Mitochondrial Modulation of \( \text{Ca}^{2+} \)-Induced \( \text{Ca}^{2+} \)-Release in Rat Sensory Neurons

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INTRODUCTION

\( \text{Ca}^{2+} \)-induced \( \text{Ca}^{2+} \)-release (CICR) was originally described for excitation-contraction coupling in cardiac myocytes (Fabiato and Fabiato 1975) and is the process by which a transient elevation of the intracellular \( \text{Ca}^{2+} \) concentration ([\( \text{Ca}^{2+} \)]) is amplified by the activation of ryanodine receptors (RyRs) on the sarcoplasmic reticulum. RyRs are also expressed in nervous tissue (Furuichi et al. 1994; Lai et al. 1992; Lokuta et al. 2002; McPherson et al. 1991), and RyR-mediated CICR from the endoplasmic reticulum (ER) has been described for neurons (Friel and Tsien 1992; Kuba 1980; Shmigol et al. 1995; Thayer et al. 1998; Usachev and Thayer 1997; Usachev et al. 1993). Indeed, RyR-mediated CICR regulates numerous neuronal processes, including synaptic plasticity (Kohda et al. 1995), neurotransmitter release and exocytosis (Peng 1996; Smith and Cunnane 1996), and differentiation and neurite outgrowth (Gomez et al. 1995; Holliday et al. 1991).

The openings of RyRs in response to \( \text{Ca}^{2+} \) are normally uncoordinated events, limited temporally and spatially (Bootman et al. 2001). These elemental events, however, have the potential to transform small, graded \( \text{Ca}^{2+} \) increases into regenerative responses by the subsequent recruitment and synchronization of \( \text{Ca}^{2+} \) release (Berridge 1998; Usachev and Thayer 1999b). In sensory neurons, regenerative CICR occurs after \( \text{Ca}^{2+} \) reaches a specific threshold at which point \( \text{Ca}^{2+} \) release no longer depends on \( \text{Ca}^{2+} \) influx (Usachev and Thayer 1997). Indeed, when RyRs are sensitized, regenerative CICR will establish stable oscillations in [\( \text{Ca}^{2+} \)], (Friel 1995; Usachev and Thayer 1999b).

\( \text{Ca}^{2+} \) regulatory processes operate interdependently to control the fidelity of information encoded in the \( \text{Ca}^{2+} \) signal (Berridge 1998; Thayer et al. 2002). CICR is particularly sensitive to \( \text{Ca}^{2+} \) regulatory processes because \( \text{Ca}^{2+} \) conveys the stimulus and mediates the response (Usachev and Thayer 1999b). Mitochondria might be expected to exert a strong influence on ER \( \text{Ca}^{2+} \) signals because of their close proximity to the ER (Rizzuto et al. 1998), their large capacity for \( \text{Ca}^{2+} \) (Werth and Thayer 1994), and their production of ATP (Hajnoczky et al. 1995; Nicholls and Budd 2000).

In this report, we studied CICR oscillations to determine how CICR is influenced by \( \text{Ca}^{2+} \) regulatory processes. We found that modulating the sensitivity of RyR to [\( \text{Ca}^{2+} \)], or modulating cellular \( \text{Ca}^{2+} \) homeostasis altered oscillation frequency and the shape of the [\( \text{Ca}^{2+} \)] spike. Mitochondria exerted a powerful influence on CICR by buffering \( \text{Ca}^{2+} \) and providing ATP. We also discovered that specific \( \text{Ca}^{2+} \) regulatory mechanisms were supported by ATP from different sources, with the ER \( \text{Ca}^{2+} \)-ATPase relying on mitochondrially generated ATP, whereas the plasma membrane \( \text{Ca}^{2+} \) pump proved insensitive to inhibition of oxidative phosphorylation. These findings illustrate the complex interplay between mitochondria and ER \( \text{Ca}^{2+} \) regulatory processes in sensory neurons.

METHODS

Cell culture

Rat dorsal root ganglion (DRG) neurons were grown in primary culture as described previously (Thayer and Miller 1990). In brief, 1- to 3-day-old Sprague-Dawley rats were killed by decapitation with sharp scissors under a protocol approved by the University of Min-
nesota Institutional Animal Care and Use Committee. Ganglia were dissected from the thoracic and lumbar regions, incubated at 37°C in collagenase-dispase (V. alginolyticus/ B. Polymyxa: 0.8 and 6.4 U/ml, respectively; Roche Diagnostics, Indianapolis, IN) for 45 min, disso-
ciated by trituration through a flame-constricted pipette, and then plated onto laminin-coated (50 mg/ml) glass coverslips (25 mm diam). Cells were grown in Ham’s F12 medium supplemented with 5% heat-inactivated horse serum, 50 ng/ml NFG-7S (mouse submaxillary gland; Sigma), 4.4 mM glucose, 2 mM l-glutamine, modified Eagle’s medium vitamins, and penicillin-streptomycin (100 U/ml and 100 mg/ml, respectively). Cultures were maintained at 37°C in a humidified atmosphere of 5% CO₂. Cells were used on the second and third days in vitro.

**Microfluorometric recording of [Ca²⁺]**

[Ca²⁺] was recorded from cultured rat DRG neurons using indo-1 or mag-indo-1-based microfluorometry (Gryniewicz et al. 1985). Cells were loaded with 2 µM indo-1 acetoxyethyl ester (AM) for 45 min at 22°C in HEPES-buffered Hank’s salt solution (HHSS) containing 0.5% bovine serum albumin or with mag-indo-1 AM at a concentra-
tion of 7.5 µM in 0.02% Pluronic F-127 at 37°C for 30 min. The cells were washed for 30 min in dye-free HHSS at 37°C before initiating recording. HHSS was composed of the following (in mM): 20 HEPES, 137 NaCl, 1.3 CaCl₂, 0.4 MgSO₄, 0.5 MgCl₂, 5.0 KCl, 0.4 KH₂PO₄, 0.6 Na₂HPO₄, 3.0 NaHCO₃, and 5.6 glucose. Cells were rinsed in HHSS and the indicator allowed to de-esterify for 10 min prior to start of the experiment. Cells were placed in a flow-through chamber (Thayer and Miller 1990) (10-s solution exchange) that was mounted on the stage of an inverted epifluorescence microscope (Nikon) equipped with a 70× objective (NA 1.15). Experiments were performed at room temperature (22°C). The dye was excited at 350 nm (10 nm band-pass), and emission was detected at 405 (20) and 490 (20) nm. Fluorescence was monitored by a pair of photomultiplier tubes (Thorn, EMI, Fairfield, NJ) operating in photon-counting mode. The output signals were then passed through a frequency to voltage converter and digitized using a Digidata 1322 a A/D Converter (Axon Instruments, Foster City, CA). Data were stored and analyzed on a personal computer.

Fluorescence changes were converted to [Ca²⁺], by using the formula $[\text{Ca}^{2+}]_i = K_d (R - R_{\text{min}})/(R_{\text{max}} - R)$, where $R$ is 405/490 nm fluorescence ratio (Gryniewicz et al. 1985). The dissociation constant ($K_d$) for indo-1 was 250 nM, and $\beta$ was the ratio of fluorescence emitted at 490 nm and measured in the absence and presence of Ca²⁺. $R_{\text{min}}$ and $R_{\text{max}}$ and $\beta$ were determined by bathing intact cells in 2 µM indo-1 in Ca²⁺-free buffer (1 mM EGTA) and saturating Ca²⁺ (5 mM Ca²⁺). Values for $R_{\text{min}}, R_{\text{max}}$, and $\beta$ were 0.4, 3.65, and 3.34, respectively. After completion of each experiment, cells were wiped from the microscope field using a cotton-tipped applicator. Background light levels were determined at each wave-
length (~5% of cell counts) and subtracted prior to calculating ratios. Potential cytoplasmic contamination with mag-indo-1 prevented us from calibrating the indicator; thus we reported $[\text{Ca}^{2+}]_i$ as the ratio $R$ of fluorescence intensity of the Ca²⁺-bound (405 nm) relative to the Ca²⁺-free form (490 nm) of the dye. To evoke action potentials in intact neurons, extracellular field stimulation was used (Piser et al. 1994). Exponential functions were fitted to the data using a nonlinear, least-squares curve fitting algorithm (Origin 4.1 software; OriginLab, Northampton, MA) (Usachev et al. 2006). Data are presented as means ± SE.

**Simultaneous whole cell patch-clamp and microfluorimetric recording**

Electrical measurements and [Ca²⁺], were recorded from cultured rat DRG neurons by using the whole cell patch-clamp technique (Hamill et al. 1981) in combination with indo-1-based microfluorim-
etry. Microfluorimetry was performed as described in the preceding text. Indo-1 (50 µM) was loaded into the cells via the patch pipette. Patch-clamp data were acquired using an Axopatch 200A amplifier. Both the patch clamp and the optical signals were digitized using a Digidata 1322a A/D converter, stored on a personal computer and analyzed with pClamp 9.0 (Axon Instruments). Patch pipettes were pulled from borosilicate glass (2–4 MΩ; Narishige, Tokyo, Japan) on a Sutter Instruments (Novato, CA) P-87 micropipette puller and filled with the following solution (in mM): 125 potassium gluconate, 10 KCl, 3 Mg-ATP, 1 MgCl₂, 10 HEPES, and 0.05 indo-1, pH 7.25 with KOH, 290 mosM/kg with sucrose. Extracellular recording solution contained (in mM): 140 NaCl, 5 KCl, 2 CaCl₂, 1 MgCl₂, 10 HEPES, and 10 glucose, pH 7.35 with NaOH, 310 mosM/kg with sucrose. Background light levels were collected in the cell-attached configu-
ration and subtracted prior to calculating the ratios. Data are presented as means ± SE. Background levels were collected in the cell-attached configu-
ration and subtracted prior to calculating the ratios. Data are presented as means ± SE.
Ca^{2+} oscillations. These oscillations were remarkably stable and could last several hours in the maintained presence of these agents. Oscillations were analyzed for changes in amplitude, width, and frequency. The mean amplitude for a \([\text{Ca}^{2+}]_{i}\) spike was 177 \pm 6 nM, the mean frequency was 1.5 \pm 0.3 spikes/min, and the mean width was 24 \pm 1 s (\(n = 209\)). Cells capable of regenerative CICR were identified by stable \([\text{Ca}^{2+}]_{i}\) oscillations in the presence of 25 mM K and 5 mM caffeine (Fig. 1).

We further characterized this system, testing whether oscillations could be modulated by factors influencing both influx and the release of \(\text{Ca}^{2+}\) from the ER. In Fig. 1B, we show that bath application of 100 nM ryanodine, an antagonist of the ryanodine receptor, terminated \(\text{Ca}^{2+}\) oscillations (\(n = 4\)).

The ryanodine receptor is sensitive to caffeine (Zucchi and Ronca-Testoni 1997). Consistent with this observation, caffeine concentration modulated CICR oscillations (Fig. 1C). Decreasing caffeine concentration from 5 to 2.5 mM decreased oscillation frequency by 36 \pm 11\% (\(P < 0.0001; n = 16\)) and increased inter-spike interval by 57 \pm 12\% (\(P < 0.001; n = 16\)).

Decreasing caffeine concentration increased peak width by 83 \pm 14\% (\(P < 0.001; n = 16\)). Bath application of cyclopiazonic acid (CPA; 100 nM), a reversible antagonist of the sarcoplasmic-endoplasmic reticulum \(\text{Ca}^{2+}\)-ATPase (SERCA), blocked \(\text{Ca}^{2+}\) oscillations in six of seven cells (Fig. 1D). Oscillations resumed after washout of CPA. Combined, these experiments indicate that CICR oscillations depend on the release from, and subsequent refilling of, ryanodine-sensitive \(\text{Ca}^{2+}\) stores.

An increase in \([\text{Ca}^{2+}]_{i}\) at the mouth of the ryanodine receptor triggers release of \(\text{Ca}^{2+}\) from intracellular stores. Furthermore, the stores are refilled with \(\text{Ca}^{2+}\) by uptake from the cytoplasm. Thus we hypothesized that decreasing basal \([\text{Ca}^{2+}]_{i}\) would decrease CICR oscillation frequency. In Fig. 1E, we show that reducing extracellular \([\text{Ca}^{2+}]_{i}\) from 1.26 to 0.25 mM (a 1:5 dilution) decreased oscillation frequency by 44 \pm 14\% (\(P < 0.01; n = 8\)).

**Mitochondria modulate regenerative CICR**

Mitochondria play an important role in the regulation of \([\text{Ca}^{2+}]_{i}\). They buffer \(\text{Ca}^{2+}\) transients, accumulating \(\text{Ca}^{2+}\) when \([\text{Ca}^{2+}]_{i}\) is elevated above a set point and slowly releasing \(\text{Ca}^{2+}\)
back to the cytosol when \([\text{Ca}^{2+}]\), falls below the set point (Budd and Nicholls 1998). Additionally, close apposition of the ER and mitochondria has been observed (Rizzuto et al. 1998). Thus mitochondria might be expected to alter \(\text{Ca}^{2+}\) oscillations.

\(\text{Ca}^{2+}\) accumulated by mitochondria is released via \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchange (Baron and Thayer 1997; Werth and Thayer 1994). To test the contribution of mitochondria to changes in spike duration, we applied CGP37157 (1 \(\mu\)M; IC\(_{50}\) = 0.80 \(\mu\)M), an inhibitor of mitochondrial \(\text{Na}^+\)-\(\text{Ca}^{2+}\) exchange (Cox et al. 1993), to DRG neurons undergoing CICR oscillations (Fig. 2A). Inhibition of \(\text{Na}^+\)-dependent \(\text{Ca}^{2+}\)-efflux from mitochondria decreased oscillation frequency by 26 \(\pm\) 6% \((P < 0.001; n = 15)\) and decreased spike width by 45 \(\pm\) 5% \((P < 0.001; n = 15)\). The reduced frequency may be due to CGP37157-dependent lowering of inter-spike \([\text{Ca}^{2+}]\) resulting in increased time to reach the threshold \([\text{Ca}^{2+}]\), needed to initiate a spike. The decreased spike width in the presence of CGP 37157 suggests that \(\text{Ca}^{2+}\) release from mitochondria prolongs the duration of the increase in \([\text{Ca}^{2+}]\).

We predicted that blocking mitochondrial \(\text{Ca}^{2+}\) buffering (\(\text{Ca}^{2+}\) uptake) would increase peak height, decrease peak width, and increase oscillation frequency by increasing basal \([\text{Ca}^{2+}]\). Uncoupling of electron transport depolarizes the mitochondrial inner membrane and prevents mitochondrial \(\text{Ca}^{2+}\) uptake. Application of the proton ionophore carbonyl cyanide p-trifluoromethoxy-phenyl-hydrazone (FCCP; 50 nM) resulted in a transient increase in \([\text{Ca}^{2+}]\) attributable to leak of \(\text{Ca}^{2+}\) from the mitochondria (Fig. 2B). As predicted, the amplitude of each \([\text{Ca}^{2+}]\), transient increased. The width also decreased suggesting that the release of \(\text{Ca}^{2+}\) from the mitochondria contributes in part to the duration of each spike. Contrary to our hypothesis, spike frequency decreased by 76 \(\pm\) 10%, with oscillations ceasing in two of six experiments.

Both \(\text{Ca}^{2+}\) uptake and ATP production are coupled to the mitochondrial proton gradient. To distinguish changes in \(\text{Ca}^{2+}\) buffering from changes in ATP production, we examined the effects of the ATP-synthase inhibitor oligomycin B. As seen in Fig. 2C, oligomycin (1 \(\mu\)M) decreased oscillation frequency by 69 \(\pm\) 10% \((P < 0.001; n = 10)\). Oligomycin B and FCCP applied together stopped oscillations \((n = 3); \text{data not shown}\). The slowed oscillation frequency produced by oligomycin B suggests that mitochondrially generated ATP regulates CICR oscillations.

To determine the role of mitochondrially produced ATP directly, ATP concentration was held constant at 3 mM using the whole cell configuration of the patch clamp technique (Fig. 2D). Cells with the ability to oscillate were first identified in 25 mM K\(^+\) and 5 mM caffeine. Caffeine was removed and cells allowed to equilibrate before forming a seal. Cells were held between -55 and -70 mV. Re-addition of caffeine resulted in a large transient increase in \([\text{Ca}^{2+}]\), with \([\text{Ca}^{2+}]\) oscillations starting when \([\text{Ca}^{2+}]\), recovered near basal levels. In the maintained presence of ATP, oligomycin B (1 \(\mu\)M) no longer decreased spike frequency. This result indicates that mitochondrial inhibitors slowed oscillation frequency by decreasing ATP production. This slowing might result from loss of ATP-dependent sensitization of the ryanodine receptor (Laver et al. 2001) or might be due to slowed ATP-dependent SERCA activity (Landolfi et al. 1998). When ATP concentration was held at 3 mM, FCCP increased oscillation frequency by 46 \(\pm\) 8% \((P < 0.05)\) relative to oscillations in oligomycin B, suggesting that mitochondrial \(\text{Ca}^{2+}\) uptake increases the time it takes \([\text{Ca}^{2+}]\), to rise to the threshold for regenerative \(\text{Ca}^{2+}\) release.

Caffeine but not aerobic ATP modulates the threshold for regenerative CICR

Binding sites for ATP have been identified on each of the three RyR isoforms (Zarka and Shoshan-Barmatz 1993) and ATP potentiates \(^{3}H\)-ryanodine binding to RyRs isolated from mitochondria.
brain (Mcpherson and Campbell 1993). Millimolar concentrations of ATP stimulate Ca\(^{2+}\) release and augment CICR in skinned muscle fibers (Duke and Steele 1998; Endo et al. 1970), sarcoplasmic reticulum vesicles (Meissner et al. 1986), and through single channels (McGarry and Williams 1994; Meissner et al. 1988; Rousseau et al. 1986). In Fig. 4, we examined the effects of changing caffeine concentration and of ATP depletion on the threshold for action potential-induced regenerative CICR. Previous work demonstrated that all-or-none CICR displayed a distinct threshold for activation (Usachev and Thayer 1997). In this series of experiments, neurons previously identified as capable of oscillating were treated with 5 mM caffeine in HHSS. Ca\(^{2+}\) influx was driven by action potentials delivered using extracellular field stimulation at a frequency of 1 Hz. The electrical stimulation was maintained until the Ca\(^{2+}\) increase became regenerative and thus no longer dependent on maintained influx. We defined the inflection point where the [Ca\(^{2+}\)]\(_i\) increase becomes supralinear as the threshold for regenerative CICR. In untreated neurons (Fig. 3, A and B), this threshold for CICR remained constant during repeated stimulation. The average threshold for the first three responses was 81 ± 4 nM, which was similar to the average of the last three responses (79 ± 4; n = 8). Decreasing caffeine concentration from 5 to 2.5 mM increased the [Ca\(^{2+}\)]\(_i\) threshold required to evoke a regenerative response (Fig. 3, C and D) from 134 ± 13 to 151 ± 16 nM (P < 0.05; n = 9), consistent with the sensitization of the ryanodine receptors by caffeine (Usachev and Thayer 1997). The increased threshold required a longer train of action potentials to trigger CICR (23 ± 7 to 108 ± 20 s; P < 0.001; n = 9). Thus caffeine lowers the threshold [Ca\(^{2+}\)]\(_i\) required to evoke regenerative Ca\(^{2+}\) release.

A potential mechanism by which inhibition of aerobic metabolism decreased oscillation frequency (Fig. 2C) was by lowering the local ATP concentration and thus decreasing the sensitivity of the RyR to [Ca\(^{2+}\)]\(_i\). However, oligomycin B (1 \(\mu\)M) did not significantly alter the threshold for CICR (Fig. 3, E and F).
Plasma membrane Ca$^{2+}$-ATPases do not depend on aerobically derived ATP

In rat sensory neurons, one of the principle components of Ca$^{2+}$ clearance is the plasma membrane Ca$^{2+}$-ATPase (PMCA). PMCAs exchange 2 H$^+$ for each Ca$^{2+}$ extruded. Elevating extracellular pH (pH$_0$) inhibits PMCA function by decreasing the protons available to exchange for Ca$^{2+}$ (Benham et al. 1992; Park et al. 1996). We hypothesized that increased pH$_0$ would elevate basal [Ca$^{2+}$], and thus accelerate spike frequency. As demonstrated in Fig. 4A, increasing pH$_0$ from 7.45 to 7.75 increased oscillation frequency by 73 ± 31% ($P < 0.001; n = 12$).

These results indicate that PMCAs regulate CICR by controlling basal [Ca$^{2+}$]. We decided to determine whether inhibition of mitochondrial ATP synthesis altered PMCA activity. Neurons displaying CICR oscillations were identified in the presence of caffeine and 25 mM K$^+$ and returned to HHSS. [Ca$^{2+}$], increases were elicited by extracellular field stimulation (4s, 2–6 Hz) every 4 min. In sensory neurons, these small calcium transients recover to basal levels by the combined processes of sequestration and efflux (Benham et al. 1992;
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Usachev et al. 2002). Thus to study PMCA function in isolation, SERCA-mediated Ca\(^{2+}\) was blocked by CPA (5 μM). [Ca\(^{2+}\)] transient amplitudes were kept <400 nM; under these conditions, recovery does not depend on mitochondrial uptake or Na\(^{+}\)-dependent Ca\(^{2+}\) exchange across the plasma membrane (Usachev et al. 2002; Werth and Thayer 1994). A time constant for recovery was calculated by fitting the recovery phase of the [Ca\(^{2+}\)], transients to a monoexponential decay function (τ = 23 ± 2 s; n = 16). As depicted in Fig. 4B, these action potential elicited [Ca\(^{2+}\)] responses were consistent in amplitude and rate of recovery. In Fig. 4C, normalized [Ca\(^{2+}\)] transients from the recording in Fig. 4B were superimposed to demonstrate the reproducibility of the recovery rate. The mean recovery rate for the last three responses was 97 ± 11% of the mean of the first three (n = 8). As shown in Fig. 4, D and E, treatment with oligomycin B did not significantly decrease the rate of recovery. The mean recovery rate for the last three responses in 1 μM oligomycin B was 95 ± 10% of the mean of the first three control responses (n = 8). Thus it appears that PMCA activity in sensory neurons does not depend on mitochondrially derived ATP. Furthermore, our ability to evoke action potentials in the presence of oligomycin B suggests that other plasma membrane functions remained intact. Thus with glucose present in the bathing medium (5.6 mM), glycolysis supports plasma membrane ATPases in the presence of oligomycin.

Aerobically derived ATP regulates the rate of ER refilling

We next tested the hypothesis that sequestration of Ca\(^{2+}\) into the ER by SERCA is dependent on ATP derived from mitochondrial oxidative phosphorylation. To directly visualize [Ca\(^{2+}\)]\(_{\text{ER}}\), we loaded DRG neurons with the low-affinity Ca\(^{2+}\) indicator mag-indo-1 AM (K\(_{d}\) = 35 μM) using conditions that promote sequestration of the dye into the ER (Solovyova et al. 2002; Usachev et al. 2006). The contribution of cytosolic Ca\(^{2+}\) changes to changes in mag-indo-1 fluorescence is minimal due to the low affinity of the indicator for Ca\(^{2+}\).

Application of the SERCA inhibitor, CPA (5 μM) in Ca\(^{2+}\)- free media depleted the ER of Ca\(^{2+}\) (Fig. 5A). Re-addition of Ca\(^{2+}\) to the medium refilled the ER. The time constant (τ) for refilling was calculated by fitting the time course of the recovery to a monoexponential equation (Fig. 5B). The mean time constant for refilling was 152 ± 50 s (n = 8). As shown in Fig. 5A (and superimposed in Fig. 5B), the ER could be repeatedly depleted and refilled with the refilling rate reproducible for a given cell (τ\(_{2}/\tau\(_{1}\) = 0.75 ± 0.2; n = 8). Even though the cells started with varied [Ca\(^{2+}\)]\(_{\text{ER}}\), the standard protocol employed here returned [Ca\(^{2+}\)]\(_{\text{ER}}\) to reproducible levels. The amplitude of the second refilling returned to 86 ± 10% of the original (Fig. 5E). Application of oligomycin B (1 μM) before the second refilling significantly decreased the rate of refilling (τ\(_{2}/\tau\(_{1}\) = 2.2 ± 0.5; P < 0.01; n = 7). The modest decline in the total extent of refilling in oligomycin-treated cells was not statistically significant. In Fig. 5D, traces of the first and second phases of refilling (from Fig. 5C) are superimposed on an expanded time scale to illustrate the slowed refilling in the presence of oligomycin B. These data are consistent with the hypothesis that mitochondrial derived ATP supports SERCA-mediated ER refilling and is responsible for the slowed CICR oscillations (Fig. 2C) following the inhibition of aerobic ATP synthesis.

Mitochondrial Ca\(^{2+}\) buffering creates complex CICR waveforms

In DRG neurons that oscillate, a small fraction display complex Ca\(^{2+}\) waveforms, reminiscent of burst firing of action potentials. Recovery of the [Ca\(^{2+}\)] transients to basal levels in these neurons consisted of three phases (Fig. 6A): there was an initial rapid decrease in [Ca\(^{2+}\)], a protracted plateau phase followed during which [Ca\(^{2+}\)] remained elevated and secondary, smaller Ca\(^{2+}\) spikes were observed, and finally there was a rapid decrease in [Ca\(^{2+}\)], to original resting levels. In cells spontaneously displaying these complex waveforms, the mean amplitude of the secondary [Ca\(^{2+}\)] spikes was 31 ± 5% less than the primary [Ca\(^{2+}\)]. The mean width of the primary [Ca\(^{2+}\]), transient in cells with complex waveforms was 70 ± 8 s (n = 6), substantially larger than the mean spike width displayed in the general population of oscillating neurons. We therefore hypothesized that these secondary release events were the result of prolonged elevation of [Ca\(^{2+}\)]. Decreasing caffeine concentration, a treatment that increases peak width, increased the duration of the plateau and the number of spikes per cluster (Fig. 6A).

The underlying plateau phase is reminiscent of the marked mitochondrial Ca\(^{2+}\) release phase seen in these cells after stimulation with large Ca\(^{2+}\) loads (Werth and Thayer 1994). Consistent with the idea that mitochondrial Ca\(^{2+}\) release underlies these phenomena, these doublets appeared in 4 of 18 neurons treated with CGP37157 (Fig. 6B). Furthermore, in neurons already displaying complex waveforms, CGP37157 returned oscillations to a normal (singlet) spiking pattern (n = 3; Fig. 6C). These neurons displayed a graded decrease in plateau height during CGP37157 application. Collectively, the experiments in Fig. 6, B and C, suggest that adjusting the amplitude of the mitochondrially mediated plateau [Ca\(^{2+}\)], with CGP37157 determines whether sustained mitochondrial Ca\(^{2+}\) release evokes multiple release events. To determine whether all Ca\(^{2+}\) spikes within a cluster were the result of CICR from the ER, we simultaneously measured [Ca\(^{2+}\)]\(_{\text{ER}}\) and [Ca\(^{2+}\)]\(_{\text{c}}\), during Ca\(^{2+}\) oscillations displaying this complex waveform. In the trace displayed in Fig. 6D, [Ca\(^{2+}\)]\(_{\text{c}}\), remained sufficiently elevated to elicit a secondary release. The plateau phase of the primary spike appeared to decrease over time as the spikes resolved into distinct [Ca\(^{2+}\)], transients. Simultaneously, the mag-fluo-4 fluorescence increased throughout the recording, suggesting that the ER was accumulating Ca\(^{2+}\).

DISCUSSION

Regenerative CICR transforms [Ca\(^{2+}\)] responses that are small, graded, and localized into large, all-or-none, global [Ca\(^{2+}\)] signals. We show here that a subset of sensory neurons display a robust CICR response mediated by ryanodine-sensitive Ca\(^{2+}\) stores and that in the maintained presence of Ca\(^{2+}\) influx and caffeine, these cells exhibit sustained oscillations in [Ca\(^{2+}\)]. We studied these oscillations to learn how other Ca\(^{2+}\) regulatory processes influence CICR. Modulating the sensitivity of the RyR to [Ca\(^{2+}\)], altered oscillation frequency and the shape of the [Ca\(^{2+}\)] spike. Mitochondrial Ca\(^{2+}\) buffering
affected spike shape and mitochondrial ATP production moduli
ted oscillation frequency. Aerobically produced ATP specif-
cically modulated the rate of ER refilling but did not alter
PMCA-mediated Ca\(^{2+}\) clearance or CICR threshold.

What is the role of regenerative CICR in sensory neurons?
Depletion of ER Ca\(^{2+}\) can trigger apoptosis (Hajnoczky et al.
2003; Mengesdorf et al. 2001), but the rapid refilling of the
Ca\(^{2+}\) store and the sustained nature of the oscillations suggest
a more physiological role. In peripheral neurons, Ca\(^{2+}\) release
of Ryanodine-sensitive Ca\(^{2+}\) stores activates K\(^+\) channels
that mediate a slow afterhyperpolarization (Moore et al. 1998;
Narita et al. 2000), and somatic release of neuropeptides from DRG neurons may influence other cells in the
ganglion (Harding et al. 1999). The global Ca\(^{2+}\) wave that
results from regenerative CICR in these cells propagates to the
nucleus (Usachev and Thayer 1997) and [Ca\(^{2+}\)]\(_{\text{ER}}\) oscillations
optimize the nuclear translocation of certain transcription fac-
tors (Tomida et al. 2003), suggesting that this regenerative type
of signaling might participate in coupling excitation to tran-
scription.

Oscillatory behavior was most prevalent in DRG neurons of
medium to large diameter and in cells that were in culture <3
days. Large DRG cells relay low-threshold mechanical and
propriospinoceptive stimuli (Hendry et al. 1999). We have not linked regenerative CICR to this particular sensory modality other than to suggest that it may be a mechanism to enhance information transfer in large neurons. Cells with low surface to volume ratios may be more dependent on intracellular Ca\(^{2+}\) stores to propagate [Ca\(^{2+}\)]\(_i\) signals. The reduced prevalence of oscillatory cells with age in culture suggests that oscillations may be associated with growth and extension of processes rather than with their length. Acutely dissociated DRG neurons isolated from adult rats and mice display [Ca\(^{2+}\)]\(_i\) increases in response to caffeine application (Kruglikov et al. 2004; Shmigol et al. 1996) and caffeine-induced [Ca\(^{2+}\)]\(_i\) oscillations have been described for other peripheral neurons, including neurons of the sympathetic ganglia (Friel 1995). The survival of the various DRG subtypes depends on specific trophic factors (Oakley et al. 1997; Stucky et al. 1998, 2002). However, the proportion of DRG neurons that display the [Ca\(^{2+}\)]\(_i\) oscillation phenotype was not affected by the presence of trophic factors in the cell culture medium.

Caffeine, at millimolar concentrations, sensitzizes ryanodine receptors to [Ca\(^{2+}\)]\(_i\) (Endo et al. 1970; Thayer et al. 1988), and we showed previously that caffeine concentration modulates the threshold for regenerative CICR in sensory neurons (Usachev and Thayer 1997). Here we showed that increasing the caffeine concentration increased the frequency of [Ca\(^{2+}\)]\(_i\) oscillations, consistent with the predicted lowering of the [Ca\(^{2+}\)]\(_i\) threshold for triggering CICR. Interestingly, increasing the caffeine concentration also decreased the duration of the individual transients. This observation is qualitatively similar to the observation that inhibition of RyRs by tetracaine yielded a lower frequency of spontaneous release events while simultaneously enhancing the amount of Ca\(^{2+}\) released per burst (Overend et al. 1997; Velez et al. 1997). The increased width of the [Ca\(^{2+}\)]\(_i\) spike at lower caffeine concentrations may result from an increase in the driving force for Ca\(^{2+}\) to exit the ER.

Other studies have shown a steep relationship between ER Ca\(^{2+}\) content and the magnitude of the cytosolic [Ca\(^{2+}\)]\(_i\) increase (Trafford et al. 2000). Overall, our observations support a model in which caffeine controls oscillation frequency through its effects on RyR sensitivity and that changes in frequency modulate the duration of the individual [Ca\(^{2+}\)]\(_i\) transients through secondary effects on luminal Ca\(^{2+}\) concentration.

We thought that the oscillations might serve as a useful assay to identify physiological agents that sensitize the RyR. However, we have not to date found physiological conditions that will substitute for caffeine. Other laboratories found that cyclic ADP ribose sensitizes the ER to [Ca\(^{2+}\)]\(_i\) (Currie et al. 1992; Hua et al. 1994), although we have not found conditions in which this agent affects CICR in DRG neurons (Usachev and Thayer 1997). Thus we can only speculate on the physiological conditions that might elicit the regenerative responses studied here. However, factors that modulate regenerative responses likely influence elementary Ca\(^{2+}\) release events that occur in sensory neurons with prominent RyR-mediated Ca\(^{2+}\) release (Pacher et al. 2002).

Mitochondria play a complex role in neurons; they serve as a nexus where neuronal survival and death cues are integrated, provide a mechanism for local energy supply via oxidative phosphorylation, and regulate Ca\(^{2+}\) signals (Jacobson and Duchen 2004). Mitochondria take up Ca\(^{2+}\) into the mitochondrial matrix through the Ca\(^{2+}\) uniporter by a process driven by the large membrane potential established during oxidative phosphorylation (Kirichok et al. 2004). Ca\(^{2+}\) is recycled to the cytosol via a Na\(^{+}\)-dependent Ca\(^{2+}\)-exchanger (Baron and Thayer 1997; Zhang and Lipton 1999). These two processes enable mitochondria to blunt the amplitude of [Ca\(^{2+}\)]\(_i\) increases and to translate increases in Ca\(^{2+}\) load to changes in the duration of [Ca\(^{2+}\)]\(_i\) transients (Werth and Thayer 1994). Thus depolarizing the mitochondrial membrane potential with FCCP...
increased the amplitude and shortened the duration of individual [Ca$^{2+}$] spikes. Inhibition of mitochondrial Na$^+$/Ca$^{2+}$ exchange in DRG neurons lowers the plateau phase of [Ca$^{2+}$],
recovery because the balance of Ca$^{2+}$ release from mitochondria relative to Ca$^{2+}$ clearance from the cytoplasm reaches a new steady state (Baron and Thayer 1997; Werth and Thayer 1994). Treatment with CGP37157, an inhibitor of Na$^+$/Ca$^{2+}$-dependent Ca$^{2+}$-exchange accelerated the return to basal [Ca$^{2+}$], producing, in most cells, a corresponding decrease in the spike frequency and a decrease in the spike width. However, in some cells, particularly those with prominent mitochondrial contributions to spike width, inhibition of Na$^+$/Ca$^{2+}$ exchange did not reduce the recovery to basal [Ca$^{2+}$], but rather resulted in a plateau [Ca$^{2+}$], near threshold for triggering CICR and thus increased the frequency of CICR oscillations. This observation raises some concern about potential adverse Ca$^{2+}$ signaling events that might result from pharmacologic inhibition of mitochondrial Na$^+$/Ca$^{2+}$ exchange, a strategy proposed to improve ATP production in metabolic disorders (Cox and Matlib 1993). Taken together, the results from uncoupling electron transport and inhibition of Na$^+$/Ca$^{2+}$ exchange in mitochondria identify a transfer of Ca$^{2+}$ from the ER to the matrix that is not detected by a cytoplasmic indicator. A similar shuttle of Ca$^{2+}$ between the ER and mitochondria was shown to have a pacemaker role in regulating inositol triphosphate (IP$_3$)-induced Ca$^{2+}$ oscillations in HeLa cells (Ishii et al. 2006). These observations are consistent with the close apposition of mitochondria to the ER (Rizzuto et al. 1998) and mitochondrial uptake of Ca$^{2+}$, suggesting that the decrease in CICR oscillation frequency was secondary to SERCA inhibition. That the two pumps was secondary to SERCA inhibition. That the two pumps

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The plasma membrane Ca$^{2+}$ pumps were not dependent on ATP derived from oxidative phosphorylation, consistent with a similar finding reported for peripheral nerve terminals (Gover et al. 2004). Glycolytic activity preferentially drives a number of plasma membrane ion transport systems, including the K$_{ATP}$ channel (Weiss and Lamp 1987, 1989), the Na$^+$/K$^+$ pump, (Glitsch and Tappe 1993), and the Na$^+$/H$^+$ exchanger (Wu and Vaughan-Jones 1994). The coupling of Ca$^{2+}$ transport systems to different metabolic pathways is likely to be broadly important to neuronal signaling and survival.

Mitochondrial regulation of CICR raises the possibility that pathological changes in cellular energy metabolism, such as those that accompany diabetic neuropathy, might have profound effects on ER Ca$^{2+}$
signaling (Kruglikov et al. 2004). Perhaps the reliance of SERCA-mediated Ca$^{2+}$ uptake on aerobic metabolism contributes to the sensitivity of the CNS to oxygen deprivation (Erecinska and Silver 2001). Ca$^{2+}$ levels within the ER lumen influence protein processing and trigger certain forms of apoptosis (Hajnoczky et al. 2003; Mengesdorf et al. 2001; Paschen 2004).

CONCLUSION

CICR oscillations illustrate the complexity and interdependence of the Ca$^{2+}$ regulatory mechanisms operating in an intact neuron. Competition between various reuptake and clearance mechanisms allows neurons to shape Ca$^{2+}$ signals, altering their spatial and temporal attributes. The interplay between mitochondria and ER Ca$^{2+}$ release may influence neuronal energy metabolism and Ca$^{2+}$-triggered changes in excitability, secretion, transcription, and apoptosis.

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