Dissection of Mitochondrial Ca\textsuperscript{2+} Uptake and Release Fluxes In Situ after Depolarization-evoked [Ca\textsuperscript{2+}]\textsubscript{i} Elevations in Sympathetic Neurons

Stephen L. Colegrove, Meredith A. Albrecht, and David D. Friel

From the Department of Neuroscience, Case Western Reserve University, Cleveland, Ohio 44106

abstract  We studied how mitochondrial Ca\textsuperscript{2+} transport influences [Ca\textsuperscript{2+}]\textsubscript{i}, dynamics in sympathetic neurons. Cells were treated with thapsigargin to inhibit Ca\textsuperscript{2+} accumulation by SERCA pumps and depolarized to elevate [Ca\textsuperscript{2+}]\textsubscript{i}; the recovery that followed repolarization was then examined. The total Ca\textsuperscript{2+} flux responsible for the [Ca\textsuperscript{2+}]\textsubscript{i} recovery was separated into mitochondrial and nonmitochondrial components based on sensitivity to the proton ionophore FCCP, a selective inhibitor of mitochondrial Ca\textsuperscript{2+} transport in these cells. The nonmitochondrial flux, representing net Ca\textsuperscript{2+} extrusion across the plasma membrane, has a simple dependence on [Ca\textsuperscript{2+}]\textsubscript{i}, while the net mitochondrial flux (J\textsubscript{mito}) is biphasic, indicative of Ca\textsuperscript{2+} accumulation during the initial phase of recovery when [Ca\textsuperscript{2+}]\textsubscript{i} is high, and net Ca\textsuperscript{2+} release during later phases of recovery. During each phase, mitochondrial Ca\textsuperscript{2+} transport has distinct effects on recovery kinetics. J\textsubscript{mito} was separated into components representing mitochondrial Ca\textsuperscript{2+} uptake and release based on sensitivity to the specific mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchange inhibitor, CGP 37157 (CGP). The CGP-resistant (uptake) component of J\textsubscript{mito} increases steeply with [Ca\textsuperscript{2+}]\textsubscript{i}, as expected for transport by the mitochondrial uniporter. The CGP-sensitive (release) component is inhibited by lowering the intracellular Na\textsuperscript{+} concentration and depends on both intra- and extramitochondrial Ca\textsuperscript{2+} concentration, as expected for the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. Above \(\sim 400\) nM [Ca\textsuperscript{2+}]\textsubscript{i}, net mitochondrial Ca\textsuperscript{2+} transport is dominated by uptake and is largely insensitive to CGP. When [Ca\textsuperscript{2+}]\textsubscript{i} is \(\sim 200–300\) nM, the net mitochondrial flux is small but represents the sum of much larger uptake and release fluxes that largely cancel. Thus, mitochondrial Ca\textsuperscript{2+} transport occurs in situ at much lower concentrations than previously thought, and may provide a mechanism for quantitative control of ATP production after brief or low frequency stimuli that raise [Ca\textsuperscript{2+}]\textsubscript{i} to levels below \(\sim 500\) nM.

title{key words:} mitochondria • calcium • calcium signaling • neurons • CGP 37157

INTRODUCTION

There is a growing interest in mitochondrial Ca\textsuperscript{2+} transport. Ca\textsuperscript{2+} uptake and release by these organelles is thought to influence the dynamics of cytosolic free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{i}) in a variety of cell types after stimuli that promote either Ca\textsuperscript{2+} entry from the extracellular medium or release from intracellular stores (for reviews see Miller, 1991, 1998; Babcock and Hille, 1998; Duchen, 1999). Modulation of [Ca\textsuperscript{2+}]\textsubscript{i}, dynamics by mitochondria may be a key factor in some forms of Ca\textsuperscript{2+} signaling (Hajnoczky et al., 1995; Budd and Nicholls, 1996; Hoth et al., 1997; Tang and Zucker, 1997; David et al., 1998). Moreover, changes in intramitochondrial free Ca\textsuperscript{2+} concentration ([Ca\textsuperscript{2+}]\textsubscript{m}) that occur as a result of stimulation are thought to regulate ATP synthesis in anticipation of cellular energy demands (McCormack and Denton, 1993; Robb-Gaspers et al., 1998).

Mitochondrial Ca\textsuperscript{2+} transport has been studied extensively in isolated mitochondria. Ca\textsuperscript{2+} uptake is controlled by a Ca\textsuperscript{2+}-sensitive uniporter (EC\textsubscript{50} \(\sim 10–20\) \(\mu\)M; Gunter and Gunter, 1994) that permits Ca\textsuperscript{2+} to flow into the matrix down its steep electrochemical gradient. Ca\textsuperscript{2+} release from neuronal mitochondria is regulated primarily by a Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (Gunter and Pfeiffer, 1990) that is distinct from the plasma membrane exchange found in many excitable cells (Crompton et al., 1978; Cox and Matlib, 1993). Overall, the magnitude and direction of net mitochondrial Ca\textsuperscript{2+} transport depends on the relative rates of Ca\textsuperscript{2+} uptake and release. When the extramitochondrial Ca\textsuperscript{2+} concentration is high, the rate of Ca\textsuperscript{2+} uptake via the uniporter greatly exceeds the maximal rate of Ca\textsuperscript{2+} release via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger (Gunter and Pfeiffer, 1990), resulting in strong net mitochondrial Ca\textsuperscript{2+} accumulation. In contrast, at lower Ca\textsuperscript{2+} concentrations, where activity of the uniporter is far below its maximum, the net mitochondrial flux should depend on the relative rates of uptake and release.

Despite the importance of mitochondrial Ca\textsuperscript{2+} uptake and release pathways in defining the rate of net mitochondrial Ca\textsuperscript{2+} transport, their individual contributions to [Ca\textsuperscript{2+}] dynamics in situ have not been determined, in part because they operate within an intracellular network of coupled transporters that makes contributions from individual transport systems difficult to resolve. Pharmacological agents have been useful in resolving this.
identifying mitochondrial contributions to depolarization-evoked \( [\text{Ca}^{2+}]_i \), responses in intact cells. Proton ionophores, such as FCCP, depolarize the inner membrane and reduce the electrochemical driving force for \( \text{Ca}^{2+} \) uptake, suppressing mitochondrial \( \text{Ca}^{2+} \) accumulation. Inhibitors of the uniporter, such as ruthenium red or its active component Ru360 (Matlib et al., 1998), directly block \( \text{Ca}^{2+} \) uptake. However, because inhibition of \( \text{Ca}^{2+} \) uptake precludes subsequent \( \text{Ca}^{2+} \) release, neither approach is suited for discriminating between mitochondrial \( \text{Ca}^{2+} \) uptake and release fluxes and their interplay in situ.

We sought to characterize the \( \text{Ca}^{2+} \) transport systems that restore resting \( [\text{Ca}^{2+}]_i \), after depolarization-induced \( [\text{Ca}^{2+}]_i \) elevations in sympathetic neurons. These cells respond to depolarization with a rise in \( [\text{Ca}^{2+}]_i \), that is initiated by \( \text{Ca}^{2+} \) entry through voltage-gated \( \text{Ca}^{2+} \) channels but is strongly influenced by mitochondrial \( \text{Ca}^{2+} \) transport (Friel and Tsien, 1994; Pivovarova et al., 1999). The net cytosolic \( \text{Ca}^{2+} \) flux was determined by measuring the rate at which \( [\text{Ca}^{2+}]_i \), declines after repolarization, and the mitochondrial and nonmitochondrial components of this flux were distinguished based on sensitivity to FCCP; cells were pretreated with thapsigargin to minimize contributions from ER \( \text{Ca}^{2+} \) transport. Separation of the net mitochondrial flux into uptake and release components was accomplished with CGP 37157 (CGP), a specific inhibitor of the mitochondrial \( \text{Na}^+/\text{Ca}^{2+} \) exchanger (Chiesi et al., 1988; Cox et al., 1993). It was found that the activity of each \( \text{Ca}^{2+} \) transport pathway depends on \( \text{Ca}^{2+} \) concentration in a distinctive manner. At high \( [\text{Ca}^{2+}]_i \), mitochondrial \( \text{Ca}^{2+} \) transport is dominated by the uptake pathway and is largely insensitive to CGP. However, mitochondrial \( \text{Ca}^{2+} \) transport also occurs at \( [\text{Ca}^{2+}]_i \) levels as low as 200–300 nM; under these conditions, the net mitochondrial flux is the sum of much larger uptake and release fluxes of similar magnitude that largely cancel. Some of these results have been presented in abstract form (Colegrove and Friel, 1998).

**MATERIALS AND METHODS**

**Cell Dissociation and Culture**

All procedures conform with guidelines established by our Institutional Animal Care and Use Committee. Sympathetic neurons were obtained as described previously (Friel and Tsien, 1992). Adult male bullfrogs (Rana catesbeiana) were killed by decapitation and pithing, after which the sympathetic chains were removed and desheathed. The chains were incubated for 40 min at 35°C in nominally Ca-free Ringer’s solution containing 3 mg/ml collagenase (Worthington, Type I) and for 10 min in Ringer’s supplemented with 1.5 mg/ml trypsin (EC 3.4.21.4, Sigma). Ringer’s consisted of (in mM): 128 NaCl, 2 KCl, 10 HEPES (N-[2-Hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid]), 10 glucose, pH 7.3, with NaOH. Normal Ringer’s contained 2 mM added CaCl₂, while low Ca²⁺ Ringer’s contained nominally Ca-free Ringer’s + 0.2 mM EGTA (ethylene glycol-bis(β-aminoethy ether) N,N’,N’-tetraacetic acid). The ganglia were washed, and cells were dispersed by trituration and plated onto poly-lysine-coated cover slips affixed with Sygird (Dow Corning) to 60-mm culture dishes, covering 20-mm-diam holes in the bottom of the dishes. Cells were cultured for up to 1 wk at room temperature (19–22°C) in a 1:1 mixture of Liebovitz’s L-15 medium (GIBCO BRL) and normal Ringer’s solution supplemented with glucose (3 μg/ml), ascorbic acid (25 μg/ml), glutathione (2.5 μg/ml), and 6,7-dimethyl-5,6,7,8-tetrahydropterine, HCl (0.25 μg/ml; Calbiochem).

**Cytosolic Calcium Measurements**

Cells were incubated with 3 μM fura-2 AM (Molecular Probes) for 40 min at room temperature with gentle agitation. Fura-2 AM was dispensed from a 1-mM stock solution in DMSO containing 25% (wt/wt) pluronic F127 (BASF Corporation) that was stored at −20°C. Cells were rinsed and recordings began after ~20 min to facilitate de-esterification of the Ca²⁺ indicator. Cells were placed on the stage of an inverted microscope (Nikon Diaphot TMD) and superfused continuously (~5 ml/min) with normal Ringer’s. Solution changes (~200 ms) were made using a system of microcapillaries (Drummond micropipats, 20 μl) mounted on a micromanipulator as described in Friel and Tsien (1992).

Cells were illuminated by light from a 150 W xenon lamp that passed through excitation filters (350 ± 5 nm, 380 ± 5 nm) mounted on a filter wheel rotating at 40–100 Hz and was focused with a 40× objective (NA 1.3; Nikon, Fluor). Emitted light passed through a long-pass dichroic mirror (400 nm) and an emission filter (510 ± 10 nm) and was detected by a photomultiplier tube (Thorn EMI 9124). A spectrophotometer (Cairn Research Limited) was used to control the filter wheel and measure fluorescence intensity at the two excitation wavelengths. Fluorescence measurements were made at 4-5 Hz and saved on a laboratory computer. \( [\text{Ca}^{2+}]_i \), was calculated according to the method of Grynkiewicz et al. (1985) as described previously (Friel and Tsien, 1992).

**Voltage Clamp**

Simultaneous measurements of depolarization-evoked \( [\text{Ca}^{2+}]_i \) elevations and voltage-sensitive \( \text{Ca}^{2+} \) currents \( (I_{\text{Ca}}) \) were made under voltage clamp in fura-2 AM loaded cells using the perforated patch technique. Patch electrodes \( (1-2 \text{ MΩ}) \) were pulled (Sutter Instruments P-97), and tips were filled with a solution containing (in mM): 125 CsCl, 5 MgCl₂, 10 HEPES, and 0–10 mM NaCl, with reciprocal changes in CsCl; pH 7.3, with CsOH. After filling, tips were back-filled with the same solution supplemented with 520 μM amphotericin B, dispersed from concentrated aliquots \( (12 \text{ mg/100 μl DMSO}) \). Amphotericin B–containing internal solutions were kept on ice and used within 2 h. After achieving a high resistance seal, series resistance declined over 5–10 min to <10 MΩ. Cells were exposed to an extracellular solution containing (in mM): 130 TEACl, 10 HEPES, 10 glucose, 2 ClCa₂, 1 MgCl₂, pH 7.3, with TEAOH. Currents were measured with an Axopatch 200A voltage clamp (Axon Instruments) using series resistance compensation (~90%) and were filtered at 5 kHz. Cells were held at −70 mV and depolarized to voltages between −15 and 0 mV, while current and fluorescence intensity were measured at 0.1–5 kHz just before and 0.2–10 s after changes in voltage, and at 4–5 Hz otherwise, and saved on a laboratory computer. Currents were corrected for a linear leak based on responses to small hyperpolarizing voltage steps. \( [\text{Ca}^{2+}]_i \), elevations evoked under voltage clamp were somewhat larger than those elicited by high K+ at comparable membrane potentials, presumably because of more rapid depolarization and more efficient \( \text{Ca}^{2+} \) channel activation under voltage clamp. However, the kinet-
ics of the [Ca$^{2+}$], recovery after repolarization were similar for the two techniques, provided that pipette solutions contained mM levels of Na$^+$. 

**Measurement of Ca$^{2+}$ Fluxes**

To study the Ca$^{2+}$ transport systems that restore [Ca$^{2+}$], to its resting level after depolarization-evoked Ca$^{2+}$ entry, cells were depolarized either by exposure to high K$^+$ Ringer’s (equimolar substitution for Na$^+$) or under voltage clamp, and the [Ca$^{2+}$], recovery that followed repolarization was examined. Cells were treated with 200–500 nM thapsigargin (Tg) for 10–20 min before beginning measurements to inhibit SERCA pump activity and minimize Ca$^{2+}$ accumulation by the endoplasmic reticulum. Such treatments rendered cells completely insensitive to other SERCA pump inhibitors, including CPA (50 µM) and BHQ (10 µM), and to caffeine (1–10 mM), each of which consistently elicited [Ca$^{2+}$], transients in cells that had not been treated with Tg.

Cells typically responded to high K$^+$ depolarization with [Ca$^{2+}$], responses that were quite reproducible, making it possible to compare, in single cells, responses elicited under several different conditions. Under voltage clamp, intracellular ion concentrations could be manipulated and Ca$^{2+}$ fluxes could be measured over a wider range of [Ca$^{2+}$], but after depolarizations in the presence of FCCP, [Ca$^{2+}$], recovered to values that were ~50–100 nM higher than those measured in the presence of FCCP before depolarization. The reason for this increase is not clear, but it is consistent with the development of a small Ca$^{2+}$ leak (~5 nM/s), which would introduce a small error in the measured FCCP-sensitive component of the total flux, leading to a slight overestimation of this flux (~5% at 500 nM [Ca$^{2+}$]).

The net cytosolic Ca$^{2+}$ flux per unit volume during the recovery ($J_i$, units: nM/s) was calculated as the time derivative of [Ca$^{2+}$], at each intermediate sample time $t_i$, according to
\[
\frac{([\text{Ca}^{2+}]_{o} - [\text{Ca}^{2+}]_{i}(t+\Delta t/2)) - ([\text{Ca}^{2+}]_{o} - [\text{Ca}^{2+}]_{i}(t-\Delta t/2))}{\Delta t}
\]
where $\Delta t$ (400–500 ms) is twice the sampling interval. For the first and last sample points, the flux was estimated by computing the slope of a fitted line over the first and last sets of three sample points, respectively, or by fitting an exponential over $4\Delta t$ and calculating the slope of the fitted exponential at the endpoints. The total Ca$^{2+}$ flux during the recovery ($J_{cont}$) was separated into mitochondrial and nonmitochondrial components based on their differing sensitivities to FCCP. The net mitochondrial flux was determined by taking the difference between the total cytosolic Ca$^{2+}$ flux in the presence and absence of FCCP at corresponding values of [Ca$^{2+}$]. The net mitochondrial Ca$^{2+}$ flux was then separated into components based on sensitivity to CGP and intracellular Na$^+$. This approach is described and validated in Results. Data were acquired at discrete times so that flux measurements in the presence and absence of an inhibitor were not always made at identical values of [Ca$^{2+}$]. Therefore, linear interpolation was used to approximate each measured flux at equally spaced values of [Ca$^{2+}$]. Before calculating difference fluxes, the measured fluxes were smoothed 1–3 times with a binomial filter that replaced each intermediate flux value $j_i$ with a weighted average of $j_i$ and its nearest neighbors ($j_{i-1} + 2j_i + j_{i+1})/4$.

**Data Analysis**

Quantifying the plateau level during the [Ca$^{2+}$], recovery. The plateau level was defined as the value of [Ca$^{2+}$] where the first inflection point occurs during the recovery. It was measured by fitting a 9–12th order polynomial to the [Ca$^{2+}$], recovery and determining where the second derivative of the fitted curve changed sign. This provided a suitable way to quantitate the plateau level and its sensitivity to CGP. At high concentrations of CGP, an inflection point was sometimes difficult to resolve, so in these cases the plateau level was defined as the value of [Ca$^{2+}$], where the second derivative fell below 0.001 nM/s$^2$.

**Statistics**

Population results are expressed as mean ± SEM and statistical significance was assessed using Student’s t test (Holm, 1971).

**Drugs**

CGP 37157 was a kind gift from Anna Suter (Novartis). Purified ruthenium red was generously provided by Dr. M. A. Matlib. Unless indicated otherwise, all other compounds were obtained from Sigma Chemical Co.

**Results**

**Mitochondrial and Nonmitochondrial Components of the Total Ca$^{2+}$ Flux**

Fig. 1 compares [Ca$^{2+}$], responses elicited by weak and strong depolarization in an intact fura-2–loaded sympathetic neuron that was pretreated with Tg to inhibit ER Ca$^{2+}$ accumulation by SERCA pumps. Exposure to a solution containing 30 mM K$^+$, which depolarizes $V_m$ from a typical resting potential of ~69.9 ± 2.5 mV to ~−35 mV (Friel and Tsien, 1992), increases [Ca$^{2+}$], from its resting level to ~300 nM (Fig. 1 A, left). After restoring [K$^+$], to 2 mM, which rapidly repolarizes $V_m$ (Friel and Tsien, 1992), [Ca$^{2+}$], declines with a nearly exponential time course (see fitted curve). During a stronger depolarization (50 mM K$^+$), which depolarizes $V_m$ to −21 ± 1.5, [Ca$^{2+}$], rises to a higher level approaching ~500 nM, and the recovery that follows repolarization is kinetically complex, consisting of four distinct phases (Fig. 1 A, middle): an initial rapid decline (i), a plateau (ii), an accelerated decline (iii), and a final slow approach to the prestimulation level (iv). Similar complex response kinetics are observed when [Ca$^{2+}$], is elevated by other means, including trains of stimulated action potentials (Friel and Tsien, 1994) and depolarization under voltage clamp (see below) and have been observed in a variety of other excitable cells (e.g., Thayer and Miller, 1990; Herrington et al., 1996; McGown et al., 1996).

Four observations indicate that mitochondria play a role in shaping [Ca$^{2+}$], responses elicited by strong depolarization in these cells. First, the responses are greatly modified if cells are stimulated during maintained exposure to the proton ionophore carbonyl cyanide p-(trifluoromethoxy) phenylhydrazone (FCCP, 1 µM; Fig. 1 A, right). In this case, [Ca$^{2+}$], rises to a higher level during stimulation, and the ensuing recovery lacks both the initial rapid decline and the slow plateau phase (30 cells). Similar modifications are observed after treatment with antimycin A$_1$ and oligomycin (see below), and after microinjection of ruthenium red (not shown). Second, rapid exposure to FCCP (10 µM) in Ca$^{2+}$-free Ringer’s elicits a large [Ca$^{2+}$], transient during the plateau phase of the recovery, but not in the same cells after [Ca$^{2+}$], returns to basal levels, ar-
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arguing that depolarization reversibly increases the Ca$^{2+}$ content of an FCCP-releasable Ca$^{2+}$ store. Third, FCCP has little or no effect on resting [Ca$^{2+}$], or on responses to weak depolarization which raise [Ca$^{2+}$], to ~300 nM or below (Friel and Tsien, 1994), indicating that the effectiveness of FCCP increases with [Ca$^{2+}$], as expected if it suppresses Ca$^{2+}$ uptake by the mitochondrial uniporter. Finally, direct measurement of total mitochondrial Ca$^{2+}$ concentration ([Ca$^m$]) in these cells using electron probe microanalysis indicates that exposure to 50 mM K$^+$ reversibly elevates [Ca$^m$] in an FCCP-inhibitable manner, and that the recovery parallels the decline in [Ca$^{2+}$], (Pivovarova et al., 1999), leaving little doubt that the FCCP-sensitive store is mitochondrial. Ca$^{2+}$ accumulation by mitochondria at high [Ca$^{2+}$] would slow the rise in [Ca$^{2+}$], during depolarization and speed the initial decline after repolarization; subsequent net mitochondrial Ca$^{2+}$ release would slow the recovery, contributing to the plateau.

To illustrate mitochondrial and nonmitochondrial contributions to the complex [Ca$^{2+}$], recovery, Fig. 1 B plots the total cytosolic Ca$^{2+}$ flux ($J = -d[Ca^{2+}]/dt$) vs [Ca$^{2+}$], for each of the three recoveries in A, (positive values of $J$ represent outward fluxes from the cytosol). In each case, $J$ is positive, indicating that Ca$^{2+}$ removal is dominant, but there is a striking difference between the recoveries that follow small and large depolarization-evoked [Ca$^{2+}$], elevations. After weak depolarization (Fig. 1 B, left), $J$ is nearly proportional to [Ca$^{2+}$], below ~300 nM, as expected if Ca$^{2+}$ is removed from the cytosol by a simple first order process. In contrast, after stronger depolarizations that elevate [Ca$^{2+}$] to higher levels (Fig. 1 B, middle), $J$ varies with [Ca$^{2+}$] in a complex manner that mirrors the temporal complexity of the recovery (Fig. 1 A, middle). During phase i, when [Ca$^{2+}$] is high, $J$ is much larger than the extrapolated linear flux (Fig. 1 B, dashed line), but then declines so that over the range of [Ca$^{2+}$], associated with the plateau (phase ii), it is smaller than the linear flux. $J$ then rises during phase (iii), approaching and ultimately coinciding with the linear flux as [Ca$^{2+}$], nears its prestimulation level (phase iv). Fig. 1 B (right) shows $J$ during the recovery after 50 K$^+$ depolarization in the presence of FCCP. For [Ca$^{2+}$], up to ~300 nM, the FCCP-resistant flux ($J_{FCCP resist}$) closely resembles the linear flux that restores [Ca$^{2+}$], to its resting level after weak depolarization (dashed line). However, at higher [Ca$^{2+}$], $J_{FCCP resist}$ is smaller than the extrapolated linear flux, indicating...
that Ca\(^{2+}\) removal by the underlying transporters becomes limited when [Ca\(^{2+}\)] is high, or that a source of Ca\(^{2+}\) is active just after repolarization (see also Herrington et al., 1996; Fig. 3 B and C). Treatment with oligomycin did not modify the effects of FCCP, arguing that ATP consumption via reverse mode ATP synthase activity does not seriously deplete ATP during treatment with FCCP in these experiments (not shown).

A simple interpretation of the complex [Ca\(^{2+}\)], recovery after large [Ca\(^{2+}\)], elevations is that the underlying Ca\(^{2+}\) flux consists of two components. One component represents Ca\(^{2+}\) removal by nonmitochondrial transporters at a rate that increases saturably with [Ca\(^{2+}\)], while the other component represents reversible net Ca\(^{2+}\) transport by mitochondria: net Ca\(^{2+}\) accumulation at high [Ca\(^{2+}\)], followed by net Ca\(^{2+}\) release at low [Ca\(^{2+}\)]. An obvious approach to separating J into mitochondrial and nonmitochondrial components is to take the difference between J in the presence and absence of FCCP at corresponding values of [Ca\(^{2+}\)], to give the FCCP-sensitive flux (J\(_{\text{FCCP-sens}}\)). This method has been used in previous studies (e.g., Herrington et al., 1996; Fierro et al., 1998), but the conditions under which it is valid have not been examined in detail. In the next section, these conditions are described and evaluated, permitting the separation of J into its mitochondrial and nonmitochondrial components.

Properties of the FCCP-resistant component of the total Ca\(^{2+}\) flux. Fig. 1 B (right) illustrates the [Ca\(^{2+}\)], dependence of the FCCP-resistant flux (J\(_{\text{FCCP-res}}\)). Since this flux is seen under conditions where Ca\(^{2+}\) transport by both mitochondria and the endoplasmic reticulum should be largely inhibited, it presumably represents the parallel combination of plasma membrane Ca\(^{2+}\) extrusion and a background leak. The net mitochondrial Ca\(^{2+}\) flux can be determined from the control flux by subtracting J\(_{\text{FCCP-res}}\) at corresponding values of [Ca\(^{2+}\)], if: (a) FCCP specifically and completely inhibits net mitochondrial Ca\(^{2+}\) transport, and (b) the rate of nonmitochondrial Ca\(^{2+}\) transport at each instant in time depends only on [Ca\(^{2+}\)], at that time. If these conditions are satisfied, J\(_{\text{FCCP-res}}\) gives the contribution of nonmitochondrial Ca\(^{2+}\) transport to the control flux. Moreover, at each time during the recovery, the control flux is the sum of the net mitochondrial flux and J\(_{\text{FCCP-res}}\) at the corresponding value of [Ca\(^{2+}\)], making it possible to calculate J\(_{\text{mito}}\), by subtracting J\(_{\text{FCCP-res}}\) from J\(_{\text{cont}}\).

To test for specificity of FCCP, cells were treated with antimycin A\(_{1}\) and oligomycin as an independent way to inhibit mitochondrial Ca\(^{2+}\) transport, and then depolarized both in the presence and absence of 1 \(\mu\)M FCCP. If FCCP directly influences nonmitochondrial Ca\(^{2+}\) transport, it should modify the recovery in cells treated with antimycin A\(_{1}\) and oligomycin. Fig. 2 A compares recoveries in a cell that was treated with antimycin A\(_{1}\) and oligomycin and then depolarized in the presence (left) and absence of FCCP (middle). FCCP has little or no effect on the recovery kinetics, which can be seen more clearly by superimposing the recoveries (right). To assess potential nonmitochondrial effects of FCCP quantitatively, recoveries in the presence and absence of FCCP were fit with two decaying exponentials and the parameters of the fitted curves were compared. FCCP did not influence any of the parameters (five cells, data not shown). Therefore, FCCP (1 \(\mu\)M) does not influence antimycin/oligomycin-resistant Ca\(^{2+}\) transport, arguing that it specifically inhibits mitochondrial Ca\(^{2+}\) transport in these cells.

To determine if J\(_{\text{FCCP-res}}\) is influenced by long lasting effects of the [Ca\(^{2+}\)], elevation that precedes the recovery, responses to strong and weak depolarization were elicited in the continued presence of 1 \(\mu\)M FCCP and the recoveries were compared (Fig. 2 B). Despite following [Ca\(^{2+}\)], elevations of very different magnitude and time course, the recoveries were essentially identical over the common range of [Ca\(^{2+}\)], (see superimposed traces, Fig. 2 B). To examine the impact of prior [Ca\(^{2+}\)], elevations on recovery kinetics quantitatively, recoveries after weak and strong depolarization were fit over the common range of [Ca\(^{2+}\)], with the sum of two decaying exponentials and the parameters of the fitted curves were compared, which indicated that the recoveries were indistinguishable (four cells, data not shown). This shows that at each time during the recovery, the rate of Ca\(^{2+}\) removal by FCCP-resistant transporters depends on [Ca\(^{2+}\)], at that time but not on the history of [Ca\(^{2+}\)], (or membrane potential). Thus, given an initial value of [Ca\(^{2+}\)], the time course of the [Ca\(^{2+}\)], recovery is determined. These results also indicate that inhibition of mitochondrial Ca\(^{2+}\) transport by FCCP is nearly complete at 1 \(\mu\)M; otherwise, the recovery after strong depolarization would be slower than that after weak depolarization.

It is concluded that J\(_{\text{FCCP-res}}\) represents the activity of nonmitochondrial Ca\(^{2+}\) transport systems that restore resting [Ca\(^{2+}\)], after depolarization. Collectively, these transporters generate an outward net Ca\(^{2+}\) flux whose magnitude at each instant in time is defined by [Ca\(^{2+}\)], at that time. Therefore, the FCCP-sensitive flux, which will be referred to below as the net mitochondrial Ca\(^{2+}\) flux (J\(_{\text{mito}}\)), can be calculated from the control flux (J\(_{\text{cont}}\)) by subtracting J\(_{\text{FCCP-res}}\) at corresponding values of [Ca\(^{2+}\)].

Properties of the mitochondrial Ca\(^{2+}\) flux. Fig. 3 compares mitochondrial and nonmitochondrial components of J\(_{\text{cont}}\) during the recovery after a 9 s 50 K\(^{-}\) depolarization from a representative cell (left column) along with collected results from 10 cells (right column). The measured fluxes (J\(_{\text{cont}}, J_{\text{FCCP-res}}\)) are shown in Fig. 3 B, while the difference flux (J\(_{\text{mito}}\)) is shown in (C). J\(_{\text{mito}}\) is large and outward at high [Ca\(^{2+}\)], but small and inward
dominated by nonmitochondrial Ca\(^{2+}\) removal systems that define the slow final phase of recovery (phase iv). Note that while \(J_{\text{mito}}\) is plotted against [Ca\(^{2+}\)], it may also depend on other quantities that change during the recovery, such as the intramitochondrial Ca concentration (see below). However, since the flux subtraction used to measure \(J_{\text{mito}}\) requires only that the nonmitochondrial flux is defined by [Ca\(^{2+}\)], it is valid even if \(J_{\text{mito}}\) depends on variables other than [Ca\(^{2+}\)].

Two main conclusions can be drawn from these results. First, mitochondrial Ca\(^{2+}\) accumulation provides the major mechanism for cytosolic Ca\(^{2+}\) clearance when [Ca\(^{2+}\)], is high, in general agreement with the findings of Herrington et al. (1996) and Xu et al. (1997) in adrenal chromaffin cells. Second, net mitochondrial Ca\(^{2+}\) transport occurs at [Ca\(^{2+}\)], levels as low as 200 nM but at a rate that is comparable to nonmitochondrial Ca\(^{2+}\) transport. Under these conditions, it is the relative rate of mitochondrial and nonmitochondrial transport that is critical in determining the total Ca\(^{2+}\) flux.

Separation of the Net Mitochondrial Ca\(^{2+}\) Flux into Uptake and Release Components

Effects of the mitochondrial Na\(^{+}/Ca^{2+}\) exchange inhibitor CGP 37157. To understand how mitochondrial Ca\(^{2+}\) transport contributes to Ca dynamics, it is necessary to separate \(J_{\text{mito}}\) into its components. Neuronal mitochondria accumulate Ca\(^{2+}\) via a uniporter and release Ca\(^{2+}\) via a Na\(^{+}/Ca^{2+}\) exchanger (Gunter and Pfeiffer, 1990). The benzothiazepine CGP 37157 (CGP) selectively inhibits Na\(^{+}\)-dependent mitochondrial Ca\(^{2+}\) release from isolated cardiac mitochondria with an IC\(_{50}\) in the range 360–800 nM (Cox et al., 1993; Chiesi et al., 1988). Therefore, actions of this compound on depolarization-evoked [Ca\(^{2+}\)], responses were examined. Fig. 4 A illustrates effects of CGP on [Ca\(^{2+}\)], responses elicited by 50 mM K\(^{+}\). CGP did not detectably modify the rise in [Ca\(^{2+}\)], during depolarization or the rapid [Ca\(^{2+}\)], decline that immediately followed repolarization, but it almost completely suppressed the [Ca\(^{2+}\)], plateau (A, see superimposed traces at right), consistent with the idea that mitochondrial Ca\(^{2+}\) release via the Na\(^{+}/Ca^{2+}\) exchanger contributes to the slow [Ca\(^{2+}\)], decline during this phase of the recovery. Similar effects of CGP on the plateau have been seen in other cells (Babcock et al., 1997; White and Reynolds, 1997; Baron and Thayer, 1997). Reproducible effects were observed after 2–3-min incubations with 2 \(\mu\)M CGP and were largely reversed within \(~\sim\) 10 min of washout.

If CGP completely inhibits mitochondrial Ca\(^{2+}\) release, it might be expected to enhance mitochondrial Ca\(^{2+}\) accumulation when [Ca\(^{2+}\)], is high enough to activate the uniporter, slowing the rise in [Ca\(^{2+}\)], during stimulation and speeding the initial phase of recovery.
after repolarization. This was not observed, arguing that when \([\text{Ca}^{2+}]_i\) is high, net mitochondrial \(\text{Ca}^{2+}\) transport is dominated by the uptake pathway. The slow \([\text{Ca}^{2+}]_i\) tail observed during the recovery in the presence of CGP may represent residual mitochondrial \(\text{Ca}^{2+}\) release due to incomplete block of the \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger or release by a CGP-insensitive pathway. The observation that the slow tail is not seen at higher CGP concentrations (see Fig. 6 C) support the former explanation.

The actions of CGP are concentration dependent. When cells are depolarized in the presence of CGP at increasing concentrations, the \([\text{Ca}^{2+}]_i\) plateau level is progressively lowered and the recovery is prolonged (Fig. 4 B). This effect was quantified by measuring the \([\text{Ca}^{2+}]_i\) level where the rate of recovery reaches a minimum (see Materials and Methods). Plotting the normalized plateau level vs CGP concentration shows that the plateau level falls with concentration in a manner consistent with a single binding site model with \(IC_{50} \approx 560 \text{ nM}\) (Fig. 4 C, smooth curve). This value agrees with the \(IC_{50}\) for CGP-induced inhibition of \(\text{Na}^+\)-dependent \(\text{Ca}^{2+}\) efflux from isolated cardiac mitochondria (360-800 nM; Chiesi et al., 1988; Cox et al., 1993). These re-
Results support the conclusion that CGP modifies \([\text{Ca}^{2+}]_i\), dynamics by inhibiting \(\text{Ca}^{2+}\) release via the mitochondrial \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger, and that drug binding has reached equilibrium before depolarization under the conditions of these experiments (see also Baron and Thayer, 1997).

The CGP-sensitive component of the recovery requires intracellular sodium. If the \([\text{Ca}^{2+}]_i\), plateau reflects \(\text{Ca}^{2+}\) release via the mitochondrial \(\text{Na}^+ / \text{Ca}^{2+}\) exchanger, both the plateau level and its sensitivity to CGP should depend on intracellular \(\text{Na}^+\). To examine this point, cells were depolarized under voltage clamp (perforated patch conditions) with or without \(\text{Na}^+\) added to the pipette solution (Fig. 5). When 10 mM \(\text{Na}^+\) was included in the pipette solution, brief depolarization elicited \([\text{Ca}^{2+}]_i\), elevations followed by recoveries showing pronounced plateaus (Fig. 5A, 323 ± 19 nM, n = 12) much like those seen in non-voltage-clamped cells after high \(K^+\) depolarization; peak \([\text{Ca}^{2+}]_i\), elevations are larger than those elicited by high \(K^+\) presumably because depolarization and \(\text{Ca}^{2+}\) channel activation are more rapid under voltage clamp, leading to higher \(\text{Ca}^{2+}\) entry rates. This concentration of \(\text{Na}^+\) would be expected to enable mitochondrial \(\text{Na}^+ / \text{Ca}^{2+}\) exchange based on studies of isolated mitochondria (half-maximal activation at ~2–3 mM \([\text{Na}^+]_i\), maximal activation at ~10 mM \([\text{Na}^+]_i\); Cox and Matlib, 1993). Plateau levels were similar when \([\text{Na}^+]_i\) = 6.5 (293 ± 9 nM, n = 6) so results with \([\text{Na}^+]_i\) = 6.5 and 10 mM were pooled (see Fig. 5D).

As with cells depolarized with high \(K^+\), the plateau observed under voltage clamp is depressed by CGP. Fig. 5B illustrates a \([\text{Ca}^{2+}]_i\), response elicited by a depolarizing step from −70 to −10 mV, first in the presence of CGP (left) and then after washout (right). In the presence of CGP, the plateau is largely suppressed and there is a prolonged tail, possibly reflecting incomplete inhibition of \(\text{Ca}^{2+}\) release and/or release via a CGP-insensitive pathway. After washing out CGP, depolarization elicits a rise in \([\text{Ca}^{2+}]_i\), followed by a pronounced plateau, even though the rise in \([\text{Ca}^{2+}]_i\), was smaller, reflecting current rundown during the washout period (see Fig. 5B, right, inset). These responses are representative of ten cells in which \(\text{Na}^+\) was included in the pipette solution: in the presence of CGP, recoveries were marked by plateaus (123 ± 13 nM) that were significantly lower than those measured in the same cells in the absence of CGP (305 ± 13 nM, \(P < 0.001\)) (Fig. 5 D).
When Na\textsuperscript{+} was not added to pipette solutions, depolarization elicited [Ca\textsuperscript{2+}]\textsubscript{i} elevations that were followed by simple recoveries (Fig. 5 C, left; n = 4) like those seen in Na\textsuperscript{+}-containing cells in the presence of CGP (Fig. 4 A). A second response elicited after treatment with CGP exhibited a recovery that was very similar to that seen in the absence of CGP (Fig. 5 C, right), indicating that CGP has little effect in the absence of intracellular Na\textsuperscript{+} (D), supporting the conclusion that CGP specifically blocks the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. The failure of [Ca\textsuperscript{2+}]\textsubscript{i} to recover completely to prestimulation levels (Fig. 5, B and C) is consistent with the development of a Ca\textsuperscript{2+} leak over these long experiments.

Relationship between the actions of CGP and FCCP. If CGP specifically inhibits mitochondrial Ca\textsuperscript{2+} release via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, it should have no additional effect on [Ca\textsuperscript{2+}]\textsubscript{i} responses elicited in the presence of FCCP. Fig. 6 A shows responses induced by high K\textsuperscript{+} before and during exposure to FCCP, and then in the combined presence of FCCP and CGP. CGP had no additional effect after treatment with FCCP, indicating that it only modifies FCCP-sensitive (mitochondrial) Ca\textsuperscript{2+} transport and does not affect nonmitochondrial Ca\textsuperscript{2+} transport systems. Similar results were obtained in each of three cells. A different conclusion was reached by Baron and Thayer (1997) based on the finding that CGP depressed high K\textsuperscript{+}-induced [Ca\textsuperscript{2+}]\textsubscript{i} responses in rat DRG neurons (~50% at 3 \mu M), suggesting that it directly blocks voltage-gated Ca\textsuperscript{2+} channels in these cells. We found that at concentrations which virtually eliminated the slow plateau phase of recovery, CGP had little or no effect on [Ca\textsuperscript{2+}]\textsubscript{i} during depolarization, or on the initial rate of recovery after repolarization (Fig. 4). Moreover, CGP did not detectably inhibit I\textsubscript{Ca} (Fig. 5). In any case, all flux measurements in this study were made after repolarization, when Ca\textsuperscript{2+} channels are closed. Therefore, potential effects of CGP on I\textsubscript{Ca} during depolarization do not influence the conclusions of this study (see below). As expected, FCCP still had a prominent effect on depolarization-induced [Ca\textsuperscript{2+}]\textsubscript{i} re-
responses during treatment with CGP (Fig. 6 B, four cells), indicating that CGP modifies some, but not all, FCCP-sensitive processes.

When FCCP is applied at a higher concentration (10 μM) in the absence of extracellular Ca\(^{2+}\) during the plateau phase of recovery, it elicits a large [Ca\(^{2+}\)] transient (Δ[Ca\(^{2+}\)] = 1,438 ± 396 nM, n = 7) but only a small [Ca\(^{2+}\)] rise when applied after [Ca\(^{2+}\)], recovers to basal levels (35 ± 14 nM, n = 7), providing another way to monitor Ca\(^{2+}\) loss from loaded mitochondria during the recovery. If CGP effectively inhibits mitochondrial Ca\(^{2+}\) release, then in the presence of the inhibitor, depolarization should still increase mitochondrial Ca\(^{2+}\) concentration, but the increase should persist even after resting [Ca\(^{2+}\)] is restored. To test this, cells were depolarized in the continued presence of a nearly saturating concentration of CGP, and then after [Ca\(^{2+}\)] recovery was elicited in the presence of CGP (4 μM), rapid application of FCCP (10 μM) in low Ca\(^{2+}\) Ringer’s (nominally Ca\(^{2+}\)-free with 0.2 mM EGTA) elicits a large [Ca\(^{2+}\)] transient, in contrast to the small response seen in the absence of CGP; collected results comparing FCCP-induced [Ca\(^{2+}\)] transients (Δ[Ca\(^{2+}\)]\(_{\text{FS}}\)) in the presence (+CGP, 2–6 μM) and absence (cont) of CGP are shown in panel D (* indicates significant difference from control, P < 0.001).
FCCP elicits a larger [Ca\textsuperscript{2+}] transient when applied at 10 μM in the presence of 4 μM CGP (Fig. 6 C) than at 1 μM in the presence of 1 μM CGP (B) probably reflects a combination of incomplete block by CGP at the lower concentration (see prolonged tail during the recovery after the second depolarization in B) and slower Ca\textsuperscript{2+} release induced by FCCP at the lower concentration. Slow FCCP-induced Ca\textsuperscript{2+} release would also explain why rapid application of the protonophore at the lower concentration during the plateau only prolongs the recovery (four cells) in contrast to the large and rapid rise elicited by 10 μM FCCP (not shown).

Properties of the CGP-sensitive and -resistant components of J\textsubscript{mito}. Taken together, the observations presented above indicate that CGP is a specific inhibitor of mitochondrial Ca\textsuperscript{2+} efflux via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger. This compound was therefore used to dissect the net mitochondrial Ca\textsuperscript{2+} flux into its components. Fig. 7 A shows [Ca\textsuperscript{2+}], responses elicited under control conditions (left), in the presence of a nearly saturating concentration of CGP (2 μM, middle), and in the presence of 1 μM FCCP after CGP washout (right). Using a strategy like that employed to separate the total Ca\textsuperscript{2+} flux into mitochondrial and nonmitochondrial components, CGP was used to separate J\textsubscript{mito} into CGP-sensitive and -resistant components that are associated with mitochondrial Ca\textsuperscript{2+} release and uptake pathways.

Fig. 7 B plots J\textsubscript{mito} and its CGP-resistant component (J\textsubscript{CGP-res}) versus [Ca\textsuperscript{2+}], during the recovery from the cell shown in panel A and collected results from ten cells (C). J\textsubscript{CGP-res} was calculated by subtracting J\textsubscript{FCCP-res} from the total flux measured in the presence of CGP (J\textsubscript{CGP}) at corresponding values of [Ca\textsuperscript{2+}], as expected for the mitochondrial uniporter under conditions where the electrochemical driving force for Ca\textsuperscript{2+} favors uptake. Importantly, this flux is non-zero even when bulk [Ca\textsuperscript{2+}] is as low as 200–300 nM. Since these measurements were made after voltage-gated Ca\textsuperscript{2+} channels have closed and radial Ca\textsuperscript{2+} gradients have largely dissipated (Hernandez-Cruz et al., 1990; Hua et al., 1993), bulk [Ca\textsuperscript{2+}], measurements should provide a reasonable estimate of the extramitochondrial Ca\textsuperscript{2+} concentration. Therefore, our results support the conclusion that mitochondrial Ca\textsuperscript{2+} uptake occurs when local [Ca\textsuperscript{2+}] is as low as 200–300 nM.

The CGP-sensitive component of J\textsubscript{mito}, determined by subtracting J\textsubscript{CGP-res} at corresponding values of [Ca\textsuperscript{2+}], is easily interpreted if CGP and J\textsubscript{CGP} satisfy conditions like those described above for FCCP and J\textsubscript{FCCP-res} (a) CGP specifically and completely inhibits mitochondrial Ca\textsuperscript{2+} release, and (b) the CGP-resistant flux depends only on the magnitude of [Ca\textsuperscript{2+}], at each instant in time during the recovery and not on the history of [Ca\textsuperscript{2+}]. If these conditions are satisfied, J\textsubscript{CGP-res} gives the rate of mitochondrial Ca\textsuperscript{2+} uptake, and the net mitochondrial flux is the sum of J\textsubscript{CGP-res} and the CGP-sensitive component of J\textsubscript{mito} (J\textsubscript{CGP-sens}) at corresponding values of [Ca\textsuperscript{2+}], making it possible to calculate J\textsubscript{CGP-sens} as the difference between J\textsubscript{mito} and J\textsubscript{CGP-res}.

Regarding specificity, the results described above indicate that CGP inhibits Na\textsuperscript{+}-dependent mitochondrial Ca\textsuperscript{2+} release, and the following observations show that if CGP influences any other Ca\textsuperscript{2+} transport systems that contribute to the recovery, its effects are small. CGP does not alter resting [Ca\textsuperscript{2+}], (1–4 μM CGP, 50 cells) and therefore has little effect on Ca\textsuperscript{2+} transporters responsible for setting this [Ca\textsuperscript{2+}], level. Also, CGP does not influence [Ca\textsuperscript{2+}], recoveries after depolarization in cells already treated with FCCP (1 μM; e.g., Fig. 6 A): in the absence of CGP, the fast and slow time constants of recovery were (s) 14.7 ± 3.8 and 249.7 ± 117.4, while in the presence of 1 μM CGP they were 17.7 ± 3.9 s and 271.3 ± 83.1, n = 3, NS). Finally, CGP does not modify [Ca\textsuperscript{2+}] recovery kinetics under perforated patch conditions when pipette solutions lack Na\textsuperscript{+} (Fig. 5 C).

Two complementary approaches were used to determine if the CGP-resistant flux is defined by [Ca\textsuperscript{2+}] during the recovery. In these experiments, J\textsubscript{CGP} was analyzed since it should be the sum of J\textsubscript{CGP-res} and J\textsubscript{FCCP-res}, and the latter flux component has already been shown to have this property. For each approach, mitochondrial Ca\textsuperscript{2+} release via the Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger was inhibited and [Ca\textsuperscript{2+}] was elevated to different levels by weak and strong depolarization so that the subsequent recoveries could be compared. The approaches differed in the way Ca\textsuperscript{2+} release was inhibited. When release was inhibited by CGP (2 μM), recoveries were nearly identical over the common range of [Ca\textsuperscript{2+}], despite being preceded by very different [Ca\textsuperscript{2+}] elevations (Fig. 7 D, see superimposed recoveries at right). Therefore, under the conditions of these experiments, the rate of Ca\textsuperscript{2+} removal in the presence of CGP depends only on the magnitude of [Ca\textsuperscript{2+}], at each time. The second approach used Na\textsuperscript{+}-free pipette solutions under voltage clamp to suppress mitochondrial Ca\textsuperscript{2+} release without relying on CGP. Large and small [Ca\textsuperscript{2+}], responses were elicited and the ensuing recoveries were compared. Under these conditions, both fast and slow components of the recoveries were indistinguishable (Fig. 7 E, recoveries are compared at right), demonstrating that, like the CGP-resistant component of the total flux, the Na\textsuperscript{+}-insensitive component depends on [Ca\textsuperscript{2+}], but not its history or the state of mitochondrial Ca\textsuperscript{2+} loading.

Fig. 7 B shows the CGP-sensitive flux (J\textsubscript{CGP-sens}) calculated by subtracting J\textsubscript{CGP} from J\textsubscript{cont} at corresponding values of [Ca\textsuperscript{2+}]; averaged results from 10 cells are presented in C. J\textsubscript{CGP-sens} represents net mitochondrial Ca\textsuperscript{2+} release and exhibits a U-shaped dependence on
As \([Ca^{2+}]\) declines during the recovery, the magnitude of \(J_{\text{CGP-sens}}\) rises from a small value near zero to a maximum when \([Ca^{2+}]\) is near the plateau level (see arrow) and then declines as \([Ca^{2+}]\) approaches the prestimulation level. The biphasic dependence of \(J_{\text{CGP-sens}}\) on \([Ca^{2+}]\) is also evident from the \([Ca^{2+}]\) responses: CGP has little effect on the recovery rate during the initial rapid phase when \([Ca^{2+}]\) is high, or on the final approach to prestimulation levels, when \([Ca^{2+}]\) is low. It is only when \([Ca^{2+}]\) is at intermediate levels that CGP-sensitive flux is a significant fraction of the total \(Ca^{2+}\) flux, rendering the recovery rate sensitive to CGP.

Components of the mitochondrial flux measured under voltage clamp. The components of the total \(Ca^{2+}\) flux were also measured under voltage clamp, which made it possible to examine the \([Ca^{2+}]\) dependence of the fluxes over a wider \([Ca^{2+}]\) range. The first set of experiments was designed to measure the nonmitochondrial \(Ca^{2+}\) flux and the uptake component of \(J_{\text{mito}}\). Fig. 8A shows...
results from a cell with low internal Na⁺ to inhibit mitochondrial Ca²⁺ release via the Na⁺/Ca²⁺ exchanger. The total Ca²⁺ flux was then measured during the recovery after raising [Ca²⁺], by a 2.3-s depolarization from −70 to −10 mV before (J_{cont}) and after exposure to 1 μM FCCP (J_{FCCP-res}). The FCCP-sensitive component of the total flux was outwardly directed and increased steeply with [Ca²⁺], closely resembling J_{CGP-res} measured in cells after high K⁺ depolarization (compare with Fig. 7, B and C). Similar results were observed in 3/3 cells. The second set of experiments examined the CGP-sensitive component of J_{mito} (Fig. 8 B). Ca²⁺ fluxes were measured in Na⁺-containing cells by depolarizing before (J_{cont}) and after exposure to CGP (J_{CGP}). The CGP-sensitive flux, obtained by subtraction, was inwardly directed and displayed a U-shaped [Ca²⁺], dependence qualitatively like that seen with J_{CGP-sens} measured in cells stimulated with high K⁺ (compare with Fig. 7, B and C). Similar results were obtained in 4/4 cells. Overall, the similarity between these results and those obtained from cells depolarized with high K⁺ indicate that the properties of the component fluxes are largely independent of the method used to evoke voltage-sensitive Ca²⁺ entry, and depend primarily on the size of the cytosolic Ca²⁺ load.

To summarize, J_{mito} can be separated into uptake and release components based on their sensitivity to CGP: Mitochondrial Ca²⁺ uptake is steeply dependent on [Ca²⁺], and becomes the dominant mitochondrial Ca²⁺ transport pathway when [Ca²⁺], is high (>~500 nM). Uptake still occurs when [Ca²⁺], is as low as 200–300 nM, but it is opposed by release at comparable rate, accounting for the small net mitochondrial Ca²⁺ flux. Under these conditions, the relative rates of uptake and release are critical in defining the net mitochondrial Ca²⁺ flux. Since the uniporter is the main pathway for mitochondrial Ca²⁺ uptake and the Na⁺/Ca²⁺ exchanger is the principle route for neuronal mitochondrial Ca²⁺ release (Gunter and Pfeiffer, 1990), J_{CGP-res} and J_{CGP-sens} will be referred to in the following as J_{uni} and J_{Na/Ca}, although it is impossible to rule out small contributions to these components from other mitochondrial Ca²⁺ transport pathways during these recoveries.

Properties of the Component Fluxes Explain the Complex Time Course of Recovery

The kinetics of the [Ca²⁺], recovery can be understood in terms of J_{cont} and its components. Fig. 9 A shows a [Ca²⁺], response elicited by a 70 mM K⁺ depolarization with the four phases of recovery (i–iv) indicated. Fig. 9 B shows the time course of J_{cont} (thick trace) and its mitochondrial and nonmitochondrial components (thin traces); Fig. 9 C illustrates J_{mito} and its components on the same scale. Also shown is the time course of the integrated mitochondrial Ca²⁺ flux (Fig. 9 A, dotted trace) which provides a measure of the change in mitochondrial Ca²⁺ concentration (∆[Ca²⁺]_m) from its resting value after recovery is complete (see Appendix). During recovery phase i (see Fig. 9, A and B), J_{cont} is large and outward because of the combined effects of strong mitochondrial Ca²⁺ uptake and weak extrusion across the plasma membrane. As a result, ∆[Ca²⁺]_m rises and [Ca²⁺], falls rapidly. As [Ca²⁺], falls, J_{FCCP-res} declines and J_{mito} changes sign to become an inward flux, which causes ∆[Ca²⁺]_m to fall and the [Ca²⁺], recovery to be slowed (phase ii). J_{mito} reaches a minimum near zero when the opposing fluxes J_{FCCP-res} and J_{mito} are nearly balanced, and then rises because the inward flux J_{mito} decays more rapidly than the outward flux J_{FCCP-res} causing the recovery to accelerate. Finally, during phase iv, J_{mito} approaches zero and the [Ca²⁺], recovery is controlled by net Ca²⁺ extrusion. Note that while the initial rapid phase of recovery is dominated by mitochondrial Ca²⁺ transport, and the final phase is dominated by net Ca²⁺ extrusion, the intermediate phases (ii–iii) are influenced similarly by net mitochondrial Ca²⁺ release and net Ca²⁺ extrusion.

Figure 8. Mitochondrial Ca²⁺ uptake and release fluxes measured under voltage clamp. (A) Fluxes measured from a representative cell under voltage clamp after depolarization from −70 to −10 mV with a Na⁺-free pipette solution, before (J_{cont}) and after exposure to 1 μM FCCP (J_{FCCP-res}). The FCCP-sensitive flux (J_{FCCP-sens}, thin trace), calculated as the difference between the total flux measured in the absence (J_{cont}) and presence of FCCP (J_{FCCP-res}), is positive and increases steeply with [Ca²⁺], much like the CGP-resistant flux seen in cells after high K⁺ depolarization. Cell ma9480. (B) Flux measurements from another cell during the recovery after a step from −70 to −10 mV (pipette solution contained 6.5 mM NaCl) before and after exposure to CGP (1 μM).
The dynamics of $J_{\text{mito}}$ can be understood in terms of its components (Fig. 9 C). During phase i, $J_{\text{mito}}$ is large and outward because $J_{\text{uni}}$ is much larger in magnitude than the inward flux $J_{\text{Na/Ca}}$, which is close to zero. Mitochondrial Ca$^{2+}$ accumulation causes [Ca$^{2+}$]$_i$ to fall, which is accompanied by a decline in $J_{\text{uni}}$ and a rise in the inward flux $J_{\text{Na/Ca}}$. Together, these changes cause $J_{\text{mito}}$ to change sign to become an inward flux. Then $J_{\text{uni}}$ and $J_{\text{Na/Ca}}$ decline at approximately the same rate, so that for a time $J_{\text{mito}}$ is a nearly constant inward flux that maintains [Ca$^{2+}$]$_i$, near the plateau level. As both fluxes approach zero during phase iii, the inward flux $J_{\text{mito}}$ declines, net Ca$^{2+}$ extrusion becomes unopposed and the recovery accelerates (see Fig. 9 B). Note that while Ca$^{2+}$ uptake via the uniporter is the dominant component of the net mitochondrial flux during phase i of the recovery, uptake and release fluxes have similar magnitudes during phases ii–iii.

The results presented above show that even when [Ca$^{2+}$]$_i$ is as low as 200–300 nM during the recovery, the mitochondrial Ca$^{2+}$ uptake pathway is active (Figs. 7 B and 9 C). Thus, uptake should occur even during weak depolarizations that elevate [Ca$^{2+}$]$_i$ to levels within this range. However, previous work has shown that FCCP has little effect under these conditions (Friel and Tsien, 1994), suggesting that the net mitochondrial Ca$^{2+}$ flux is small, requiring that ongoing uptake and release fluxes nearly balance one another. If this were true, inhibition of the release pathway should enhance net mitochondrial Ca$^{2+}$ accumulation and measurably slow the rise in [Ca$^{2+}$]$_i$ induced by weak depolarization, and additionally speed the recovery after repolarization. As a test of this prediction, small [Ca$^{2+}$]$_i$ elevations were evoked by exposure to 30 mM K$^+$ before and after treatment with 1 μM CGP (Fig. 9 D). In the presence of CGP, the rise in [Ca$^{2+}$]$_i$ was greatly slowed, the apparent steady-state [Ca$^{2+}$]$_i$ elevation was reduced, and the recovery after repolarization was accelerated when compared with the control response elicited in the absence of CGP over the same [Ca$^{2+}$]$_i$ range, effects that were reversed by FCCP (3/3 cells).

**DISCUSSION**

**Summary of Main Results**

This study describes the interplay between Ca$^{2+}$ transport systems that restore the resting distribution of Ca$^{2+}$.
in sympathetic neurons after depolarization-evoked Ca\textsuperscript{2+} entry. Cells were treated with thapsigargin to inhibit SERCA pump activity and minimize contributions from ER Ca\textsuperscript{2+} transport, and the remaining Ca\textsuperscript{2+} flux was separated into mitochondrial and nonmitochondrial components. It was found that mitochondria are powerful Ca\textsuperscript{2+} sequestration organelles (Brinley et al., 1978) and provide the major Ca\textsuperscript{2+} clearance system when [Ca\textsuperscript{2+}]\textsubscript{i} is high during the initial rapid phase of recovery (see also Herrington et al., 1996; Xu et al., 1997). The same conclusion is reached when SERCA pumps are operational, since SERCA inhibitors have a much smaller effect on [Ca\textsuperscript{2+}]\textsubscript{i}, recovery kinetics after strong depolarization than does FCCP; this is expected, since when [Ca\textsuperscript{2+}]\textsubscript{i} is high the rate of Ca\textsuperscript{2+} uptake by the uniporter greatly exceeds the rate of uptake via SERCA pumps (unpublished observations). It was also found that during the later phases of the recovery, when [Ca\textsuperscript{2+}]\textsubscript{i} is lower, the relative rates of net mitochondrial release and net Ca\textsuperscript{2+} extrusion are critical. During the plateau phase of recovery, these rates are similar, causing [Ca\textsuperscript{2+}]\textsubscript{i} to be nearly steadily elevated for a period of time that depends on mitochondrial Ca\textsuperscript{2+} load (see accompanying study). During the subsequent phases of recovery, net Ca\textsuperscript{2+} extrusion becomes the dominant Ca\textsuperscript{2+} clearance system, and is primarily responsible for restoring resting [Ca\textsuperscript{2+}]\textsubscript{i}. When SERCA pumps are operational, the later phases of recovery are also influenced by net ER Ca\textsuperscript{2+} accumulation (Friel and Tsien, 1992) followed by net Ca\textsuperscript{2+} release (Colegrove, S.L., and D.D. Friel, unpublished observations).

The net mitochondrial Ca\textsuperscript{2+} flux was separated into components representing distinct Ca\textsuperscript{2+} uptake and release components. It was found that mitochondrial Ca\textsuperscript{2+} uptake is steeply dependent on [Ca\textsuperscript{2+}]\textsubscript{i}, as expected for the mitochondrial uniporter, and occurs even when [Ca\textsuperscript{2+}]\textsubscript{i} is as low as 200–300 nM. Mitochondrial Ca\textsuperscript{2+} release requires intracellular Na\textsuperscript{+} and is blocked by CGP 37157, a specific inhibitor of the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger, indicating that release depends on activity of this transporter. Because of its high transport rate and steep [Ca\textsuperscript{2+}]\textsubscript{i} dependence, the uptake pathway dominates the net mitochondrial Ca\textsuperscript{2+} flux when [Ca\textsuperscript{2+}]\textsubscript{i} is high (>\textasciitilde400–500 nM), while both uptake and release pathways make comparable contributions to the net flux when [Ca\textsuperscript{2+}]\textsubscript{i} is lower. Mitochondrial Ca\textsuperscript{2+} transport also occurs during weak depolarization when [Ca\textsuperscript{2+}]\textsubscript{i} rises to low levels (~300 nM). Under these conditions, the net mitochondrial Ca\textsuperscript{2+} flux is small, representing the sum of much larger uptake and release fluxes that nearly cancel one another. Recent studies have shown that similar [Ca\textsuperscript{2+}]\textsubscript{i} elevations stimulate mitochondrial Ca\textsuperscript{2+} accumulation in heart cells (e.g., Zhou et al., 1998).

Properties of the Measured Fluxes

The FCCP-resistant flux was measured after treatment with Tg and FCCP, so it probably represents predominantly net Ca\textsuperscript{2+} transport across the plasma membrane. Additional support for this conclusion is provided by the finding that J\textsubscript{FCCP-res} is reduced by \textasciitilde90% after removal of extracellular Na\textsuperscript{+} and addition of La\textsuperscript{3+} (1–5 mM, unpublished observations). This flux increases with [Ca\textsuperscript{2+}]\textsubscript{i}, and levels off at high [Ca\textsuperscript{2+}]\textsubscript{i}, possibly indicating saturation of the underlying extrusion systems. Since J\textsubscript{FCCP-res} was defined by [Ca\textsuperscript{2+}]\textsubscript{i}, at each instant in time during the recovery, the underlying transport systems appear to have little intrinsic time dependence under the conditions of our experiments.

The CGP-resistant component of the net mitochondrial Ca\textsuperscript{2+} flux represents mitochondrial Ca\textsuperscript{2+} uptake and shows a steep dependence on [Ca\textsuperscript{2+}]\textsubscript{i} (Hill coefficient \textasciitilde2, see accompanying study) as expected for the mitochondrial uniporter (Scarpa and Graziotti, 1973). One of the most important findings is that Ca\textsuperscript{2+} uptake occurs even when [Ca\textsuperscript{2+}]\textsubscript{i} is as low as 200–300 nM. It is unlikely that these measurements grossly underestimate [Ca\textsuperscript{2+}]\textsubscript{i}, near the majority of mitochondria since they were made long after Ca\textsuperscript{2+} channels have closed and radial [Ca\textsuperscript{2+}]\textsubscript{i} gradients have dissipated (Hernandez-Cruz et al., 1990; Hua et al., 1993). Activity of the Ca uniporter at such low [Ca\textsuperscript{2+}]\textsubscript{i} may seem surprising in view of the high EC\textsubscript{50} for activation of the uniporter (~10–20 \mu M; Gunter and Pfeiffer, 1990), but is expected based on the properties of the transporter in isolated mitochondria (Carafoli 1979; Gunter and Gunter, 1994; see discussion in Pivovarova et al., 1999). Activity of the uniporter at low [Ca\textsuperscript{2+}]\textsubscript{i}, reconciles the low affinity and steep [Ca\textsuperscript{2+}]\textsubscript{i} dependence of this transporter with the widely reported finding that mitochondria accumulate Ca\textsuperscript{2+} even when [Ca\textsuperscript{2+}]\textsubscript{i} < 1 \mu M. Like J\textsubscript{FCCP-res} J\textsubscript{uni} did not appear to have a strong intrinsic time dependence.

The CGP-sensitive flux represents mitochondrial Ca\textsuperscript{2+} release and shows an apparent U-shaped dependence on [Ca\textsuperscript{2+}]\textsubscript{i}. However, even though J\textsubscript{NM, Ca} varies with [Ca\textsuperscript{2+}]\textsubscript{i}, it is not clear that it actually depends on [Ca\textsuperscript{2+}]\textsubscript{i}, (i.e., is a function of [Ca\textsuperscript{2+}]\textsubscript{i}) and a dependence on other factors is likely, such as the concentration of intramitochondrial free Ca ([Ca\textsuperscript{2+}]\textsubscript{m}). Wingrove and Gunter (1986) showed that with constant extramitochondrial Ca\textsuperscript{2+} concentration, the rate of Ca\textsuperscript{2+} release from liver mitochondria increases saturably with [Ca\textsuperscript{2+}]\textsubscript{m}. However, this is difficult to reconcile with the measured flux which has the same small magnitude initially after depolarization, and after recovery is nearly complete, even though mitochondrial Ca concentration is very different at these times (Fig. 9, A and C). One possible explanation is provided by the finding that the mitochondrial Na\textsuperscript{+}/Ca\textsuperscript{2+} exchanger can be inhibited by submicromo-
lar concentrations of external Ca\(^{2+}\) (Hayat and Cromp-
ton, 1982). Dual regulation of \(J_{\text{Na/Ca}}\) by \([\text{Ca}^{2+}]_m\) and 
\([\text{Ca}^{2+}]_i\), provides a possible explanation for the apparent 
U-shaped dependence on \([\text{Ca}^{2+}]_i\). During the initial 
phase of recovery, \([\text{Ca}^{2+}]_i\), may be large enough to in-
hibit \(J_{\text{Na/Ca}}\) despite high \([\text{Ca}^{2+}]_m\). Inhibition would then 
be relieved as \([\text{Ca}^{2+}]_i\), declines so that \(J_{\text{Na/Ca}}\) subsequently 
depends primarily on \([\text{Ca}^{2+}]_m\). Using \(\Delta[\text{Ca}^{2+}]_m\) as a ba-
sis for estimating changes in mitochondrial Ca concentra-
tion, such a model provides a reasonable quantitative 
description of \(J_{\text{Na/Ca}}\), although regulation by other fac-
tors (e.g., intramitochondrial Na\(^+\)) is also possible (see 
accompanying study).

### Interplay between the Components of the Total \(\text{Ca}^{2+}\) Flux

This study illustrates how net mitochondrial \(\text{Ca}^{2+}\) trans-
port and \(\text{Ca}^{2+}\) transport across the plasma membrane 
contribute to \([\text{Ca}^{2+}]_i\), dynamics. It also shows how the rate of net mitochondrial \(\text{Ca}^{2+}\) transport depends on 
the relative rates of uptake and release. When \([\text{Ca}^{2+}]_m\), is 
high (\(<\sim 300-400\) nM) uptake is much more powerful 
than release, accounting for strong mitochondrial \(\text{Ca}^{2+}\) 
accumulation. When \([\text{Ca}^{2+}]_i\), is low (\(<\sim 200-300\) nM), 
mitochondrial \(\text{Ca}^{2+}\) uptake and release occur at com-
parable rates, accounting for the small net mitochondrial 
\(\text{Ca}^{2+}\) flux. Moreover, since the component fluxes 
are large compared with the net flux, modulation of ei-
ther the uptake or release rate would have a large im-
port on the net mitochondrial \(\text{Ca}^{2+}\) flux. Also, since the 
rate of release depends on the intramitochondrial \(\text{Ca}^{2+}\) 
concentration, which in turn depends on the history of 
\([\text{Ca}^{2+}]_i\), (see accompanying study), the net mito-
chondrial \(\text{Ca}^{2+}\) flux at low \([\text{Ca}^{2+}]_i\), should be sensitive to stim-
ulus history (see accompanying study).

The interplay between net mitochondrial \(\text{Ca}^{2+}\) trans-
port and net \(\text{Ca}^{2+}\) extrusion across the plasma mem-
brane is also important in determining the \([\text{Ca}^{2+}]_i\), level 
reached during depolarization, but in this case the rela-
tive rates of mitochondrial \(\text{Ca}^{2+}\) accumulation and net 
\(\text{Ca}^{2+}\) entry are critical. Assuming an initial steady-state 
before depolarization in which \([\text{Ca}^{2+}]_i\), is at its resting 
level and the net mitochondrial flux is zero, a small 
steady rise in \([\text{Ca}^{2+}]_i\), induced by weak depolarization 
would be expected to stimulate the mitochondrial \(\text{Ca}^{2+}\) 
uptake pathway, creating an imbalance between uptake 
and release which leads to net \(\text{Ca}^{2+}\) accumulation. The 
resulting increase in \([\text{Ca}^{2+}]_m\), would be expected to in-
crease the rate of release, eventually leading to a new 
steady-state in which release and uptake balance. In-
deed, during maintained exposure to 30 mM K\(^+\) which 
raises \([\text{Ca}^{2+}]_i\), to \(<\sim 300\) nM, mitochondrial Ca accumu-
lation does occur (Pivovarova et al., 1999). Paradoxically, 
proton ionophores have little effect on the magnitude of 
\([\text{Ca}^{2+}]_i\), responses elicited by such weak stimuli (Friel 
and Tsåen, 1994; Herrington et al., 1996). However, as 
shown in the accompanying study, this is expected for 
weak stimuli that raise \([\text{Ca}^{2+}]_i\), and \([\text{Ca}^{2+}]_m\), toward new 
steady-state levels. With stronger stimuli that elevate 
\([\text{Ca}^{2+}]_m\), to levels where uptake via the unipporter ex-
ceeds the maximal rate of release, the rate of mito-
chondrial \(\text{Ca}^{2+}\) accumulation would increase until it 
balances net entry and then become constant. For ex-
ample, during 45–120 s exposure to 50 mM K\(^+\), mito-
chondrial Ca accumulation continues at a nearly con-
stant rate even though \([\text{Ca}^{2+}]_m\), is essentially constant at 
\(<\sim 500-800\) nM (Pivovarova et al., 1999). This would 
explain why such strong depolarizations elicit much 
larger \([\text{Ca}^{2+}]_i\), elevations during treatment with FCCP.

In the accompanying study, \(J_{\text{FCCP/\text{res}}}\), \(J_{\text{uni}}\) and \(J_{\text{Na/Ca}}\) are 
described quantitatively and incorporated into a model of 
\(\text{Ca}^{2+}\) dynamics. The model reproduces the recovery time 
course with its four distinct phases, the effects of graded 
inhibition of the Na\(^+\)/Ca\(^{2+}\) exchanger by CGP (Fig. 4), 
and accounts for the actions of CGP and FCCP on re-
sponses to weak depolarization. The model also clarifies 
the relationship between the \([\text{Ca}^{2+}]_i\), plateau level and the 
previously described mitochondrial set-point.

### Comparison with Other Studies

We found that mitochondria accumulate Ca at a rate of 
\(<\sim 400\) nM/s (nmol Ca\(^{2+}\)/li effective cytosolic vol/s) 
when \([\text{Ca}^{2+}]_i\), \(<\sim 0.8\) \(\mu\)M (Fig. 8 A), in general agree-
ment with results from rat chromaffin cells (Herrington et 
al., 1996). The net mitochondrial \(\text{Ca}^{2+}\) fluxes in this 
study can also be compared with direct measurements 
of the rate of total Ca accumulation obtained with elec-
tron probe microanalysis in the same cell type under 
the same conditions of stimulation (see Appendix, Eq. 
2). During exposure to high K\(^+\) (50 mM, 2 min), which 
elevates \([\text{Ca}^{2+}]_m\), to \(<\sim 500-800\) nM, mitochondria accu-
mulate Ca at an approximately constant rate, \(<\sim 184\) 
\(\mu\)M/s (Pivovarova et al., 1999). Since the ratio of mito-
chondrial and cytosolic volumes in sympathetic neu-
rons is \(<\sim 0.1\) (0.09 ± 0.10; Friel, D.D., and S.B. An-
drews, unpublished data), this would give a total cytoso-
lic Ca flux of \((184)(0.1) = 18.4\) \(\mu\)M/s (i.e., the rate of 
mitochondrial Ca accumulation referred to cytosolic 
volume). Estimating the ratio \((\kappa \uparrow, \text{see Appendix})\) of to-
tal to free cytosolic Ca concentration (\(~200\); Friel, 
D.D., unpublished observations) provides an estimate 
of the free cytosolic Ca\(^{2+}\) flux of 92 nM/s, placing a 
lower limit on the rate of uptake via the unipporter that 
is consistent with the value obtained in the present 
study, \(~100\) nM/s at \(~500\) nM, (Figs. 7 B and 8 A).

Our measurements of \(J_{\text{uni}}\) and \(J_{\text{Na/Ca}}\) can only be com-
pared with results obtained from isolated mitochondria, 
since measurements of these fluxes in situ have not been 
reported previously. At a membrane potential of 150 mV 
and external \(\text{Ca}^{2+}\) concentration of 500 nM, uptake by 
isolated rat liver mitochondria occurs at \(~4\) nmol/mg
of [Ca\(^{2+}\)] in that would be predicted from the electron 
port attenuates and prolongs stimulus-evoked [Ca\(^{2+}\)], by interacting with binding proteins according to the 
cytosolic [Ca\(^{2+}\)] buffering strength as 
\(13 \times 184 \mu \text{M/s} = 2.4 \text{ mM} \). 

What Is the Physiological Role of Mitochondrial Calcium Transport at Low [Ca\(^{2+}\)]? 

Mitochondrial Ca\(^{2+}\) transport may play a role in modulating cytosolic [Ca\(^{2+}\)] signals (Thayer and Miller, 1990; 
Friel and Tsien, 1994; Hajnoczky et al., 1995; Jouaville et al., 1995; McGown et al., 1996; Babcock et al., 1997; 
Hoth et al., 1997; Peng, 1998), in buffering potentially cytotoxic Ca\(^{2+}\) loads (Werth and Thayer, 1994; White 
and Reynolds, 1995) and in regulating ATP synthesis so that it meets cellular energy demands (McCormack 
and Denton, 1993; Robb-Gaspers et al., 1998). In the context of [Ca\(^{2+}\)], signaling, mitochondrial Ca\(^{2+}\) trans-
port attenuates and prolongs stimulus-evoked [Ca\(^{2+}\)], signals. Since Ca\(^{2+}\) produces many of its cellular effects 
by interacting with binding proteins according to the principle of mass action (Wier, 1990), such changes in 
the [Ca\(^{2+}\)], signal are likely to influence the impact of excitatory stimuli on [Ca\(^{2+}\)]-sensitive processes within 
the cell, such as action potential generation, synaptic transmission (Tang and Zucker, 1997) and gene expres-
sion (Finkbeiner and Greenberg, 1998). Our results confirm those of Baron and Thayer (1997) who showed 
that the mitochondrial Na\(^{+}\)/Ca\(^{2+}\) exchanger is an important determinant of the plateau level. Modulation 
of the exchanger, for example by intracellular Na\(^{+}\), Mg\(^{2+}\), Ca\(^{2+}\) or spermine (reviewed in Gunter and 
Gunter, 1994) would be expected to modify the kinetics of recovery after stimulation and thereby modify activity 
of Ca\(^{2+}\)-sensitive effectors within the cytosol.

Traditionally, mitochondrial Ca\(^{2+}\) uptake has been viewed as a low affinity process that comes into play only when [Ca\(^{2+}\)], reaches high levels (>\(0.5-1 \mu \text{M}\)). However, results in the present study indicate that mito-
chondrial Ca\(^{2+}\) uptake via the uniporter occurs even when [Ca\(^{2+}\)], is much lower (200–300 nM). What is the 
physiological role of Ca\(^{2+}\) uptake at such low [Ca\(^{2+}\)],? One possibility arises in the context of Ca\(^{2+}\)-regulated mitochondrial ATP production (McCormack and Den-
ton, 1993; Robb-Gaspers et al., 1998). As pointed out by 
Nicholls and Akerman (1982), ongoing mitochondrial 
Ca\(^{2+}\) transport would be more sensitive to modulation 
(for example by Ca\(^{2+}\)) than transport requiring large 
suprathreshold [Ca\(^{2+}\)], elevations for activation. Also, in 
excitable cells, most mitochondria within the cell body lie far from plasma membrane Ca\(^{2+}\) channels that 
are the principal sites of Ca\(^{2+}\) entry; this contrasts with many nonexcitable cells, where receptor-mediated 
Ca\(^{2+}\) release may occur in close proximity to many mi-
tochondria (Rizzuto et al., 1998). Thus, brief suprath-
reshold excitatory stimuli would evoke large [Ca\(^{2+}\)], elevations only near those mitochondria situated close to 
the plasma membrane, with the vast majority of mito-
chondria being exposed to lower [Ca\(^{2+}\)], via diffusion 
from the Ca\(^{2+}\) source. Based on studies in sympathetic 
neurons, radial [Ca\(^{2+}\)], gradients would be dissipated 
within a few seconds after a stimulus (Hernandez-Cruz 
et al., 1990; Hua et al., 1993). Subsequently, slow Ca\(^{2+}\) 
clearance would restore [Ca\(^{2+}\)], to basal levels. During 
the intervening time, [Ca\(^{2+}\)], levels beyond ~200 nM 
would create an imbalance between mitochondrial 
Ca\(^{2+}\) uptake and release that favors net Ca\(^{2+}\) accumu-
lation and a rise in [Ca\(^{2+}\)],. A small but prolonged in-
crease in [Ca\(^{2+}\)], within the vast majority of mito-
tochondria could make a significant contribution to overall 
ATP production. Moreover, during repetitive stimula-
tion that leads to frequency-dependent increases in bulk [Ca\(^{2+}\)], <500 nM, small increases in [Ca\(^{2+}\)], 
could contribute to ATP production in anticipation of energy 
demands for processes triggered by periodic [Ca\(^{2+}\)],, elevations, such as gene transcription (Fields et 
al., 1997).
APPENDIX

Relationship between Mitochondrial and Cytosolic Ca Fluxes

The net mitochondrial Ca$^{2+}$ flux ($J_{\text{mito}}$) measured in this study can be compared with the rate of total mitochondrial Ca transport deduced from electron probe microanalysis (Pivovarova et al., 1999). Let $J$ be the total net Ca$^{2+}$ flux between all mitochondria and the cytosol at an instant in time (e.g., in mmol/s). This flux would cause mitochondrial total Ca concentration to change at a rate $J / v_m$ (e.g., mM/s), where $v_m$ is the mitochondrial volume. It would also cause the total cytosolic Ca concentration to change at a rate $J / v$ (as is the cytosolic volume). If Ca$^{2+}$ binding to cytosolic buffers reaches equilibrium rapidly and conforms to a single binding site model, this flux would cause the cytosolic free Ca concentration to change rapidly and conforms to a single binding site model. The change in total Ca concentration that accompanies a change in $[\text{Ca}^{2+}]_m$ (Eq. 1):

$$\kappa_i^T = (1 + \Sigma_j B_{\text{total},j} K_{d,j}/[\text{Ca}^{2+}])^{2}.$$  (1)

$B_{\text{total},j}$ is the total concentration of the $j$th cytosolic buffer and $K_{d,j}$ is the corresponding dissociation constant. The superscript specifies that $\kappa_i^T$ is the ratio of total to free Ca concentration to distinguish it from the bound to free ratio (e.g., Neher and Augustine, 1992). The relationship between $J_{\text{mito}}$ and the mitochondrial total Ca flux ($J / v_m$) is then:

$$J_{\text{mito}} = (v_m/v_i^T)(J / v_m).$$  (2)

If $[\text{Ca}^{2+}] < < K_{d,j}$ for each buffer (Eq. 3),

$$v_i^T = (1 + \Sigma_j B_{\text{total},j} K_{d,j}),$$  (3)

so that $\kappa_i^T$ is independent of $[\text{Ca}^{2+}]$. Studies in several cell types have provided evidence that $\kappa_i^T$ is approximately constant when $[\text{Ca}^{2+}] < 1 \mu\text{M}$ (e.g., Tse et al., 1994), so for simplicity this will be assumed when estimating changes in $[\text{Ca}]_m$ that accompany changes in $[\text{Ca}^{2+}]$. Nevertheless, a rigorous test of this assumption is warranted.

The time integral of $J_{\text{mito}}$ during the recovery provides information about the change in mitochondrial Ca concentration $[\text{Ca}]_m$ from the instant of repolarization ($t = 0$) to time $t$. It is convenient to define the difference $\Delta[\text{Ca}]_m(t)$ between $[\text{Ca}]_m$ at time $t$ and the resting value measured when $t$ is large $[\text{Ca}]_m(\infty)$ (Eq. 4):

$$\Delta[\text{Ca}]_m(t) = [\text{Ca}]_m(t) - [\text{Ca}]_m(\infty)$$  (4)

$$= \int_{-\infty}^{t} (J / v_m) dt'.$$  (5)

Solving for $J / v_m$ in Eq. 2 above and substituting in Eq. 5 gives Eq. 6:

$$\Delta[\text{Ca}]_m(t) = (v_i^T/v_m) \int_{-\infty}^{t} J_{\text{mito}} dt'.$$  (6)

Assuming that the ratio of changes in total to free intra-mitochondrial Ca concentration ($\kappa_m^T$) is constant, the change in free mitochondrial Ca concentration is (Eqs. 7 and 8):

$$\Delta[\text{Ca}^{2+}]_m(t) = [\text{Ca}^{2+}]_m(t) - [\text{Ca}^{2+}]_m(\infty)$$  (7)

$$= (v_i^T/v_m \kappa_m^T) \int_{-\infty}^{t} J_{\text{mito}} dt'$$  (8)

where $\gamma = (v_m/v_i) (\kappa_i^T/\kappa_m^T)$ is the ratio of effective mitochondrial and cytoplasmic volumes. Multiplying both sides of this equation by $\gamma$ shows that the integral of $J_{\text{mito}}$ gives the change in $[\text{Ca}^{2+}]_m$ that would result if the total mitochondrial flux from time 0 to $t$ were deposited in a closed compartment having the same effective volume as the cytosol:

$$\int_{-\infty}^{t} J_{\text{mito}} dt' = \gamma \Delta[\text{Ca}^{2+}]_m(t)$$  (9)

where the superscript (i) identifies this as a change in cytosolic Ca concentration that accompanies a change in $[\text{Ca}]_m$.

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