Advancing age alters rapid and spontaneous refilling of caffeine sensitive calcium stores in sympathetic superior cervical ganglion cells

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Abstract

Intracellular calcium ([Ca\textsuperscript{2+}]) release from SER stores plays an important role in cell signaling. These stores are rapidly refilled via influx through voltage gated calcium channels or spontaneously via store-operated calcium channels (SOCC), and subsequent pumping by smooth endoplasmic reticulum Ca\textsuperscript{2+} -ATPases (SERCA). We measured [Ca\textsuperscript{2+}]i transients in isolated Fura-2 loaded superior cervical ganglion (SCG) cells from 6, 12, 20 and 24 month-old F-344 rats. For rapid refilling, [Ca\textsuperscript{2+}]i transients were elicited by a 5 sec exposure to K\textsuperscript+ (S\textsubscript{1}), caffeine to release Ca\textsuperscript{2+} from SER stores (S\textsubscript{2}), K\textsuperscript+ to refill SER Ca\textsuperscript{2+} stores (S\textsubscript{3}), and caffeine (S\textsubscript{4}). The % difference between the peak and rate of rise of the first and second caffeine-evoked [Ca\textsuperscript{2+}]i transient significantly declined over the age range of 12-24 months. To estimate spontaneous refilling, cells were depolarized for 5 sec with 68 mM K\textsuperscript+ (control), followed by a 10 sec exposure to 10 mM caffeine "conditioning stimulus" to deplete [Ca\textsuperscript{2+}]i stores. Caffeine was then rapidly applied for 5 sec at defined intervals from 60 to 300 sec. Integrated caffeine-evoked [Ca\textsuperscript{2+}]i transients were measured and plotted as a percentage of the K\textsuperscript+ response vs. time. The derivative of the refilling time curves significantly declined over the age range from 12-24 months. Overall, these data suggest that the ability of SCG cells to sustain release of [Ca\textsuperscript{2+}]i, following rapid or spontaneous refilling declines with advancing age. Compromised ability to sustain calcium signaling may possibly alter the overall function of adrenergic neurons innervating the cerebrovasculature.

Key words: Store operated calcium channels, aging and calcium release, aging and refilling of neuronal calcium stores, aging and function of superior cervical ganglia.
Introduction

A prominent factor for increased risk of stroke during aging is rising systolic blood pressure (1, 24). Adrenergic nerves arising from the superior cervical ganglia (SCG) serve to dampen increased cerebral blood flow in response to hypertension or increased intracranial pressure and reduces the risk of blood brain barrier disruption (3, 6, 7, 12, 18). Thus, age-related changes in adrenergic nerve function is emerging as an important area in the physiology of aging (20, 41).

The function of neurons depends in part on the release of calcium from the smooth endoplasmic reticulum (SER) in response to an elevation in intracellular calcium ([Ca\textsuperscript{2+}]\textsubscript{i}) mediated by voltage-gated Ca\textsuperscript{2+} channels (46-48). This process has been termed calcium induced calcium release (CICR) and is relevant in processes such as release of neurotransmitters and hormones (14, 31, 38, 47).

To sustain calcium release during neuronal activity requires refilling of the SER calcium through calcium influx and subsequent uptake into the SER via smooth endoplasmic reticulum calcium ATPase (SERCA) pumps (22, 37, 49). Thus, buffering of [Ca\textsuperscript{2+}]\textsubscript{i} transients and refilling [Ca\textsuperscript{2+}]\textsubscript{i} stores by SERCA suggest that calcium release and [Ca\textsuperscript{2+}]\textsubscript{i} buffering are intimately related processes. In SCG and sensory neurons, SER Ca\textsuperscript{2+} stores can be rapidly refilled by activation of voltage gated calcium channels with high K\textsuperscript{+} or they can spontaneously refill within 3-10 min following depletion with caffeine via activation of store operated calcium channels (SOCC) (2, 10, 11, 36, 37, 47).

Aging in all creatures is inexorable and the "why" of aging has been suggested to be a combination of developmental changes, genetic defects, environmental influences and an inborn aging process (8, 16, 17, 39, 42). However, these studies render little explanation in terms of "how" normal aging alters function of critical organ and neuronal systems or the vulnerability of particular physiological processes to advancing age. We have shown that there is an age-related decline in SERCA function with a subsequent increased reliance on
mitochondria and plasmalemma Ca\textsuperscript{2+}-ATPases (PMCA) to control high K\textsuperscript+-evoked [Ca\textsuperscript{2+}]i transients with advancing age (4, 32, 34, 44). Overall these data suggest that despite an age-related decline in the function of SERCA, cells may adapt to this loss by increased function of remaining [Ca\textsuperscript{2+}]i buffering mechanisms. Consistent with our studies in SCG cells others have shown that the function of SERCA declines with age in skeletal and heart muscle cells (13, 50). Thus, in peripheral excitable cells a subtle decline in overall SERCA function may be a common feature of the aging process.

Given that SERCA function declines with age in the SCG we studied how aging may alter the refilling and release of Ca\textsuperscript{2+} from the SER. We tested two hypotheses in this study as illustrated in Figure 1. The first hypothesis is that an age-related decline in SERCA mediated Ca\textsuperscript{2+} uptake alters rapid depolarization induced refilling of Ca\textsuperscript{2+} into the SER following caffeine-evoked depletion of SER Ca\textsuperscript{2+} stores. The second hypothesis is that an age-related decline in SERCA mediated Ca\textsuperscript{2+} uptake alters the spontaneous refilling of SER Ca\textsuperscript{2+} stores following caffeine-evoked depletion.
Methods

Experimental animals

Male Fischer-344 (F-344) rats aged 6 mo (young adult), 12 mo (mature adult), 20 mo (old) and 24 mo (senescent) were obtained from NIH-NIA breeding colony (Harlan Sprague-Dawley Incorporated, Indianapolis, IN, USA). The age-range designation comes from other studies showing the median life span in F-344 rats is approx 24 months (26). The animals were allowed to eat and drink at will and were maintained on a 12 hr light/dark cycle under controlled temperature (72–77 °F). All procedures used in this study were approved by the Institutional Animal Care and Use Committee (IACUC) at Loma Linda University and the approved guidelines were adhered to throughout the study.

Superior cervical ganglion preparation

Rats were anaesthetized with CO₂ (45 sec) followed by decapitation. The dissection of the superior cervical ganglia and preparation of isolated cells has been described previously (34). Briefly SCG were dissected from the carotid artery bifurcation and placed in cold Tyrode's solution which contained 150 mM NaCl, 4 mM KCl, 2 mM CaCl₂, 2 mM MgCl₂, 10 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES), and 10 mM glucose. The ganglia were then acutely dissociated in 5 ml of Earle's balanced salt solution (EBSS) containing: trypsin (6000 U/ml), collagenase D (1 mg/ml), DNase-1 type IV (0.1 mg/ml), HEPES (20 mM), glucose (10 mM), NaHCO₃ (10 mM) and adjusted to pH 7.4 with NaOH (1M). After incubation in a shaking water bath for 45 min at 34 °C, the digestion reaction was stopped by the addition of 5 ml of modified Hank's balanced salt solution (HBSS) with 10% fetal calf serum, 1.3 mM CaCl₂, 5 mM HEPES and adjusted to pH 7.4 with NaOH (1M). Dissociated cells were centrifuged at 600 rpm for 5 min and re-suspended in 5 ml of fresh HBSS. Cells were centrifuged again at 600 rpm for 5 min and dispersed in 0.5 ml of HBSS with 10% fetal calf serum, 5 mM HEPES adjusted to
pH 7.4 with NaOH (1M) onto Cell-Tak (BD Bioscience, Bedford, MA,) coated glass cover slips, (3.5 µg/cm²). Cover slips were modified by attaching a 2-cm Teflon ring to the surface with Sylgard adhesive (Dow Corning, Inc. MI). Dissociated cells on the cover-slips were incubated for 12-14 hrs at room temperature to allow cells to attach to the Cell-Tak protein coat before they were used in the experiments.

**Measurement of intracellular calcium**

SCG cells were loaded with 10 µM fura-2 acetoxy-methylester (fura-2/AM) for 20 min at room temperature, then washed with low K⁺ tyrodes buffer containing: 138 mM NaCl, 2 mM CaCl₂, 1 mM MgCl₂, 5 mM KCl, 10 mM HEPES, and 10 mM glucose, adjusted to pH 7.4 with NaOH (1M). Incubation was continued for an additional 20 min to allow intracellular esterases to convert the fura-2/AM dye into the free acid form (40). A potential problem with loading procedures is that there may be an age-related difference in the amount of Fura-2 taken up by the SCG cells or a difference in the activity of non-specific esterase's that convert Fura-2/AM to the free salt. To assess this possibility, the intensity of the fluorescence signal at 510 nM when the dye is activated at 380 nM is proportional to the amount of loaded dye (30). In this study we monitored the 510 nM emission fluorescence signal when fura-2 is excited at 380 nm (F₃₈₀) in resting SCG cells in each age group. The F₃₈₀ was not significantly different in SCG cells from 6-24 month-old animals. The specific values for F₃₈₀ following loading were, 160.04 ± 5.6, 169.80 ± 5.0, 164.57 ± 11, 169.90 ± 7.0 in SCG cells from 6, 12, 20 and 24 month-old animals respectively. These data are consistent with our previous studies showing that the F₃₈₀ does not change with age suggesting that dye loading is equivalent in SCG cells from each age group (32, 33).

Cover slips were mounted into a superfusion chamber, which was attached to the stage of a Nikon inverted microscope (Nikon Instruments, Tokyo, Japan). The microscope was attached to a Universal Imaging System running MetaFluor version 6.2 (Universal Imaging Corporation, a
subsidiary of Molecular Devices, West Chester, PA, USA). The perfusion system allowed the chamber volume (~250µl) to be exchanged at the rate of 500 µL (i.e. 2 times per second). A xenon lamp illuminated the fura-2 probe, and fluorescence was excited alternately at wavelengths 340 and 380 nm by a Lamda DG-4 (Sutter Instruments, Novato, CA, USA) hyper switch. The fura-2 emission fluorescence was measured at 510 nm and recorded by a Photometric Cool Snap 12-bit digital camera (Roper Scientific, AZ, USA). Adjusting the microscope stage to a point with no cells in the field of view and capturing a background image before the start of the experiment corrected for background light levels. Prior to fura-2/AM loading, cellular autofluorescence was examined in SCG cells. Autofluorescence was found to be undetectable by our imaging system and did not significantly alter our [Ca^{2+}] measurements. During the experiment, 340 and 380 fluorometric signal were collected, corrected for background fluorescence, calcium concentration calculated and the data logged to an exel file at a rate of ~300 msec. During dye loading and data collection, ambient light levels were minimized and SCG cells were only illuminated during data acquisition to minimize bleaching and potential photo damage of the dye.

Intracellular calcium was estimated by both in vitro and in vivo calibration methods. The in vitro method was performed using a calcium calibration kit (Molecular Probes, Eugene, OR, USA) with known [Ca^{2+}] ranging from 0 to 40 µM. Each prepared calcium solution was loaded with 4 µM fura-2 pentapotassium salt. A droplet of each [Ca^{2+}] was placed onto a glass slide and the fluorescent intensities from 340 and 380 excitation were measured and a curve of 340/380 ratio (R) versus [Ca^{2+}] was plotted. The in vivo method was performed on SCG cells by decreasing extracellular calcium concentration to 0 mM and fluorescence from 380 (F_{min}), and 340/380 ratio (R_{min}) was recorded for 1 min. The extracellular medium was then replaced with 10 mM [Ca^{2+}] with ionomycin (1 µM) and depolarized with high potassium (68 mM). Values were then recorded for 1 min to obtain F_{max} and R_{max}. The values for F_{min}, R_{min}, F_{max} and R_{max} were
remarkably similar between the *in vitro* and *in vivo* methods. Our *in vitro* calibration for *Kd* is comparable with *in vivo* values in neuronal cells (28, 31). Furthermore, we applied the same *Kd* for both young and old SCG neurons, since previous reports have shown no significant change in fura-2 *Kd* values for young and old neurons (28). Since our *in vitro* method correlated well with *in vivo* measurements, the values obtained from the *in vitro* calibration were used to convert the experimental fluorescent intensity ratios (*R*) to \([\text{Ca}^{2+}]\) over the physiological range of \([\text{Ca}^{2+}]\) by iterative fit to the equation: 

\[
[\text{Ca}^{2+}] = K_d \left( \frac{R - R_{\text{min}}}{R_{\text{max}} - R} \right) S_f.
\]

*Rmin* is the 340/380 ratio at zero \([\text{Ca}^{2+}]\) and \(R_{\text{max}}\) is the 340/380 ratio at 40 \(\mu\text{M} [\text{Ca}^{2+}]\). The value of *Kd* is the dissociation constant of fura-2, while *Sf* is a correction factor relating the ratio \(F_{\text{min}}/F_{\text{max}}\), which is the emission intensity at 380 nm when fura-2 is in the free \((F_{\text{min}}, 0 \mu\text{M} [\text{Ca}^{2+}])\) or bound \((F_{\text{max}}, 40 \mu\text{M} [\text{Ca}^{2+}])\) form (15). For this report we used multiple calibrations and the \([\text{Ca}^{2+}]\) was estimated using averaged *in vitro* values for *Sf* (11.3), *Rmin* (0.39), *Rmax* (2.5) and *Kd* (270 nM).

**Protocol 1: Measurement of K⁺-evoked (fast) Refilling of [Ca²⁺]i stores.**

Figure 2A shows representative data in a single SCG cell from a 6 month-old animal demonstrating the response to sequential additions of high K⁺ and caffeine to release and refill SER [Ca²⁺]i stores. Specifically, cells were exposed for 5 sec to 68 mM K⁺ (S₁) to insure uniform loading of SER [Ca²⁺]i stores (27). Next cells were exposed for 5 sec to 10 mM supermaximal, caffeine, (S₂), to release calcium from SER [Ca²⁺]i stores. Next a 5 sec exposure to high K⁺ to rapidly refill the SER [Ca²⁺]i stores (S₃) and caffeine to once again release calcium from the SER (S₄). These data show that we can reproducibly generate [Ca²⁺]i transients with different treatments and that in young cells high K⁺ is able to refill the SER Ca²⁺ stores. Figure 2B illustrates that caffeine selectively releases calcium from SER calcium stores in SCG cells. Note that caffeine still evokes an [Ca²⁺]i transient in the absence of extracellular calcium. These data
are consistent with other studies demonstrating that caffeine selectively evokes release of calcium from SER \([\mathrm{Ca}^{2+}]_i\) stores in SCG cells (11).

**Protocol 2: Measurement of Spontaneous Refilling of \([\mathrm{Ca}^{2+}]_i\) stores**

Figure 3 illustrates the protocol in a single SCG cell used to measure how aging may alter the spontaneous refilling of \([\mathrm{Ca}^{2+}]_i\) stores following caffeine-evoked depletion. This protocol was derived from a previous study in acutely dissociated dorsal root ganglion cells (45). Previous studies have shown that the initial responses of isolated neurons to caffeine can be quite variable suggesting variability in the loading of \([\mathrm{Ca}^{2+}]_i\) stores (2, 27). This variability to caffeine can be markedly reduced by initially depolarizing the cells to insure equal loading of \([\mathrm{Ca}^{2+}]_i\) stores. Thus, the cells were exposed for 5 sec to high \(K^+\) (S1) as a normalization control (data not shown) and to insure a more uniform loading of \([\mathrm{Ca}^{2+}]_i\) stores (2, 27). Next caffeine sensitive SER \([\mathrm{Ca}^{2+}]_i\) stores were depleted by a 10 sec exposure to 10 mM caffeine (S2) called the “conditioning response” (data not shown). Following the conditioning response, the SER calcium stores were allowed to spontaneously refill and cells were briefly exposed to 10 mM caffeine for 5 sec at the intervals indicated until the maximal response to caffeine following the “conditioning response” was obtained.

**Data analysis**

Calcium transients were analyzed using Origin 6.1 software in all age groups. In protocol 1 peak \([\mathrm{Ca}^{2+}]_i\) was determined by subtracting basal \([\mathrm{Ca}^{2+}]_i\) from the maximum \(K^+\) or caffeine-evoked \([\mathrm{Ca}^{2+}]_i\) transients. Rate of rise of \([\mathrm{Ca}^{2+}]_i\) was determined by linear fit \((r = 0.99 \pm .07)\) from basal \([\mathrm{Ca}^{2+}]_i\) to the maximum \(K^+\) or caffeine-evoked \([\mathrm{Ca}^{2+}]_i\). Rate of recovery of \([\mathrm{Ca}^{2+}]_i\) transients was determined using a first order exponential fit \((r = 0.99 \pm .06)\). In protocol 2 caffeine-evoked \([\mathrm{Ca}^{2+}]_i\) transients following the conditioning response for were analyzed by taking the total area under the curve and normalization to the area under the curve of the \(K^+\) control. Thus, data is expressed as integrated caffeine-evoked \([\mathrm{Ca}^{2+}]_i\) as a percentage of the \(K^+\) control. These data
were plotted vs time after the conditioning response to estimate the spontaneous refilling of [Ca\textsuperscript{2+}]i stores as shown in Figure 8A. The rate of spontaneous refilling following caffeine-evoked release of calcium from [Ca\textsuperscript{2+}]i stores was estimated by taking the derivative of the curves in Figure 8A using Origin 6.1 and is expressed in Figure 8B.

Statistics

The impact of age on all measured parameters was determined using ANOVA and Fischer PLSD test. All data in each age group were analyzed for heterogeneity of variance using the Cochrans test. If the variances were significantly different then values were log transformed and statistical tests were repeated (51).
Results

Properties of high [K⁺ and caffeine-evoked [Ca²⁺]ᵢ transients

Using the protocol in figure 2A we calculated the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]ᵢ transients in each age group as shown in Figure 4. There is a clear age related effect on the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]ᵢ transients. These parameters increased in SCG cells from 6-12 month-old animals and then progressively declined from 12-24 months. Despite the decline in these parameters from 12-24 months the consistency of the dynamic response between the first and second high K⁺-evoked [Ca²⁺]ᵢ transients appears to be maintained with advancing age. Again using the protocol in figure 2A we calculated the peak and rate of rise of the first and second caffeine-evoked [Ca²⁺]ᵢ transients in each age group as shown in Figure 5. In a similar fashion to high K⁺, these parameters increased in SCG cells from 6-12 month-old animals and significantly declined from 12-24 months. In contrast to the high K⁺-evoked [Ca²⁺]ᵢ transients, the peak and rate of rise of caffeine-evoked [Ca²⁺]ᵢ transients appears decline from the first and second exposure to caffeine in SCG cells from 12-24 month-old animals.

To clarify how advancing age may alter the consistency of the peak and rate of rise of high K⁺ and caffeine-evoked [Ca²⁺]ᵢ transients, we analyzed the percentage difference between the first and second high K⁺ or caffeine-evoked [Ca²⁺]ᵢ transients. When the data were analyzed in this manner, a clearer pattern emerges as shown in figure 6. There is no significant age-related decline in the percentage difference in the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺]ᵢ transients. In contrast to high K⁺, there is a significant age-related decline in the percentage difference in the peak and rate of rise of the first and second caffeine-evoked [Ca²⁺]ᵢ transient.
Spontaneous refilling of \([\text{Ca}^{2+}]_i\) stores following caffeine-evoked depletion

To enhance the rigor of our study on the impact of age on spontaneous refilling of \([\text{Ca}^{2+}]_i\) stores (figure 3) we performed a series of validation controls as shown in figure 7. In figure 7A we demonstrate that \([\text{Ca}^{2+}]_i\) stores in resting SCG cells will refill in the presence of the L and N-type voltage gated calcium channel antagonists nifedipine and \(\omega\)-conotoxin respectively, following caffeine-evoked depletion of \([\text{Ca}^{2+}]_i\) stores. Note that in the presence of the voltage-gated calcium channel antagonists, cells still exhibit robust caffeine-evoked release of \([\text{Ca}^{2+}]_i\).

In figure 7B we show the efficacy of the L and N-type channel calcium antagonists on high K+-evoked peak \([\text{Ca}^{2+}]_i\) transients. Note that both nifedipine and \(\omega\)-conotoxin block approximately 97% of the K+-evoked Ca\(^{2+}\) response suggesting that L and N-type channel subtypes predominate in the SCG as previously shown (23). Figure 7C demonstrates the activation of spontaneous calcium influx as shown by a rise in \([\text{Ca}^{2+}]_i\) following caffeine-evoked depletion of and blockade of the SERCA mediated refilling of \([\text{Ca}^{2+}]_i\) stores. However, in the continued presence of the SERCA blocker THAPS, caffeine no longer elicits a response. Figure 7D demonstrates that La\(^{3+}\) abolishes the spontaneous calcium influx following caffeine-evoked depletion of \([\text{Ca}^{2+}]_i\) and blockade of SERCA by THAPS. Under these conditions \([\text{Ca}^{2+}]_i\) no longer rises after depletion of \([\text{Ca}^{2+}]_i\) stores and subsequent responses of SCG cells to caffeine are abolished.

Figure 8 demonstrates that advancing age alters the spontaneous refilling of SER Ca\(^{2+}\) stores via SOCC and SERCA activity following caffeine-evoked depletion of \([\text{Ca}^{2+}]_i\) stores. After caffeine evoked depletion (S\(_2\)), the cells were exposed for 5 sec to caffeine at the time intervals shown in figure 3. The data in figure 8A show that there is a significant age-related decline in the response to caffeine at each time point after the conditioning response. However, at the 240 sec time interval, there is no longer any significant difference in the response to caffeine in any age group. To estimate the rate of spontaneous refilling of \([\text{Ca}^{2+}]_i\) stores following caffeine-
evoked depletion we calculated the derivative of the curves in figure 8A, and plotted these values as a function of age (Figure 8B). The derivatives were found to be significantly lower in SCG cells from 12, 20, and 24 month-old animals as compared to 6-months (Figure 8B).

Measurement of Basal $[Ca^{2+}]_i$ and rate of recovery of high K$^+$ and caffeine-evoked $[Ca^{2+}]_i$ transients.

Table 1 shows the impact of age on basal $[Ca^{2+}]_i$ following successive exposures to high K$^+$ and caffeine using the protocol in figure 2A. Under all treatment conditions basal $[Ca^{2+}]_i$ significantly increases in SCG cells from 6-12 month-old animals and then significantly declines from 12-24 months. Table 2 shows the impact of age on the recovery rate constant following exposure to high K$^+$ or caffeine as shown in the protocol in figure 2A. There is no significant age-related change in the magnitude of the recovery rate constants with any of the successive exposures to high K$^+$ or caffeine.
Discussion

The most important and straightforward findings in this study is that release of calcium from [Ca$^{2+}$]i stores and high K$^+$-evoked and spontaneous refilling of these stores declines with advancing age in SCG cells. The magnitude and shape of stimulation-evoked increases in [Ca$^{2+}$]i are modulated by both influx of and release of calcium in neurons (25, 46-48). Sustaining the release of calcium from intracellular stores during ongoing activity requires the refilling of these stores following the release. Overall, the data in this study suggest that the ability to sustain release of calcium from intracellular stores declines with age and may have implications as to the function of sympathetic neurons during the aging process. As refilling of [Ca$^{2+}$]i stores is dependent on both influx and uptake into the SER via SERCA the decline in refilling may reflect and age related decline in the function of both mechanisms. Indeed, we have shown that SERCA function declines with age in sympathetic neurons (32, 33, 43, 44). Thus, at least one mechanism that may account for the decline in refilling of [Ca$^{2+}$]i stores is an age-related decline in the function of SERCA pumps. As adrenergic nerves from the SCG serve to protect the CNS from blood brain barrier disruption (3, 5, 7, 12, 18), these data suggest that the ability of the SCG to sustain its protective function may possibly be altered with advancing age.

Aging and fast refilling of [Ca$^{2+}$]i stores

The data reported in this study (Fig. 2B) as well as published data suggest that selective release of calcium from intracellular stores in dorsal root ganglion (DRG) and SCG cells can be accomplished with caffeine (11, 25, 35, 47). In addition, these stores are rapidly refilled by depolarization of the neurons following exposure to caffeine (11, 25, 35, 47). Thus, we utilized a protocol to determine if advancing age alters repeated caffeine-evoked release of [Ca$^{2+}$]i after high K$^+$-evoked refilling (Fig 2A). There was no age-related difference in the [Ca$^{2+}$]i dynamics between the first and second high K$^+$-evoked [Ca$^{2+}$]i transient (Fig. 6A,B). Thus, although the dynamics of K$^+$-evoked [Ca$^{2+}$]i transients decline with age, they remain constant within each age group. In contrast to K$^+$-evoked [Ca$^{2+}$]i transients, advancing age caused a significant reduction
in the \([\text{Ca}^{2+}]_i\) dynamics between the first and second caffeine exposure within the oldest age groups (Fig. 6C,D). There are numerous studies demonstrating the impact of age on depolarization-evoked \([\text{Ca}^{2+}]_i\) transients (4, 33, 34, 44, 49). However, to our knowledge this is the first study demonstrating that the ability of peripheral neurons to sustain the release of calcium following rapid depolarization-evoked refilling, declines with age.

Interpretation of the overall age-related decline of \(\text{K}^+\)-evoked \([\text{Ca}^{2+}]_i\) transients is complex (Fig. 4) as depolarization-evoked \([\text{Ca}^{2+}]_i\) transients reflects both influx and release of calcium (46). Using patch clamp methods coupled with measurement of \([\text{Ca}^{2+}]_i\) with fura-2, one study showed that stimulation-evoked calcium influx increases but the measured \([\text{Ca}^{2+}]_i\) by fura-2 declines with age (28). The measurement of \(\text{K}^+\)-evoked \([\text{Ca}^{2+}]_i\) in this study does not directly distinguish between the contributions made to the calcium signal by influx, and release of calcium from intracellular stores. Thus, it is possible that at least one mechanism that may account for the decline in \(\text{K}^+\)-evoked \([\text{Ca}^{2+}]_i\) dynamics is reduced release. However, these data do not rule out changes in calcium influx mediated through voltage gated calcium channels.

**Validation Controls**

Validation control experiments (Fig. 7) were done to demonstrate that spontaneous refilling of \([\text{Ca}^{2+}]_i\) stores following caffeine-evoked release occurred independently of voltage-gated calcium channels and appears to be dependent on the activation of calcium influx and SERCA as shown in previous studies (2, 45, 47). When SER calcium stores were depleted and SERCA were blocked with THAPS, \([\text{Ca}^{2+}]_i\) begins to rise in SCG cells in a similar manner as shown in previous studies (2, 47). Indeed this rise in \([\text{Ca}^{2+}]_i\) was abolished by La\(^{3+}\) and possibly suggests that this calcium influx may be mediated by opening of SOCC channels (2, 47). An interesting observation is that the time for activation of presumed SOCC in our preparation appears slow relative to activation of SOCC in other cell models (19). Overall these data demonstrate that spontaneous refilling of \([\text{Ca}^{2+}]_i\) stores in resting SCG cells following the release of \([\text{Ca}^{2+}]_i\) appears to be mediated by both SOCC and SERCA activity (2, 47).
Aging and spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores

There are studies demonstrating that in SCG and DRG cells the $[\text{Ca}^{2+}]_i$ stores can spontaneously refill within 3-10 min following depletion with caffeine (11, 25, 45, 47). This spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores requires two interdependent mechanisms. These are the spontaneous influx of calcium through SOCC and subsequent pumping of calcium into the SER via SERCA (2, 47). The function of SOCC channels can be blocked with ions such as $\text{La}^{3+}$ and SERCA function is blocked by THAPS (2, 47). Since we have previously shown that SERCA function declines with advancing age in isolated SCG cells (32, 34, 44), we developed an experimental protocol to study the impact of age on the spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores following caffeine-evoked depletion (Fig. 3). In order to validate that the spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores is independent of VOCC and mediated by SOCC and SERCA activity we performed a series of validation experiments (Fig. 7). These control data suggest that in isolated resting SCG cells spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores appears to be mediated by influx of calcium via SOCC and subsequent uptake into the SER via SERCA pumps. These data are consistent with previous studies demonstrating that spontaneous refilling of $[\text{Ca}^{2+}]_i$ store is mediated by SOCC and SERCA function (2, 2, 47).

To our knowledge this is the first study to demonstrate that spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores slows with age in isolated SCG cells (Fig. 8). Interestingly, it appears that SCG cells from senescent animals will eventually refill to similar levels if given an appropriate amount of time. Thus, SCG cells from healthy senescent animals maintain some ability to release and spontaneously refill $[\text{Ca}^{2+}]_i$ stores, implying that some level of function of sympathetic neurons is maintained with age. Using various antagonists of SERCA we have shown that SERCA function declines in both SCG cells and sympathetic nerve endings (32, 34, 43, 44). We propose that at least one mechanism that may account for a decline in spontaneous refilling of $[\text{Ca}^{2+}]_i$ stores is reduced SERCA function. However, these data do not rule out age-related changes in SOCC function. We are currently developing protocols to study how aging may alter SOCC function.
Aging and basal [Ca$^{2+}$]i levels and rate of recovery of [Ca$^{2+}$]i

Surprisingly we found that basal [Ca$^{2+}$]i levels rose from 6-12 months and then steadily declined with age (Table 1). The increase in basal [Ca$^{2+}$]i from 6-12 months may reflect late maturational changes. In our previous studies using only two age-groups we found no significant difference in basal [Ca$^{2+}$]i levels in SCG cells from 6 and 20 month-old animals (32, 34). Comparison of the current data with our previous studies succinctly demonstrates the necessity of using more than two age groups in aging studies. Using multiple age groups appears to be essential as the range from maturity to senescence provides a clearer overview of how advancing age affects the parameters of interest (9). Since we have shown that SERCA function declines with age in SCG cells one might predict higher resting levels of [Ca$^{2+}$]i. Indeed our data stand in contrast to age-related increases in basal [Ca$^{2+}$]i in DRG neurons (21). These data may suggest that the aging process may not have uniform effects on all neuronal models. Since SERCA function depends on both the level of cytosolic calcium and modulation by other factors such as phosphorylation (50), reduced levels of cytosolic calcium may possibly contribute to lower loading levels of [Ca$^{2+}$]i stores.

Depolarization-evoked [Ca$^{2+}$]i transients depend on influx and release of [Ca$^{2+}$]i (46, 47), and caffeine-evoked [Ca$^{2+}$]i transients reflect calcium released from [Ca$^{2+}$]i stores. Thus, lower loading levels of [Ca$^{2+}$]i stores may possibly contribute to an age-related decline in both high K$^+$ and caffeine-evoked release of [Ca$^{2+}$]i observed in these studies (Fig. 4,5). In future studies we will use calcium indicators such as furaptra to determine the impact of age on the levels of SER [Ca$^{2+}$]i stores similar to studies accomplished in smooth muscle cells (29).

Since SERCA function declines with age in SCG cells (32, 44) it is remarkable that there is no age-related change rate of recovery of high K$^+$- or caffeine-evoked [Ca$^{2+}$]i transients. However, rate of recovery of high [Ca$^{2+}$]i transients in SCG cells is complex as we have shown that SERCA, mitochondrial calcium uptake and PMCA all contribute to the rate of recovery of high [Ca$^{2+}$]i transients in SCG cells (4, 32, 34, 44). In addition, we have shown that the age-
related decline in SERCA function is accompanied by an apparent increased reliance on mitochondrial calcium uptake and PMCA function (4, 32, 34). Taken together our current and past studies suggest that in the face of an age-related decline in SERCA function, the rate of recovery is maintained by increased activity of other calcium uptake and extrusion systems. These data suggest that with advancing age buffering systems do not necessarily “fall apart” as a decline in one system may be compensated for by increased function of another system. Thus old SCG cells appear to have inherent adaptive responses to maintain some vitality in the face of age related declines the function of particular calcium regulatory mechanisms.

In conclusion, the data presented in this study suggest that with advancing age there is an age-related decline in the ability of SCG cells to sustain release of calcium from \([\text{Ca}^{2+}]_{i}\) stores. These data may have implications for the function of sympathetic autonomic neurons as animals undergo the normal aging process. This inability to sustain calcium signaling may possibly alter the neuronal firing rate, leading to altered neuronal processes such as neurotransmission, and plasticity.
Acknowledgements

The authors wish to acknowledge the technical expertise of Mr. Charles Hewitt in the development and execution of measurement of \([\text{Ca}^{2+}]_i\) with our imaging system. This work was supported in part by grants from the American Heart Association, National Center (#0040021N) and NIH P01 31226.


Figure Legends

**Fig. 1.** Model illustrating overall experimental design of this study. The measured variable is relative cytosolic calcium concentration in response to various protocols used within the study. The overall governing hypothesis is that an age-related decline in SERCA function alters the SER calcium levels and their refilling following depletion. Abbreviations: Smooth endoplasmic reticulum (SER). Smooth endoplasmic reticulum calcium ATP-ase (SERCA). Voltage gated calcium channels (VOCC). Store operated calcium channels (SOCC).

**Fig. 2.** (A) Representative data of protocol 1 demonstrating caffeine-evoked release of [Ca^{2+}]i from SER stores and fast K+-evoked refilling of [Ca^{2+}]i stores in a single Fura-2 loaded SCG cell from a 6-month animal. An [Ca^{2+}]i transient (S1) was evoked by 5 sec exposure to 68 mM K+ followed by 2 min equilibration. A second [Ca^{2+}]i transient (S2) was evoked by 5 sec exposure to 10 mM caffeine to release calcium from [Ca^{2+}]i stores followed by 2 min equilibration. The third [Ca^{2+}]i transient (S3) was evoked by 5 sec exposure to 68 mM K+ to refill [Ca^{2+}]i stores followed by two min equilibration. A final [Ca^{2+}]i transient (S4) was evoked by 5 sec exposure to 10 mM caffeine followed by 2 min equilibration. (B) Data derived from a single Fura-2 loaded SCG cell from a 6 month animal demonstrating selective caffeine evoked Ca^{2+} release from [Ca^{2+}]i stores. An [Ca^{2+}]i transient was evoked by 5 sec exposure to buffer containing 68 mM K+ and 2 mM extracellular Ca^{2+} followed by 2 min equilibration. A second [Ca^{2+}]i transient was evoked by 5 sec exposure to buffer containing 10 mM caffeine and zero extracellular Ca^{2+} (3 mM EGTA) followed by 2 min equilibration. Next the cell was exposed for 5 sec to buffer containing 68 mM K+ and zero extracellular Ca^{2+} (3 mM EGTA) followed by two min equilibration. A final [Ca^{2+}]i transient was evoked by 5 sec exposure to buffer containing 68 mM K+ and 2 mM extracellular Ca^{2+}. 
Fig. 3. Example of protocol 2 demonstrating the spontaneous refilling of SER calcium stores following caffeine-evoked emptying in a single SCG cell. Cells were exposed for 5 sec to 68 mM K⁺ and then for 10 sec to 10 mM caffeine representing the control and conditioning response respectively (data not shown). At the time points indicated following the conditioning response, 10 mM caffeine was applied for 5 sec until the maximum response to caffeine was achieved.

Fig. 4. (A, B) Peak [Ca^{2+}]i evoked by the first and second exposure to high K⁺ in isolated SCG cells from animals aged 6-24 months. (C, D) Rate of rise of [Ca^{2+}]i evoked by the first and second exposure to high K⁺ in isolated SCG cells from animals aged 6-24 months. These data were derived from the protocol shown in figure 2A. Peak [Ca^{2+}]i was measured by subtracting basal [Ca^{2+}]i from the maximum K⁺-evoked [Ca^{2+}]i transient. Rate of rise of [Ca^{2+}]i from baseline to maximum was determined by linear fit using Origin 6.1. Data represent the mean ± S.E. n = 21-42 cells from each age group. ** = significantly different from two other age groups, P<0.05. *** = significantly different from three other age groups, P<0.05.

Fig. 5. (A, B) Peak [Ca^{2+}]i evoked by the first and second exposure to 10 mM caffeine in isolated SCG cells from animals aged 6-24 months. (C, D) Rate of rise of [Ca^{2+}]i evoked by the first and second exposure to 10 mM caffeine in isolated SCG cells from animals aged 6-24 months. These data were derived from the protocol shown in figure 2A. Peak [Ca^{2+}]i was measured by subtracting basal [Ca^{2+}]i from the maximum caffeine-evoked [Ca^{2+}]i transient. Rate of rise of [Ca^{2+}]i from baseline to maximum was determined by linear fit using Origin 6.1. Data represent the mean ± S.E. n = 21-42 cells from each age group. ** = significantly different from two other age groups, P<0.05. *** = significantly different from three other age groups, P<0.05.

Fig. 6. (A, B) Impact of age on the percentage difference between the first and second K⁺-evoked peak and rate of rise of [Ca^{2+}]i transients. (C, D) Impact of age on the percentage
difference between the first and second caffeine-evoked \([Ca^{2+}]_i\) transients. The percentage difference was calculated as the difference between the first and second exposure to K\(^+\) or caffeine divided by the first exposure to K\(^+\) or caffeine times 100. Data represent the mean ± S.E. \(n = 21-42\) cells from each age group. * = significantly different from one other age group, \(P<0.05\). ** = significantly different from two other age groups, \(P<0.05\). *** = significantly different from three other age groups, \(P<0.05\).

**Fig. 7.** Validation controls to determine that SOCC channels and SERCA mediate spontaneous refilling of SER calcium stores following caffeine-evoked release. (A) SER calcium stores refill in the presence of calcium channel blockers, nifedipine and \(\omega\)-conotoxin. Cells were exposed for 5 sec to high K\(^+\) buffer (S\(_1\)) followed by a 10 sec exposure to a buffer containing 10 mM caffeine to release \([Ca^{2+}]_i\) stores (S\(_2\)). Next the cells were continually exposed to a buffer containing 10 \(\mu\)M nifedipine and 1 \(\mu\)M \(\omega\)-conotoxin to block L and N-type calcium channels respectively. As indicated the cells were exposed to again exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average for 5 cells from a 6 month-old animal. (B) L and N-type channel antagonists, nifedipine and \(\omega\)-conotoxin block K\(^+\)-evoked \([Ca^{2+}]_i\) transients in SCG cells. Cells were exposed for 5 sec to high K\(^+\) buffer (control). Next cells were exposed for 5 sec to high K\(^+\) buffer containing 10 \(\mu\)M nifedipine and then for 5 sec to high K\(^+\) buffer containing 10 \(\mu\)M nifedipine and 1 \(\mu\)M \(\omega\)-conotoxin. Data represent the mean ± S.E for 6 cells from a 6 month-old animal. (C) Activation of SOCC channels occur following the caffeine-evoked depletion of SER calcium stores and blockade of SERCA with thapsigargin (THAPS). Cells were exposed for 5 sec to high K\(^+\) (S\(_1\)) and then to 10 mM caffeine for 10 sec (S\(_2\)). Next cells were continually exposed to a buffer containing the SERCA antagonist THAPS, 1 \(\mu\)M. At the times indicated the cells were exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average of 4 cells from a 6 month-old animal. (D) Activation of SOCC channels is blocked with La\(^{3+}\).
following caffeine-evoked depletion of SER. Cells were exposed for 5 sec to a buffer containing high K⁺ (S₁). Next cells were exposed for 10 sec to a buffer containing 10 mM caffeine (S₂). Following S₂ cells were continually exposed to a buffer containing THAPS (1 µM) and La³⁺ (100 µM). At the times indicated cells were exposed for 5 sec to a buffer containing 10 mM caffeine. Data represent the average for 5 cells from a 6 month-old animal.

**Fig. 8.** (A) Aging alters the spontaneous refilling of SER calcium stores following caffeine-evoked depletion. Using protocol 2 (Figure 3), data were plotted as integrated caffeine-evoked release of [Ca²⁺]ᵢ as a percentage of the K⁺ control vs time after conditioning response. Data represent the mean ± S.E. n = 13-29 cells from 6-24 month-old animals. ** = significantly different from two other age groups, P<0.01. *** significantly different from three other age groups, P<0.01. (B) Aging alters the derivative of caffeine-evoked [Ca²⁺]ᵢ transients as a percentage of K⁺ control. Data derived for each individual cell in (A) were plotted and fitted by a Boltzmann fit and the derivatives calculated using Origin 6.1. Data represent the mean ± S.E. n = 13-29 cells from 6-24 month-old animals. *** = significantly different from three other age groups, P<0.001. ** = significantly different from two other age groups, P<0.01.
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**Table 1:** Basal $[\text{Ca}^{2+}]_{i}$ significantly declines from 12-24 months following each treatment in protocol 1. Data represent the mean ± S.E. $n = 21-42$ cells from each age group. $P<0.05$. 
<table>
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**Table 2.** Rate of recovery of [Ca^{2+}]_i transients with each treatment in protocol 1 in isolated SCG cells from 6-24 month-old animals. Rate of recovery was calculated by obtaining the first order time constant (Tc) using Origin 6.1. The reciprocal of Tc yields the rate constant. N= 21-42 cells from 12-20 animals.
Figure 1

Hypothesis 1: An age-related decline in SERCA-mediated Ca\(^{2+}\) uptake alters voltage-dependent rapid refilling after caffeine depletion.

Hypothesis 2: An age-related decline in SERCA-mediated Ca\(^{2+}\) uptake alters spontaneous refilling after caffeine depletion.

SERCA-mediated Ca\(^{2+}\) uptake

Caffeine-evoked depletion

Measured Variable

Fura2-Ca\(^{2+}\)

(SOCC)

Measured Variable
Figure 2: Protocol 1

A.

B.
Figure 3: Protocol 2

[Diagram showing calcium concentration over time with annotations for 60 sec after conditioning, 120 sec after conditioning, 180 sec after conditioning, and 240 sec after conditioning.]
Figure 4

A. 1\textsuperscript{st} KCL

B. 2\textsuperscript{nd} KCL

C. 1\textsuperscript{st} KCL

D. 2\textsuperscript{nd} KCL
Figure 6

A. Peak K⁺-evoked [Ca²⁺]i (nM)

B. Rate of Rise of K⁺-evoked [Ca²⁺]i (nM)

C. Peak Caffeine-evoked [Ca²⁺]i (nM)

D. Rate of Rise of Caffeine-evoked [Ca²⁺]i (nM)
Figure 7

A. Refill SER Ca²⁺ stores via SOCC’s and SERCA’s

B. Peak K⁺-Evoked [Ca²⁺]i Transients (nM)

C. Block SERCA’s and SOCC’s

D. Activation of SOCC

Time (sec)
Figure 8

A. Integrated Caffeine-evoked [Ca^{2+}] as a % K^+

B. Derivative of Caffeine-evoked [Ca^{2+}] as a % K^+