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Sustained Ca^{2+}-induced Ca^{2+}-release underlies the post-glutamate lethal Ca^{2+} plateau in older cultured hippocampal neurons

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Abstract

Several studies have shown that a prolonged Ca^{2+} elevation follows a glutamate-mediated excitotoxic insult in cultured neurons, and may be associated with impending cell death. Recently, we showed that the prolonged Ca^{2+} elevation that emerges as neurons age in culture is specifically linked to an age-related increase in excitotoxic vulnerability. However, the multiple sources of Ca^{2+} that contribute to Ca^{2+} elevation during and after glutamate exposure are not well understood. Here, we examined the Ca^{2+} sources of the age-related prolonged Ca^{2+} elevation in cultured hippocampal neurons. Studies with caffeine showed that the ryanodine receptor-dependent releasable pool of Ca^{2+} from intracellular stores was similar in older and younger neurons. Thapsigargin, which inhibits intracellular store refilling, did not mimic the age-related prolonged Ca^{2+} elevation and, in fact, partially reduced it. Ryanodine, which blocks Ca^{2+}-induced Ca^{2+}-release (CICR) from stores, completely blocked the age-related prolonged Ca^{2+} elevation following glutamate exposure but did not alter maximal Ca^{2+} elevation during the glutamate exposure. Thus, we conclude that sustained CICR plays a selective and key role in generating the lethal, age-related, prolonged Ca^{2+} elevation, and is the likely mechanism underlying age-related, enhanced vulnerability to excitotoxicity in neurons.

Keywords: Caffeine; Ryanodine; Thapsigargin; Aging; Excitotoxicity; Endoplasmic reticulum; Ca^{2+} imaging

1. Introduction

It is well established that excessive *N*-methyl-D-aspartate (NMDA) receptor activation, induced by exposure to glutamate or NMDA, is toxic to neurons (“excitotoxicity”) (Olney, 1986; Rothman and Olney, 1987), and that much of this effect is dependent on rapid increases in cytosolic Ca^{2+} (Choi, 1992; Lipton and Rosenberg, 1994). In addition to the Ca^{2+} influx during the glutamate insult, however, Ca^{2+} influx following exposure to glutamate plays an important role in excitotoxicity (Choi, 1992). During this post-glutamate period, prolonged Ca^{2+} elevations have been described, which in some studies have been correlated with impending cell death (Randall and Thayer, 1992; Dubinsky, 1993; Tymianski et al., 1993; Limbrick et al., 1995; Cheng et al., 1999). However, there appear to be multiple reversible and irreversible components of prolonged Ca^{2+} elevations, and it is not clear which are causes and which are effects of cell death (Randall and Thayer, 1992; Tymianski et al., 1993; Limbrick et al., 2001). In addition, the Ca^{2+} sources that contribute to the various components and sequelae of glutamate-induced Ca^{2+} elevations have not been clearly elucidated.

It also has been recognized for some years that neuronal cultures maintained in vitro over extended periods (e.g., 2–4 weeks) become increasingly more vulnerable to excitotoxic challenges (Choi et al., 1987; Peterson et al., 1989; Frandsen and Schousboe, 1990; Mattson et al., 1991; Xia et al., 1995; Adamec et al., 1998; Cheng et al., 1999; Toescu and Verkhratsky, 2000; Attucci et al., in press). Recently, using hippocampal neurons maintained in culture for different durations (i.e., 7–27 days in vitro), we determined that a large prolonged Ca^{2+} elevation develops in response to glutamate in specific temporal association with the appearance of enhanced, age-dependent vulnerability (Attucci et al., in press). That is, older neurons in culture display substantially more prominent prolonged Ca^{2+} elevations and are also much more vulnerable to excitotoxic insults than younger neurons. Furthermore, specific blockade of the

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prolonged Ca^{2+} elevation without blocking the glutamate exposure-associated Ca^{2+} elevation, using a type I metabotropic glutamate (mglu) receptor antagonist, conferred neuroprotection to older neurons. This age-dependent increase in the prolonged Ca^{2+} elevation did not appear to be associated with the amplitude of the glutamate Ca^{2+} elevation during the insult, as we maintained those elevations at similar levels in both age groups. Moreover, selective modulation of the prolonged Ca^{2+} elevation by a type I mglu receptor antagonist suggests that it may be generated by different Ca^{2+} sources from the glutamate-associated Ca^{2+} elevation. Thus, the emergence of a prominent prolonged Ca^{2+} elevation in older neurons in culture appears to be a key specific element in age-dependent vulnerability (Attucci et al., in press).

As noted, the sources of Ca^{2+} for this age-dependent prolonged Ca^{2+} elevation are not clear. Upon glutamate exposure, primary transmembrane Ca^{2+} influx contributes to Ca^{2+} elevations, but also recruits other Ca^{2+} sources, for example, via the mechanism of Ca^{2+}-induced Ca^{2+}-release (CICR) from the ryanodine receptor (Llano et al., 1994; Verkhratsky and Petersen, 1998). Blockade of Ca^{2+} release from endoplasmic reticulum Ca^{2+} stores also has been found to be neuroprotective, strongly suggesting that CICR is a source for some of the Ca^{2+} load associated with neurotoxicity (Frandsen and Schousboe, 1991; Mody and Macdonald, 1995; Korkotian and Segal, 1996; Jonas et al., 1997; GepdIremen et al., 2001). Some evidence also links intracellular release to the prolonged Ca^{2+} elevation. Korkotian and Segal (1996) found that perturbation of Ca^{2+} stores significantly decreased a nuclear form of the prolonged Ca^{2+} elevation. Conversely, however, Limbrick et al. (2001) reported no significant alterations in Ca^{2+} responses following an insult under conditions of store depletion. Therefore, it is still unclear whether the age-dependent prolonged Ca^{2+} elevation depends in part on elevated CICR and, if so, how this source is altered in older cultured neurons.

Age-dependent changes in Ca^{2+} regulation and vulnerability of neurons in culture also appear to have potential relevance to brain aging in vivo. Despite the large temporal differences in age courses, we and others have found that older hippocampal neurons in culture share many properties of aged neurons in vivo, including increased L-type Ca^{2+} channel activity, altered Ca^{2+} regulation, and astrocyte hypertrophy (Thibault and Landfield, 1996; Porter et al., 1997; Thibault et al., 1998; Blalock et al., 1999; Cheng et al., 1999; Toescu and Verkhratsky, 2000). Consequently, it seems interesting that Ca^{2+} stores also may be altered in physiological aging, and appear to be either empty or somewhat "reluctant" to refill (Kirischuk and Verkhratsky, 1996; Murchison and Griffith, 1999; Pottorf et al., 2000). On the other hand, in Alzheimer's disease models, release of Ca^{2+} from intracellular sources appears increased (Ito et al., 1994; Gibson et al., 1996; Leissring et al., 1999; Mattson et al., 2000).

In the present studies, therefore, we tested whether CICR of endoplasmic reticulum Ca^{2+} stores via the ryanodine receptor plays a major and specific role in generating the age-dependent prolonged Ca^{2+} elevation in older neurons in culture. Further, we addressed the mechanistic questions of whether there are concomitant changes in release, refilling, or Ca^{2+} content within the stores. Our results show that CICR is a major and selective source of Ca^{2+} for the prolonged Ca^{2+} elevation, but not for the glutamate-associated Ca^{2+} elevation, likely because of sustained activation of the ryanodine receptor in older neurons.

2. Material and methods

2.1. Hippocampal cultures

Primary hippocampal neuronal cultures were prepared as previously described (Porter et al., 1997). All procedures were performed in accordance with the regulations of the Institutional Animal Care and Use Committee at the University of Kentucky as well as international guidelines on the ethical use of animals. Briefly, fetal hippocampal tissue was taken from E18 Sprague–Dawley rat pups and placed in ice-cold Ca^{2+}- and Mg^{2+}-free Hank's Balanced Salt Solution (HBSS). The hippocampal tissue was then transferred to 10 ml HBSS containing 0.25% trypsin and 1 mM EDTA and treated enzymatically for 10 min at room temperature. The hippocampi were subsequently washed three times with 15 ml volumes of Minimum Essential Medium (MEM; supplemented with additional 30 mM glucose) and then dispersed by repeated trituration. The cell suspension was diluted with MEM to a final concentration of $3-5 \times 10^5$ cells/ml and 1 ml added to poly-L-lysine-coated (1 mg/ml) glass-bottom plastic culture dishes (35 mm; Mattek) which contained 1 ml MEM with 10% fetal bovine and 10% horse sera (previously incubated overnight at 36 °C in a humidified atmosphere of 5% CO_2 and 95% air). Cells were placed in the incubator and on the next day, half of the medium was exchanged for medium containing only MEM and 10% horse serum (MEM/H). To inhibit proliferation of non-neuronal cells, at 3 days in vitro, half the medium was replaced with MEM/H containing 5-fluoro-2′-deoxy-uridine (15 μg/ml); uridine (35 μg/ml) was also included to prevent the inhibition of RNA synthesis. Because evaporation occurs in cultures maintained for longer periods, culture dishes were supplemented with 0.2 ml 26 mM $NaHCO_3$ at 10 and 24 days in vitro. In addition, at 17 days in vitro, cultures were fed by removing 0.8 ml of medium and replacing with an equal amount of MEM/2.5% H. We have found this feeding schedule to be optimal for maintaining neurons over longer periods. For a typical experiment, approximately half of the cells plated were imaged at 6–10 days in vitro (younger cells). The remaining sister cultures were left in the incubator and imaged at 27–31 days in vitro (older cells).

2.2. Intracellular Ca^{2+} measurement

2.2.1. Loading of hippocampal neurons with Indo-1 or Fluo-3

Cultures were placed at room temperature in the dark for a 20-min incubation period in the following loading solution (in mM): 145 NaCl, 2.5 KCl, 10 HEPES, 10 glucose, 2 $CaCl_2$, 1 $MgCl_2$, 10 μM glycine, and either 2 μM acetoxymethyl ester (AM) of Indo-1 (Indo-1-AM) or 0.5 μM Fluo-3 (Fluo-3-AM). Both indicators were dissolved in dimethyl sulfoxide (DMSO) complemented with 20% Pluronic F-127 (w/v). Final DMSO and Pluronic F-127 concentrations were 0.04% and 0.01%, respectively. Cells were then washed three times with indicator-free loading solution (artificial cerebrospinal fluid (ACF)), and incubated for an additional 15 min to allow for indicator de-esterification. We estimated the intracellular Indo-1 concentration to be less than 50 μM, by comparing the emitted light intensities of AM-loaded cells as described above, with K^+ salt-loaded cells (see calibration below). All experiments were performed at room temperature.

2.2.2. Indo-1-based Ca^{2+} imaging

For studies of the age-dependent prolonged Ca^{2+} elevation, Ca^{2+} transients were acquired on an RCM 8000 UV-compatible confocal laser-scanning microscope (Nikon, New York) equipped with a Nikon diaphot 300 and a 40 × water immersion objective. Depth discrimination with the pinhole size used was 1.2 μm. For ratiometric Indo-1 imaging, two emitted wavelengths (<400 and >500 nm) were acquired simultaneously through a dichroic mirror centered at 445 nm. Excitation was provided by an Argon-laser generating multiline UV light (351–364 nm). Final images were obtained using signal averaging of 64 frames, each image corresponding to a 2-s segment. All signals were background-subtracted from an area adjacent to the cells of interest. The area of interest in which $[Ca^{2+}]_i$ was measured was drawn by hand using Metamorph imaging software (Universal Imaging, West Chester, PA) and consisted of the entire somatic region. Ratios of the two wavelength images were constructed in Metamorph and analyzed based on an in situ calibration (see below). Ratios were acquired immediately prior to glutamate (Glu) application (rest) and at 2, 10, 30 s, and 5 min during Glu application, as well as at 1, 5, 15, 30, and 45 min post-Glu (e.g., Fig. 1).

2.2.3. Fluo-3-based Ca^{2+} imaging

Because caffeine quenches Indo-1 fluorescence (O'Neill et al., 1990), we chose the single wavelength Ca^{2+} indicator Fluo-3 to monitor Ca^{2+} changes in caffeine experiments. Fluo-3 Ca^{2+} imaging and analysis were performed as with Indo-1 imaging, except that a single excitation wavelength (488 nm) was used, and a single emission wavelength (522 nm) was monitored. For Fluo-3 imaging, the change in fluorescence from the average resting fluorescence (F) of a particular cell was used to analyze percent Ca^{2+} changes

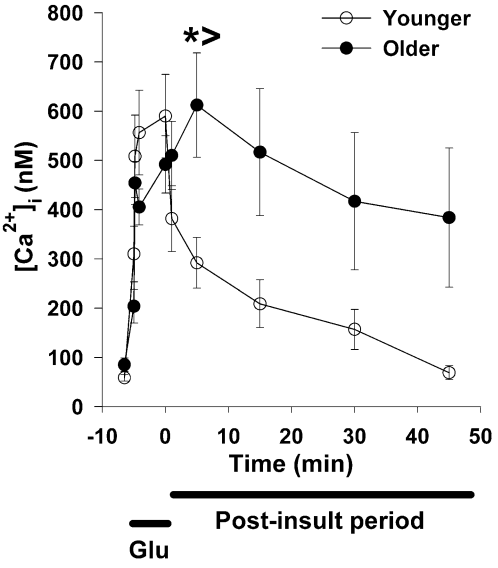

Fig. 1. Ca^{2+} responses to glutamate exposure in younger and older neurons. Ca^{2+} concentrations were monitored before, during, and after a 5-min glutamate application (Glu). Although similar peak $[Ca^{2+}]$ values were attained during glutamate exposure in both groups, a prolonged Ca^{2+} elevation followed the glutamate exposure in older cells. Because of major differences in Glu sensitivity, younger cells were exposed to 400 μM Glu while older cells were exposed to 10 μM Glu. Values represent means ± S.E.M. for somatic $[Ca^{2+}]$ measures. *>This point and every point thereafter were significantly different with culture age ($p<0.05$; post hoc test).

(%ΔF/F). For each cell, the average resting fluorescence was determined by calculating the mean of four images acquired before drug application. Single images were then acquired immediately upon caffeine application and at 5-s intervals for the duration of the application (30 s, seven measures), as well as during the post-application washout period at 30 s, 1 min, and 3.5 min (e.g., Fig. 2).

2.2.4. In situ calibration in hippocampal cultures

Estimates of Ca^{2+} concentrations were derived from the background-subtracted ratios of the two emitted wavelengths calibrated in situ. Hippocampal neurons, prepared and maintained as above, were loaded with a series of different Ca^{2+} concentrations (0 to 39.8 μM free Ca^{2+}, 1 mM Mg^{2+}, Molecular Probes standards, Eugene, OR) using patch pipettes for microinjection and an Axoclamp 200 amplifier in voltage-clamp mode. Cells were held at −70 mV. Indo-1 (pentapotassium salt, Molecular Probes) was dialyzed at two concentrations (50 or 100 μM) to determine the extent of intracellular Indo-1 loading. The bathing solution consisted of (in mM): 130 $LiCl_2$, 5 CsCl, 2 $MgCl_2$, 10 glucose, 10 HEPES, 0.001 TTX, and 0.1 D-(−)2-amino-4-phosphonopentanoic acid (AP5). $LiCl_2$ was used instead of $NaCl_2$ in order to decrease the natural driving force on Na^+ and, hence, the activity of the Na^+/Ca^{2+} exchanger. ATP was not included in the patch pipettes to reduce the activity of plasmalemmal Ca^{2+}–ATPase pumps (Bassani et al., 1995). AP5 and TTX were used to inhibit firing and subsequent transmitter release. Five to eight cells per Ca^{2+}

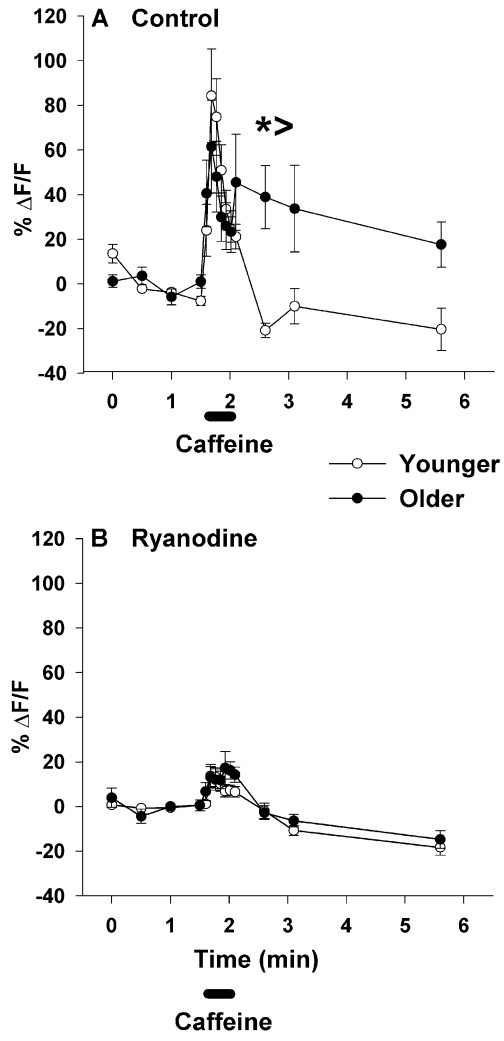

Fig. 2. Ca^{2+} responses to caffeine administration in younger and older neurons. (A) Caffeine administration (10 mM, 30 s) evoked similar peak responses during exposure in younger and older cells. However, slower recovery to baseline was seen in older neurons. (B) Pretreatment with 100 μM ryanodine for 15 min essentially blocked the caffeine-induced response in both younger and older neurons. Values are reported as means ± S.E.M. for $\%\Delta F/F$ measures. *>This point and every point thereafter were significantly different with culture age ($p < 0.05$; post hoc test).

standard solution were imaged to create a calibration curve. Ratios were converted to $[Ca^{2+}]$ by the equation $[Ca^{2+}]_i = K_d\beta(R - R_{min})/(R_{max} - R)$, in which R is the 400/500 nm fluorescence emission ratio of a particular cell and R_{max}, R_{min}, $K_d\beta$ were determined from the in situ calibration (Grynkiewicz et al., 1985; Poenie, 1990). Separate calibration curves were analyzed for younger and older cells and no differences in the parameters were found. The in situ calibration yielded values of 1.3 for R_{min}, 4.3 for R_{max}, and 0.8 for $K_d\beta$ (in μM).

2.3. Drug application

Ryanodine and thapsigargin were added to cultures throughout the 15-min de-esterification incubation and were also included in glutamate exposure and wash solutions for the remainder of the 1-h-long experiments. Glu was administered for 5 min at different concentrations to the younger and older neurons. Because of the major differences in sensitivity to excitotoxins of the two age groups, older cells were exposed to 10 μM Glu and younger cells to 400 μM Glu, concentrations which induce approximately equivalent magnitudes of peak $[Ca^{+}]$ elevation in the two groups (Attucci et al., in press). In a typical experiment, a perfusion fast-step system (SF-77A: Warner Instruments, Hamden, CT) was used to rapidly deliver Glu with or without ryanodine or thapsigargin. This system was also used in caffeine experiments. For rapid delivery, all drugs were dissolved in the above-mentioned ACF solution and delivered via square glass tubes, each 800 μm wide, micropositioned 400 μm above the cells of interest. In our cultures, most pyramidal cell somata are smaller than 20 μm and, therefore, the 800-μm-wide drug delivery square tube easily controlled the immediate environment bathing the cells of interest. Control/wash or drug solutions were applied through separate tubes by a gravity fed, manifold system. A step mechanism rapidly aligned one of the solutions (for example, vehicle or drug) above a neuron by using laterally incremented steps (800 μm in either direction). In addition, perfusion of the entire culture dish with ACF solution containing either vehicle or drug was given throughout the 5-min Glu exposure and the post-Glu period to maintain a constant volume as well as to wash out excess Glu.

It seemed important to maintain background synaptic activity intact in the cultures because network communication between cells significantly increases with days in vitro (Basarsky et al., 1994; Scholz and Miller, 1995) and could influence the Ca^{2+} store content at rest. Thus, TTX was not used in these experiments. Caffeine (10 mM) and ryanodine (100 μM) were dissolved in ACF solution and thapsigargin (100 nM) was dissolved from a 500 μM stock in 100% EtOH. A final EtOH concentration of 0.02% was used as a vehicle control in all thapsigargin experiments. A relatively low thapsigargin concentration was used to prevent blockade of plasma membrane Ca^{2+} channels (Shmigol et al., 1995).

2.4. Statistics

All differences were tested for significance at the $p < 0.05$ level, using two-way repeated measure analysis of variance (ANOVA) and Tukey post hoc analyses.

3. Results

As shown in Fig. 1, a major prolonged Ca^{2+} elevation develops following glutamate application in older but not in younger neurons in culture. In general, prolonged Ca^{2+} elevations can be blocked by NMDA receptor antagonists (Randall and Thayer, 1992; Dubinsky, 1993; Tymianski et

al., 1993) and are also completely blocked by dizocilpine (MK-801) in older cultures (Attucci et al., in press). Both younger and older neurons in the present study displayed similar peak [Ca^{2+}] levels during glutamate exposure (Fig. 1), yet the prolonged Ca^{2+} elevation was seen only in older neurons. Measures of the overall [Ca^{2+}] elevation obtained up to 45 min following the glutamate insult (Fig. 1) were significantly larger in older neurons ($F(1,9)=5.73$; $p<0.001$; $n=10$ younger and 11 older cells). The aging effect on the prolonged Ca^{2+} elevation was significant at 5 min post-insult and at every point thereafter ($p<0.05$; post hoc comparison).

Typically, older neurons in culture exhibit significantly larger Ca^{2+} responses than younger neurons during a glutamate exposure insult (Thibault et al., 1997; Cheng et al., 1999; Attucci et al., in press) apparently because of increased density of NMDA receptors (Xia et al., 1995; Cheng et al., 1999). However, our results show that induction of an age-related prolonged Ca^{2+} elevation does not depend directly on the magnitude of the [Ca^{2+}] elevation during the glutamate exposure. Glutamate-induced Ca^{2+} elevations in the present study were equalized in both age groups by utilizing different glutamate concentrations. The [Ca^{2+}] elevations were triggered with 400 μM glutamate in younger and 10 μM glutamate in older cells. Under these conditions, no significant differences were seen in maximal [Ca^{2+}] elevations during the insult. Thus, despite comparable prolonged Ca^{2+} elevations, younger neurons restored [Ca^{2+}] almost to pre-insult levels within 15 min after the insult, whereas [Ca^{2+}] remained significantly elevated in older neurons.

3.1. Caffeine

To test whether the ryanodine receptor-mediated releasable pool of Ca^{2+} differed in younger and older neurons, we exposed neurons to caffeine, a ryanodine receptor agonist, and measured Ca^{2+} responses using the single wavelength Ca^{2+} indicator, Fluo-3. A 30-s exposure to 10 mM caffeine evoked a clear release of Ca^{2+} from caffeine-sensitive stores in younger and older neurons ($n=8-9$ neurons/group, Fig. 2A). Peak Ca^{2+} and area under the curve values during the caffeine exposure were not significantly different in younger vs. older cells ($F(1,15)=0.6$; $p>0.5$). Although it is not possible from these results to separate the contributions of store content from sensitivity of the ryanodine receptor to caffeine, the lack of overall difference suggests that neither differed with age. Similarly, no age-dependent differences in caffeine sensitivity were observed previously in studies of dissociated basal forebrain neurons (Murchison and Griffith, 1999). Despite the brief (30 s) activation period, however, a significant lengthening of the Ca^{2+} recovery process was seen in older neurons beginning immediately after the caffeine application (Fig. 2A). This observation suggests that some form of the prolonged Ca^{2+} elevation can be triggered by ryanodine receptor activation even in the absence of NMDA receptor activation.

Ryanodine blocked the caffeine-induced release almost completely (Fig. 2B), indicating that the caffeine-induced Ca^{2+} elevation was mediated by activation of the ryanodine receptors. A 15-min pretreatment of cells with 100 μM ryanodine, which at this concentration should inhibit the ryanodine receptor channel (McPherson and Campbell, 1993), inhibited the major fraction of the caffeine-induced Ca^{2+} response in younger ($F(1,22)=12.6$; $p<0.005$) and older neurons ($F(1,16)=7.2$; $p<0.02$), as seen in Fig. 2B ($n=8-14$ neurons/group). Thus, at the concentrations used in this study, ryanodine appeared able to block nearly all ryanodine receptor-mediated Ca^{2+} release in both younger and older neurons in culture.

3.2. Ryanodine effects on the age-related prolonged Ca^{2+} elevation

In order to test the proposition that CICR from intracellular stores contributes to the age-related prolonged Ca^{2+} elevation, we treated younger and older neurons with the same concentration of ryanodine (100 μM) that inhibited release from the ryanodine receptor channel. In comparison to vehicle controls, [Ca^{2+}] responses following the glutamate insult were reduced dramatically in ryanodine-treated cells. This blocking effect was seen to a small degree in younger neurons (Fig. 3A, middle rows), but was especially pronounced on the large prolonged Ca^{2+} elevations that develop in the older neurons (Fig. 3B, middle rows). Quantitative analyses ($n=13-14$ younger and $6-7$ older neurons) revealed a highly significant effect of ryanodine on Ca^{2+} levels irrespective of age ($F(1,1)=8.9$; $p<0.01$). However, post hoc analyses showed that only the last two time points differed in the younger neurons (Fig. 4A, $p<0.05$), whereas all post-glutamate exposure points differed with ryanodine treatment in older neurons (Fig. 4B, $p<0.05$). In older neurons, ryanodine blocked the large prolonged Ca^{2+} elevation essentially completely, making the overall Ca^{2+} response curve nearly identical to that in younger neurons (Fig. 4A and B). The small effect on the post-insult response in younger neurons (Fig. 4A) shows that the prolonged Ca^{2+} elevation has already begun to develop in some younger neurons (e.g., by 9–11 days in vitro, Attucci et al., in press).

Particularly notable was the observation that ryanodine did not alter the maximal Ca^{2+} elevation during the glutamate exposure (Fig. 4A and B). However, ryanodine exerted a clear trend to reduce the prolonged Ca^{2+} elevation by the 5-min glutamate exposure point ($p<0.05$, in younger neurons). Ryanodine exerted no effect on resting [Ca^{2+}] in older or younger neurons.

3.3. Thapsigargin effects on the age-related prolonged Ca^{2+} elevation

To determine whether altered refilling of intracellular stores and resultant sustained elevation of cytosolic Ca^{2+} might play a role in generating the age-related prolonged

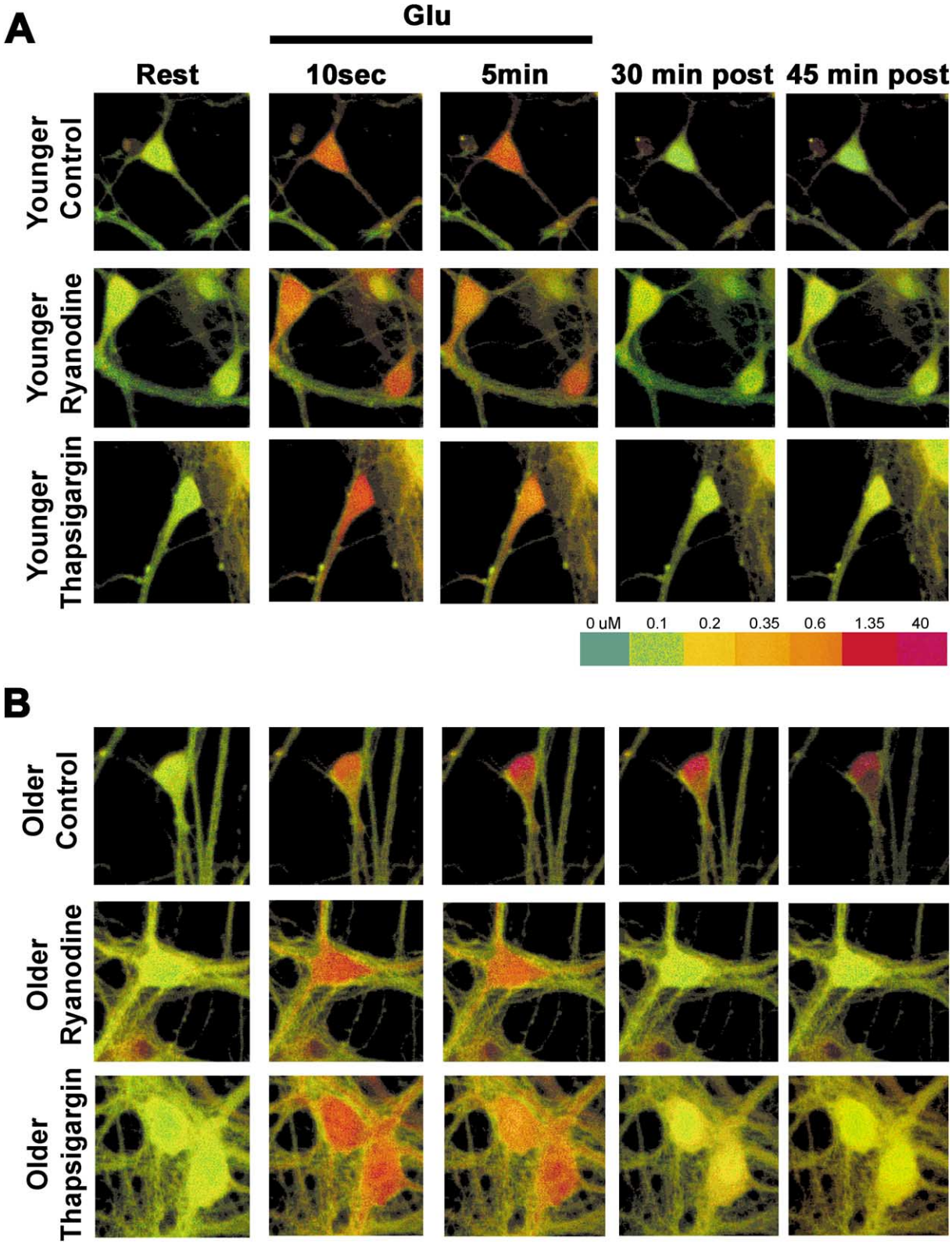

Fig. 3. Ryanodine and thapsigargin selectively attenuated the prolonged Ca^{2+} elevation. Younger (A) and older neurons (B) were exposed to 400 and 10 μM glutamate, respectively, for 5 min in the presence of either control solution (top row), ryanodine (middle row), or thapsigargin (bottom row). Representative pseudocolor images are presented at rest, during the first 10 s and the last 10 s of the 5-min glutamate exposure (bar), as well as at 30 and 45 min after the glutamate exposure. Note that in younger neurons, $[Ca^{2+}]$ elevations are restored to near baseline during the post-insult period (A). However, in the older control neurons, elevated $[Ca^{2+}]$ persisted and cell lysis and dye leakage were seen occasionally at 45 min post-insult (B). Ryanodine and thapsigargin both facilitated recovery of post-glutamate $[Ca^{2+}]$ elevations in older neurons.

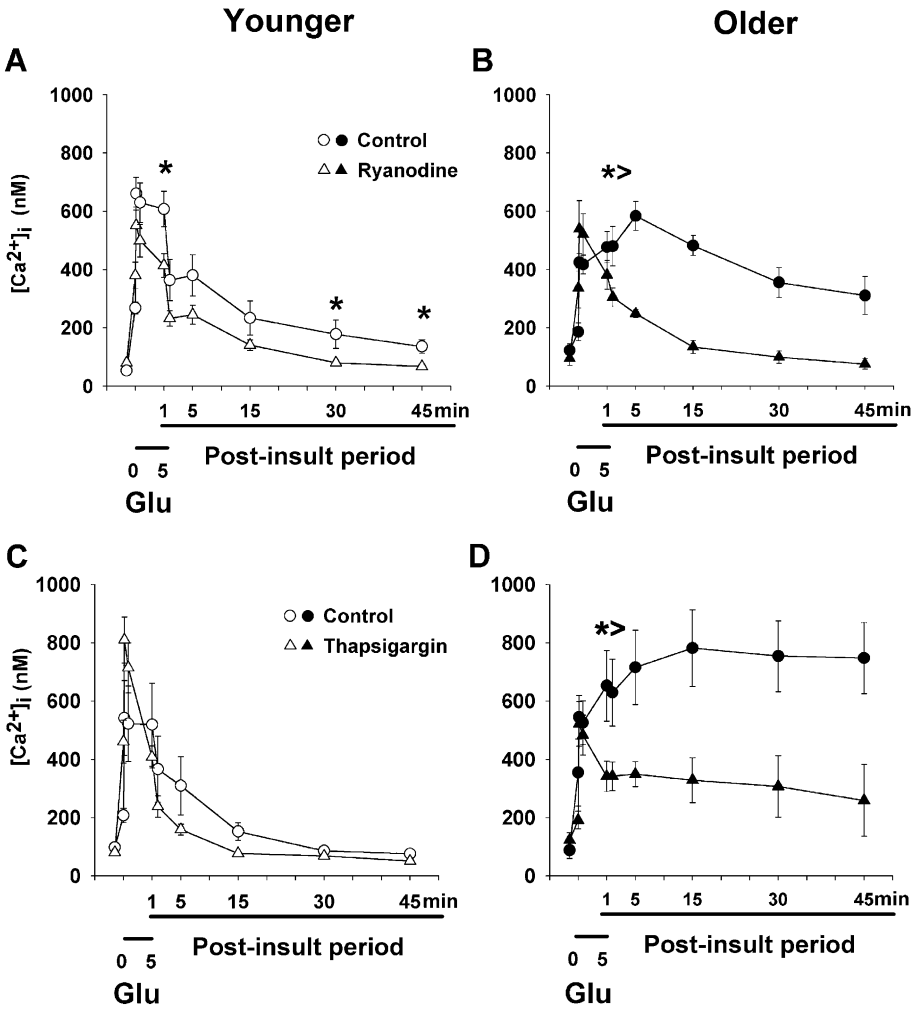

Fig. 4. Mean ± S.E.M. $[Ca^{2+}]$ measures in control, ryanodine, and thapsigargin groups. Younger (A) and older (B) cells were treated with ryanodine 15 min prior to, during, and after a 5-min glutamate insult. Ryanodine resulted in significant reductions in $[Ca^{2+}]$ elevations attained in the post-insult period in older neurons (B), but did not affect peak $[Ca^{2+}]$ responses during the 5-min glutamate exposure. * $p<0.05$ by post hoc test. Younger (C) and older (D) cells treated with thapsigargin using the same protocol showed a similar decrease in post-insult $[Ca^{2+}]$ elevation (D), although not to the same degree as older ryanodine-treated cells (B). Neither ryanodine nor thapsigargin affected $[Ca^{2+}]$ responses during the 5-min glutamate exposure, except at the 5-min point (A, D). *>This point and every point thereafter were significantly different with drug treatment ($p<0.05$; post hoc test; except for 5-min Glu-associated Ca^{2+} elevation and 1-min age-related prolonged Ca^{2+} elevation (D) which were nearly significant at $p<0.07$ level).

Ca^{2+} elevation, we also examined the effects of treatment with thapsigargin. Thapsigargin (100 nM) exposure irreversibly inhibits Ca^{2+} uptake into the endoplasmic reticulum via inhibition of sarcoplasmic-endoplasmic reticulum Ca^{2+}–ATPase (SERCA) pumps (Thastrup et al., 1990; Shmigol et al., 1995). This effect also leads to store depletion because of Ca^{2+} leakage (Verkhratsky and Petersen, 1998). If reduced SERCA function contributes to the prolonged Ca^{2+} elevation, therefore, inhibition of SERCAs by thapsigargin should tend to enhance or mimic such elevations.

As shown in Fig. 3A and B (bottom rows), the effect of thapsigargin on glutamate-induced Ca^{2+} responses was similar in direction but somewhat reduced in comparison to ryanodine (middle rows). Thapsigargin decreased but did not completely block the prolonged Ca^{2+} elevation in older neurons. Two-way ANOVA revealed a significant interaction between age of the culture and thapsigargin's reduction in Ca^{2+} levels ($F(1,36)=6.1$; $p<0.02$). In younger neurons, thapsigargin did not significantly affect post-glutamate Ca^{2+} responses (Fig. 4C). However, thapsigargin significantly reduced the prolonged Ca^{2+} elevation in older cells ($F(1,12)=4.6$; $p<0.05$; Fig. 4D). Again, thapsigargin had no effect on resting $[Ca^{2+}]$ or maximal $[Ca^{2+}]$ elevations during the glutamate insult ($n=7-15$ neurons/group), although it essentially ($p<0.06$) reduced the glutamate-associated Ca^{2+} elevation at 5 min of glutamate in the older cells (Fig. 4D). As with ryanodine pretreatment, therefore, thapsigargin's effect was substantially more pronounced in older cells that exhibit a large prolonged Ca^{2+} elevation. Thus, thapsigargin did not increase the prolonged Ca^{2+} elevation in younger or older neurons, as should be predicted if impaired SERCA function contributes significantly to generating the age-related prolonged Ca^{2+} eleva-

tion. Rather, thapsigargin reduced the age-related prolonged Ca^{2+} elevation, much as did ryanodine receptor inhibition.

4. Discussion

4.1. Release from ryanodine-sensitive stores contributes to the prolonged Ca^{2+} elevation but not the glutamate-associated Ca^{2+} elevation

Our results suggest that intracellular Ca^{2+} stores contribute significantly to age-dependent [Ca^{2+}] elevations in the period following exposure to glutamate. As shown here, generation of the age-related prolonged Ca^{2+} elevation is almost completely ryanodine-sensitive (Fig. 4B). Further, the presence of a significant Ca^{2+} plateau following caffeine application in older neurons (Fig. 2) supports the idea that in older cells, activation of the ryanodine receptor may induce significant and prolonged [Ca^{2+}] elevation, irrespective of whether such activation results from Ca^{2+} influx via the NMDA receptor or from direct caffeine stimulation. In addition, particularly striking was the observation that ryanodine exerted no effects on [Ca^{2+}] elevation during the early phases of the glutamate exposure-associated Ca^{2+} elevation (Fig. 4A and B). These findings support the proposition (Attucci et al., in press) that the post-glutamate Ca^{2+} elevation is regulated selectively and differently, and is generated by different Ca^{2+} sources, from the glutamate-associated Ca^{2+} elevation.

The finding that neither ryanodine nor thapsigargin significantly altered the early maximal glutamate exposure-associated Ca^{2+} elevation is somewhat surprising considering that synaptic activation and single action potentials can give rise to CICR in hippocampal neurons (Alford et al., 1993; Miller et al., 1996; Jacobs and Meyer, 1997; Sandler and Barbara, 1999), and neurons exposed to excitotoxic challenges depolarize and fire action potentials during the early part of the insult (Coulter et al., 1992; Norris et al., 2000). Furthermore, it has been reported that Ca^{2+} store depletion can affect both rapid (Segal and Manor, 1992) and longer lasting NMDA-induced [Ca^{2+}] elevations (i.e., 2 min; Simpson et al., 1993). Nevertheless, here, we did not detect a significant effect of ryanodine or thapsigargin during the early glutamate exposure. These apparent discrepancies may be related to several explanations, including that different neuronal cell types exhibit different CICR responses (Shmigol et al., 1994; Verkhratsky and Petersen, 1998), that different regions of neurons may show different patterns of CICR, or that large Ca^{2+} influxes during intense glutamate stimulation may overwhelm and obscure CICR in the early phases of exposure. On the other hand, our observations that ryanodine and thapsigargin inhibit the [Ca^{2+}] elevation at the 5-min point of glutamate exposure (Fig. 4A and D) suggest that a delayed or progressively activating CICR response develops over several minutes in hippocampal neurons under our exposure conditions. Thus, our data are consistent with the view that CICR appears to be slowly activated in hippocampal neurons but, in older cells, is also substantially prolonged and non-self-terminating. As noted, however, alternative mechanisms might also account for the prolonged Ca^{2+} elevation. These are discussed further below.

4.2. Caffeine-sensitive Ca^{2+} store contents appear similar in younger and older neurons

The almost complete blockade of the caffeine-induced Ca^{2+} rise by a ryanodine pretreatment (Fig. 2B) indicates that caffeine's mode of action was primarily restricted to the activation of the ryanodine receptor. In addition, caffeine's specificity for the ryanodine receptor in such conditions has been established previously in experiments showing that caffeine's effects on [Ca^{2+}] do not result from its actions on intracellular phosphorylation status (Sandler and Barbara, 1999) or membrane potential (Akaike and Rhee, 1997; Garaschuk et al., 1997).

Based on this specificity, therefore, our caffeine experiments indicated that younger and older hippocampal neurons have similar ryanodine receptor-activated Ca^{2+} stores (Fig. 2A). Further, the similar responses to the same concentration of caffeine suggest that both Ca^{2+} store content and ryanodine receptor sensitivity were relatively similar in younger and older neurons. However, this conclusion will need to be confirmed in concentration–response studies with a ratiometric indicator.

4.3. Altered store refilling as a possible basis for the prolonged Ca^{2+} elevation

As noted, impaired SERCA function might well generate a prolonged Ca^{2+} elevation because of inability to clear the large Ca^{2+} transients resulting from NMDA receptor activation. However, impaired SERCA function does not appear to be the basis for the prolonged Ca^{2+} elevation in this preparation, as thapsigargin, which blocks SERCA function, did not generate a prolonged Ca^{2+} elevation in younger neurons or a larger prolonged Ca^{2+} elevation in older neurons. Instead, thapsigargin reduced the prolonged Ca^{2+} elevation, much like ryanodine, strongly suggesting that this effect also resulted from reduced CICR arising from thapsigargin's well established effect on store depletion. Moreover, the observation that thapsigargin did not completely block the age-related prolonged Ca^{2+} elevation may well reflect recent findings that the stores cannot be completely depleted (Solovyova et al., 2002). Thus, even in the presence of thapsigargin, sufficient Ca^{2+} apparently remains in the stores to generate a partial prolonged Ca^{2+} elevation via CICR. Nevertheless, blocked SERCA function was not able to generate (Fig. 4C) or amplify (Fig. 4D) the age-related prolonged Ca^{2+} elevation, even in conjunction with the large [Ca^{2+}] elevations induced by glutamate exposure. However, these

conclusions must be tempered by the potentially confounding effects of thapsigargin on store depletion. That is, partial store depletion could prevent observation of an enhanced prolonged Ca^{2+} elevation in thapsigargin-treated older neurons (Fig. 4D). Experiments in which thapsigargin is administered only in the post-insult period will more definitively resolve this question.

The converse question may also be raised, namely, whether increased SERCA function might develop in older neurons. This could conceivably accelerate refilling and lead to greater CICR and, in turn, generation of a prolonged Ca^{2+} elevation. However, this possibility also seems unlikely because, in that case, thapsigargin at the concentration used should fully block SERCAs and, therefore, also block the prolonged Ca^{2+} elevation completely. Instead, a significant age-related prolonged Ca^{2+} elevation remained in the presence of thapsigargin (Fig. 4D).

4.4. Alternative mechanisms

We show here an almost complete block of the prolonged Ca^{2+} elevation in older neurons treated with ryanodine. Therefore, it appears clear that Ca^{2+} released from ryanodine receptors contributes critically to the age-related prolonged Ca^{2+} elevation. In addition, thapsigargin did not either mimic or completely block the age-related prolonged Ca^{2+} elevation, indicating that altered SERCA function is not the basis for the age-related prolonged Ca^{2+} elevation. However, alterations in other Ca^{2+} handling/buffering systems could also contribute, either directly or indirectly, to the appearance of a prolonged Ca^{2+} elevation in older neurons. That is, even if ryanodine receptor and SERCA function were normal, impaired clearance or sequestration of Ca^{2+} by other processes could result in cytosolic accumulation of Ca^{2+} released from the ryanodine receptor. In fact, multiple mechanisms have been shown and/or proposed to play a role in lengthening of Ca^{2+} transients with age, including weaker Na^+/Ca^{2+} exchangers (Segal and Manor, 1992; Khodorov et al., 1993), Na^+ overload and subsequent reversal of the Na^+/Ca^{2+} exchangers (Choi, 1992), altered mitochondrial Ca^{2+} sequestration possibly followed by sustained release (Kiedrowski and Costa, 1995; Murchison and Griffith, 2000), mitochondrial depolarization (Vergun et al., 1999; Murchison and Griffith, 2000; Toescu et al., 2000), and weakened plasma membrane and intracellular Ca^{2+}–ATPases (Mironov, 1995; Michaelis et al., 1996).

Under our conditions, however, two treatments (thapsigargin and ryanodine) that reduce CICR via the ryanodine receptor by very different mechanisms, reduced or blocked the age-related prolonged Ca^{2+} elevation. In addition, activation of the ryanodine receptor by caffeine elicited a prolonged Ca^{2+} response. As noted, therefore, it seems clear that CICR is the source of Ca^{2+} for the age-related prolonged Ca^{2+} elevation, even if some altered clearance process provides the mechanistic basis for the prolongation of Ca^{2+} transients. Nonetheless, if the latter were the case, it is not clear why that altered clearance would not also result in a prolongation of the glutamate exposure-associated Ca^{2+} elevation in the face of ryanodine-dependent suppression of prolonged Ca^{2+} elevation (Fig. 4B).

4.5. Sustained CICR appears to underlie the age-related Ca^{2+} elevation

Based on the above considerations, we suggest that the most parsimonious and consistent hypothesis is that the age-related Ca^{2+} elevation depends largely, if not solely, on a mechanism of sustained CICR. As noted, a number of studies have shown that, under excitotoxic conditions, the amplification of primary Ca^{2+} influx pathways (e.g., NMDA and L-type Ca^{2+} channels) by CICR appears to increase toxicity or the duration/amplitude of toxic Ca^{2+} elevations (Bouchelouche et al., 1989; Frandsen and Schousboe 1991; Simpson et al., 1993; Mody and MacDonald, 1995; Korkotian and Segal, 1996). Here, however, we provide novel evidence that this major Ca^{2+} source contributes to Ca^{2+} elevations predominantly and specifically in the period following the insult and does so almost exclusively in older neurons. Thus, CICR appears to contribute little to the initial Ca^{2+} transients during excitotoxic insult in cultured hippocampal neurons.

Two main alternative mechanisms appear able to account for sustained CICR. Either the properties of the ryanodine receptor are altered (e.g., decreased inactivation), or the source of Ca^{2+} that triggers CICR, or fills the stores via non-SERCA pathways, is increased. The evidence of a plateau following caffeine application in older neurons (Fig. 2A) suggests that the ryanodine receptor responses in older neurons may well be altered. On the other hand, there is substantial evidence that Ca^{2+} influx is greater in older cells. The density of L-type Ca^{2+} channels increases with age both in vivo (Thibault and Landfield, 1996) and in vitro (Porter et al., 1997; Blalock et al., 1999). These L-type channels couple to Ca^{2+} stores (Chavis et al., 1996) and contribute to store-mediated toxicity (GepdIremen et al., 2001) and, therefore, could increasingly activate the CICR or accelerate store filling. This possibility is also consistent with evidence that an mglu receptor-dependent process couples L-type Ca^{2+} channels and ryanodine receptors (Chavis et al., 1996), because a group I mglu receptor antagonist (1-aminoindan-1, 5-dicarboxylic acid; (AIDA)), significantly diminishes the age-related prolonged Ca^{2+} elevation and protects older neurons against excitotoxicity (Attucci et al., in press). Further, the neuroprotective effects of dantrolene were shown to be enhanced by concurrent application of the L-type Ca^{2+} channel antagonist nimodipine (GepdIremen et al., 2001). Nonetheless, L-type Ca^{2+} channels are subject to voltage-dependent inactivation, and it is not clear that L-type channels would remain functional during the prolonged depolarization that accompanies the 5-

min excitotoxic insult and the post-insult period (Coulter et al., 1992; Norris et al., 2000).

Clearly, another potential source of increased Ca^{2+} influx as a trigger for sustained CICR is the NMDA receptor. NMDA receptor density increases in older neurons in culture (Cheng et al., 1999) as does NMDA receptor-induced current (Brewer et al., 2000). We recently found that the age-related post-insult Ca^{2+} elevation depends on sustained NMDA receptor-mediated depolarization during the post-insult period (Norris et al., 2000), suggesting that prolonged Ca^{2+} influx via increased NMDA receptors, and perhaps increased L-type Ca^{2+} channels, in older neurons could induce sustained CICR. On the other side, this mechanism does not appear able by itself to account for prolonged Ca^{2+} elevations in response to caffeine (Fig. 2). Thus, it seems conceivable that sustained CICR in older neurons arises from a complex interaction between both increased Ca^{2+} influx and altered properties of ryanodine receptors.

4.6. Functional implications

In contrast to older cultured neurons, aging hippocampal neurons in vivo do not exhibit increased NMDA-mediated currents (Barnes et al., 1997; Magnusson, 1998). Thus, any mechanism that depends on enhanced NMDA receptor activation may not have high functional relevance for normal brain aging.

However, because increased Ca^{2+} influx, regardless of its source, may result in increased CICR, the increased density/activity of L-type Ca^{2+} channels in aged hippocampal neurons in vivo as well as in older neurons in vitro (Thibault and Landfield, 1996; Porter et al., 1997; Blalock et al., 1999; Norris et al., 2002) could represent a mechanism for boosting Ca^{2+} elevations via increased CICR during brain aging.

Recent studies that imaged $[Ca^{2+}]$ in adult and aged hippocampal neurons during repetitive synaptic stimulation seem consistent with this possibility. During stimulation, $[Ca^{2+}]$ was elevated more in aged rat neurons, but only after several seconds of suprathreshold stimulation (Thibault et al., 2001). Those results suggest that major Ca^{2+} dyshomeostasis did not develop until $[Ca^{2+}]$ elevations reached some critical value. Given that we found a significant delay before Ca^{2+} from stores begins to affect $[Ca^{2+}]$ (Fig. 4A and D), the delayed enhancement of $[Ca^{2+}]$ in aged neurons (Thibault et al., 2001) could well reflect induction of CICR.

Thus, the present evidence of sustained CICR in hippocampal neurons appears to raise the possibility that elevated L-type Ca^{2+} channel activity in aged hippocampal neurons may also have the potential to trigger deleterious increases in CICR. If so, this mechanism for amplifying altered Ca^{2+} homeostasis could have substantial implications for normal brain aging and/or Alzheimer's disease (Disterhoft et al., 1994; Thibault et al., 1998; Verkhratsky and Toescu, 1998; Paschen and Frandsen, 2001).

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