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Mitochondrial Ca^{2+} influx and efflux rates in guinea pig cardiac mitochondria:Low and high affinity effects of cyclosporine A☆

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ABSTRACT

Ca^{2+} plays a central role in energy supply and demand matching in cardiomyocytes by transmitting changes in excitation–contraction coupling to mitochondrial oxidative phosphorylation. Matrix Ca^{2+} is controlled primarily by the mitochondrial Ca^{2+} uniporter and the mitochondrial Na^+/Ca^{2+} exchanger, influencing NADH production through Ca^{2+}-sensitive dehydrogenases in the Krebs cycle. In addition to the well-accepted role of the Ca^{2+}-triggered mitochondrial permeability transition pore in cell death, it has been proposed that the permeability transition pore might also contribute to physiological mitochondrial Ca^{2+} release. Here we selectively measure Ca^{2+} influx rate through the mitochondrial Ca^{2+} uniporter and Ca^{2+} efflux rates through Na^+-dependent and Na^+-independent pathways in isolated guinea pig heart mitochondria in the presence or absence of inhibitors of mitochondrial Na^+/Ca^{2+} exchanger (CGP 37157) or the permeability transition pore (cyclosporine A). cyclosporine A suppressed the negative bioenergetic consequences ($\Delta\Psi_m$ loss, Ca^{2+} release, NADH oxidation, swelling) of high extramitochondrial Ca^{2+} additions, allowing mitochondria to tolerate total mitochondrial Ca^{2+} loads of >400 nmol/mg protein. For Ca^{2+} pulses up to 15 μM, Na^+-independent Ca^{2+} efflux through the permeability transition pore accounted for ~5% of the total Ca^{2+} efflux rate compared to that mediated by the mitochondrial Na^+/Ca^{2+} exchanger (in 5 mM Na^+). Unexpectedly, we also observed that cyclosporine A inhibited mitochondrial Na^+/Ca^{2+} exchanger-mediated Ca^{2+} efflux at higher concentrations ($IC_{50}=2$ μM) than those required to inhibit the permeability transition pore, with a maximal inhibition of ~40% at 10 μM cyclosporine A, while having no effect on the mitochondrial Ca^{2+} uniporter. The results suggest a possible alternative mechanism by which cyclosporine A could affect mitochondrial Ca^{2+} load in cardiomyocytes, potentially explaining the paradoxical toxic effects of cyclosporine A at high concentrations. This article is part of a Special Issue entitled: Mitochondria and Cardioprotection.

1. Introduction

Ca^{2+} is the central player in excitation–contraction coupling in cardiac myocytes, but it also serves as an important signaling molecule between cytosol and mitochondria. In the heart, mitochondria take up Ca^{2+} from the cytosol through the calcium uniporter (mCU) and extrude it primarily through the mitochondrial Na^+/Ca^{2+} exchanger (mNCE) [1,2]. Mitochondrial calcium ($Ca^{2+}{}_m$) regulates NADH production by activating Ca^{2+}-sensitive dehydrogenases in the TCA cycle [3] and may stimulate oxidative phosphorylation at other sites as well [4,5]. By changing the driving force for Ca^{2+} efflux through the mNCE, alterations in cytosolic Na^+ ($Na^+{}_i$) will significantly impact the rate of mitochondrial Ca^{2+} accumulation when the amplitude or frequency of the cytosolic Ca^{2+} transient changes. For example, $Na^+{}_i$ is elevated in chronic heart failure [6–8] and during ischemia–reperfusion injury [9–12], as well as during treatment with cardiac glycosides, and we have shown that high $Na^+{}_i$ blunts $Ca^{2+}{}_m$ accumulation in cardiac myocytes subjected to a rapid increase in work [13–16]. Moreover, the oxidation of NAD(P)H associated with inadequate mitochondrial Ca^{2+} signaling contributes to increased oxidative stress, arrhythmias and contractile dysfunction [15,16]. These detrimental effects are prevented by treatment with an mNCE inhibitor, or by lowering $Na^+{}_i$, emphasizing the importance of mitochondrial Ca^{2+} efflux as a modulator of excitation–contraction–bioenergetic coupling.

Na^+-independent mitochondrial Ca^{2+} efflux can also play a role in mitochondrial Ca^{2+} regulation, particularly in certain non-cardiac tissues in which a H^+/Ca^{2+} antiporter is active [17]. Alternatively, Gunter and Pfeiffer [18] proposed that the transient and reversible opening of the mitochondrial permeability transition pore (PTP) could be an energetically favorable way to release mitochondrial Ca^{2+} in a Na^+-independent manner, albeit with the proviso that the

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concomitant loss of mitochondrial membrane potential ($\Delta\Psi_m$) would have to be rapidly reversed in order to maintain ATP production. In this light, Altschuld et al. [19] reported that cyclosporine A (CsA), an inhibitor of the PTP [20,21], increased mitochondrial Ca^{2+} load in rat ventricular myocytes, possibly explaining the toxic effects of CsA on electrically-paced rat cardiomyocytes [22]. In a recent study, we too observed enhanced mitochondrial Ca^{2+} loading in response to pacing in the presence of CsA (see Supplemental data Fig. S2 in Ref. [14]). These observations could be interpreted as evidence for a physiological role for reversible PTP opening during Ca^{2+} cycling or that pathophysiological activation of the PTP is occurring in a fraction of mitochondria in the network of the isolated cardiomyocyte. However, based on the behavior of isolated mitochondria, unless there is significant metabolic stress [23,24], mitochondria should be able to tolerate rather large Ca^{2+} loads without activation of the PTP because of the robust Ca^{2+} buffering capacity of the matrix due to the formation of calcium phosphate precipitates [25,26].

In the context of the outstanding questions mentioned above, and in order to understand mitochondrial Ca^{2+} handling in both normal and disease states, quantitative measurements of unidirectional Ca^{2+} uptake and efflux rates are necessary. In this study, Ca^{2+} fluxes through mCU, mNCE and PTP were measured in normal isolated guinea pig heart mitochondria. The effects of variables such as $Ca^{2+}{}_i$, $Na^+{}_i$, and CsA on the mitochondrial Ca^{2+} transport were explored. The results indicate that the steady state extramitochondrial Ca^{2+} concentration is strongly influenced by $Na^+{}_i$ and that, unexpectedly, CsA, at concentrations higher than that required to inhibit the PTP, has an inhibitory effect on *both* PTP- and mNCE-mediated mitochondrial Ca^{2+} efflux. These kinetic measurements also provide essential information for refinement of computational models of mitochondrial Ca^{2+} handling, with the ultimate goal of interpreting the influence of mitochondria on cellular Ca^{2+} handling, redox potential and energetics.

2. Methods

Guinea pig heart mitochondria were isolated using a protocol described previously [27]. The extramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{out}$) was measured using the Ca^{2+}-sensitive fluorescent probe, CaGreen-5N, hexapotasssium salt (Molecular Probes, Invitrogen) in a fluorometer (Quantamaster, Photon Technologies International) at room temperature. Mitochondria (~0.5 mg) were suspended in a potassium-based buffer solution consisting of 137 mM KCl, 2 mM KH_2PO_4, 20 μM EGTA, 20 mM HEPES, and 5 mM glutamate/malate (G/M) at pH 7.15. Calcium green-5N (0.1 μM) fluorescence was recorded at excitation and emission wavelengths of 505 nm and 535 nm [28]. Mitochondrial 90° light scattering was monitored at 540 nm with a second detector and NADH fluorescence was recorded with excitation at 350 nm and emission at 450 nm. Mitochondrial membrane potential was monitored by the ratiometric method of Scaduto and Grotyohann [29] using tetramethylrhodamine methyl ester (TMRM) at excitations of 546 nm and 573 nm and emission at 590 nm. Mitochondrial protein concentrations were determined by the BCA assay (Thermo Scientific Pierce).

Free calcium in the buffer solution was calculated using MaxChelator (http://www.stanford.edu/~cpatton/maxc.html) and a standard curve was constructed in the presence of mitochondria, but with Ca^{2+} uptake blocked (see Supplemental Figure S1) relating the CaGreen-5N signal to the free Ca2+ concentration in the buffer solution by fitting to the Grynkiewicz equation [30].

$$\left[Ca^{2+}\right]_{free} = K_d\left[\frac{F-F_{min}}{F_{max}-F}\right]$$

where F is the fluorescence intensity of calcium green at the experimental calcium level, F_{min} is the fluorescence intensity without calcium, F_{max} is the fluorescence intensity of CaGreen-5N saturated with calcium, and the K_d (14 μM) was the solution dissociation constant of CaGreen-5N provided by the manufacturer.

To study CsA effects on Ca^{2+} transport by mitochondria, cyclosporine A (Sigma-Aldrich) was added directly to the mitochondrial suspension from a 4 mM dimethyl sulfoxide (DMSO) stock solution. Amounts of DMSO alone, equivalent to those of the largest amount used in a given experiment (usually <1% of total volume), had no effect on the measured parameters.

2.1. Statistical analysis

The results are presented as mean ± SEM. An unpaired Student's *t*-test was used to evaluate the significance of the differences between means of CsA-treated versus untreated mitochondrial Ca^{2+} fluxes using either Origin (Microcal) or Matlab statistical toolbox. Statistical significance was assumed at $P<0.05$.

3. Results

The capacity of guinea pig heart mitochondria to accumulate Ca^{2+} was tested by multiple additions of Ca^{2+} (first addition was 15 μM and subsequent ones were 25 μM each) while monitoring extramitochondrial Ca^{2+} concentration ($[Ca^{2+}]_{out}$), NADH, light scattering and $\Delta\Psi_m$ (Fig. 1). The first addition of Ca^{2+} evoked rapid Ca^{2+} uptake from the medium and a small (~5 mV) depolarization of $\Delta\Psi_m$ corresponding to the energetic cost of Ca^{2+} entry (that is, Ca^{2+} uptake, coupled to Na^+/Ca^{2+} exchange, coupled to Na^+/H^+ exchange), a transient oxidation of NADH, and a decrease in light scattering corresponding to an increase in mitochondrial volume (Fig. 1A and B). In the absence of CsA, by the third or fourth Ca^{2+} addition, a secondary release of Ca^{2+} is evident and is accompanied by a larger and sustained decrease in $\Delta\Psi_m$ and NADH, but the ability of the suspension to regulate extramitochondrial Ca^{2+} is still partially maintained (Fig. 1A). After the sixth Ca^{2+} addition, however, corresponding to a total mitochondrial Ca^{2+} load of more than 400 nmol Ca^{2+}/mg mitochondrial protein, $\Delta\Psi_m$ collapses, NADH is oxidized, additional swelling occurs, and Ca^{2+} is released to the medium (Fig. 1A). In striking contrast, in the presence of CsA (4 μM), the mitochondria readily take up eight additions of Ca^{2+} while maintaining $\Delta\Psi_m$, NADH, and mitochondrial volume. Steady-state $[Ca^{2+}]_{out}$ was regulated at a constant setpoint (Fig. 1B) with no evidence of a permeability transition.

Since PTP activation was not a factor for the response to a single addition of Ca^{2+}, we focused on measuring the mitochondrial Ca^{2+} influx and efflux rates under various conditions for additions of Ca^{2+} no greater than 20 μM. For a single Ca^{2+} addition of 15 μM, in the absence of Na^+, mitochondrial Ca^{2+} uptake was rapid and $[Ca^{2+}]_{out}$ was lowered to <0.32 μM (Fig. 1C; left panel) in less than 200 s. The Ca^{2+} addition had minimal effects on $\Delta\Psi_m$ (it decreased by only 2 mV) (Fig. 1C; left panel) and NADH (Fig. 1C; right panel). The effects of sequentially increasing Na^+ on steady state $[Ca^{2+}]_{out}$ were then tested. Increasing extramitochondrial Na^+ concentration to 5, 10 and 15 mM Na^+ increased the $[Ca^{2+}]_{out}$ setpoints to 0.80 μM, 1.28 μM, and 1.34 μM, respectively (Fig. 1C; left panel). For Na^+ greater than 15 mM, however, $[Ca^{2+}]_{out}$ paradoxically decreased to 1.12 μM (30 mM) and 0.76 μM (60 mM) presumably because the capacity of the mitochondria to regulate matrix Na^+ through Na^+/H^+ exchange may have been saturated, thus allowing matrix Na^+ levels to rise and decreasing the driving force for Ca^{2+} extrusion through the mNCE.

Mitochondrial calcium uptake and extrusion in cardiac mitochondria are mediated mainly through mCU and mNCE under normal conditions in the absence of PTP activation [18]. A protocol was developed to study the individual Ca^{2+} uptake and efflux rates by loading mitochondria with a single 15 μM Ca^{2+} pulse and then selectively blocking mCU with Ru360 (mCU inhibitor) [31–33], mNCE with CGP-37157 (CGP) [34] or PTP with CsA [21]. With 5 mM Na^+ in

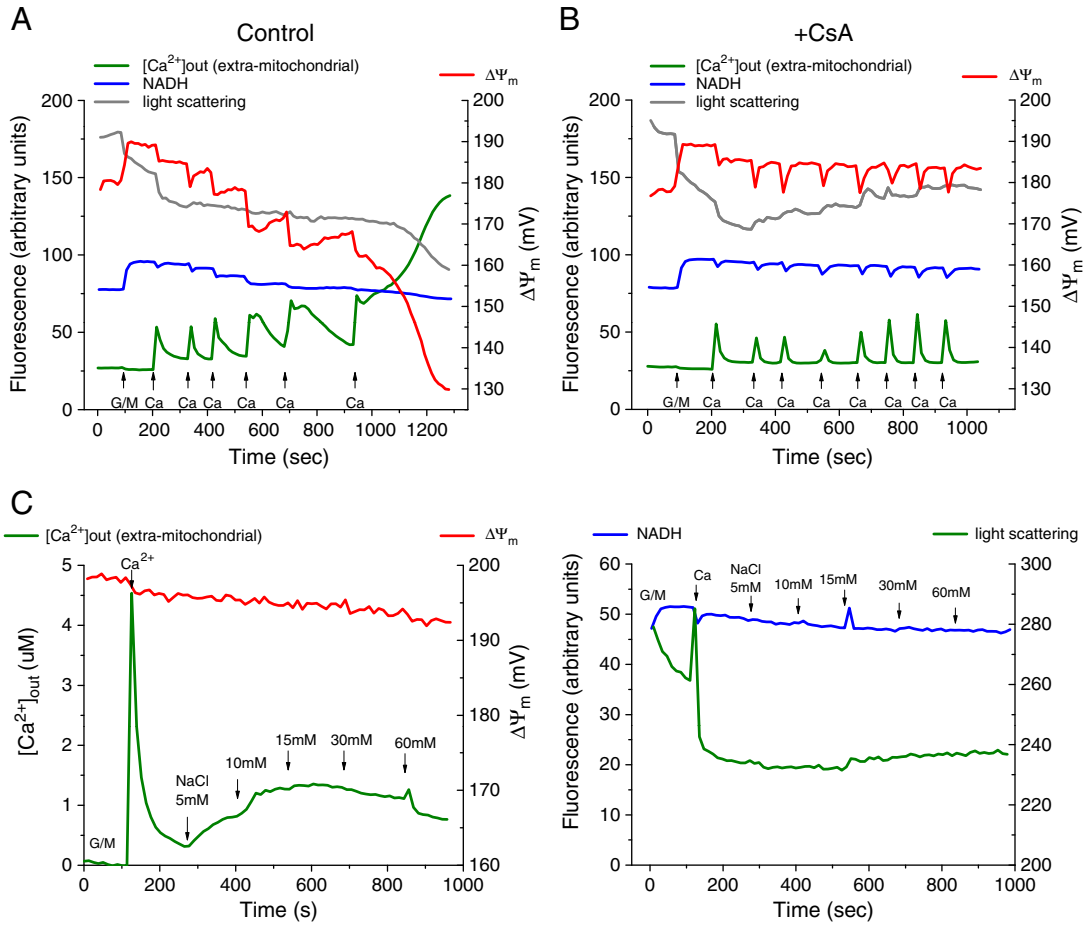

Fig. 1. Effects of sequential Ca^{2+} additions on mitochondrial Ca^{2+} uptake and energetic parameters. Mitochondria were suspended in KCl-based solution with 5 mM NaCl and 5 mM G/M and the mitochondrial membrane potential (ΔΨm), NADH, volume change(from light scattering) and extra-mitochondrial Ca^{2+} level were simultaneously monitored. (A) In the absence of cyclosporine A (CsA), the mitochondrial permeability transition pore (PTP) opens after a train of calcium pulses accompanied by mitochondrial swelling, membrane potential depolarization, and efflux of Ca^{2+} from the mitochondria. The first calcium pulse is 15 μM free Ca^{2+} and each pulse afterward was 25 μM. The total free Ca^{2+} load tolerated before full PTP opening in this experiment was 400 nmol/mg. (B) 4 μM cyclosporine A augmented mitochondrial calcium uptake capacity by preventing PTP opening. (C) After the Ca2+ addition, multiple pulses of Na^+ were added from 5 mM to 60 mM to examine the effects of increased Na^+-dependent efflux on steady state extramitochondrial Ca^{2+}.

the buffer, Ca^{2+} was added to energized mitochondria pretreated with either CGP (10 μM), CsA (10 μM), or CGP + CsA. In all cases, mitochondrial Ca^{2+} uptake was rapid and a steady state representing the balance between Ca^{2+} uptake and efflux rates was achieved approximately 200 s after the pulse (Fig. 2; phase i). The steady state $[Ca^{2+}]_{out}$ (Ca_{SS}) was modestly decreased by CsA and more significantly lowered by CGP or CGP + CsA (to ~0.5 μM). The unidirectional Ca^{2+} efflux rate was then measured after application of Ru360 (Fig. 2; phase ii). CGP suppressed the majority of the Ca^{2+} efflux in 5 mM Na^+ (Fig. 2; blue trace). Interestingly, in addition to blocking the remaining small CGP-insensitive Ca^{2+} efflux (CGP + CsA; Fig. 2; green trace), CsA also inhibited a significant fraction of total Ca^{2+} efflux (Fig. 2; red trace), prompting further investigation of its effects.

The Na^+ dependence of Ca_{SS} and the Ca^{2+} efflux rate in the presence and absence of CsA was analyzed by varying Na^+ in the buffer from 0 to 60 mM (Fig. 3A). Several parameters were measured for this protocol (Fig. 3B): the initial Ca^{2+} uptake rate (rate 1; Fig. 3C) after the addition of Ca^{2+}; the net Ca^{2+} extrusion rate after Ru360 addition (rate 2; Fig. 3D); and the Ca_{SS} (at the end of phase i; Fig. 3E). The average mitochondrial Ca^{2+} load after a single Ca^{2+} addition (15 μM) was calculated as the total amount of Ca^{2+} added to the cuvette minus Ca_{SS}, normalized to the mitochondrial protein concentration (Fig. 3F).

Ca_{SS} (Fig. 3E) increased from 0.6 μM to 2 μM as extra-mitochondrial Na^+ concentration increased from 5 mM to 15 mM by increasing net Ca^{2+} extrusion (Fig. 3D) and reducing mitochondria Ca^{2+} load (Fig. 3F). When $[Na^+]_{out}$ was higher than 30 mM, Na^+ had the opposite effect and mitochondrial Ca^{2+} load increased (i.e., Ca_{SS} decreased). CsA (10 μM) also decreased Ca_{SS} (Fig. 3E) and increased Ca^{2+} load in the mitochondria (Fig. 3F). The net unidirectional Ca^{2+} efflux rate was significantly reduced by CsA (Fig. 3D) but there was no effect of CsA on the Ca^{2+} uptake rate (Fig. 3C).

A second protocol was employed to more selectively measure individual fluxes from mCU, mNCE and PTP (Fig. 4A). A single Ca^{2+} addition was made under zero-Na^+ conditions and then Ru360 was added to block the mCU, leaving active only the Na^+-independent Ca^{2+} efflux pathway [35]. Na^+ was then added to activate mNCE [17,36]. The maximum uptake rate of mCU measured in guinea pig heart mitochondria with a 15 μM free Ca^{2+} addition was 0.49 ± 0.04 nmol/s/mg (Fig. 4B). The Na^+-independent Ca^{2+} extrusion rate was 0.003 ± 0.0004 nmol/s/mg and the Na^+-dependent (plus Na^+-independent) extrusion rate with 5 mM Na^+ was 0.018 ± 0.003 nmol/s/mg (Fig. 4B). CsA (10 μM) reduced Na^+-independent Ca^{2+} efflux measured in phase ii from 0.003 to 0.001 nmol/s/mg, consistent with a small Ca^{2+} leak mediated by the PTP [37,38]. However, in the presence of CsA, a 40% inhibition of the *Na^+-dependent* Ca^{2+} efflux measured in phase iii, was also observed (CsA decreased the Na^+-dependent efflux rate to 0.011 ± 0.001 nmol/s/mg; Fig. 4B).

The dependence of the Ca^{2+} transport rates on the size of the extramitochondrial Ca^{2+} addition ($[Ca^{2+}]_{added}$), ranging from 2 μM to 20 μM, using the second protocol was determined (Fig. 4C). The maximal Ca^{2+} uptake rate increased linearly as a function of $[Ca^{2+}]_{added}$ from

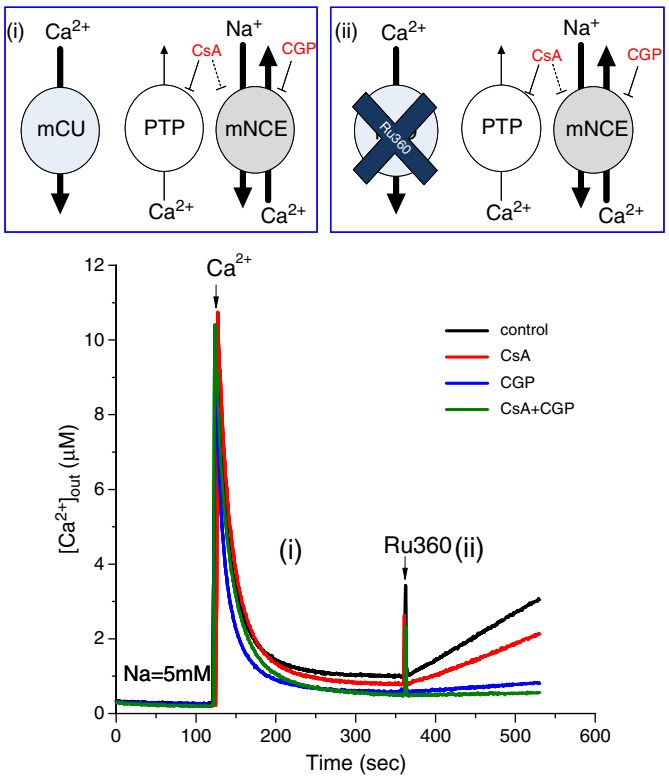

Fig. 2. Selective block of mCU following a Ca^{2+} addition with Ca^{2+} uptake and efflux active. Phase (i): in the presence of 5 mM NaCl and 5 mM G/M, a 15 μM Ca^{2+} pulse was given and there is a net uptake of Ca^{2+}. A steady state extramitochondrial Ca^{2+} concentration is attained when the Ca^{2+} influx and efflux rates are equal. Phase (ii): after the addition of Ru360, influx through the mCU is blocked and net Ca^{2+}efflux occurs via the Na^+-dependent pathway (mNCE) and the Na^+-independent pathway (PTP). CsA partially inhibited efflux, which was mainly contributed by the mNCE, as indicated by its CGP-37157 sensitivity. Inhibitor concentrations: 5 nM Ru360, 10 μM CsA, and 10 μM CGP.

0.05 nmol/s/mg to 0.6 nmol/s/mg and was not significantly altered by CsA. The mNCE flux (with 5 mM NaCl) also increased from ~0.015 to 0.03 nmol/s/mg as $[Ca^{2+}]_{added}$ was increased (Fig. 4C), corresponding to mitochondrial Ca^{2+} loads after the Ca^{2+} uptake phase of 4.5, 19, 37, 57, and 77 nmol/mg for $[Ca^{2+}]_{added}$ levels of 2, 5, 10, 15, and 20 μM, respectively. In the presence of 10 μM CsA, the mNCE rate was approximately 0.012 nmol/s/mg, and interestingly, this rate did not increase as a function of $[Ca^{2+}]_{added}$ (Fig. 4C). The Na^+-independent Ca^{2+} efflux was minimally increased by raising $[Ca^{2+}]_{added}$ over the range of 2-15 μM (from 0.001 to 0.002 nmol/s/mg), but a larger CsA-sensitive Na^+-independent Ca^{2+} efflux (0.006 nmol/s/mg) was evoked after a 20 μM Ca^{2+} addition (Fig. 4C).

The effects of Na^+, in the range of 2.5 mM–60 mM (covering both the physiological and pathophysiological range of Na^+_i [14,16,39]), on the mNCE rate (phase iii) were investigated after Ca^{2+} loading with 15 μM Ca^{2+} (Fig. 4D). The mNCE rate increased from 0.01 nmol/s/mg to 0.07 nmol/s/mg as $[Na^+]_{out}$ increased from 2.5 mM to 15 mM then decreased at higher $[Na^+]_{out}$ from 30 mM to 60 mM. Again, CsA partially inhibited mNCE flux, reducing the rates by about 0.01 nmol/s/mg over the full range of $[Na^+]_{out}$.

The instantaneous mCU rate as a function of $[Ca^{2+}]_{out}$ could also be determined during active mitochondrial Ca^{2+} uptake from single Ca^{2+} additions (Fig. 5). A relationship similar to that determined above from the maximal Ca^{2+} uptake rates for various Ca^{2+} additions (Fig. 4C) was obtained.

The concentration dependence of the CsA inhibition of Ca^{2+} efflux was investigated for a range of [CsA] from 0.05 μM to 40 μM (Fig. 6). Maximal inhibition of mNCE flux (15 μM Ca^{2+} loading pulse; 5 mM Na^+ addition) by CsA was 40% (reduced from 0.020 nmol/s/mg to 0.012 nmol/s/mg) for [CsA] ≥ 10 μM (10–40 μM range equivalent to 40–160 nmol CsA/mg). The half-maximal inhibitory concentration (IC_{50}) for inhibition of the mNCE by CsA was 2 μM. Inhibition of the Na^+-independent Ca^{2+} efflux, presumably mediated by the PTP, was maximal at a lower CsA concentration (0.05 μM; 200 pmol/mg), which corresponds to the effective inhibitory concentration of CsA for the PTP reported previously [20,21,40].

4. Discussion

The present work provides the first quantitative measurements of the unidirectional Ca^{2+} uptake and extrusion rates of mitochondria from the guinea pig heart and analyzes the influence of $[Ca^{2+}]_{out}$, $[Na^+]_{out}$, CsA, CGP-37157 and Ru360 on Ca^{2+} transport. Maximal Ca^{2+} uptake rates through the Ru360-sensitive mCU for Ca^{2+} additions of 2–20 μM ranged from 0.05 to 0.6 nmol/s/mg and the steady-state extramitochondrial Ca^{2+} level was dependent on concomitant Ca^{2+} efflux, primarily through the mNCE. Both Na^+-independent and Na^+-dependent Ca^{2+} efflux pathways were present, with the mNCE rate predominating (roughly 10-fold higher than the Na^+-independent rate). The mNCE had a biphasic dependence on Na^+; its rate increasing over the range of 2.5–15 mM and then decreasing at 30–60 mM. In addition to preventing PTP activation for large mitochondrial Ca^{2+} loads exceeding 400 nmoles/mg, CsA increased the mitochondrial Ca^{2+} load for single lower Ca^{2+} pulses by inhibiting the Na^+-independent Ca^{2+} efflux pathway with high affinity (pmol/mg range) and partially inhibiting Na^+-dependent Ca^{2+} efflux with a lower affinity (~2 μM IC_{50}; nmol/mg range).

Mitochondrial Ca^{2+} transport in the heart is a topic of increasing interest and frequent controversy [41–43]. In terms of physiological regulation, Ca^{2+} uptake during EC coupling provides a crucial feedforward signal to mitochondrial oxidative phosphorylation to increase NADH production and ATP supply to meet the demands of contractile activation and ion transport. On the other hand, excessive mitochondrial Ca^{2+} loading, by activating the PTP, is a key event leading to necrotic or apoptotic cell death [24,44]. Understanding the balance between the positive and negative effects of mitochondrial Ca^{2+} requires a detailed understanding of the factors modulating the Ca^{2+} uptake and efflux pathways under normal and pathophysiological conditions. Early work by Chance [5] showed that Ca^{2+} additions to mitochondria evoked rapid (<100 ms) changes in the redox potential of the respiratory chain carrier cytochrome b and transient oxidation followed by reduction of the pyridine nucleotide (NADH) pool, as well as transient stimulation of mitochondrial respiration. A decrease in light scattering (mitochondrial swelling) was also observed upon Ca^{2+} addition, and when multiple Ca^{2+} additions were made, a threshold was reached at which NADH completely oxidized and swelling was maximal. These early observations of large amplitude mitochondrial swelling are quite similar to what is observed using the "standard" PTP assay, as shown in Fig. 1 for multiple Ca^{2+} additions. Notably, the CsA-inhibitable Ca^{2+} release and reuptake evident after the third, fourth, and fifth Ca^{2+} additions in Fig. 1A are followed by a complete release of Ca^{2+} from the mitochondrial matrix after the sixth addition (total Ca^{2+} added was 140 μM, or 400 nmoles/mg protein), which also corresponded to the collapse of $\Delta\Psi_m$, NADH oxidation, and large amplitude swelling. Prior to the sustained activation of the PTP, the CsA-sensitive partial release and reuptake of Ca^{2+} could be interpreted in several ways. First, the response could be due to a reversible or transient opening of the PTP, which has been previously proposed [37]. Alternatively, the transient Ca^{2+} release could represent irreversible opening of the PTP in a fraction of the mitochondrial population and reuptake of Ca^{2+} by the remaining intact mitochondria. Third, a combination of the two effects could be occurring, i.e., Ca^{2+} release from mitochondria in which the PTP is open, followed by PTP closure as their Ca^{2+} load is decreased

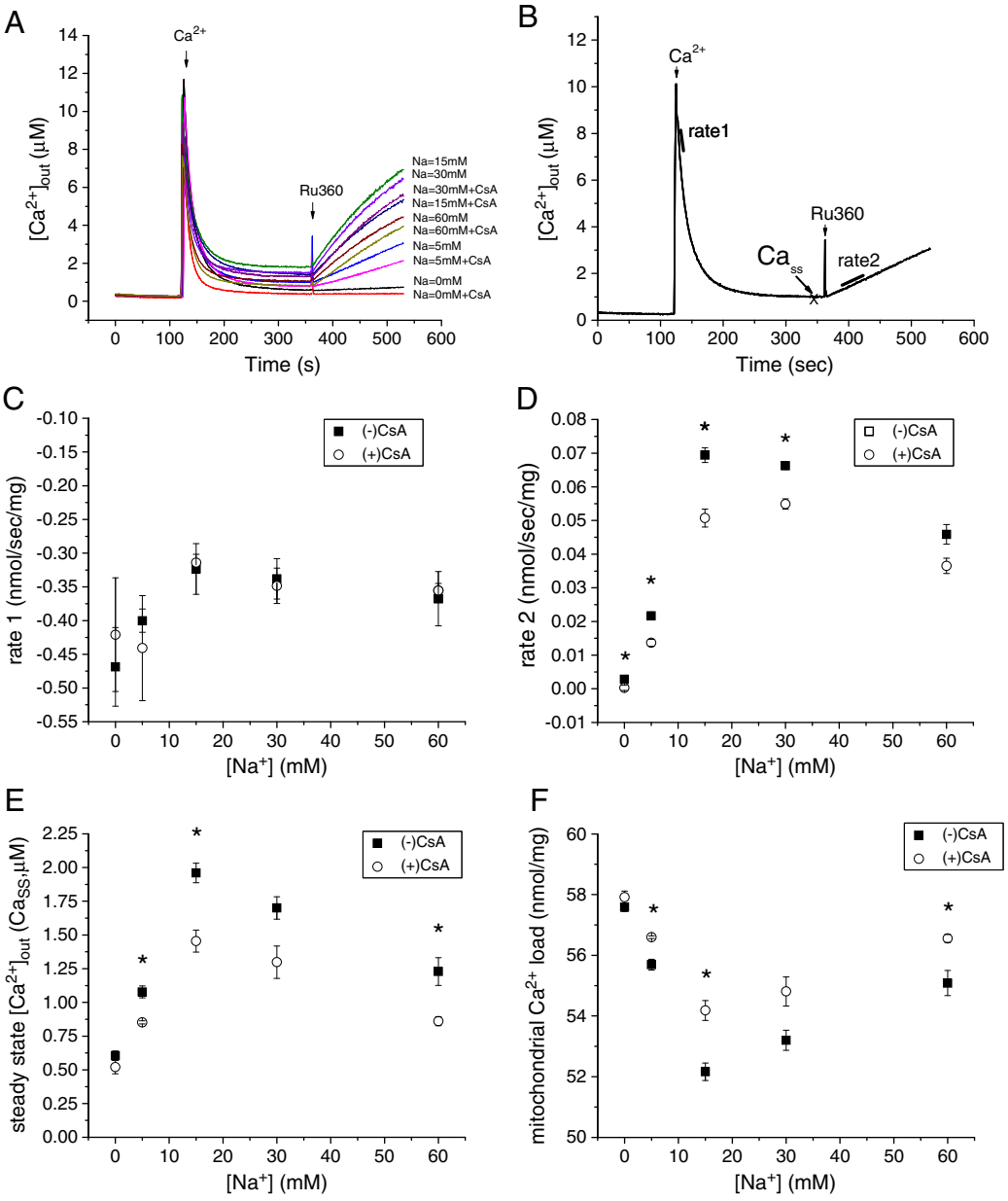

Fig. 3. Effects of extramitochondrial Na^+ on net Ca^{2+} uptake and extrusion. (A) Superimposed traces of $[Ca^{2+}]_{out}$ with the experimental protocol shown in Fig. 2. Isolated mitochondria were equilibrated in buffer solutions containing 0–60 mM Na^+, with or without 10 μM CsA. (B) Mitochondrial parameter measurements: Ca^{2+} uptake slope at 130–140 s (rate 1), Ca^{2+} efflux slope at 380–390 s (rate 2), steady state extramitochondrial Ca^{2+} at 350 s (Ca_{SS}). (C–E) Na^+ effects on rate 1, rate 2 and Ca_{SS}. (F) The mitochondria Ca^{2+} load calculated from the difference between the Ca^{2+} added and the Ca_{SS}. Data presented as mean ± SEM, $n = 3$. *P<0.05, (−)CsA vs (+)CsA. Filled symbols: control data, open symbols: CsA (10 μM) treatment.

[45]. Arguing against the idea that the PTP opening is reversed is the observation that depolarization of $\Delta\Psi_m$ and oxidation of the NADH pool is sustained even after the released Ca^{2+} is taken back up, supporting the interpretation that a fraction of the mitochondria have undergone irreversible PTP activation. Interestingly, in the presence of CsA, the initial swelling evoked by Ca^{2+} is almost completely reversed in a stepwise manner by subsequent Ca^{2+} additions, indicating that some type of Ca^{2+}-mediated volume regulation has been activated (see light scattering recording in Fig. 1B). Further investigation will be required to characterize the mechanism responsible for this effect.

For smaller single Ca^{2+} additions ≤15 μM, corresponding to mitochondrial Ca^{2+} loads of 57 nmol/mg, PTP activation was not a significant factor, and the maximal Ca^{2+} uptake rate in the absence of Na^+ was not altered by CsA. This indicates that we were truly measuring the unidirectional flux through the mCU, which was completely inhibited by Ru360. The Ca^{2+} uptake rates that we measured were ~10-fold higher than the Na^+-dependent Ca^{2+} efflux rate and were similar in magnitude to, but slightly higher than, those reported previously in guinea pig heart mitochondria under less selective conditions [46]. The relatively low affinity of the mCU for Ca^{2+} (10s of μM [46]) relative to the range of diastolic (0.1 μM) and systolic (1 μM) $[Ca^{2+}]$ evident during the cytosolic transient has always raised the question of the relevance of mitochondrial Ca^{2+} uptake in the beat-to-beat regulation of Ca^{2+}[43]. Nevertheless, there is strong evidence that the close juxtaposition of mitochondria and the sites of SR Ca^{2+} release (as close as 37 nm [47]) creates a local Ca^{2+} microdomain that could support significant Ca^{2+} uptake by the mitochondria surrounding the diad [13,48,49]. Computational studies of the local Ca^{2+} in the dyadic cleft during EC coupling indicate that, at the peak of triggered SR Ca^{2+} release, $[Ca^{2+}]$ at the center of the cleft may approach 600 μM, while 200 nm away at the periphery of the cleft $[Ca^{2+}]$ may be as high as 100 μM, declining back

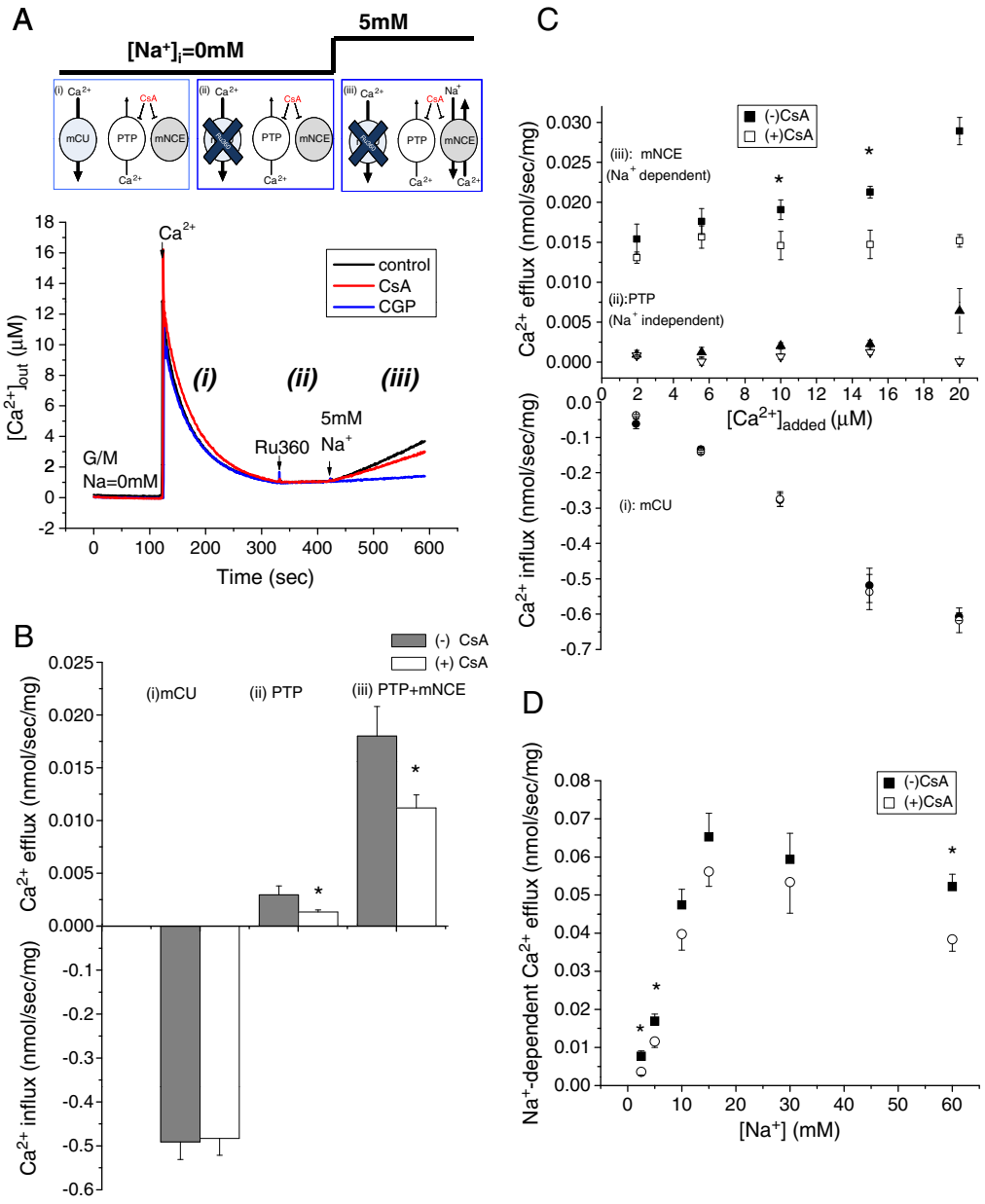

Fig. 4. Unidirectional Ca^{2+} flux rates through the mCU, PTP, and mNCE (A) A single Ca^{2+} pulse (15 μM) is given in 0 mM Na^+ solution (i: mCU-mediated Ca^{2+} uptake) and then Ru360 (5 nM) is added to block the Ca^{2+} uptake pathway, leaving only Na^+-independent Ca^{2+} extrusion (ii: includes PTP-mediated leak). 5 mM Na^+ is then added to activate Na^+-dependent Ca^{2+} extrusion (iii: mNCE). (B) Summary data for mitochondrial Ca^{2+} transport rates in the presence and absence of 10 μM CsA. Data presented as mean ± SEM, $n = 11$. *$P<0.05$. (C) Ca^{2+} dependence of the Ca^{2+} influx and efflux rates for Ca^{2+} additions between 2 and 20 μM using the same protocol. Data presented as mean ± SEM, $n = 5$. (D) Na^+-dependence of the initial mNCE rate with additions of different concentrations of Na^+ (2.5 mM–60 mM). Data presented as mean ± SEM, $n = 5$. Filled symbols: control data, open symbols: CsA (10 μM) treatment.

to the diastolic level over 150 ms [50]. This would certainly be sufficient to support fast Ca^{2+} uptake through the mCU, although other cytosolic factors, such as Mg^{2+},[36,51], adenine nucleotides [52], or endogenous polyamines [53] could shift the apparent K_m for Ca^{2+} uptake (see Supplemental Fig. S3 for uptake rates in the presence of MgATP). The kinetic data obtained in the present work will be vital for refining such models of local Ca^{2+} transport to include mitochondria near the junctional microdomain.

The effect of extramitochondrial Na^+ on the rate of Ca^{2+} efflux through the mNCE was shown to be biphasic, increasing for Na^+ up to 15 mM and then decreasing for Na^+ in the range of 30–60 mM. The latter was true in the experiments where both influx and efflux were active, in which case the extramitochondrial Ca^{2+} setpoint was affected (Figs. 1C and 3), or when only Ca^{2+} efflux was active (Fig. 4D). The steady state $[Ca^{2+}]_{out}$ levels varied from 1 to 2 μM as $[Na^+]_{out}$ was increased from 5 mM to 15 mM and then decreased to ~1.25 μM at 60 mM $[Na^+]_{out}$. The suppressive effect on Ca^{2+} efflux at higher Na^+ was not due to generalized degradation of energetic functions, since $\Delta\Psi_m$, NADH, and volume were not significantly altered (Fig. 1C). We hypothesize that the suppression of the rate at $Na^+ > 30$ mM may be due to Na^+ loading of the mitochondrial matrix, which would decrease the driving force for Ca^{2+} extrusion. This could occur if Na^+ influx exceeds the capacity of the proton pumps to support Na^+ extrusion coupled to Na^+/H^+ exchange. A sigmoidal fit of the mNCE rates as Na^+ was varied over the range of 5–30 mM yielded a K_m for Na^+ of 7.5 mM, close to the previously reported value of 8 mM obtained for mNCE protein reconstituted in lipid vesicles [54].

The normal range of Na^+_i reported in unstimulated guinea pig cardiomyocytes is 5–8 mM and it increases to 15–20 mM in heart failure [14]; therefore, based on the present findings, approximately a

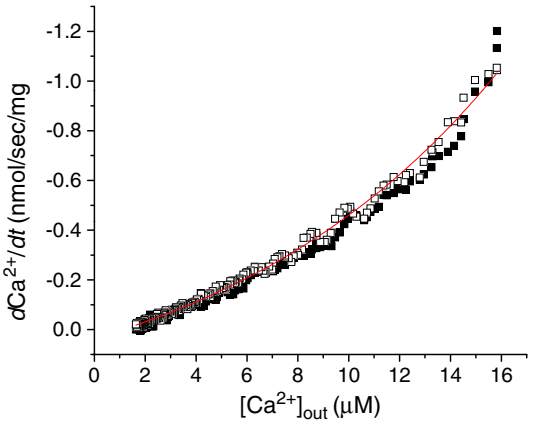

Fig. 5. Instantaneous relationship between mCU-mediated Ca^{2+} influx rate dCa^{2+}/dt and extramitochondrial Ca^{2+}.

4-fold increase in the mitochondrial Ca^{2+} efflux rate would be expected. Consistent with this effect, we have previously reported that elevated Na^{+}_{i} (15 mM) accelerates the decay rate and amplitude of mitochondrial Ca^{2+} transients and decreases the overall mitochondrial Ca^{2+} loading during a train of electrically-evoked Ca^{2+} transients in intact guinea pig myocytes—this results in insufficient activation of NADH production to compensate for increased energy demand [13]. A similar impairment of energy supply and demand exists during the development of chronic heart failure [14], or after inhibition of the sarcolemmal Na^{+} pump with the cardiac glycoside ouabain, which also elevates Na^{+}_{i} to greater than 15 mM [16]. During the course of these earlier studies, we also noted that mitochondrial Ca^{2+} accumulation during a train of Ca^{2+} transients was moderately potentiated by CsA (see Supplementary Fig. S2 in Ref. [14]), which motivated the present investigation of the effects of CsA on unidirectional mitochondrial Ca^{2+} influx and efflux rates.

For a single Ca^{2+} addition, CsA decreased steady state extramitochondrial Ca^{2+}, indicative of an increase in mitochondria Ca^{2+} accumulation. Hence, we examined whether CsA influenced mCU-mediated Ca^{2+} influx, Na^{+}-independent Ca^{2+} efflux, and Na^{+}-dependent Ca^{2+} efflux. The maximal Ca^{2+} uptake rate through the mCU was not affected by CsA (up to 10 μM). The rate of Na^{+}-independent Ca^{2+} efflux was less than 5% of the Na^{+}-dependent flux for Ca^{2+} additions up to 15 μM and this pathway was inhibited by just 50 nM CsA. This suggests that there may be some small role for PTP opening as a Ca^{2+} efflux pathway under "normal" conditions; however, one would also expect that other factors present in the cytoplasm, such as ATP [55], would further suppress this residual activation of the PTP. The larger effect of CsA on Ca^{2+} flux, in the absence of PTP activation, was to inhibit the Na^{+}-dependent Ca^{2+} efflux rate, with an IC_{50} of 2 μM. Alterations in the energy state of the mitochondria by CsA could not account for this effect, as no significant differences in $\Delta\Psi_m$, NADH, or volume were observed in the presence of CsA. The possibility that CsA could have been acting by suppressing a Na^{+}-dependent potentiation of PTP opening could also be ruled out, based on the observation that all of the Na^{+}-dependent Ca^{2+} efflux could be blocked by the mNCE inhibitor 1 μM CGP 37157, which has an IC_{50} of approximately 0.36 μM [34]. Thus, we conclude that the inhibitory effect of CsA on Na^{+}-dependent mitochondrial Ca^{2+} efflux was due to inhibition of the mNCE. The nature of this inhibition is presently unknown, but it does not appear to be a direct competitive effect of CsA on the transport site, since the fractional inhibition of mNCE by CsA decreased at higher concentrations of Na^{+}. A kinetic analysis of the data shows that the CsA effect is consistent with a "mixed type" inhibition (see Fig. S2). Notably, the CsA inhibition of Na^{+}-dependent Ca^{2+} efflux was larger in the presence of higher mitochondrial Ca^{2+} loads (Fig. 4C), which might indicate that CsA is interfering with an intramitochondrial mNCE regulatory process. By analogy with the sarcolemmal NCX, CsA could be inhibiting internal Ca^{2+} activation of the transporter (this occurs via a distinct module in the intracellular regulatory loop of the sarcolemmal NCX). Alternatively, there may be multiple isoforms of mNCE present, one of which is activated at higher mitochondrial Ca^{2+} loads and is inhibited by CsA, with the other forms insensitive to CsA inhibition. These speculative hypotheses can be tested in future studies, aided by the recent discovery of a molecular candidate for mNCE (NCLX) [56].

While the kinetics of mitochondrial Ca^{2+} fluxes for the mCU, mNCE, and PTP have been previously been characterized in mitochondria from various tissues and species [2,32,35,36,54,56–58], a systemic and quantitative assessment of the unidirectional influx and efflux rates obtained under identical conditions is required to improve our understanding of mitochondrial Ca^{2+} transport as a whole. These data will therefore be invaluable for integrated model development in the future. From the perspective of pathophysiology, there is an emerging view that inhibition of PTP opening protects against acute ischemic injury [24,59], as well as progressive degenerative diseases such as muscular dystrophy [60,61] and Alzheimer's [62]. The present findings showing an additional effect of CsA on mNCE suggest that high concentrations of CsA can influence mitochondrial Ca^{2+} dynamics by a PTP-independent mechanism. Inhibition of mNCE could be beneficial in instances where impaired mitochondrial Ca^{2+} loading is a problem, such as in heart failure [14,15]; however, increased mitochondrial Ca^{2+} loading could also have toxic effects, such as those reported by Olbrich et al. [22] for CsA concentrations

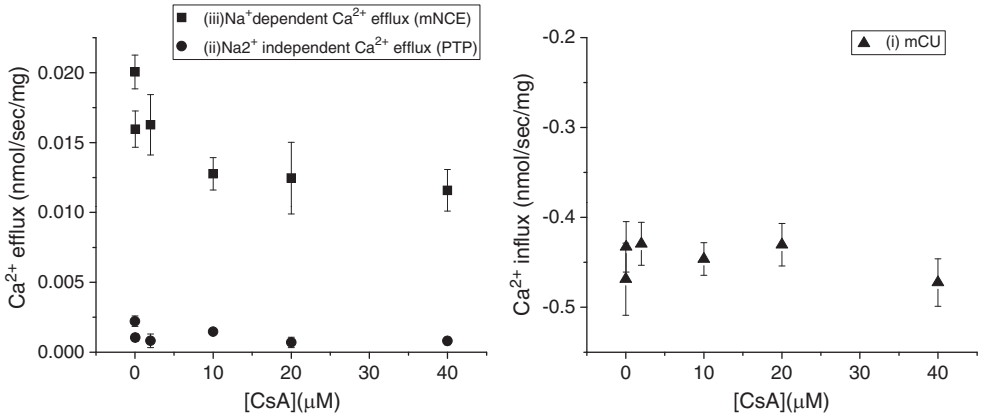

Fig. 6. Concentration-dependence of CsA inhibition of PTP- or mNCE-mediated Ca^{2+} efflux (left panel). Lack of effect of CsA on mCU rate (right panel). Mitochondria were incubated with different concentrations of cyclosporine A (0, 0.05, 2, 10, 20, 40 μM) in a KCl-based buffer solution with 5 mM G/M. Mitochondria then were given a 15 μM Ca^{2+} loading pulse and a 5 mM Na^{+} addition after a Ru360 addition. Data presented as mean ± SEM, $n = 5$–6.

ranging from 2 to 8 μM in electrically-paced rat cardiomyocytes. In addition, previous studies of the concentration dependence of CsA-mediated effects on ischemia–reperfusion injury indicated that 0.2 μM CsA, but not 2 μM CsA, was protective [63]. Thus, increased mitochondrial Ca^{2+} loading could potentially contribute to detrimental effects of CsA at concentrations above that required to inhibit the PTP. Further studies will be required to examine the mechanism of the CsA effect on mNCE and whether it requires the presence of cyclophilin D, as does PTP inhibition [64].

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