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Roles of mitochondria and temperature in the control of intracellular calcium in adult rat sensory neurons

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SUMMARY

We recorded Ca^{2+} current and intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) in isolated adult rat dorsal root ganglion (DRG) neurons at 20 and 30 °C. In neurons bathed in tetraethylammonium and dialyzed with cesium, warming reduced resting average $[\text{Ca}^{2+}]_i$ from 87 to 49 nM and the time constant of the decay of $[\text{Ca}^{2+}]_i$ transients (τ_T) from 1.3 s to 0.99 s ($Q_{10} = 1.4$). The Buffer Index, the ratio between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$ ($J_{\text{Ca}} \cdot dt / \Delta[\text{Ca}^{2+}]_i$), increased 2- to 3-fold with warming. Neither inhibition of the plasma membrane Ca^{2+} -ATPase by intracellular sodium orthovanadate nor inhibition of Ca^{2+} uptake by the endoplasmic reticulum by thapsigargin plus ryanodine were necessary for the effects of warming on these parameters. In contrast, inhibition of the mitochondrial Ca^{2+} uniporter by intracellular ruthenium red largely reversed the effects of warming. Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (500 nM) increased resting $[\text{Ca}^{2+}]_i$ at 30 °C. 10 mM intracellular sodium prolonged the recovery of $[\text{Ca}^{2+}]_i$ transients to 10–40 s. This effect was reversed by an inhibitor of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange (CGP 37157, 10 μM). Thus, mitochondrial Ca^{2+} -uptake is necessary for the temperature-dependent increase in Ca^{2+} buffering and mitochondrial Ca^{2+} fluxes contribute to the control of $[\text{Ca}^{2+}]_i$ between 50 and 150 nM at 30 °C.

Keywords

plasma membrane Ca^{2+} ATPase; endoplasmic reticulum; carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone; FCCP; ruthenium red; sodium-calcium exchange; Q_{10}

INTRODUCTION

Primary afferent sensory neurons with cell bodies located in the sensory ganglia of the brainstem and the dorsal root ganglia (DRGs) conduct information from the periphery into the central nervous system. The spherical cell bodies of these neurons are separated from the long axonal processes linking the periphery to the central nervous system by relatively short T-stem axons. Thus, the cell bodies do not directly influence the conduction of action potentials into the spinal cord or brain stem. Nevertheless, action potentials are conducted into the cell bodies where voltage-gated Ca^{2+} channels mediate activity-dependent Ca^{2+} influx that raises intracellular Ca^{2+} ($[\text{Ca}^{2+}]_i$) thereby regulating ion channels [1;2], activating secretion of neurotransmitters [3], and regulating gene expression [4;5]. These activities are controlled by the limitation and localization of increases in $[\text{Ca}^{2+}]_i$ by cytoplasmic Ca^{2+} -buffering

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mechanisms including Ca^{2+} -binding, Ca^{2+} -sequestration, and Ca^{2+} -extrusion. Studies of these mechanisms in DRG neurons at room temperature found the restoration of resting $[\text{Ca}^{2+}]_i$ following Ca^{2+} influx is relatively slow (time constants ranging from 5 to 20 s) [6–9] and that the plasma membrane Ca^{2+} ATPase is the principal mechanism that restores resting $[\text{Ca}^{2+}]_i$ following modest increases in $[\text{Ca}^{2+}]_i$ [10–12]. This work also led to the views that Ca^{2+} uptake by the mitochondria restrains the increase in $[\text{Ca}^{2+}]_i$ if Ca^{2+} entry raises $[\text{Ca}^{2+}]_i$ above ≈ 300 nM and mediates a rapid phase of recovery of $[\text{Ca}^{2+}]_i$ to ≈ 300 nM, and that mitochondrial Ca^{2+} release slows the final restoration of resting $[\text{Ca}^{2+}]_i$, and that Ca^{2+} uptake and release have little effect on resting $[\text{Ca}^{2+}]_i$ [6;7;13;14].

The majority of these studies were done on DRG neurons isolated from neonatal rats and examined room temperature. Thus, there is relatively little information on the control of $[\text{Ca}^{2+}]_i$ in DRG neurons of adult rats, the primary experimental model for studies of somatic sensation including nociception, at warmer temperatures. That temperature might be an important parameter is suggested by reports that warming increases Ca^{2+} buffering by mitochondria [15–18]. A temperature-dependent increase in Ca^{2+} buffering by the mitochondria is expected to limit the ability of Ca^{2+} influx to raise global $[\text{Ca}^{2+}]_i$. In this case, relatively large increases in global $[\text{Ca}^{2+}]_i$ observed at room temperature would be physiologically irrelevant and the direct effects of Ca^{2+} influx will be limited to the cytoplasm near the plasma membrane.

We investigated these issues by recording membrane currents and $[\text{Ca}^{2+}]_i$ in isolated adult rat DRG neurons at 20 and 30 °C using conventional patch-clamp and fluorescence methods. We found that warming increased the ability of the neurons to buffer a Ca^{2+} influx by 2- to 3-fold and that the mitochondrial Ca^{2+} uptake was necessary for this temperature-dependence. Further, we found evidence that mitochondrial Ca^{2+} fluxes contribute to the control of $[\text{Ca}^{2+}]_i$ between 50 and 150 nM at 30 °C.

Materials and Methods

Preparation of neurons

DRGs were collected from adult male Sprague Dawley rats (90 – 200 g) killed by CO_2 asphyxiation as approved by the Institutional Animal Care and Use Committee at the University of Nevada, Reno. We dissociated the DRG neurons by incubating the ganglia in a Ca^{2+} -free Puck's solution containing 16.5 units/ml of papain (Worthington, www.worthington-biochem.com) for 20 minutes at 37 °C. The ganglia were then washed in F-12 medium (Invitrogen, www.invitrogen.com) augmented with 0.1 ml/l horse serum (Invitrogen), 2 mM L-glutamine (Invitrogen), 250 ng/ml nerve growth factor (Harlan Bioproducts for Science, www.hbps.com), 0.1 mg/ml normocin (Invivogen, www.invivogen.com), 0.3 mg/ml 5-fluoro-2-deoxyuridine, 0.7 mg/ml uridine, and 1.18 g/l of NaH_2CO_3 and then incubated in this medium plus 1.25 mg/ml collagenase for 15 minutes at 37 °C. The ganglia were then collected by centrifugation, washed in the F12 medium, and dissociated mechanically. The DRG neurons were collected by centrifugation, washed in the F12 medium, and plated onto squares of aclar (a non-fluorescent plastic, Ted Pella, Inc., www.tedpella.com) coated with poly-D-lysine and laminin. Neurons were incubated in 3% CO_2 at 37 °C until they were used for experiments (within 36 h for patch-clamp, 48 h for fura-AM protocols).

The day after plating, a portion of aclar with neurons was placed in a chamber on the stage of a Nikon TE 2000 inverted microscope (www.nikonusa.com). The bottom of the chamber was a 0.12 mm thick coverslip with a thin indium tin oxide coating that acted as a resistive heater controlled by a feedback circuit (TC2bip, Cell Microcontrols, www.cellmc.com). The temperature in the chamber was sensed by a thermistor (TH-2Km, Cell Microcontrols) placed

<10 mm downstream from the cells. The solution entering the chamber was warmed by a feedback controlled preheater driven by the TC2bip. In order to set the temperature to 20 °C the solution was first cooled by looping the inflow tube through an ice bath and then warmed to 20 °C. All data were collected from isolated adult rat DRG neurons <30 µm in diameter.

Patch-clamp recording

Neurons were bathed in one of three extracellular solutions. In order to isolate Ca^{2+} currents, we used a “TEA bath” containing (in mM) 160 tetraethylammonium chloride, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose, 10 mannitol (pH = 7.4). In order to record $[\text{Ca}^{2+}]_i$ under more physiological conditions, we used either an “NaCl bath” containing (in mM) 140 NaCl, 2 CaCl_2 , 1 MgCl_2 , 10 HEPES, 10 glucose (pH = 7.4) or an “NaCl + TEA bath” in which 10 mM NaCl was replaced by tetraethylammonium chloride. Test compounds were applied to the neurons either by bath exchange (FCCP and thapsigargin plus ryanodine) or by a puffer pipette (FCCP and CGP 37157).

Patch pipettes were pulled (PP-83, Narishige Scientific Instruments, www.narishige.co.jp) from Corning 7052 glass (Warner Instruments, www.warneronline.com) and fire polished to final resistances of 2 – 3 MΩ when filled with one of the following pipette solutions. In order to isolate Ca^{2+} currents, we used a “ Cs^+ -citrate” pipette solution containing (in mM) 135 CsCl, 10 citrate, 5 ATP (magnesium salt), 0.5 GTP (sodium salt), 10 HEPES, plus Ca^{2+} indicator (see below). In order to examine the effects of intracellular Na^+ , we used a “ Cs^+ -citrate + 10 mM Na^+ ” pipette solution in which 10 mM of CsCl was replaced by NaCl. The pH of these solutions was set with CsOH to 7.2. In order to record $[\text{Ca}^{2+}]_i$ under more physiological conditions, we used a “ K^+ -citrate + 10 mM Na^+ ” pipette solution in which the CsCl of the Cs^+ -citrate pipette solution was replaced by 125 mM KCl plus 10 mM NaCl and the pH was set with KOH. The Ca^{2+} indicator was either 30 µM bis-fura-2 (Invitrogen) or (in a limited series of experiments) 100 µM fura-2 (Invitrogen).

In designing these solutions we considered the relationship between the Ca^{2+} -binding ratios of the components and $[\text{Ca}^{2+}]_i$, i.e. $\kappa_B = d[\text{BCa}]/d[\text{Ca}^{2+}]_i$ [19;20]. For free Ca^{2+} between 10 and 100 nM, κ_B for 100 µM fura-2 is between 400 and 200, i.e. comparable to the endogenous Ca^{2+} -binding ratio of adult rat DRG neurons ($\kappa_s = 370$ Zeilhofer *et al.* [21]). In contrast, because of its lower affinity for Ca^{2+} and lower concentration, κ_B for 30 µM bis-fura-2 over this range of $[\text{Ca}^{2+}]_i$ is relatively constant at ≈ 60 , i.e. less than one-third of κ_s in adult rat DRG neurons. Thus, one expects more accurate assessment of endogenous Ca^{2+} -buffering with 30 µM bis-fura-2. As the non-chloride anion, we chose citrate as a very low affinity Ca^{2+} buffer ($K_d = 491$ µM at 30 °C, pH = 7.2, ionic strength = 0.140, Maxchelator www.stanford.edu/~cpatton/maxc.html [22]) that contributes a Ca^{2+} -binding ratio of ≈ 20 for free Ca^{2+} between 10 and 100 nM. Thus, citrate will have little impact on the $[\text{Ca}^{2+}]_i$ transients we describe below. Nevertheless, calculations with Maxchelator indicate that 10 mM citrate along with 5 mM ATP ($\kappa_B \approx 25$) and 30 µM bis-fura-2 will buffer free Ca^{2+} to ≈ 71 nM in a pipette solution containing 8.5 µM contaminating Ca^{2+} . Thus, the Cs^+ -citrate and K^+ -citrate pipette solutions achieve a low κ_B without burdening the neuron with an exogenous Ca^{2+} load at resting $[\text{Ca}^{2+}]_i$.

Membrane currents were recorded using an Axopatch 200 amplifier, a Digidata 1200 interface, and pClamp6 (Molecular Devices, www.moleculardevices.com). Membrane potentials were corrected for liquid junction potentials (10 mV for the Cs^+ -citrate pipette solutions, 6 mV for the K^+ -citrate pipette solution) calculated using a spreadsheet implementing the equations described by Barry [23] available in the *Primer on Junction Potentials for the Patchologist* at <http://www.unr.edu/physio/fackenyon.html>). The analog circuitry of the Axopatch amplifier was used to compensate for >90% (typically 95%) of the membrane capacitance and series resistance.

Fluorescence measurement of $[Ca^{2+}]_i$

In patch-clamp experiments, fura-2 and bis-fura-2 were loaded into the neurons by dialysis via the patch pipette. To measure $[Ca^{2+}]_i$ in intact neurons, the cells were washed in the NaCl bath and loaded with indicator by incubation in this solution plus 10 μ M fura-2-AM (Invitrogen) at room temperature for 60 minutes (AM-loaded). The neurons were then washed in the NaCl bath to remove the fura-2 ester and incubated for at least 30 minutes to allow for de-esterification. $[Ca^{2+}]_i$ was recorded during superfusion with the NaCl bath.

The Ca^{2+} indicators were excited at 340 and 380 nm with light from a Sutter DG-4 light source. The widefield fluorescence emission at 510 nm was imaged by a Hamamatsu Orca camera and normalized to the fluorescence produced by Fluoresbrite beads (Polysciences Inc. #18340, www.polysciences.com) as described by Zhou and Neher [24]. The excitation and emission were conditioned by filters obtained from Chroma Technology Corp (www.chroma.com). The excitation, imaging, and initial analysis were done using Simple PCI version 5 (Compix Inc., www.cimaging.net).

The global fluorescence of the Ca^{2+} indicators was measured by averaging the fluorescence signal from the pixels representing the neuron under study, exporting the data to Excel, and converting the fluorescence to $[Ca^{2+}]_i$ as described by Neher [25] using parameters determined in DRG neurons at 20 and 30 °C. Briefly, the relationship between the fluorescence ratio (R) and $[Ca^{2+}]_i$ is:

$$[Ca^{2+}]_i = K_{eff} \left(\frac{R - R_{min}}{R_{max} - R} \right) \quad \text{Equation 1}$$

R_{min} and R_{max} were determined by measuring the fluorescence ratio of fura-2 or bis-fura-2 in neurons dialyzed with a modified version of the Cs^+ -citrate pipette solution containing either 10 mM EGTA or 10 mM $CaCl_2$. K_{eff} was determined from R_{min} and R_{max} plus measurements of R_{mid} , the fluorescence ratio of the indicator at a known $[Ca^{2+}]_i$ established in neurons dialyzed with a modified Cs^+ -citrate pipette solution containing 10 mM EGTA plus $CaCl_2$ to give a free Ca^{2+} of 256 nM at 20 °C and 138 nM at 30 °C (determined using a Ca^{2+} -selective mini-electrode [26]). Initial measurements of R_{mid} were variable and unexpectedly low suggesting that the EGTA plus $CaCl_2$ was unable to buffer cytoplasmic $[Ca^{2+}]_i$. More consistent and higher R_{mid} values were obtained in bath solutions in which we replaced glucose by deoxy-D-glucose and added 1 μ M carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and we took these as our best estimates of R_{mid} . K_{eff} values were 0.96 μ M for fura-2 at 20 °C, 2.4 μ M for bis-fura-2 at 20 °C, and 1.2 μ M for bis-fura-2 at 30 °C.

Our limited interest quantifying fura-2 measurements at 30 °C did not justify a similar effort and we used R_{min} and R_{max} determined at 20 °C (these parameters are temperature-insensitive) and determined K_{eff} from our measurements of the resting fura-2 fluorescence ratio at 30 °C and the assumption that resting Ca^{2+} in these neurons was 49 nM, i.e. the same as resting $[Ca^{2+}]_i$ measured using bis-fura-2.

Analysis

We assessed the relationship between Ca^{2+} influx and $[Ca^{2+}]_i$ using a “DURA175” voltage-clamp protocol in which the membrane potential was held at -70 mV and stepped to 0 mV to elicit a nearly maximal Ca^{2+} current for durations between 10 and 175 ms in 8 increments of 23.6 ms. In neurons dialyzed with Cs^+ -citrate under control conditions, 20 s intervals between depolarizations were adequate for recovery of Ca^{2+} current and resting $[Ca^{2+}]_i$. However, under conditions where the recovery of resting $[Ca^{2+}]_i$ was slowed (i.e. ruthenium red and in the presence of Na^+) intervals were increased.

From the data obtained from the DURA175 protocol, we measured the Ca^{2+} influx during each depolarization as the time integral of the current ($\int I_{\text{Ca}} \cdot dt$), resting $[\text{Ca}^{2+}]_i$ before each depolarization, the change in $[\text{Ca}^{2+}]_i$ ($\Delta[\text{Ca}^{2+}]_i$) caused by each depolarization, and the largest peak $[\text{Ca}^{2+}]_i$ during the protocol. Resting $[\text{Ca}^{2+}]_i$ for a given DURA175 execution was taken as the average resting $[\text{Ca}^{2+}]_i$ before the last three pulses for all protocols except those examining ruthenium red where the $[\text{Ca}^{2+}]_i$ before the first pulse was taken. As described below, the restoration of resting $[\text{Ca}^{2+}]_i$ in neurons dialyzed with the Cs^+ -citrate pipette solution was well described by a single time constant independent of the amplitude of the $[\text{Ca}^{2+}]_i$ transient. Accordingly, the time constant for the restoration of resting $[\text{Ca}^{2+}]_i$ for a given DURA175 execution (τ_r) was taken as the average of the time constants associated with the last three pulses.

In order to assess the Ca^{2+} buffering ability of the neurons, we determined the relationship between $\int I_{\text{Ca}} \cdot dt$ and $\Delta[\text{Ca}^{2+}]_i$ for each DURA175 depolarization (cf. Verkhratsky [27]). In addition, we calculated the “Buffer Index”, the ratio between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$ ($\int I_{\text{Ca}} \cdot dt / \Delta[\text{Ca}^{2+}]_i$), as a quantitative measure of Ca^{2+} buffering. In common with the endogenous Ca^{2+} -binding ratio (κ_s , [19;20]) a larger Buffer Index indicates more powerful Ca^{2+} buffering. However, the Buffer Index lacks a quantitative theoretical justification and carries no implication with regard to mechanism. It does provide a quantitative comparison of Ca^{2+} buffering by Ca^{2+} -binding molecules, Ca^{2+} sequestration, and Ca^{2+} extrusion in DRG neurons of similar size and resting $[\text{Ca}^{2+}]_i$.

Sources and statistics

Statistical analyses (t-tests, one-way and two-way ANOVAs, log-rank test) were done using Prism (GraphPad Software, www.graphpad.com) with $P < 0.05$ taken as statistically significant. Analysis of membrane currents and curve fitting were done using Origin 7.0 (OriginLab Corp., www.originlab.com). Unless noted above, all chemicals were obtained from Sigma-Aldrich (www.sigmaaldrich.com).

Results

Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ at 20 °C

We recorded Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ in response to the DURA175 protocol at 20 °C in 22 neurons using the Cs^+ -citrate pipette solution plus 30 μM bis-fura-2. Results from a typical neuron are shown in Figures 1A1 – 1A3 where membrane potential and Ca^{2+} currents are shown in panel A1 and $[\text{Ca}^{2+}]_i$ is shown in Panel A2. The rightmost plot in Panel A2 shows the transient elicited by the 151 ms depolarization and an exponential curve fitted to the recovery phase at an expanded time scale. The relationship between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$ is illustrated in Panel A3. Typically, $\Delta[\text{Ca}^{2+}]_i$ increased linearly with Ca^{2+} influx showing little tendency to saturate at the highest Ca^{2+} influxes (straight line in Panel A3) similar to the relationship reported in neonatal DRG neurons at room temperature for Ca^{2+} influxes less than 300 pCoul [6;28;29]. Average values for resting $[\text{Ca}^{2+}]_i$, largest peak $[\text{Ca}^{2+}]_i$, and τ_r for 22 neurons studied in this protocol are listed in Table 1. In agreement with others [30; 31], we found that $[\text{Ca}^{2+}]_i$ transients in adult rat DRG neurons at 20 °C recovered 3 to 5 times faster than $[\text{Ca}^{2+}]_i$ transients of similar magnitude in neonatal rat DRG neurons at room temperature [6;8;9]. We tested the possibility that this difference was caused by the lower exogenous Ca^{2+} -binding ratio in our experiments by recording Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ in response to the DURA175 protocol in 6 neurons dialyzed with 100 μM fura-2. Neither the resting $[\text{Ca}^{2+}]_i$, largest peak $[\text{Ca}^{2+}]_i$, nor τ_r differed from the values we obtained using 30 μM bis-fura-2 (Table 1). Thus, the distinct kinetics of the $[\text{Ca}^{2+}]_i$ transients we observed cannot be attributed to the lower exogenous Ca^{2+} buffering in our experiments. Lastly, the similarity of

the values obtained with the two indicators indicates that the calibration parameters for the two indicators are internally consistent.

Ca²⁺ influx and [Ca²⁺]_i at 30 °C

We recorded Ca²⁺ currents and [Ca²⁺]_i in response to the DURA175 protocol at 30 °C in 21 neurons using the Cs⁺-citrate pipette solution plus 30 μM bis-fura-2. This temperature was chosen because it provided a convenient temperature range that minimized the activation of Ca²⁺ permeable TRPV and TRPN1 channels expressed in DRG neurons. Of the known temperature sensing channels, only TRPM8 is activated over this range [32]. Results from a typical neuron are shown in Figure 1B1 – 1B3 (using the format of Figure 1A1 – 1A3). On average, the peak Ca²⁺ currents were not significantly different from those recorded at 20 °C in either the 100 μM fura-2 or the 30 μM bis-fura-2 experiments. However, the relationship between Ca²⁺ influx and Δ[Ca²⁺]_i (Panel B3) differed qualitatively from that observed at 20 °C by bending down for larger Ca²⁺ influx such that the data were reasonably described by an exponential: $y = B \cdot (1 - \exp(-C \cdot x))$ (smooth line in Panel B3).

Average values for resting [Ca²⁺]_i, largest peak [Ca²⁺]_i, and τ_r for 21 neurons are listed in Table 1. Resting [Ca²⁺]_i measured at 30 °C with 30 μM bis-fura-2 was significantly lower than that observed at 20 °C with bis-fura-2 ($P < 0.001$) or with 100 μM fura-2 ($P < 0.001$). Similarly, the largest peak [Ca²⁺]_i measured at 30 °C using 30 μM bis-fura-2 was significantly lower than that observed at 20 °C using bis-fura-2 ($P < 0.001$) or using 100 μM fura-2 ($P < 0.05$). Thus, the ability of Ca²⁺ influx to elevate [Ca²⁺]_i is significantly attenuated by warming. This is illustrated in Figure 1C1 where we have plotted the relationship between Ca²⁺ influx and Δ[Ca²⁺]_i for each of the neurons examined at 20 and 30 °C using 30 μM bis-fura-2 with the DURA175 protocol. For Ca²⁺ influxes greater than 100 pCoul the Δ[Ca²⁺]_i values for the two temperatures are clearly separated. As with the individual experiments, the trends of the 20 °C and 30 °C ensemble data were reasonably described by a straight line or exponential respectively. Further, the 30 °C data were tighter and Δ[Ca²⁺]_i flattened out for influx values greater than about 150 pCoul. That is, at 30 °C the DURA175 protocol could not raise [Ca²⁺]_i by more than about 150 nM. In order to make a statistical comparison of these results, we binned the Buffer Index values obtained at the two temperatures by the Ca²⁺ influx values (50 pCoul bin width), calculated the mean and standard error (n = number of neurons), and plotted the results as a function of Ca²⁺ influx (open symbols in Figure 1C2). The Buffer Index values at both 20 and 30 °C increased with increasing Ca²⁺ entry. However, the increase was steeper at 30 °C and the Buffer Index values were 2- to 3-fold higher than at 20 °C ($P < 0.001$ for all bins, 2-way ANOVA). Other data plotted in Figure 1C2 are discussed below. In addition to increasing the Buffer Index, warming also reduced τ_r (Table 1) consistent with a Q_{10} for the rate of Ca²⁺ clearance from the cytoplasm of approximately 1.4.

In order to identify the temperature-dependent mechanism or mechanisms that underlie the temperature-dependencies of resting [Ca²⁺]_i, τ_r , and Buffer Index, we used inhibitors of the plasma membrane Ca²⁺ ATPase, Ca²⁺ uptake by the endoplasmic reticulum, and Ca²⁺ uptake by the mitochondria as described below. The rationale behind these experiments was that a reversal of the effects of warming by the inhibition of a given mechanism would identify that mechanism as necessary for the temperature-dependence of the control of [Ca²⁺]_i.

Ca²⁺ extrusion by the Plasma Membrane Ca²⁺-ATPase is not necessary for the temperature-dependencies of resting [Ca²⁺]_i, τ_r , and Buffer Index

We assessed the requirement for Ca²⁺ extrusion by the plasma membrane Ca²⁺-ATPase for the temperature-dependencies of resting [Ca²⁺]_i, τ_r , and the Buffer Index by recording Ca²⁺ currents and [Ca²⁺]_i at 30 °C in 8 adult rat DRG neurons dialyzed with Cs⁺-citrate pipette solution plus 30 μM bis-fura-2 and 200 μM sodium orthovanadate, a nonspecific inhibitor of

ATPases including the plasma membrane Ca^{2+} -ATPase. 200 μM intracellular orthovanadate slows the restoration of resting $[\text{Ca}^{2+}]_i$ in neonatal rat DRG neurons dialyzed with K^+ [11] or with Cs^+ [33] at room temperature. Typical Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ records obtained after extensive dialysis of an adult rat DRG neuron are shown in Panels A and B of Figure 2. On average, the peak Ca^{2+} currents were not significantly different from those observed in the absence of orthovanadate. Importantly, the resting $[\text{Ca}^{2+}]_i$, largest peak $[\text{Ca}^{2+}]_i$, and τ_r were not significantly different from the values obtained in the absence of orthovanadate (Table 1, One-way ANOVA). The relationship between Ca^{2+} influx and $[\text{Ca}^{2+}]_i$ in neurons dialyzed with orthovanadate was similar to described above for neurons at 30 °C as shown in Figure 2C where we have plotted the data points for each of the 8 neurons exposed to orthovanadate over the smooth line obtained from the fit to the 30 °C data in Figure 1C1. Consistent with these observations, the Buffer Index values (solid boxes in Figure 1C2) were not significantly different from those obtained in the absence of orthovanadate at 30 °C ($P > 0.05$ for all bins, 2 way ANOVA).

Our failure to detect significant differences between control and vanadate-dialyzed neurons is not sufficient to conclude that the plasma membrane Ca^{2+} -ATPase does not contribute to the control of $[\text{Ca}^{2+}]_i$ in adult rat DRG neurons at 30 °C for a number of reasons. The contribution may be too small for our analysis to detect or could be masked by Ca^{2+} buffering by other mechanisms, particularly increased Ca^{2+} buffering by the mitochondria in these Na^+ -poor conditions (see below). Nevertheless, our data imply that a temperature-dependent increase in Ca^{2+} extrusion by the plasma membrane Ca^{2+} -ATPase is not necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and Buffer Index summarized in Figure 1 and Table 1.

Ca^{2+} uptake by the endoplasmic reticulum is not necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and Buffer Index

We assessed the requirement for Ca^{2+} uptake by the endoplasmic reticulum for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and the Buffer Index by recording Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ at 30 °C in adult rat DRG neurons dialyzed with Cs^+ -citrate pipette solution plus 30 μM bis-fura-2 by testing the effects of 500 nM thapsigargin (an irreversible inhibitor of the SERCA pump) plus 10 μM ryanodine (an irreversible inhibitor of Ca^{2+} -induced Ca^{2+} release). Results from a typical experiment are shown in Panels A through D in Figure 3. Following the control responses to the DURA175 protocol (Panels A and C) the neuron was exposed to thapsigargin plus ryanodine and the protocol repeated (Panels B and D). In each of the experiments, the Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ transients recorded in the presence of the inhibitors were smaller than the control responses, consistent with the direct inhibition of Ca^{2+} currents described by Shmigol *et al.* [34]. However, as shown by the filled and closed circles in Panel E, treatment with thapsigargin plus ryanodine did not have a consistent affect on the relationship between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$. Similarly, as shown in the rightmost panels of C and D, τ_r was little affected by this treatment.

Similar results were obtained in each of 5 neurons tested in this protocol (Table 2). During the exposure to thapsigargin plus ryanodine, resting $[\text{Ca}^{2+}]_i$ was slightly (but significantly) higher than control, and the largest peak $[\text{Ca}^{2+}]_i$ was significantly smaller (paired t-test). However, we did not detect an effect of thapsigargin plus ryanodine on τ_r (paired t-test) or on the Buffer Index (two-way ANOVA, Figure 3F). In order to examine the effects of longer exposure, we pre-incubated 5 other neurons for >30 minutes in 500 nM thapsigargin plus 10 μM ryanodine in the HEPES buffered NaCl solution. Resting $[\text{Ca}^{2+}]_i$, largest peak $[\text{Ca}^{2+}]_i$, and τ_r obtained from these neurons were not significantly different from those obtained in the presence of thapsigargin plus ryanodine in the paired protocol (Table 2). Similarly, there was no difference in the Buffer Index when compared to the control or test values obtained in the paired experiments (two-way ANOVA and Figure 3F, also compare Figure 1C2).

Interpretation of these data is subject to limitations with regard to the power of the data to detect small effects and compensation by other Ca^{2+} buffering mechanisms noted above. They are further complicated by the small but significant increase in resting $[\text{Ca}^{2+}]_i$ detected in one experiment that suggests a possible contribution by the endoplasmic reticulum to the control of $[\text{Ca}^{2+}]_i$. However, this effect was small and there was no detectable effect of thapsigargin plus ryanodine on τ_r or the Buffer Index implying that a temperature-dependent increase in Ca^{2+} -uptake by the endoplasmic reticulum is not necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and the Buffer Index summarized in Figure 1 and Table 1..

Ca^{2+} uptake mediated by the mitochondrial Ca^{2+} uniporter is necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and the Buffer Index

We assessed the role of mitochondrial Ca^{2+} uptake in the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and the Buffer Index by recording Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ at 30 °C using the DURA175 protocol in adult rat DRG neurons dialyzed with Cs^+ -citrate plus 30 μM bis-fura-2 and either 2 μM (8 neurons) or 4 μM (4 neurons) ruthenium red, an effective [6;35] albeit non-specific [36;37] inhibitor of the mitochondrial Ca^{2+} uniporter. In contrast to our recordings described to this point, inclusion of ruthenium red in the pipette solution caused Ca^{2+} currents to run down markedly and resting $[\text{Ca}^{2+}]_i$ to increase during a DURA175 run. This occurred with both concentrations but was faster with 4 μM where effects of ruthenium red were detectable within minutes of establishing whole cell recording and the Ca^{2+} current was lost and resting $[\text{Ca}^{2+}]_i$ increased above 200 nM after one or two runs of the DURA175 protocol. The slower onset of the effects at 2 μM facilitated the experiments with typical results shown in Figure 4. Membrane currents and $[\text{Ca}^{2+}]_i$ recorded during the first DURA175 run (Panels A and C) resemble typical control recordings with the exception of the marked run down of the Ca^{2+} current. After an additional 14 minutes of dialysis the DURA175 protocol was repeated finding that Ca^{2+} currents and $[\text{Ca}^{2+}]_i$ transients were strongly reduced (Panels B and D). Importantly, τ_r was increased (right most trace in panel D), and resting $[\text{Ca}^{2+}]_i$ was higher at the start of the run and failed to recover between depolarizations. This trend continued in a third DURA175 run with the Ca^{2+} current falling below 600 pA and resting $[\text{Ca}^{2+}]_i$ increasing above 200 nM (not shown). The relationships between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$ in the early and late runs (Panel E) were similar and both were well described by a straight line.

Average measurements obtained by applying the DURA175 protocol shortly after obtaining whole cell access and repeating the protocol >14 minutes later in 8 neurons dialyzed with 2 μM ruthenium red are listed in Table 3. Resting $[\text{Ca}^{2+}]_i$ increased significantly, the peak $[\text{Ca}^{2+}]_i$ transient decreased significantly, and τ_r doubled during dialysis with 2 μM Ruthenium Red. The Buffer Index values determined in the later runs (solid diamonds in Figure 1C2) fell between those obtained at 20 °C and 30 °C in the absence of ruthenium red. Specifically, the Buffer Index values in 2 μM ruthenium red were significantly smaller than those in the absence of ruthenium red at 30 °C for Ca^{2+} entry greater than 75 pCoul ($P < 0.01$, two-way ANOVA) and slightly but significantly larger than those in the absence of ruthenium red at 20 °C for all Ca^{2+} entries ($P < 0.05$ to $P < 0.001$). We note that the effects of ruthenium red increased during dialysis until the recordings were lost and that the results described here are not steady state effects of this relatively low concentration (cf. [6;36]). Dialysis with 4 μM ruthenium red caused the rapid development of similar effects in 4 neurons. Indeed, substantial increases in resting $[\text{Ca}^{2+}]_i$ and reduction of Ca^{2+} current compromised the analysis of these experiments. Nevertheless, the trends were similar to those in 2 μM ruthenium red and the τ_r after <5 minutes of dialysis averaged 4.1 ± 0.42 s ($n = 4$).

Thus, micromolar ruthenium red significantly increased resting $[\text{Ca}^{2+}]_i$ and τ_r whilst reducing the Buffer Index partially reversing the effects of warming on these parameters. In addition, ruthenium red linearized the relationship between Ca^{2+} influx and $\Delta[\text{Ca}^{2+}]_i$ (see also Thayer

and Miller [6]) again reversing an effect of warming. Although ruthenium red is not specific for the mitochondrial Ca^{2+} uniporter (it also inhibits the plasma membrane Ca^{2+} -ATPase [36] and ryanodine receptors [37]), our observations that these mechanisms are not necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , or the Buffer Index, suggest strongly that the effects of ruthenium red are due to the inhibition of the mitochondrial Ca^{2+} uniporter. Accordingly, we conclude that mitochondrial Ca^{2+} uptake mediated by the uniporter is necessary for the temperature-dependencies of resting $[\text{Ca}^{2+}]_i$, τ_r , and Buffer Index. Further, within the limitations of our ability to detect small contributions by the plasma membrane Ca^{2+} -ATPase and the endoplasmic reticulum, our data imply that mitochondrial Ca^{2+} uptake is necessary and sufficient for the maintenance of resting $[\text{Ca}^{2+}]_i$, the restoration of $[\text{Ca}^{2+}]_i$, and normal Ca^{2+} buffering by adult rat DRG neurons at 30 °C under the conditions of these measurements. The caveat is important because, as we describe below, dialysis with low Na^+ pipette solution in these experiments enhanced mitochondrial Ca^{2+} uptake thereby reducing the relative contributions of other mechanisms that may make significant contributions under physiological conditions (see Discussion).

Depolarization of mitochondria by FCCP raises resting $[\text{Ca}^{2+}]_i$ in adult rat DRG neurons

As a second approach to assess the role of mitochondria in controlling $[\text{Ca}^{2+}]_i$, we examined the effects of 500 nM FCCP, a protonophore that depolarizes mitochondria thereby removing the driving force for Ca^{2+} accumulation and retention. Figure 5A shows a recording of membrane currents (upper panels) and $[\text{Ca}^{2+}]_i$ (lower panel) from an adult rat DRG neuron dialyzed with Cs^+ -citrate plus 30 μM bis-fura-2 and superfused with the TEA bath solution at 30 °C. Membrane potential was held at -70 mV and stepped to 0 mV for 50 ms every 20 s. Control responses (numbers 1–4) were stable. Shortly before the fifth depolarization, 500 nM FCCP was applied via puffer pipette (gray bar) whereupon $[\text{Ca}^{2+}]_i$ increased from 17 nM to 275 nM for the duration of the FCCP exposure. The Ca^{2+} currents and the $[\text{Ca}^{2+}]_i$ transients during the period of elevated $[\text{Ca}^{2+}]_i$ (numbers 6 and 7) were substantially reduced, recovering partially during washout (numbers 8 – 10). On average, 500 nM FCCP caused resting $[\text{Ca}^{2+}]_i$ to increase by 797 ± 154 nM ($n = 12$). The variance of these data was large with responses ranging from 149 to nearly 2000 nM (Figure 6).

Although increases in resting $[\text{Ca}^{2+}]_i$ similar to our observations have been reported in response to micromolar protonophore in patch-clamped rat DRG neurons in Na^+ -poor conditions [6] and in mouse DRG neurons [13;38] and chromaffin cells [39;40] in physiological solutions, the consensus observation is that incubation in micromolar protonophore for tens of minutes has little or no effect on resting $[\text{Ca}^{2+}]_i$ in neonatal and adult rat DRG neurons loaded with Ca^{2+} indicator by incubation in the acetoxymethyl ester (AM-loaded) [6;7;14;41;42]. This led us to consider whether the different responses to FCCP reflected a methodological difference between our work and previous efforts and to further characterize the response to protonophore. We first tested whether the increase in resting $[\text{Ca}^{2+}]_i$ in response to FCCP required the Ca^{2+} influx during the 50 ms depolarizations preceding the exposure to 500 nM FCCP. Figure 5B shows a typical result from an experiment in which membrane potential was stepped once from -70 to 0 mV for 10 ms shortly after establishing the patch clamp to establish the condition of the neuron (not shown). Membrane potential was then held at -70 mV for over 5 minutes. Subsequent application of 500 nM FCCP (gray bar) caused $[\text{Ca}^{2+}]_i$ to increase from 50 to 1000 nM for the duration of the exposure. On average, 500 nM FCCP caused resting $[\text{Ca}^{2+}]_i$ to increase by 889 ± 210 nM ($n = 8$) in this protocol, similar to the increases observed when the neurons were depolarized at 20 s intervals (as in Figure 5A). Thus, voltage-gated Ca^{2+} entry is not required for an increase in resting $[\text{Ca}^{2+}]_i$ in response to 500 nM FCCP. We next tested whether the increase in resting $[\text{Ca}^{2+}]_i$ required warming the neurons to 30 °C by applying the protocol used in Figure 5A to 4 neurons at 20 °C (data not shown). On average, 500 nM FCCP

caused resting $[Ca^{2+}]_i$ to increase by 2490 ± 745 nM. Thus, warming is not required for an increase in resting $[Ca^{2+}]_i$ in response to 500 nM FCCP.

We next considered the possibility that the increase in resting $[Ca^{2+}]_i$ in response to 500 nM FCCP required the replacement of intracellular K^+ by Cs^+ and the removal of Na^+ from the intracellular and extracellular solutions. We first recorded the responses of 18 adult rat DRG neurons loaded with fura-2 via incubation in the acetoxymethyl ester (AM-loaded) and superfused with the NaCl bath at 30 °C. Eleven AM-loaded neurons responded with substantial increases in $[Ca^{2+}]_i$ with a typical result shown in Figure 5C where exposure to 500 nM FCCP (gray bar) caused resting $[Ca^{2+}]_i$ to increase to 407 nM for the duration of the exposure. However, seven neurons responded with only modest or insignificant increases in $[Ca^{2+}]_i$ as shown in Figure 5D. On average, 500 nM FCCP caused resting $[Ca^{2+}]_i$ in fura-AM-loaded neurons to increase by 312 ± 84 nM ($n = 18$) with the amplitudes of the responses ranging from 0 to slightly over 1000 nM (Figure 6). We next examined the responses of adult rat DRG neurons dialyzed with the K^+ -citrate + 10 mM Na^+ pipette solution plus 30 μ M bis-fura-2 and superfused with NaCl + TEA bath at 30 °C with a typical result shown in Figure 5E. Exposure of this neuron to 500 nM FCCP (gray bar) caused resting $[Ca^{2+}]_i$ to increase from 65 to 545 nM. On average, 500 nM FCCP caused resting $[Ca^{2+}]_i$ in neurons dialyzed with K^+ -citrate + 10 Na^+ to increase by 468 ± 59 nM ($n = 31$). Here again there was a broad distribution in the amplitudes of the responses with 6 of the 31 neurons showing only modest increases in $[Ca^{2+}]_i$ (Figure 6).

The amplitudes of the responses to FCCP are summarized in Figure 6. Quantitative analysis of these data is complicated by the use of two Ca^{2+} indicators (see Methods) and by the large variance and non-Gaussian distributions in the amplitudes of the responses. Comparison of the three columns in Figure 6A by ANOVA (Kruskal-Wallis test and Dunn's post-test) revealed that the effect of 500 nM FCCP on $[Ca^{2+}]_i$ in the fura-AM-loaded neurons was significantly smaller than the effect observed in neurons dialyzed with the Cs^+ -citrate pipette solution ($P < 0.01$). No other significant differences between treatments were detected. However, analysis of the cumulative distributions in Figure 6B by the log-rank test (cf. McGehee and Oxford [43]) determined that the responses of neurons dialyzed with Cs^+ -citrate pipette solution (Na^+ -poor) tended to be larger than those of neurons dialyzed with K^+ -citrate (10 mM Na^+) pipette solution neurons ($P < 0.04$).

The distributions and analyses of the data in Figure 6 reveal two important observations. First, an increase in resting $[Ca^{2+}]_i$ in response to 500 nM FCCP did not require the replacement of intracellular K^+ by Cs^+ and the removal of Na^+ from the intracellular and extracellular solutions. However, they also reveal a modest but significant trend in the data such that K^+ - and Na^+ -containing neurons bathed in Na^+ -containing solution (Fura-AM and Na^+ +TEA / K^+) were more likely to show little or no increase in $[Ca^{2+}]_i$ (in closest agreement with the consensus described above [6;7;14;41;42]) than were Cs^+ -containing neurons bathed in Na^+ -free solution. Thus, the choice of protocol has a quantitative effect on the distribution of the amplitude of response (see Discussion).

The abilities of FCCP and ruthenium red to raise resting $[Ca^{2+}]_i$ imply that in resting adult rat DRG neurons at 30 °C mitochondrial Ca^{2+} fluxes contribute to the maintenance of resting $[Ca^{2+}]_i$. We investigated this further by examining the effects of activation and inhibition of the mitochondrial Na^+/Ca^{2+} -exchanger on resting $[Ca^{2+}]_i$ and the recovery of resting $[Ca^{2+}]_i$ following Ca^{2+} influx.

Mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange influences $[\text{Ca}^{2+}]_i$ between 50 and 150 nM in adult rat DRG neurons

The experiments using the Cs^+ -citrate pipette solution were done in Na^+ -poor conditions to enable measurement of Ca^{2+} currents. However, the absence of Na^+ inactivates $\text{Na}^+/\text{Ca}^{2+}$ -exchangers located in the plasma membrane [30] and in the mitochondrial inner membrane [44;45] and might have contributed to the dominance of the mitochondrial in the control of $[\text{Ca}^{2+}]_i$ that we observed. We investigated this by dialyzing adult rat DRG neurons with Cs^+ -citrate + 10 Na^+ or K^+ -citrate + 10 Na^+ pipette solutions. In preliminary experiments recording $[\text{Ca}^{2+}]_i$ using these solutions and the TEA-bath resting $[\text{Ca}^{2+}]_i$ increased steadily during recording, presumably due to Ca^{2+} entry mediated by the plasma membrane $\text{Na}^+/\text{Ca}^{2+}$ -exchanger. In contrast, $[\text{Ca}^{2+}]_i$ was stable in neurons superfused with the NaCl bath or NaCl + TEA bath solutions and we used these bath studies in the following experiments. However, this resulted in large inward Na^+ currents that precluded measurement of Ca^{2+} currents.

10 mM intracellular Na^+ had little if any effect on resting $[\text{Ca}^{2+}]_i$ (average value of 57 ± 7 nM, $n = 14$). However, it dramatically slowed and altered the time course of the restoration of resting $[\text{Ca}^{2+}]_i$ following Ca^{2+} influx as shown in Figure 7 (see Panels B and E for expanded time scales). Specifically, recovery of resting $[\text{Ca}^{2+}]_i$ occurred in two phases: a rapid phase lasting 1 to 2 s that brought $[\text{Ca}^{2+}]_i$ down to near 100 nM followed by a plateau phase lasting tens of seconds. A similar time course was observed by Verdru *et al.* [30], also in adult rat DRG neurons in the presence of intracellular Na^+ . On average, the transition from the rapid phase to the plateau occurred at a $[\text{Ca}^{2+}]_i$ of 118 ± 18 nM ($n = 14$) for neurons dialyzed with Cs^+ -citrate + 10 Na^+ and 116 ± 6 ($n = 4$) for neurons dialyzed with K^+ -citrate + 10 Na^+ .

Qualitatively similar biphasic recoveries of $[\text{Ca}^{2+}]_i$ have been observed in neonatal rat DRG neurons and frog sympathetic neurons. In these neurons, the transition between phases has been suggested to reflect a Ca^{2+} set-point at which the mitochondria change from net importers to net exporters of Ca^{2+} with the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange mediating at least a portion of the export (see Discussion). We examined whether this model was appropriate in adult rat DRG neurons by applying 2.5 μM CGP 37157, a specific inhibitor of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange. CGP 37157 caused a modest but significant decrease in resting $[\text{Ca}^{2+}]_i$ in neurons dialyzed with Cs^+ -citrate + 10 Na^+ (57 ± 7 nM to 35 ± 3 nM, $n = 14$, $P < 0.0001$, Figure 7G) and in neurons dialyzed with K^+ -citrate + 10 Na^+ (50 ± 3 to 40 ± 1 nM, $n = 4$, $P < 0.04$) implying that the release of Ca^{2+} via the mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange raises steady state $[\text{Ca}^{2+}]_i$ in neurons dialyzed with 10 mM Na^+ . The effect of CGP 37157 on the time course of the restoration of resting $[\text{Ca}^{2+}]_i$ was more striking as CGP 37157 abolished the plateau phase of the recovery and reduced the time taken to restore resting $[\text{Ca}^{2+}]_i$ in 13 of 14 neurons dialyzed with Cs^+ -citrate + 10 Na^+ and each of 4 neurons dialyzed with K^+ -citrate + 10 Na^+ (typical results are shown in Figure 7, panels A through F). We quantified this effect by measuring the time taken for recovery of 80% of the peak $[\text{Ca}^{2+}]_i$ ($T_{80\%}$). CGP 37157 reduced $T_{80\%}$ from 15 ± 1.9 s to 1.1 ± 0.7 s ($P < 0.0001$, Figure 7H) in Cs^+ -citrate + 10 Na^+ dialyzed neurons and from 10 ± 3 s to 1.1 ± 0.14 s ($P < 0.01$) in K^+ -citrate + 10 Na^+ dialyzed neurons. Furthermore, the time course of the recovery of $[\text{Ca}^{2+}]_i$ was well described by a single exponential the presence of CGP 37157 (Panels C and F) with an average value of 0.68 ± 0.04 s ($n = 14$) in Cs^+ -citrate + 10 Na^+ dialyzed neurons and 0.76 ± 0.19 s ($n = 4$) in K^+ -citrate + 10 Na^+ dialyzed neurons, i.e. similar to τ_r determined in Na^+ -poor conditions (see Table 1) suggesting that the principal difference in the control of $[\text{Ca}^{2+}]_i$ in Na^+ -poor and Na^+ -rich conditions is the contribution of mitochondrial $\text{Na}^+/\text{Ca}^{2+}$ -exchange.

These results are in qualitative agreement with data obtained from neonatal rat DRG neurons [44] and frog sympathetic neurons [45]. Applying the interpretation developed by these authors, we suggest that the transition between net mitochondrial Ca^{2+} uptake and net mitochondrial Ca^{2+} release in adult rat DRG neurons at 30 °C occurs at $[\text{Ca}^{2+}]_i$ near 100 nM,

i.e. substantially lower than the transition near 300 nM $[Ca^{2+}]_i$ in neonatal rat DRG neurons and frog sympathetic neurons at room temperature.

Discussion

Warming increases Ca^{2+} buffering in adult rat DRG neurons

At 20 °C the relationship between Ca^{2+} influx and $[Ca^{2+}]_i$ was linear with Ca^{2+} influxes of 400 – 500 pCoul raising $[Ca^{2+}]_i$ by 500 to 600 nM. In contrast, at 30 °C the increase in $[Ca^{2+}]_i$ saturated such that Ca^{2+} influxes over this range could not raise $[Ca^{2+}]_i$ by more than 100 – 150 nM. We quantified this effect determining that increasing the temperature from 20 to 30 °C increased the Buffer Index by 2- to 3-fold over the observed range of Ca^{2+} influx. Along with the temperature-dependent increase in the Buffer Index, we also found that warming significantly reduced resting $[Ca^{2+}]_i$ and τ_r . The latter effect was consistent with a Q_{10} for the rate of clearance of Ca^{2+} from the cytoplasm of 1.4. Thus, the ability of the cell bodies of adult rat DRG neurons to resist a change in $[Ca^{2+}]_i$ is significantly increased by warming, an effect that must be considered when evaluating the ability of Ca^{2+} influx to activate membrane conductances, transmitter release, and gene expression.

To develop information on the mechanism underlying these temperature-dependencies, we tested the abilities of inhibitors of the plasma membrane Ca^{2+} -ATPase, Ca^{2+} uptake and release by the endoplasmic reticulum, and Ca^{2+} uptake by the mitochondria to reverse the effects of warming in Na^+ -poor conditions that inactivated Na^+/Ca^{2+} -exchange. Only inhibition of mitochondrial Ca^{2+} uptake by 2 μ M ruthenium red significantly reversed the reduced $[Ca^{2+}]_i$, τ_r , and increased Buffer Index observed at 30 °C providing substantial evidence that mitochondrial Ca^{2+} uptake is necessary for the observed temperature-dependencies of $[Ca^{2+}]_i$, τ_r , and the Buffer Index in Na^+ -poor conditions. This observation is consistent with previous work by ourselves [15] and others [16–18] pointing to a temperature-sensitivity in mitochondrial control of $[Ca^{2+}]_i$.

The mitochondrial Ca^{2+} set-point is near resting $[Ca^{2+}]_i$ in adult rat DRG neurons at 30 °C

The lack of contribution by the plasma membrane Ca^{2+} -ATPase and the endoplasmic reticulum in the results summarized above is at odds with previously reported results on the physiological control of $[Ca^{2+}]_i$ (see Introduction) suggesting that the conditions of our experiments artificially minimized the contributions of these mechanisms relative to the contribution the mitochondria. A likely candidate here is the Na^+ -poor condition we used to isolate Ca^{2+} currents. That is, the absence of cytoplasmic Na^+ will inhibit Na^+/Ca^{2+} -exchange-mediated Ca^{2+} efflux from the mitochondria [44;46] thereby increasing uptake of Ca^{2+} by the mitochondria at all $[Ca^{2+}]_i$ and the absolute and relative contribution of the mitochondria to the control of $[Ca^{2+}]_i$. This idea is supported by the effects of intracellular Na^+ on the response to 500 nM FCCP (see below) and on the time course of recovery of resting $[Ca^{2+}]_i$. Accordingly, we suggest that Na^+ -poor conditions contributed to the dominant role of the mitochondria in maintaining and restoring resting $[Ca^{2+}]_i$ that we observed in patch-clamp experiments using TEA bath and Cs^+ -citrate pipette solution. We discuss this issue further below.

Whatever the mechanism, our data document the potential of the mitochondria of adult rat DRG neurons to absorb a substantial Ca^{2+} load and to rapidly lower $[Ca^{2+}]_i$ below 100 nM. Three lines of evidence imply that this potential is realized under physiological conditions. First, depolarization of the mitochondria with FCCP increased resting $[Ca^{2+}]_i$ in the majority of neurons tested including AM-loaded neurons in the presence of Na^+ . Second, inhibition of the mitochondrial Na^+/Ca^{2+} -exchanger in neurons dialyzed with physiological intracellular Na^+ caused a modest but significant decrease in resting $[Ca^{2+}]_i$. Both observations imply a steady state mitochondrial Ca^{2+} uptake that contributes to the maintenance of resting $[Ca^{2+}]_i$.

Third, the restoration of resting $[Ca^{2+}]_i$ following a Ca^{2+} influx in the presence of intracellular Na^+ was greatly prolonged and followed a complex time course with a rapid phase followed by a plateau starting near 100 nM. A qualitatively similar time course with a transition between rapid and plateau phases near 300 nM in rat DRG neurons and frog sympathetic neurons [6; 44;46] was interpreted as indicating that mitochondria are net importers of Ca^{2+} at $[Ca^{2+}]_i$ above 300 nM and net exporters of Ca^{2+} at $[Ca^{2+}]_i$ below 300 nM. That is, there is a Ca^{2+} set-point of 300 nM (reviewed by Nicholls and Budd [47]). Applying this interpretation to our data, we suggest that the Ca^{2+} set-point in adult rat DRG neurons at 30 °C is near 100 nM, i.e. substantially lower than in rat or frog neurons at room temperature and within about 50 nM of resting $[Ca^{2+}]_i$. Although we have not investigated systematically the mechanism underlying the lower Ca^{2+} set-point in our experiments, in 6 adult rat DRG neurons loaded by incubation in fura-2-AM and depolarized by exposure to 50 mM K^+ at room temperature the average plateau $[Ca^{2+}]_i$ was 279 ± 32 nM, i.e. similar to the plateau found by others in this protocol [6;41;42;44;46] and higher than the plateau we observed using 50 ms voltage-clamp depolarizations at 30 °C. Thus, the Ca^{2+} set-point may be sensitive to temperature, the size of the Ca^{2+} load, or both. A lowering of the Ca^{2+} set-point with warming is consistent with a mitochondrial contribution to the increased Buffer Index described above.

A Ca^{2+} set-point between 50 and 150 nM $[Ca^{2+}]_i$ in adult rat DRG neurons at 30 °C implies that the powerful mitochondrial Ca^{2+} buffering observed in Na^+ -poor conditions will accumulate or release Ca^{2+} to bring $[Ca^{2+}]_i$ to this set-point under physiological conditions. It also implies that mitochondrial Ca^{2+} buffering is optimized when $[Ca^{2+}]_i$ is near its resting value just as a pH buffer is optimized when the pH is near its pK_a . Lastly, we note that our findings are consistent with recent observations in other systems that mitochondria can take up Ca^{2+} when $[Ca^{2+}]_i$ is between 50 and 150 nM (reviewed by Szanda *et al.* [48]).

The interpretation of the effect of protonophores on $[Ca^{2+}]_i$

As described above, numerous studies have reported that micromolar concentrations of protonophore have little if any effect on resting $[Ca^{2+}]_i$ in rat DRG neurons [6;7;14;41;42]. These data have led to the consensus that mitochondria do not contribute to the control of resting $[Ca^{2+}]_i$ and are depleted of Ca^{2+} at rest (reviewed in [47;47]). In contrast, we and others [6;13;38–40] have observed increases in resting $[Ca^{2+}]_i$ in response to protonophores. A critical examination of the protocol provides an explanation for these variant results and suggests that the consensus view is not well supported by the data. That is, the ability of a protonophore to raise $[Ca^{2+}]_i$ depends on the existence and size of a pool of releasable Ca^{2+} within the mitochondria, the rate of Ca^{2+} release from the mitochondria in response to the protonophore, and the rate of Ca^{2+} clearance by Ca^{2+} -binding, Ca^{2+} sequestration, and Ca^{2+} extrusion. Thus, an observation that protonophore does not raise $[Ca^{2+}]_i$ implies only that non-mitochondrial mechanisms were able to compensate for any release of Ca^{2+} from the mitochondria. It is not evidence that the mitochondria are depleted of Ca^{2+} or that the mitochondria do not contribute to the control of $[Ca^{2+}]_i$. In contrast, our observations that a low concentration of FCCP causes large, sustained increases in $[Ca^{2+}]_i$ in the majority of neurons tested under a variety of conditions are strong evidence that the mitochondria are contributing to the control of resting $[Ca^{2+}]_i$, particularly when supported by similar results from other laboratories [6;13;38–40] and our results obtained with ruthenium red and CGP 37157.

This understanding implies that small variations in the size of the releasable pool and the relative rates of Ca^{2+} uptake, release, and clearance can result in large variations in the amplitude of the response to FCCP. Thus, we have a mechanism that can account for the large variance and non-Gaussian distributions in the amplitudes of the responses shown in Figure 6 and the variability of responses to protonophore in the literature. Similarly, our observations that K^+ -containing neurons bathed in Na^+ -containing solution (Fura-AM and Na^+ +TEA / K^+

in Figure 6) were more likely to show little or no increase in $[Ca^{2+}]_i$ compared with Cs^+ -containing neurons bathed in Na^+ -free solution are consistent with increased Ca^{2+} sequestration and extrusion under the more physiological conditions. On this basis, we suggest that the probability of observing little or no effect of protonophore on resting $[Ca^{2+}]_i$ is greater if the protonophore is applied gradually (i.e. via bath exchange as in Figure 5 D vs. puffer as in Figure 5C) to intact neurons with physiological extracellular and intracellular Na^+ at room temperature (where mitochondrial Ca^{2+} uptake is reduced).

Intracellular Na^+ influences $[Ca^{2+}]_i$ via two Na^+/Ca^{2+} -exchangers in adult rat DRG neurons

The roles of intracellular Na^+ and Na^+/Ca^{2+} -exchange in controlling $[Ca^{2+}]_i$ in rat DRG neurons have received relatively little attention since initial observations found little or no effect of extracellular Na^+ on resting $[Ca^{2+}]_i$ or $[Ca^{2+}]_i$ transients [6;8;13;30;33]. However, more recent work demonstrated the existence and function of Na^+/Ca^{2+} -exchangers in both the plasma membrane [30] and the inner mitochondrial membrane [44]. Our findings that intracellular Na^+ prolonged the $[Ca^{2+}]_i$ transient ten-fold or more and that inhibition of the mitochondrial Na^+/Ca^{2+} -exchanger by CGP 37157 caused a modest (but significant) reduction in resting $[Ca^{2+}]_i$ whilst fully reversing the prolongation of the $[Ca^{2+}]_i$ transient provide further support for the importance of physiological intracellular Na^+ in controlling $[Ca^{2+}]_i$. Specifically, at $[Ca^{2+}]_i$ between 50 and 100 nM, millimolar cytoplasmic Na^+ activates Ca^{2+} flux into the cytoplasm across both the plasma membrane and the inner mitochondrial membrane via Na^+/Ca^{2+} -exchange. Further investigation, including investigation of the effects of Na^+ influx on intracellular Na^+ , is required to establish the physiological consequences of the activities of these Na^+/Ca^{2+} -exchangers. One interesting possibility is that Na^+ entry via voltage-gated Na^+ channels, particularly via TTX-resistant channels that mediate the majority of Na^+ influx in primary afferent nociceptors [49], might increase intracellular Na^+ thereby raising the mitochondrial Ca^{2+} set-point and $[Ca^{2+}]_i$.

Our observation that $[Ca^{2+}]_i$ increased steadily in neurons dialyzed with 10 mM Na^+ and superfused with Na^+ -free solution is apparently at odds with reports by others in mammalian sensory neurons (cf. [30;33]). We cannot explain this difference but note that Benham *et al.* [33] used neurons from neonatal rats at room temperature, made their measurements in under 15 minutes of dialysis, and reported resting $[Ca^{2+}]_i$ of about 136 nM (i.e. near $[Ca^{2+}]_i$ where we observed the transition from fast to slow recovery). Similarly, it is not clear that the increases in resting $[Ca^{2+}]_i$ reported by Verdru *et al.* [30] in a subset of adult rat DRG neurons dialyzed with 10 mM Na^+ and superfused with Na^+ -free solution were steady-state observations. Thus, a combination of differences in development and protocols might account for the different observations. In order to record a stable resting $[Ca^{2+}]_i$ with 10 mM intracellular Na^+ we used a physiological Na^+ gradient. However, in this situation two experimental difficulties arose that precluded investigation of the relationship between Ca^{2+} influx and $[Ca^{2+}]_i$. First, substantial TTX-resistant Na^+ currents precluded reliable measurement of Ca^{2+} influx. Second, the activation of mitochondrial Na^+/Ca^{2+} -exchange resulted in approximately a 10-fold increase in the time needed to restore resting $[Ca^{2+}]_i$. As a practical matter, we found it impossible to record a reliable DURA175 protocol in the presence of a physiological Na^+ gradient.

Summary

The major findings of our work are that warming reduces resting $[Ca^{2+}]_i$ and τ_r while increasing the Buffer Index in adult rat DRG neurons and that mitochondrial Ca^{2+} uptake is necessary for these effects. In addition, we find that mitochondrial Ca^{2+} fluxes contribute to the control of Ca^{2+} at $[Ca^{2+}]_i$ between 50 and 150 nM in adult rat DRG neurons at 30 °C and that the mitochondrial Ca^{2+} uniporter and Na^+/Ca^{2+} -exchanger contribute to this function. Lastly, our observations that inhibition of mitochondrial Ca^{2+} uptake or release caused sustained increases

or decreases in $[Ca^{2+}]_i$ respectively contradict the view that mitochondrial Ca^{2+} uptake does not contribute to the maintenance of resting $[Ca^{2+}]_i$. Rather, they imply a steady state uptake of Ca^{2+} by this organelle and raise the question of the final disposition of that Ca^{2+} . That is, in order for mitochondrial Ca^{2+} uptake to influence resting $[Ca^{2+}]_i$ the mitochondria must be able to move Ca^{2+} out of the cell by a path that bypasses the bulk cytoplasm. Such a mechanism is consistent with the growing literature documenting exchanges of Ca^{2+} between subcellular compartments (cf. [50;51]) and merits investigation.

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Figure 1.

Membrane potential and currents (Panels A1 and B1) and $[Ca^{2+}]_i$ (Panels A2 and B2) recorded from two DRG neurons superfused with TEA bath and dialyzed with Cs^+ -citrate pipette solution plus 30 μM bis-fura-2 at either 20 °C (Panels A1 – A3) or 30 °C (Panels B1 – B3) in response to the DURA175 protocol. 1 nA and 50 ms calibration bars are for Panels A1 and B1. Panels A2 and B2 show the sequence of $[Ca^{2+}]_i$ transients elicited by this protocol with the 7th transient plotted at an expanded time scale in the rightmost plot of each panel. The smooth lines are exponential curve fits to recovery of resting $[Ca^{2+}]_i$ with time constants of 0.81 and 0.70 s respectively. Panels A3 and B3 show the relationship between Ca^{2+} influx and $\Delta[Ca^{2+}]_i$ for these two neurons. The straight line in Panel A3 is $y = A \cdot x$ fit to the data ($A = 1.33$ nM / pCoul). The smooth line in Panel B3 is a single exponential of the form $y = B \cdot (1 - \exp(-C \cdot x))$ fit to the data ($B = 196$ nM and $C = 0.0067$ pCoul⁻¹). The fits in Panels A3 and B3 are drawn to highlight the trends of the data and have no theoretical significance. Panel C1 shows the pCoul and $\Delta[Ca^{2+}]_i$ measured for each step from the DURA175 protocols from 22 neurons at 20 °C (open circles) and from 21 neurons at 30 °C (open boxes) listed in Table 1. The straight line and the curved line are fits to the data as in Panels A3 and B3 ($A = 1.63$ nM/pCoul, $B = 160.9$ nM, and $C = 0.00795$ pCoul⁻¹) to highlight the trends of the data at the two temperatures. They have no theoretical significance. Panel C2 shows the Buffer Index values (mean $\pm$ standard error of the mean) for four conditions. The open symbols were obtained by calculating the Buffer Index for each point in Panel C1 (open squares are 30 °C data, open circles are 20 °C data), binning them by the pCoul of Ca^{2+} influx (bin width = 50 pCoul), and calculating the average and standard error of the mean. Some error bars are smaller than the symbols. The solid symbols were obtained similarly from neurons exposed to 200 μM sodium orthovanadate (8 neurons, solid boxes) or to 2 μM ruthenium red (8 neurons, solid diamonds) at 30 °C as described in the text. (see Tables 1 and 3).

Figure 2.

Membrane potential and currents (Panel A) and $[Ca^{2+}]_i$ (Panel B) recorded from a single DRG neuron superfused with TEA bath and dialyzed with Cs^+ -citrate pipette solution plus 30 μM bis-fura-2 plus 200 μM sodium orthovanadate at 30 °C. Format is similar to Figure 1 except that Panel C shows the pCoul and $\Delta[Ca^{2+}]_i$ for every DURA175 depolarization of the 8 neurons we studied using this protocol. The smooth line in Panel C is the fit to the 30 °C data in Figure 1C1. The Buffer Index values obtained from these data are plotted as the solid squares in Figure 1C2.

Figure 3.

Membrane currents (Panels A and B) and $[Ca^{2+}]_i$ (Panels C and D) recorded from a single adult rat DRG neuron superfused with TEA bath and dialyzed with Cs^+ -citrate pipette solution plus 30 μ M bis-fura-2 at 30 $^{\circ}$ C. Panels A and C are control. Panels B and D are in the presence of 500 nM thapsigargin plus 10 μ M ryanodine. The circles in Panel E show the pCoul and $\Delta[Ca^{2+}]_i$ for every DURA175 depolarization of the 5 neurons we studied using this protocol (solid circles are control and open circles test). The open squares in Panel E are data from 5 additional neurons incubated in thapsigargin plus ryanodine as described in the text. The smooth line in Panel E is the fit to the 30 $^{\circ}$ C data in Figure 1C1. Panel F shows the Buffer Index values obtained from the data in Panel E binned and analyzed as in Figure 1C2. The

Buffer Index in the presence of thapsigargin plus ryanodine was not significantly different from control (two-way ANOVA).

Figure 4.

Membrane currents (Panels A and B) and $[Ca^{2+}]_i$ (Panels C and D) recorded from a single adult rat DRG neuron superfused with TEA bath and dialyzed with Cs^+ -citrate pipette solution plus 30 μM bis-fura-2 plus 2 μM ruthenium red at 30 °C. Panels A and C are the first DURA175 run immediately after gaining access while Panels B and D were recorded 14 minutes later. Panel E shows the relationship between Ca^{2+} influx and $\Delta[Ca^{2+}]_i$ for these two runs. The solid straight line (slope = 1.15 nM/pCoul) is the least squares fit to the data from the late run. It is extended in the dashed line to highlight the lack of change in this relationship with dialysis. Panel F shows the pCoul and $\Delta[Ca^{2+}]_i$ for each DURA175 depolarization after 14 minutes of dialysis from the 8 neurons studied using this protocol. The smooth line is the fit to the 30 °C

data shown in Figure 1C1. The Buffer Index values obtained from these data are plotted as the solid triangles in Figure 1C2.

Figure 5.

Panel A shows $[Ca^{2+}]_i$ (lower record) and membrane currents (upper records) from a neuron superfused with the TEA bath solution and dialyzed with Cs^+ -citrate pipette solution, held at -70 mV, depolarized to 0 mV for 50 ms at 20 s intervals, and exposed to 500 nM FCCP via puffer (gray bar). The numbers identify matching currents and $[Ca^{2+}]_i$ transients. This record showing a relatively small increase in $[Ca^{2+}]_i$ in the presence of FCCP was chosen for clarity (see text). Panel B shows $[Ca^{2+}]_i$ from a neuron superfused with the TEA bath solution dialyzed with the Cs^+ -citrate pipette solution, held at -70 mV, and exposed to 500 nM FCCP via puffer (grey bar). Panels C and D show $[Ca^{2+}]_i$ from DRG neurons loaded via AM ester and superfused with NaCl bath (no patch-clamp) and exposed to 500 nM FCCP via puffer (Panel C) or by bath exchange (Panel D). In panel D, after 5 minutes incubation in FCCP the neuron was exposed to 500 nM FCCP plus 50 mM K^+ (replacing Na^+) for 20 s (arrow) causing $[Ca^{2+}]_i$ to rise rapidly and transiently to about 1 μ M. Panel E shows $[Ca^{2+}]_i$ from a DRG neuron superfused with the NaCl + TEA bath solution and dialyzed with K^+ -citrate + 10 mM Na^+ pipette solution, held at -70 mV and exposed to 500 nM FCCP via puffer (grey bar). All experiments were done at $30^\circ C$.

Figure 6.

Panel A summarizes the effects of 500 nM FCCP on resting $[Ca^{2+}]_i$. The superfusate for the fura-AM-loaded neurons was the NaCl bath. The superfusate for the K^+ -citrate + 10 mM Na^+ dialyzed neurons was the NaCl + TEA bath (TEA // K). The superfusate for the Cs^+ -citrate dialyzed neurons was TEA bath (TEA // Cs). The TEA // Cs data combine data obtained using the pulsing protocol (e.g. Figure 5A) and the holding protocol (e.g. Figure 5B). Some neurons were first subjected to the holding protocol and later to the pulsing protocol. We took the response during the holding protocol for this plot. An ANOVA of these data determined that the average response in Fura-AM was significantly smaller than the response in TEA // Cs. No other significant differences were detected as indicated on the graph. Panel B shows the data in Panel A plotted as cumulative distributions with standard errors.

Figure 7.

Data from two adult rat DRG neurons illustrating the responses to 2.5 μM CGP 37157 marked by the horizontal bars. Panels A through C are from a neuron superfused with NaCl + TEA bath and dialyzed with Cs^+ -citrate plus 10 mM Na^+ showing a typical plateau phase in the recovery of resting $[\text{Ca}^{2+}]_i$. Panel A shows the complete experiment while Panels B and C show control and CGP 37157 records at an expanded time scale. Panels D through F are from a neuron superfused with NaCl + TEA bath and dialyzed with K^+ -citrate plus 10 mM Na^+ . The arrows in Panels B and E indicate $[\text{Ca}^{2+}]_i$ at the beginning of the plateau. The smooth lines in Panels C and F are a single exponential fit to the recovery of resting $[\text{Ca}^{2+}]_i$. Panels G and H show summary data from 14 neurons dialyzed with Cs^+ -citrate plus 10 mM Na^+ and exposed

to 2.5 μM CGP 37157. Panel G shows the effect of CGP 37157 on resting $[\text{Ca}^{2+}]_i$. Panel H shows the effect of CGP 37157 on $T_{80\%}$.

Table 1

Data from DURA175 protocols. Columns A vs. B, A vs. C, B vs. C, and C vs. D were compared using by one way ANOVA with Bonferoni's post-test for Resting $[Ca^{2+}]_i$ and Kruskal-Wallis nonparametric analysis with Dunn's post-test for Largest Peak $[Ca^{2+}]_i$ and τ_r . # denotes significantly different from column C. No other significant differences were detected.

	100 μM fura-2		30 μM bis-fura-2	
	A, 20 °C	B, 20 °C	C, 30 °C	D, 30 °C Orthovanadate
n	6	22	21	8
Resting $[Ca^{2+}]_i$ (nM)	87 $\pm$ 8#	87 $\pm$ 5#	49 $\pm$ 4	37 $\pm$ 3
Largest Peak $[Ca^{2+}]_i$ (nM)	373 $\pm$ 38#	447 $\pm$ 33#	193 $\pm$ 8	159 $\pm$ 13
τ_r (s)	1.5 $\pm$ 0.16#	1.3 $\pm$ 0.08#	0.99 $\pm$ 0.12	0.67 $\pm$ 0.04

Table 2

Data from DURA175 protocols examining 500 nM thapsigargin plus 10 μ M ryanodine. Columns 1 and 2 summarize data from 5 neurons recorded in control and in the presence of thapsigargin plus ryanodine. These values were compared by paired t-test with asterisks in column 2 indicating significant difference from control ($P < 0.01$). Column 3 summarizes data from 5 additional neurons after 10 minutes incubation in thapsigargin plus ryanodine. These values were not significantly different from those in column 2 (unpaired t-test).

	Control	500 nM Thapsigargin 10 μ M Rvan	500 nM Thapsigargin 10 μ M Rvan
n		5 (paired)	5
Resting $[Ca^{2+}]_i$ (nM)	28 ± 5	$35 \pm 5^*$	36 ± 4
Max Peak $[Ca^{2+}]_i$ (nM)	224 ± 22	$148 \pm 13^*$	170 ± 14
τ_r (s)	0.67 ± 0.05	0.70 ± 0.05	0.76 ± 0.05

Table 3

Data from DURA175 protocols examining 20 μ M Ruthenium Red. Asterisks denote significantly different from Early ($P < 0.004$) by paired t-test

	Early	14 min dialysis
Resting $[Ca^{2+}]_i$ (nM)	55 ± 6	$90 \pm 12^*$
Max Peak $[Ca^{2+}]_i$ (nM)	419 ± 28	$93 \pm 13^*$
τ_r (s)	$0.78 \pm .04$	$1.70 \pm 0.18^*$