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

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Vanterpool, Conwin K., William J. Pearce, and John N. Buchholz. Advancing age alters rapid and spontaneous refilling of caffeine-sensitive calcium stores in sympathetic superior cervical ganglion cells. J Appl Physiol 99: 963–971, 2005. First published April 21, 2005; doi:10.1152/japplphysiol.00343.2005.—Intracellular calcium concentration ([Ca\textsuperscript{2+}]i) release from smooth endoplasmic reticulum (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 and subsequent pumping by SER Ca\textsuperscript{2+}-ATPases. We measured [Ca\textsuperscript{2+}]i transients in isolated fura 2-loaded superior cervical ganglion cells from 6-, 12-, 20-, and 24-mo-old Fischer 344 rats. For rapid refilling, [Ca\textsuperscript{2+}]i transients were elicited by a 1) 5-s exposure to K\textsuperscript{+}, 2) caffeine to release Ca\textsuperscript{2+} from SER stores, 3) K\textsuperscript{+} to refill SER Ca\textsuperscript{2+} stores, and 4) caffeine. The percent 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 mo. To estimate spontaneous refilling, cells were depolarized for 5 s with 68 mM K\textsuperscript{+} (control), followed by a 10-s exposure to 10 mM caffeine “conditioning stimulus” to deplete [Ca\textsuperscript{2+}]i stores. Caffeine was then rapidly applied for 5 s at defined intervals from 60 to 300 s. 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 mo. Overall, these data suggest that the ability of superior cervical ganglion 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.

store-operated calcium channels; calcium release; refilling of neuronal calcium stores; function of superior cervical ganglia

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 reduce the risk of blood-brain barrier disruption (3, 6, 7, 12, 18). Thus age-related changes in adrenergic nerve function are 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 concentration ([Ca\textsuperscript{2+}]i) mediated by voltage-gated Ca\textsuperscript{2+} channels (46–48). This process has been termed calcium-induced calcium release 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 SER calcium ATPase (SERCA) pumps (22, 37, 49). Thus buffering of [Ca\textsuperscript{2+}]i transients and refilling [Ca\textsuperscript{2+}]i stores by SERCA suggest that calcium release and [Ca\textsuperscript{2+}]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 (SOCs) (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. Our laboratory has 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 Fig. 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 National Institutes of Health-National Institute on Aging breeding colony (Harlan Sprague-Dawley, Indianapolis, IN). The age range designation comes from other studies.
showing the median life span in F-344 rats is ~24 mo (26). The animals were allowed to eat and drink at will and were maintained on a 12:12-h 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 at Loma Linda University, and the approved guidelines were adhered to throughout the study.

**SCG Preparation.** Rats were anesthetized with CO2 (45 s) followed by decapitation. The dissection of the SCG and preparation of isolated cells have been described previously (34). Briefly, SCG were dissected from the carotid artery bifurcation and placed in cold Tyrode solution, which contained 150 mM NaCl, 4 mM KCl, 2 mM CaCl2, 2 mM MgCl2, 10 mM HEPES, and 10 mM glucose. The ganglia were then acutely dissociated in 5 ml of Earle’s balanced salt solution containing trypsin (6,000 U/ml), collagenase D (1 mg/ml), DNAse-1 (1 mM MgCl2, 5 mM KCl, 10 mM HEPES, and 10 mM glucose, 1 mM MgCl2, 2 mM CaCl2, 2 mM HEPES, and 10 mM glucose, 10 mM HEPES, and NaHCO3 (10 mM) were added to pH 7.4 with NaOH (1 M). 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, and 5 mM HEPES and adjusted to pH 7.4 with NaOH (1 M). Dissociated cells were centrifuged at 600 rpm for 5 min and resuspended 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 and 5 mM HEPES, adjusted to pH 7.4 with NaOH (1 M) onto Cell-Tak (BD Bioscience, Bedford, MA) coated glass coverslips (3.5 cm²/cm²). Coverslips were modified by attaching a 2-cm Teflon ring to the surface with Sylgard adhesive (Dow Corning). Dissociated cells on the coverslips were incubated for 12–14 h at room temperature to allow cells to attach to the Cell-Tak protein coat before they were used in the experiments.

**Measurement of [Ca²⁺].** SCG cells were loaded with 10 μM fura 2-AM for 20 min at room temperature, and then they were washed with low-K⁺ Tyrode 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 (1 M). 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 nonspecific 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- to 24-mo-old animals. The specific values for F380 following loading were 160.04 ± 5.6, 169.80 ± 5.0, 164.57 ± 11, and 169.90 ± 7.0 in SCG cells from 6-, 12-, 20-, and 24-mo-old animals, respectively. These data are consistent with our laboratory’s 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). Coverslips 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, a subsidiary of Molecular Devices, West Chester, PA). The perfusion system allowed the chamber volume (~250 μl) 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 Lambda DG-4 (Sutter Instruments, Novato, CA) hyperswitch. The fura 2 emission fluorescence was measured at 510 nm and recorded by a Photometric Cool Snap 12-bit digital camera (Roper Scientific). 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. Before 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 [Ca²⁺], measurements. During the experiment, 340-nm and 380-nm fluorometric signals were collected and corrected for background fluorescence, calcium concentration was calculated, and the data were logged to an excel file at a rate of ~300 ms. 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. [Ca²⁺], was estimated by both in vitro and in vivo calibration methods. The in vitro method was performed by using a calcium calibration kit (Molecular Probes, Eugene, OR) with known calcium buffers ranging from 0 to 40 μM. Each prepared calcium solution was loaded with 4 μM fura 2 pentapotassium salt. A droplet of each calcium buffer was placed onto a glass slide, and the fluorescent intensities from 340- and 380-nm excitation were measured, and a
curve of 340-to-380-nm ratio (R) vs. [Ca\(^{2+}\)] was plotted. The in vivo method was performed on SCG cells by decreasing extracellular [Ca\(^{2+}\)] to 0 mM and fluorescence from 380 nm (F\(_{\text{min}}\)), and 340-to-380-nm ratio (R\(_{\text{min}}\)) was recorded for 1 min. The extracellular medium was then replaced with 10 mM [Ca\(^{2+}\)] with ionomycin (1 uM) and depolarized with high potassium (68 mM). Values were then recorded for 1 min to obtain emission intensity at 380 nm when fura 2 is in the bound (40 uM [Ca\(^{2+}\)]) form (F\(_{\text{max}}\)) and 340-to-380-nm ratio at 40 uM [Ca\(^{2+}\)] (R\(_{\text{max}}\)). The values for F\(_{\text{min}}, R_{\text{min}}, F_{\text{max}}\), and R\(_{\text{max}}\) were remarkably similar between the in vitro and in vivo methods. Our in vitro calibration for dissociation constant of fura 2 (K\(_{d}\)) is comparable with in vivo values in neuronal cells (28, 31). Furthermore, we applied the same K\(_{d}\) for both young and old SCG neurons, because previous reports have shown no significant change in fura 2 K\(_{d}\) values for young and old neurons (28). Because 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 [Ca\(^{2+}\)] over the physiological range of [Ca\(^{2+}\)], by iterative fit to the equation: [Ca\(^{2+}\)] = K\(_{d}\) (R - R\(_{\text{min}}\)) (15), where R\(_{\text{min}}\) is a correction factor relating the K\(_{d}\) ratio F\(_{\text{min}}\)/F\(_{\text{max}}\). For this report, we used multiple calibrations, and the [Ca\(^{2+}\)] was estimated using averaged in vitro values for S\(_{p}\) (11.3), R\(_{\text{min}}\) (0.39), R\(_{\text{max}}\) (2.5), and K\(_{d}\) (270 nM).

Protocol 1: measurement of K\(^{+}\)-evoked (fast) refilling of [Ca\(^{2+}\)] stores. Figure 2A shows representative data in a single SCG cell from a 6-mo-old animal demonstrating the response to sequential additions of high K\(^{+}\) and caffeine to release and refill SER [Ca\(^{2+}\)] stores. Specifically, cells were exposed for 5 s to 68 mM K\(^{+}\) (S\(_{1}\)) to ensure uniform loading of SER [Ca\(^{2+}\)] stores (27). Next cells were exposed for 5 s to 10 mM supramaximal caffeine (S\(_{2}\)) to release calcium from SER [Ca\(^{2+}\)] stores. Next there was a 5-s exposure to high K\(^{+}\) to rapidly refill the SER [Ca\(^{2+}\)] stores (S\(_{3}\)) and caffeine to once again release calcium from the SER (S\(_{4}\)). These data show that we can reproducibly generate [Ca\(^{2+}\)] transients with different treatments and that, in young cells, high K\(^{+}\) is able to refill the SER Ca\(^{2+}\) stores. Figure 2B illustrates that caffeine selectively releases calcium from SER calcium stores in SCG cells. Note that caffeine still evokes an [Ca\(^{2+}\)] transient in the absence of extracellular calcium. These data are consistent with other studies demonstrating that caffeine selectively evokes release of calcium from SER [Ca\(^{2+}\)] stores in SCG cells (11).

Protocol 2: measurement of spontaneous refilling of [Ca\(^{2+}\)] stores. Figure 3 illustrates the protocol in a single SCG cell used to measure how aging may alter the spontaneous refilling of [Ca\(^{2+}\)] stores following caffeine-evoked depletion. This protocol was derived from a previous study in acutely dissociated dorsal root ganglion (DRG) 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 [Ca\(^{2+}\)] stores (2, 27). This variability to caffeine can be markedly reduced by initially depolarizing the cells to ensure equal loading of [Ca\(^{2+}\)] stores. Thus the cells were exposed for 5 s to high K\(^{+}\) (S\(_{1}\)) as a normalization control (data not shown) and to ensure a more uniform loading of [Ca\(^{2+}\)] stores (2, 27). Next caffeine-sensitive SER [Ca\(^{2+}\)] stores were depleted by a 10-s exposure to 10 mM caffeine (S\(_{2}\)) 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 s at the intervals indicated until the maximal response to caffeine following the “conditioning response” was obtained.

Data analysis. Calcium transients were analyzed using Logger Pro 6.1 software in all age groups. In protocol 1, peak [Ca\(^{2+}\)], was determined by subtracting basal [Ca\(^{2+}\)] from the maximum K\(^{+}\) or caffeine-evoked [Ca\(^{2+}\)] transients. Rate of rise of [Ca\(^{2+}\)] was determined by linear fit (r = 0.99 ± 0.07) from basal [Ca\(^{2+}\)] to the maximum K\(^{+}\) or caffeine-evoked [Ca\(^{2+}\)]. Rate of recovery of [Ca\(^{2+}\)] transients was determined by using a first-order exponential fit (r = 0.99 ± 0.06). In protocol 2, caffeine-evoked [Ca\(^{2+}\)] transients following the conditioning response were analyzed by taking the total area under the curve and normalizing to the area under the curve of the K\(^{+}\) control. Thus data are expressed as integrated caffeine-evoked [Ca\(^{2+}\)], as a percentage of the K\(^{+}\) control. These data were plotted vs. time after the conditioning response to estimate the spontaneous refilling of [Ca\(^{2+}\)] stores as shown in Fig. 4A. The rate of spontaneous refilling following caffeine-evoked release of calcium from [Ca\(^{2+}\)], stores was estimated by taking the derivative of the curves in Fig. 4A using Origin 6.1 and is expressed in Fig. 4B.

Statistics. The impact of age on all measured parameters was determined by using ANOVA and Fisher paired least significant difference test. All data in each age group were analyzed for heterogeneity of variance using the Cochran’s test. If the variances were significantly different, then values were log transformed and statistical tests were repeated (51).

RESULTS

Properties of high K\(^{+}\) concentration and caffeine-evoked [Ca\(^{2+}\)], transients. Using the protocol in Fig. 2A, we calculated the peak and rate of rise of the first and second high
Aging and Refilling of Caffeine-Sensitive Stores

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 s to 68 mM K⁺ and then for 10 s 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 were applied for 5 s until the maximum response to caffeine was achieved.

K⁺-evoked [Ca²⁺], transients in each age group, as shown in Fig. 4. There is a clear age-related effect on the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺], transients. These parameters increased in SCG cells from 6- to 12-mo-old animals and then progressively declined from 12 to 24 mo. Despite the decline in these parameters from 12 to 24 mo, the consistency of the dynamic response between the first and second high K⁺-evoked [Ca²⁺], transients appears to be maintained with advancing age. Again using the protocol in Fig. 2A, we calculated the peak and rate of rise of the first and second caffeine-evoked [Ca²⁺], transients in each age group, as shown in Fig. 5. In a similar fashion to high K⁺, these parameters increased in SCG cells from 6- to 12-mo-old animals and significantly declined from 12 to 24 mo. In contrast to the high K⁺-evoked [Ca²⁺], transients, the peak and rate of rise of caffeine-evoked [Ca²⁺], transients appear to decline from the first and second exposure to caffeine in SCG cells from 12- to 24-mo-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⁺], transients, we analyzed the percent difference between the first and second high K⁺ or caffeine-evoked [Ca²⁺], transients. When the data were analyzed in this manner, a clearer pattern emerges as shown in Fig. 6. There is no significant age-related decline in the percent difference in the peak and rate of rise of the first and second high K⁺-evoked [Ca²⁺], transients. In contrast to high K⁺, there is a significant age-related decline in the percent difference in the peak and rate of rise of the first and second caffeine-evoked [Ca²⁺], transient.

Spontaneous refilling of [Ca²⁺], stores following caffeine-evoked depletion. To enhance the rigor of our study on the impact of age on spontaneous refilling of [Ca²⁺], stores (Fig. 3), we performed a series of validation controls as shown in Fig. 7. In Fig. 7A, we demonstrate that [Ca²⁺], stores in resting SCG cells will refill in the presence of the L- and N-type voltage-gated calcium channel antagonists nifedipine and ω-conotoxin, respectively, following caffeine-evoked depletion of [Ca²⁺], stores. Note that, in the presence of the voltage-gated calcium channel antagonists, cells still exhibit robust caffeine-evoked release of [Ca²⁺],. In Fig. 7B, we show the efficacy of the L- and N-type channel calcium antagonists on high K⁺-evoked peak [Ca²⁺], transients. Note that both nifedipine and ω-conotoxin block ~97% of the K⁺-evoked Ca²⁺ 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 [Ca²⁺], following caffeine-evoked depletion.
and blockade of the SERCA-mediated refilling of \([Ca^{2+}]_i\) stores. However, in the continued presence of the SERCA blocker thapsigargin (THAPS), caffeine no longer elicits a response. Figure 7D demonstrates that La^{3+} abolishes the spontaneous calcium influx following caffeine-evoked depletion of \([Ca^{2+}]_i\) and blockade of SERCA by THAPS. Under these conditions, \([Ca^{2+}]_i\) no longer rises after depletion of \([Ca^{2+}]_i\) 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 \([Ca^{2+}]_i\) stores. After caffeine-evoked depletion \(S_2\), the cells were exposed for 5 s to caffeine at the time intervals shown in Fig. 3. The data in Fig. 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-s 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 \([Ca^{2+}]_i\) stores following caffeine-evoked depletion, we calculated the derivative of the curves in Fig. 8A and plotted these values as a function of age (Fig. 8B). The

**Fig. 5.** A and B: peak \([Ca^{2+}]_i\) evoked by the first and second exposure, respectively, to 10 mM caffeine in isolated SCG cells from animals aged 6–24 mo. C and D: rate of rise of \([Ca^{2+}]_i\) evoked by the first and second exposure, respectively, to 10 mM caffeine in isolated SCG cells from animals aged 6–24 mo. These data were derived from the protocol shown in Fig. 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. Values are means ± SE; \(n = 21–42\) cells from each age group. *Significantly different from 1 other age group, \(P < 0.05\). **Significantly different from 2 other age groups, \(P < 0.05\). ***Significantly different from 3 other age groups, \(P < 0.05\).

**Fig. 6.** A and B: impact of age on the percent difference between the first and second, respectively, \(K^+\)-evoked peak and rate of rise of \([Ca^{2+}]_i\) transients. C and D: impact of age on the percent difference between the first and second, respectively, caffeine-evoked \([Ca^{2+}]_i\) transients. The percent 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. Values are means ± SE; \(n = 21–42\) cells from each age group. *Significantly different from 1 other age group, \(P < 0.05\). **Significantly different from 2 other age groups, \(P < 0.05\). ***Significantly different from 3 other age groups, \(P < 0.05\).
derivatives were found to be significantly lower in SCG cells from 12-, 20-, and 24-mo-old animals compared with 6 mo (Fig. 8B).

Measurement of basal [Ca\(^{2+}\)] and rate of recovery of high K\(^+\) and caffeine-evoked [Ca\(^{2+}\)] transients. Table 1 shows the impact of age on basal [Ca\(^{2+}\)] following successive exposures to high K\(^+\) and caffeine using the protocol in Fig. 2A. Under all treatment conditions, basal [Ca\(^{2+}\)] significantly decreases in SCG cells from 6- to 12-mo-old animals and then significantly declines from 12 to 24 mo. 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 Fig. 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 are that release of calcium from [Ca\(^{2+}\)] stores and high K\(^+\)-evoked and spontaneous refilling of these stores decline with advancing age in SCG cells. The magnitude and shape of stimulation-evoked increases in [Ca\(^{2+}\)] are modulated by both influx 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\(^{2+}\)] stores is dependent on both influx and uptake into the SER via SERCA, the decline in refilling may reflect an age-related decline in the function of both mechanisms. Indeed, our laboratory has 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\(^{2+}\)] stores is an age-related decline in the function of SERCA pumps. As adrenergic nerves from the SCG serve to protect the central nervous system 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\(^{2+}\)] stores.** The data reported in this study (Fig. 2B), as well as published data, suggest that selective release of calcium from intracellular stores in 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 whether advancing age alters repeated caffeine-evoked release of [Ca\(^{2+}\)] after high K\(^+\)-evoked refilling (Fig. 2A). There was no age-related difference in the [Ca\(^{2+}\)] dynamics between the first and second high K\(^+\)-evoked [Ca\(^{2+}\)] transient (Fig. 6, A and 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 $[Ca^{2+}]_i$ dynamics between the first and second caffeine exposure within the oldest age groups (Fig. 6, C and D). There are numerous studies demonstrating the impact of age on depolarization-evoked $[Ca^{2+}]_i$ 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 $K^+$-evoked $[Ca^{2+}]_i$ transients is complex (Fig. 4), as depolarization-evoked $[Ca^{2+}]_i$ transients reflect both influx and release of calcium (46). Using patch-clamp methods coupled with measurement of $[Ca^{2+}]_i$ with fura 2, one study showed that stimulation-evoked calcium influx increases but that the measured $[Ca^{2+}]_i$ by fura 2 declines with age (28). The measurement of $K^+$-evoked $[Ca^{2+}]_i$ 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 $K^+$-evoked $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ stores following caffeine-evoked release occurred independently of voltage-gated calcium channels and appear 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, $[Ca^{2+}]_i$ begins to rise in SCG cells in a similar manner, as shown in previous studies (2, 47). Indeed, this rise in $[Ca^{2+}]_i$, 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 $[Ca^{2+}]_i$ stores in resting SCG cells following the release of $[Ca^{2+}]_i$ appears to be mediated by both SOCC and SERCA activity (2, 47).

Aging and spontaneous refilling of $[Ca^{2+}]_i$ stores. There are studies demonstrating that, in SCG and DRG cells, the $[Ca^{2+}]_i$ stores can spontaneously refill within 3–10 min following depletion with caffeine (11, 25, 45, 47). This spontaneous refilling of $[Ca^{2+}]_i$ 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 La$^{3+}$, and SERCA function is blocked by THAPS (2, 47). Because our laboratory has 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 $[Ca^{2+}]_i$, stores following caffeine-evoked depletion (Fig. 3). The rate of activation of SERCA2 (2, 47), in these cells is independent of voltage-gated $Ca^{2+}$ channels and

Table 1. Basal intracellular calcium concentration significantly declines from 12 to 24 mo following each treatment in protocol 1

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<th>6 Mo</th>
<th>12 Mo</th>
<th>20 Mo</th>
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<td>224.2±12</td>
<td>133.7±12.5</td>
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<td>1st Caffeine</td>
<td>135.6±8.5</td>
<td>183.1±9</td>
<td>120.5±12</td>
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<td>249.6±11</td>
<td>162.3±12</td>
<td>99.8±13</td>
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<tr>
<td>2nd Caffeine</td>
<td>158.6±9.2</td>
<td>209.1±7.5</td>
<td>138±10</td>
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</table>

Values are means ± SE; n = 21–42 cells from each age group. P < 0.05.

Table 2. Rate of recovery of intracellular calcium concentration transients with each treatment in protocol 1 in isolated superior cervical ganglia cells from 6- to 24-mo-old animals

<table>
<thead>
<tr>
<th></th>
<th>6 Mo</th>
<th>12 Mo</th>
<th>20 Mo</th>
<th>24 Mo</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st KCl</td>
<td>0.16±0.01</td>
<td>0.19±0.01</td>
<td>0.16±0.01</td>
<td>0.17±0.01</td>
</tr>
<tr>
<td>1st Caffeine</td>
<td>0.14±0.01</td>
<td>0.18±0.02</td>
<td>0.13±0.01</td>
<td>0.15±0.01</td>
</tr>
<tr>
<td>2nd KCl</td>
<td>0.15±0.01</td>
<td>0.18±0.01</td>
<td>0.16±0.01</td>
<td>0.16±0.01</td>
</tr>
<tr>
<td>2nd Caffeine</td>
<td>0.14±0.01</td>
<td>0.15±0.01</td>
<td>0.12±0.01</td>
<td>0.13±0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 21–42 cells from 12–20 animals. Rate of recovery was calculated by obtaining the first-order time constant using Origin 6.1. The reciprocal of the first-order time constant yields the rate constant.
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 [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 [Ca\(^{2+}\)]\(_i\) store is mediated by SOCC and SERCA function (2, 4, 47).

To our knowledge, this is the first study to demonstrate that spontaneous refilling of [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 [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, 44). We propose that at least one mechanism that may account for a decline in spontaneous refilling of [Ca\(^{2+}\)]\(_i\) stores is reduced SERCA function. However, these data do not rule out age-related changes in 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 to 12 mo and then steadily declined with age (Table 1). The increase in basal [Ca\(^{2+}\)]\(_i\) from 6 to 12 mo may reflect late maturational changes. In our laboratory’s 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-mo-old animals (32, 34). Comparison of the present 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). Because 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. Because 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 (Figs. 4 and 5).

Because 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 present 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 in 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 [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.

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**REFERENCES**

AGING AND REFILLING OF CAFFEINE-SENSITIVE STORES


46. Usachev YM and Thayer SA. All-or-none Ca$^{2+}$ release from intracellular stores triggered by Ca$^{2+}$ influx through voltage-gated Ca$^{2+}$ channels in rat sensory neurones. *J Neurosci* 17: 7404–7414, 1997.

47. Usachev YM and Thayer SA. Ca$^{2+}$ influx in resting rat sensory neurones that regulates and is regulated by ryanodine-sensitive Ca$^{2+}$ stores. *J Physiol* 519: 115–130, 1999.


