was suggested that Ca^{2+} was extruded as H^+ influx caused H^+ ions to compete with Ca^{2+} ions for mitochondrial binding sites (Gambassi et al., 1993). We furnish direct evidence for a link between Ca^{2+} efflux with H^+ influx in mammalian cardiac muscle mitochondria, when Na^+ is absent and the MCU is blocked after adding $CaCl_2$.

NADH levels remained unchanged after adding DNP and $CaCl_2$ (**Figures 8A,B**); this likely reflects the faster state 2 respiration (**Supplementary Figure S2**) since the inward H^+ leak by DNP was balanced by H^+ pumping from complex V as well as from complexes I, III, and IV. Only at 100 μM DNP with $CaCl_2$, which fully depolarized $\Delta\Psi_m$ (**Figures 4A,B**), did DNP result in a lower pH_m (**Figures 7A,B**) and a more oxidized redox state, i.e., a decrease in NADH (**Figures 8A,B**). It is likely that an increase in F_OF_1-ATPase activity plus a faster TCA cycle turnover (increased $NADH/NAD^+$ ratio) can result in maintained NADH levels despite the DNP-induced H^+ leak. In the presence of OMN, however, NADH was gradually oxidized (**Figures 8C,D**) along with the fall in pH_m (**Figures 7C,D**); this scenario likely occurred because the additional H^+ pumping by complex V to support $\Delta\Psi_m$ was blocked. We observed that adding $CaCl_2$ alone did not significantly change NADH levels in this model, which is consistent with our earlier study (Haumann et al., 2010). Although an increase in $[Ca^{2+}]_m$ can stimulate NADH producing dehydrogenases (Denton et al., 1980; McCormack and Denton, 1980; Wan et al., 1989; Brandes and Bers, 1997), our experiments were conducted at maximal $[Ca^{2+}]_m$ values below the $K_{0.5}$ of 1 μM Ca^{2+} at which these dehydrogenases are reported to be activated (Denton et al., 1980; McCormack and Denton, 1980).

What Is the Functional Role of CHE_m: How Is Net mCa^{2+} Efflux Modified by mCa^{2+} Influx via MCU?

The net Ca^{2+} driving force for ions across the IMM can be estimated by Nernst equilibrium potentials for given estimates of $\Delta\Psi_m$. Under conditions of 20 μM DNP, 25 μM $CaCl_2$, and in the absence of OMN, when $[Ca^{2+}]_m$ slowly increased, we calculated Nernst equilibrium potentials of approximately −8 and +18 mV, respectively, for $[Ca^{2+}]$ and $[H^+]$ at 700 s. We estimated $\Delta\Psi_m$ as −110 to −120 mV at 700 s (based on our values for % of minimal and maximal depolarization (R-123 fluorescence) and curve fitting for approximating conversion to $\Delta\Psi_m$ (Huang et al., 2007)). This indicated that the driving force for both Ca^{2+} and H^+ would remain inward despite H^+ pumping at complex V to attempt to re-establish the ΔpH_m gradient by compensating for the DNP-mediated H^+ influx. Based on our estimated $\Delta\Psi_m$ and the calculated Ca^{2+} and H^+ equilibrium potentials driving both Ca^{2+} and H^+ inward, we conclude that the outward H^+ pumping by complex V (in addition to complexes I, III, IV) was

sufficient to compensate for the continued inward influx of H$^+$ mediated by DNP thus restoring the ΔpH_m gradient, but not the *pmf*, and thus preventing activation of CHE$_m$. Ru360 blocked this additional uptake of mCa^{2+} by the MCU so that [Ca^{2+}]$_e$ did not continue to fall.

We predict that the major conduit for both fast and slow mCa^{2+} influx under our experimental conditions occurs primarily via the MCU. The efflux of Ca^{2+} via the CHE$_m$ is slow so we expect the re-uptake of Ca^{2+} via the MCU also would be slow. Although the J_{CHE} flux equation alone predicted that slow mCa^{2+} influx could have occurred via CHE$_m$ this is unsustainable if [H$^+$]$_m$ < [H$^+$]$_e$. It is likely that voltage-dependent transport of net Ca^{2+} inward is mostly responsible if there is at least a partially maintained $\Delta\Psi_m$ (Nernst potentials) despite mCa^{2+} extrusion via CHE$_m$. Interestingly, under the condition of a fully polarized $\Delta\Psi_m$ (no DNP and no OMN) (**Figures 4A–D**), [Ca^{2+}]$_m$ did not rise as it did in the presence of DNP (**Figures 5A,B**) when pH$_m$ was maintained (**Figures 7A,B**). This suggests that the secondary, slow uptake of mCa^{2+} is indirectly related to H$^+$ pumping due to the decline in [H$^+$]$_m$ to support the *pmf*; the additional, slow mCa^{2+} uptake by the MCU occurs because of the remaining charge gradient ($\Delta\Psi_m$) and Ca^{2+} chemical gradient.

In contrast, in the presence of OMN the kinetics of the delayed, slow mCa^{2+} efflux via CHE$_m$ under conditions of reduced $\Delta\Psi_m$ and low pH$_m$ are different. Our estimates of $\Delta\Psi$ (Huang et al., 2007) of −60 to −70 mV at 700 s with OMN present are much lower than without OMN; this is likely due to dissipation of both ΔpH_m and $\Delta\Psi_m$ gradients because H$^+$ pumping by complex V to support ΔpH_m (and $\Delta\Psi_m$) was blocked. With OMN present, we estimated Nernst potentials of +13 and +6 mV, respectively, for Ca^{2+} and H$^+$ (calculated at 700 s). Based on these Nernst potentials the driving forces for both Ca^{2+} and H$^+$ would remain inward with OMN present, although their Nernst potentials are reversed compared to those in the absence of OMN. With the slow inward driving force for H$^+$, unmatched by H$^+$ pumping at complex V, pH$_m$ approached pH$_e$ and net [Ca^{2+}]$_m$ became lowered due to CHE$_m$. Because inhibiting the MCU with Ru360 caused a robust increase in [Ca^{2+}]$_e$, this indicated the Ca^{2+} effluxed via CHE$_m$ re-enters via the MCU unless this pathway is blocked. Under the unique condition of collapsed $\Delta\Psi_m$ (100 μM DNP) and ΔpH_m gradients, the secondary, slow uptake of mCa^{2+} is absent (**Figures 5A,B**, black lines) so that the decline in [Ca^{2+}]$_m$ via CHE$_m$ is fully observed (**Figures 6A,B**). Thus, a fall in pH$_e$ strongly supports net mCa^{2+} efflux via CHE$_m$ even though the Nernst potentials indicate continued slow mCa^{2+} influx (via MCU), which indeed occurs if there is remaining $\Delta\Psi_m$. This means that net Ca^{2+} efflux due to CHE$_m$ (**Figures 1**, **2** and **Supplementary Figure S.6**) can be exposed by blocking the MCU after the initial bolus of CaCl$_2$ to prevent further mCa^{2+} uptake. CHE$_m$ is predicted by the J_{CHE} equation to favor mCa^{2+} efflux in exchange for mH$^+$ influx based on matrix and buffer ion concentrations obtained with OMN present (**Supplementary Figure S.10**). Our prediction assumes that Ca^{2+} is exchanged for 2H$^+$ with equal affinities for both cations, or a higher affinity for H$^+$.

Does Transient, Low Conductance mPTP Also Shuttle Ca^{2+} Across the IMM in These Experiments?

Inducing a partial $\Delta\Psi_m$ depolarization was reported to cause a slow influx of mCa^{2+} through low conductance mPTP opening (Saotome et al., 2005). CsA prevented both an increase in mCa^{2+} and the release of the small molecule calcein during simulated ischemia in cardiomyocytes suggesting that transient mPTP opening during ischemia allowed mCa^{2+} influx (Seidlmayer et al., 2015). In the present study adding CaCl$_2$ in the presence of DNP or an acidic buffer caused falls in $\Delta\Psi_m$, so could low conductance mPTP opening have contributed to the secondary, slow increase or decrease in $_m$[Ca^{2+}] we observed in the absence or presence of OMN? We doubt this for the following reasons: (1) ROS, adenine nucleotide levels, and other factors are believed to contribute to mPTP formation during IR injury. But in our study we did not utilize IR to induce increases in Ca^{2+} and ROS or decreases in pH$_m$ or $\Delta\Psi_m$; (2) Altering just the driving force for protons across the IMM using DNP or external pH to exchange Ca^{2+} ion for H$^+$ ions is not compatible for a mechanism to cause or prevent formation of mPTP but it is for inducing mCHE activity; (3) Transient mPTP formation is controversial and based largely on the utility of calcein or other small particles to mark mitochondrial release of small molecules with free flowing ions such as Ca^{2+} (Petronilli et al., 1999); (4) CsA-sensitive transient mPTP opening in individual mitochondria of cardiac myocytes is quite rare even with elevated m[Ca^{2+}] or exposure to H$_2$O$_2$ (Lu et al., 2016); (4) CsA, or its inhibition of the peptidyl prolyl *cis–trans* isomerase activity of cyclophilin D, has known and unknown effects on mitochondrial function that may be unrelated to mPTP formation (Giorgio et al., 2010). Some interpretations on effects of cyclophilin D, via CsA, may pertain to changes in Ca^{2+} flux due to mCHE rather than transitional mPTP opening.

CSA Ceases Activation of CHE$_m$

CsA unexpectedly stopped the secondary CaCl$_2$-induced effects attributed to CHE$_m$. CsA ceased all apparent CHE$_m$ activity after adding CaCl$_2$ when pH$_e$ was 6.9 or 7.15, as assessed by measurements of extra-matrix [Ca^{2+}]$_e$, pH$_m$, and $\Delta\Psi_m$ (**Supplementary Figures S.1A–C**). CsA did not blunt the partial $\Delta\Psi_m$ depolarization induced by DNP alone at pH$_e$ 7.15, but did delay full $\Delta\Psi_m$ depolarization induced by adding CaCl$_2$ after DNP (**Supplementary Figures S.7A,B**). We do not believe the slow, attenuated decreases in extrusion of Ca^{2+} or slow fall in matrix pH observed in the presence of CsA are directly related to inhibition of permanent or transient mPTP opening. CsA did not directly prevent the $\Delta\Psi_m$ depolarization that occurs during CHE$_m$ or with addition of DNP alone. In the absence of CsA (**Figures 1A–C**), the observed changes in pH$_m$, external [Ca^{2+}]$_e$, and $\Delta\Psi_m$, induced by adding CaCl$_2$ at extra-matrix pH 6.9, occurred very slowly over 25–30 min; this is indicative of slow cation exchange activity, not mPTP. Moreover, full $\Delta\Psi_m$ depolarization was incomplete. CsA or its inhibition of cyclophilin D may obviate the conditions for matrix H$^+$ influx or mCa^{2+} efflux as well as Ca^{2+} recycling via the MCU. CsA

may prevent dissipation of the ΔpH gradient when the external pH is low. Since the results obtained in the presence of CsA are not compatible with preventing or delaying mPTP opening, the effects of CsA in this setting are unclear. Additional experiments will be needed to delineate the mechanism of CsA on preventing CHE_m.

Other Potential Limitations of the Study

One important limitation of our study is the lack of a selective inhibitor of CHE_m to aid in defining a more precise mechanism of action. Since the gene code for LETM1 and its protein sequence are known, point mutations (Tsai et al., 2014) and knockdowns (Jiang et al., 2013; Doonan et al., 2014) in mammalian models will be helpful to assess mechanisms and kinetics of this cation antiporter; but it remains unclear if LETM1 mediates CHE_m exclusively, or at all. Another limitation is that mitochondria were examined outside their normal milieu so that the contributions of ATP synthesis by glycolysis and ATP hydrolysis for cellular metabolic support could not be assessed. Experiments were conducted at room temperature at which metabolism would be lower and buffering capacity different than at 37°C. The activity of CHE_m during cardiac IR is unknown and mCa^{2+} efflux in cardiac mitochondria may occur primarily via the NCE_m and not CHE_m. Nevertheless, induction of CHE_m could occur *in vivo* during IR injury under very specific circumstances of trans-IMM cationic imbalance. Evaluation of CHE_m activity in cardiac myocytes after IR injury should be helpful to design protective strategies using this mechanism.

CONCLUSION

This study furnishes new insights into the bioenergetic and dynamic mechanisms in cardiac cell mitochondria of delayed, slow mCa^{2+} influx via the MCU, and mCa^{2+} efflux via the pH_m-dependent CHE_m. We demonstrate the kinetics of slow changes in mCa^{2+} loading/unloading that are linked to unblocked *vs.* blocked ATP_m hydrolysis to decrease *vs.* increase pH_m, respectively, after partial depolarization by DNP. We found that after an initial $CaCl_2$ bolus there is slow mCa^{2+} influx (Ca^{2+} leak) through a Ru360-sensitive pathway if H^+ pumping counteracts a H^+ leak; however, there is net slow mCa^{2+} efflux that overrides $\Delta\Psi_m$-mediated Ca^{2+} influx that is activated via CHE_m if there is a high ΔpH_m gradient. In cardiac mitochondria, the rapid and slow mode of uptake of mCa^{2+} appears to be dependent primarily on the trans-membrane $[Ca^{2+}]$ and $\Delta\Psi_m$ gradients if outward H^+ pumping counteracts inward H^+ entry. In contrast, slow extrusion of mCa^{2+} by CHE_m appears to be dependent primarily on the $[\Delta H^+]_m$ gradient induced by H^+ influx/leak by DNP or by an acidic pH_e. Importantly, if NCE_m

and NHE_m are inactivated, blocking complex V might prevent delayed Ca^{2+} overload and instead stimulate Ca^{2+} extrusion via CHE_m if there is an inward H^+ leak. In intact cells, this can also serve to preserve TCA cycle-generated ATP, i.e., substrate level phosphorylation. Such passive homeostatic balance of $\Delta[Ca^{2+}]_m$ may occur during cardiac injury when there is mCa^{2+} loading accompanied by declines in NADH redox state, pH_m and Ψ_m. We conclude that the differences in the rate and magnitude of mCa^{2+} influx/efflux in partially depolarized mitochondria, in the presence or absence of $F_OF_{1-}ATPase$ activity, can be ascribed to the underlying changes in *pmf* components, ΔpH_m and $\Delta\Psi_m$, after rapid mCa^{2+} loading.

AUTHOR CONTRIBUTIONS

DS proposed the study and its initial design. JH conducted most experiments, carried out initial statistical analysis, constructed initial figures, and participated in design, interpretation and writing. AG, AB, CB, CN, and MB conducted supporting experiments. AC, W-MK, and RD participated in theoretical interpretation of the results and text editing. DS and AC supervised the team in subsequent experimental designs, interpretation of results, and manuscript construction and writing.

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SUPPLEMENTARY MATERIAL

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