Caffeine Releasable Stores of Ca\(^{2+}\) Show Depletion Prior to the Final Steps in Delayed CA1 Neuronal Death

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Abstract

Caffeine releasable stores of Ca\(^{2+}\) show depletion prior to the final steps in delayed CA1 neuronal death. J Neurophysiol 92: 2960–2967, 2004. First published June 16, 2004; 10.1152/jn.00015.2004. In addition to their role in signaling, Ca\(^{2+}\) ions in the endoplasmic reticulum also regulate important steps in protein processing and trafficking that are critical for normal cell function. Chronic depletion of Ca\(^{2+}\) in the endoplasmic reticulum has been shown to lead to cell degeneration and has been proposed as a mechanism underlying delayed neuronal death following ischemic insults to the CNS. Experiments here have assessed the relative content of ryanodine receptor-gated stores in CA1 neurons by measuring cytoplasmic Ca\(^{2+}\) increases induced by caffeine. These measurements were performed on CA1 neurons, in slice, from normal gerbils, and compared with responses from this same population of neurons 54–60 h after animals had undergone a standard ischemic insult: 5-min bilateral occlusion of the carotid arteries. The mean amplitude of responses in the postischemic population were less than one-third of those in control or sham-operated animals, and 35% of the neurons from postischemic animals showed very small responses that were ~10% of the control population mean. Refilling of these stores after caffeine challenges was also impaired in postischemic neurons. These observations are consistent with our earlier finding that voltage-gated influx is sharply reduced in postischemic in CA1 neurons and the hypothesis that the resulting depletion in endosomal Ca\(^{2+}\) is an important cause of delayed neuronal death.

INTRODUCTION

Much of the literature in the field of neuronal degeneration deals with conditions of Ca overload and chronically elevated intracellular Ca\(^{2+}\) levels as being direct or indirect effectors of cell death (Choi and Rothman 1990; Kistian and Siesjo 1998; Lipton 1999; Shuttleworth and Connor 2001). While this is not in question in many important situations where death is more or less immediate, there is a least one CNS model system, delayed neuronal death in CA1 hippocampus after global ischemia, that does not fit the high Ca\(^{2+}\) pattern. Here, there is very little immediate change in the morphology or physiology of neurons in the hours immediately following an ischemic insult as opposed to the rapid damage observed in areas such as the striatum (Kirino 1982; Pulsinelli et al. 1982). It is only after 2–5 days, depending on the severity of the insult, that area CA1 becomes severely compromised. In the intervening time period, where neurons have near normal electrical function, we have shown that activity generated Ca\(^{2+}\) influx; hence, Ca\(^{2+}\) loading is severely depressed, not elevated (Connor and Shuttleworth 2001; Connor et al. 1999). In the extreme, such a depletion would result in impaired protein synthesis (Brostrom and Brostrom 1990; Paschen et al. 1996) and interfere with protein folding (DeGracia et al. 2002; Treiman 2002; Wileman et al. 1991), which over an extended time course, could underlie cell pathology (see also Paschen and Doutreil 1999; Paschen et al. 1998). In this study, we have probed for differences in Ca content of the ER in CA1 neurons from normal animals and from postischemic animals ~2 days after a standard 5-min global occlusion of blood flow in the Mongolian gerbil. Ca content of the ER has been measured by assessing cytosol Ca\(^{2+}\) increases following pulse application of the commonly used reagent caffeine.

METHODS

Ischemia

Transient bilateral common carotid artery occlusion was performed on adult male Mongolian gerbils (55–70 g) anesthetized with 2% halothane (Lin et al. 1990). Carotid arteries were exposed and clipped, using atraumatic vascular clamps. The clips were removed after 5 min, and restoration of flow was verified. The temperature at rectal and temporialis muscle recording sites was maintained at 37–38°C throughout the procedure. Animals were allowed to recover for 50–56 h before death for brain slice preparation. Results from postischemic animals (n = 9) were compared with data from sham-operated gerbils (n = 2) and from unoperated control animals (n = 10). The person performing the experiments was different from the person performing the surgeries and was blinded to whether animals were sham-operated or postischemic.

The effectiveness of the occlusion protocol was reconfirmed by cresyl violet histochemistry, performed on five postischemic and two sham-operated gerbils. Four of the five hippocampi from postischemic animals showed almost complete loss of CA1 pyramidal neurons with

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preservation of CA3 pyramidal neurons, as described previously in rat (Pulsinelli et al. 1982) and in gerbil (Kirino 1982). The fifth showed unilateral loss of CA1 pyramidal neurons.

Slice preparation

Gerbils were anesthetized using ketamine/xylazine (40/5 mg/kg, im) and decapitated, and the brains were removed and placed in ice cold cutting solution. Cutting solution contained (in mM) 3 KCl, 1.25 NaH2PO4, 6 MgSO4, 26 NaHCO3, 0.2 CaCl2, 10 glucose, 220 sucrose, and 0.43 ketamine and was equilibrated with 95% O2-5% CO2. Coronal sections (350 μm) were cut using a Vibratome (Technical Products International, St. Louis, MO), and slices were transferred into room temperature artificial cerebrospinal fluid (ACSF) containing in mM: 126 NaCl, 3 KCl, 1.25 NaH2PO4, 1 MgSO4, 26 NaHCO3, 2 CaCl2, and 10 glucose, equilibrated with 95% O2-5% CO2. Cutting and recording solutions were both 315 mOsmol. After warming to 34°C and holding for 1 h, ACSF was changed again, and slices were held at room temperature until used for recording. Individual slices were transferred to the recording chamber and were superfused with warmed (35°C), oxygenated ACSF at 2 ml/min.

Fura-2 imaging

Cells were loaded with fura-2 using local infusion of the acetyloxy methyl ester form of the indicator, fura-2 AM. The approach follows a method first described by Regehr and Tank (1991) and has been used previously to load gerbil CA1 neurons (Connor et al. 1999). Pressure ejection pipettes were fabricated from capillary tubing (1.5/0.86 mm OD/ID) that was pulled in two stages (PP-83, Narishigi) and superfluted with warmed (35°C), oxygenated ACSF at 2 ml/min.

RESULTS

Responses to caffeine fura-2-AM–loaded slices

Figure 1 shows a band of distinct cell bodies that corresponds to the pyramidal cell layer as determined from inspection under transmitted illumination. The cell body fluorescence is superimposed on a diffuse fluorescence that arose largely from cells deeper in the slice that were out of focus. Figure 1B shows an excitation ratio image (350/380 nm) of this region prior to caffeine application. In this slice, as in many, there were cells that showed high prestimulus ratios (Fig. 1B, arrows). These cells were excluded from analysis because there was the possibility that they had been damaged by the slicing procedure. Resting intracellular Ca2+ levels measured in zero Ca2+ ACSF (Fig. 1B) were uniformly low, with a mean fura-2 ratio of 0.94 ± 0.03 recorded from 25 control preparations. No spontaneous oscillations in fura-2 signal were observed over 10-min control recording periods. To allow clear attribution of signals to intracellular release, each preparation was exposed to zero Ca2+ ACSF (with EGTA) for 7 min, before the first caffeine challenge (Fig. 1E, timeline). Caffeine was provided by pressure ejection (3 s, 10 mM) from a pipette tip positioned above the cells of interest (see METHODS). Figure 1C shows the peak response to the initial caffeine application in zero Ca ACSF. Numerical data were taken from the region shown by the outline in the ratio pictures (see METHODS) The analysis region was always chosen to be in the maximally responding area (Fig. 1C), centered around the stream of caffeine-saline flow. Figure 1D shows the time course of the Ca2+ transient measured in the outlined region of interest following the initial caffeine challenge.

To allow comparison of caffeine responses between slices and animals, stringent criteria were applied to ensure reproducible electrode placement and ejection flow (see METHODS). Therefore we could obtain the population data for caffeine responses in the control slices presented below.

Assessment of Ca2+ release from caffeine-sensitive stores

Brief exposures to the agonist caffeine were used to assess the content of ER Ca2+ stores. Since these stores discharge relatively rapidly, slow bath exchange of caffeine in the perfusate leads to underestimation of store content (data not shown). We therefore used local application of caffeine from microelectrodes placed close to the cells of interest. For caffeine application, ejection pipettes were prepared as described above for fura-2 loading and were filled with ACSF containing 10 mM caffeine. In experiments where challenges were made in zero Ca2+, the pipette contained zero Ca/EGTA ACSF. Pipette tips were positioned around 30 μm from the CA1 pyramidal layer (basal side). Caffeine challenge was a 3-s pulses at ~1 psi, delivered by a Picospitzer (General Valve). Fluid ejection was verified by observing slight movement of the fluorescently labeled pyramidal cell somata during the pressure pulses and their rapid return after termination of the stimulus. Pressure pulses sufficient to produce larger movements when ACSF alone was ejected produced no change in the fura-2 ratio. Results are means ± SE. Differences between populations were assessed using appropriate versions of Student’s t-test, with P < 0.05 being considered significant.
Emptying and reloading of caffeine-sensitive stores in control slices

Representative time courses for the Ca\(^{2+}\)/H\(^{11001}\) responses are shown in Fig. 2A. For all slices, two consecutive caffeine challenges were administered in zero Ca\(^{2+}\)/H\(^{11001}\) ACSF, with a 5-min intertrial interval. In most control preparations (18/23) intracellular Ca\(^{2+}\) transients evoked by the second challenge were significantly smaller than the initial responses. This run-down is consistent with depletion of the content of intracellular stores, such that the bulk of the store content was released with the first caffeine challenge, and a fraction of the small residual store content was released by the second challenge. In some slices (5/23), the second response was of comparable amplitude to that of the initial caffeine challenge. Because of this variability, we grouped the first two responses together to provide a better readout of the store content (see Fig. 3).

If it is assumed that the two caffeine exposures in zero Ca\(^{2+}\) ACSF largely deplete the available caffeine-sensitive stores, the ability of the responses to recover when caffeine challenges are repeated in normal Ca\(^{2+}\) ACSF should reflect the ability of these stores to refill. This re-filling could occur either from the extracellular volume, or from other compartments within the neurons. Following re-introduction of normal ACSF, resting Fura-2 ratio significantly increased to 1.04 ± 0.03 (P < 0.005, n = 23), and responses to caffeine challenge recovered rapidly to initial values, implying robust refilling of store content (see also Fig. 4).

Responses to caffeine in postischemic slices

Slices prepared from animals 54–60 h following ischemia contain CA1 neurons that are destined to die over the next few days, but at this time-point, they are robust under physiological recording conditions and maintain adequate esterase activity to...
permit effective loading with the fura-2-AM method (see also Connor et al. 1999). In fact, with identical loading conditions, postischemic slices showed no significant difference from the control population in raw fura-2 fluorescence values, and small, but significant increases in resting fura-2 ratio (0.94 ± 0.03 vs. 1.04 ± 0.04; P < 0.04, n = 25 and 23 in control and postischemic, respectively). Previous comparisons of resting ratios in fura-2-microinjected neurons showed a small (but not significant) difference between control and postischemic neurons; however, in that study, control values were slightly higher than postischemic population (Connor et al. 1999). It is possible that factors such as differential trapping of unprocessed/AM indicator in the extracellular space may contribute to the small difference in resting ratios in this study.

When challenged with caffeine, the peak amplitude of responses to the initial caffeine application in zero Ca$^{2+}$ ACSF from the postischemic slices was much smaller than responses in slices from controls. Figure 2B shows time courses of the Ca$^{2+}$ response in a postischemic slice under the same protocol as Fig. 2A. While peak responses were significantly smaller in the postischemic slices, in other respects, responses to caffeine were similar, e.g., time to peak response was similar in the two populations, as was recovery, as measured by $t_{1/2}$. This metric showed considerable variation between slices of both populations (22.4 ± 1.9 s, control; 25.4 ± 2.6 s); the difference was not significant (P = 0.34). It was found previously in single-cell studies that the time course of Ca$^{2+}$ regulation (kinetics of Ca$^{2+}$ transients evoked by voltage-dependent influx, after
normalization for amplitude) is not changed during this postischemic period (Connor et al. 1999).

Figure 3A summarizes the population responses of the control and postischemic groups in zero Ca\(^{2+}\)/H\(_{11001}\) ACSF tests. Figure 3B compares the responses of the single, maximally responding cell in each slice for the two groups. Bars represent the number of slices (or cells) whose summed, first and second responses, to caffeine fell within incremental bins of 0.2 units of ratio increase. Results of the two metrics are in substantial agreement. Figure 3C shows the population responses of the two groups in normal ACSF. Here, bars represent the number of slices in incremental bins of 0.4. In both zero Ca\(^{2+}\)/H\(_{11001}\) ACSF and normal ACSF, the responses were much smaller in the postischemic slices ($P < 0.001$). Average of the summed responses from sham animals in 0 Ca\(^{2+}\)/H\(_{11001}\) ACSF was 1.53 ($n = 3$ slices from 2 animals), well within the range of the control animals.

**Store depletion and reloading in postischemic animals**

In almost all postischemic slices examined in zero Ca\(^{2+}\)/H\(_{11001}\) ACSF (22/23), responses to the second caffeine challenge was substantially smaller than the first response. This suggests that, like the situation in control slices, the bulk of release occurred with the first caffeine challenge. In addition to the smaller response amplitude in the postischemic population, which had
been initially hypothesized, a second difference emerged from the responses in normal Ca²⁺ saline. In the control population, there was a strong tendency to "recharge" the caffeine releasable stores when the slices were bathed in normal ACSF. In contrast, in the postischemic population, responses in normal Ca²⁺ saline did not recover back to initial levels. Even after 12 min in normal ACSF, responses remained significantly reduced compared with the initial caffeine challenge. Population data are summarized in Fig. 4. This suggests that there is less effective filling from the extracellular Ca²⁺ pool. Assuming that constitutive or electrically driven activation of voltage gated calcium channels (VGCCs) is ≥1 factor in refilling stores, the finding is consistent with our previous results showing that VGCC activity is compromised in the days following an ischemic insult.

**Discussion**

Using noninvasive loading of CA1 pyramidal neurons in brain slice, we have shown that caffeine application produces much smaller cytosolic Ca²⁺ increases in postischemic slices than in slices from control or sham-operated animals. Experiments were conducted at 54–60 h after ischemic insults, 6–12 h after preceding experiments had shown large reductions in activity driven Ca²⁺ influx had occurred (Connor et al. 1999; Shuttleworth et al. 2000), but before significant cell death or deterioration by other assays had occurred. We hypothesize that the reduced Ca²⁺ responses to caffeine, especially in 0 Ca²⁺ ACSF, reflect decreased Ca²⁺ content of intracellular stores in postischemic neurons.

Loading of intracellular Ca²⁺ stores in mature hippocampal pyramidal neurons appears to depend strongly on voltage-dependent Ca²⁺ influx pathways for filling (Pozzo-Miller et al. 1996). Given that there is little evidence for ER stores-operated Ca²⁺ entry channels in fully differentiated CNS neurons, as opposed to a wealth of evidence from somatic cells (Putney and McKay 1999), it seems reasonable to propose that a reduction in activity driven Ca²⁺ influx could lead to a reduction in the Ca content of the ER in postischemic neurons as seen here. The fact that stores did not reload as well after reintroduction into regular Ca²⁺ ACSF is also consistent with a reduction in voltage-dependent Ca²⁺ entry into postischemic neurons.

An alternative hypothesis that could explain the results of this study is that the content of ER Ca stores is unaffected by ischemia, but that a reduction in ryanodine receptor (RyR) density results in suppressed responses to caffeine exposure. It has been shown that ryanodine binding is reduced following global ischemia in gerbil CA1 at time-points soon after the initial insult (30 and 120 min) (Nozaki et al. 1996, 1999). Unfortunately, there seem to be no published studies that determine RyR density at later time points, during the progression of delayed cell death processes. However, if RyR density were in fact decreased 2 days following ischemia, this alternative hypothesis would predict much less partial depletion of store content with repetitive caffeine challenges in postischemic slices. In fact, the opposite was seen. There was a significant decrease in Ca²⁺ release with the second caffeine challenge in postischemic slices, and the degree of decrease was similar to (or greater than) the decrement in control slices. This, together with the weak recharge of stores following re-introduction into Ca/ACSF, suggests that the smaller responses in postischemic slices are not limited by RyR density, but rather by the reduced starting content of the stores themselves.

Determining the mechanisms underlying long-delayed neuronal death, as in CA1 hippocampus following brief global ischemia or in forebrain neurons after focal ischemia, has proven to be complex undertaking. Several ameliorating treatments have been identified on the basis of a large number of outcome studies, i.e., an agent is applied following an ischemic insult, or in some cases before the insult, and the reduction of cell damage is assessed. Examples from an extensive literature are 1) preischemia administration of platelet derived growth factor-BB produced “almost complete inhibition of delayed neuronal death” (Kawabe et al. 1997; Koji et al. 1997); 2) administration of other factors such as estrogen or human albumin following an ischemic insult has been shown to significantly reduce neuronal damage (Belayev et al. 2001; Jover et al. 2002); and 3) administration of inhibitors targeted to certain enzyme cascades. Among the most effective of these are inhibitors of cysteine protease, which can be administered up to a few hours after the ischemic insult in animal models. Several protease targets have been hypothesized (Tontchev and Yamashima 1999; Yamashima et al. 1998; Lee et al. 1991; Chen et al. 1998; Haru et al. 1997).

The administration window for significant effectiveness of the cysteine protease inhibitors is on the order of 8–9 h after the insult, with the greatest efficacy being achieved for treatment at <3 h. While such inhibition does reduce the activity of the cell death pathway(s), it is unclear how near these actions are to the irreversible steps in the process since cell death does not occur until 2 or 3 days later. It has recently been shown (Tanaka et al. 2004) that sublethal ischemic preconditioning, a protocol that is highly protective against normally lethal insults (Kirino et al. 1991; Kitagawa et al. 1991), does not interfere with caspase 3 activation within the above treatment window, but does give a large reduction at later time-points compared with non-preconditioned animals. There are also other out-
comes of ischemic preconditioning that are potentially neuroprotective (Hayashi et al. 2003).

The experiments here are a continuation of a different approach to the problem; that of trying to identify actual, delayed, physiological changes in the affected neurons that might give rise to degeneration. Based on our observation of a reduced Ca\(^{2+}\) influx and the lack of finding any evidence for chronically elevated Ca\(^{2+}\) levels in the postischemic neurons (Connor and Shuttleworth 2001; Connor et al. 1999), we proposed depletion of intraluminal Ca as a likely contributing cause of CA1 neuronal degeneration. Ca\(^{2+}\) influx characteristics do not change in transient ischemia-resistant CA3 neurons (Shuttleworth et al. 2000).

There is accumulating evidence that persistent ER Ca depletion is an important causative factor in delayed neuronal death. Sustained decreases in ER Ca content can lead to inhibition of protein synthesis (Brostrom and Brostrom 1990; Mizuno 1989), and inhibition of neuronal protein synthesis following store depletion has been reported (Paschen et al. 1996, 1998). Notably, the Paschen et al. (1996) study used caffeine to produce transient depletion of ER stores. Following transient global ischemia, vulnerable CA1 neurons show marked ER proliferation (Kirino et al. 1992), disaggregation of polyribosomes (Deshpande et al. 1987; Kirino et al. 1984), and a persisting inhibition of protein synthesis in vulnerable CA1 neurons but not in CA3 or dentate gyrus neurons that survive the insult (Bodsch et al. 1986; Cooper et al. 1977; Thilmann et al. 1986).

Treatment with the endosomal Ca-ATPase inhibitor thapsigargin, a common technique for nonrecoverable depletion of intracellular stores, causes cell death in a wide variety of cells over a period of 24–48 h. The mechanism of cell death following store depletion in cell lines or primary neuronal cultures exhibits characteristics of apoptosis (Nath et al. 1997), and the overexpression of Bcl-2 gives some protection against cell death produced by the treatment (Wei et al. 1998). Recent work has shown that raising intracellular Ca\(^{2+}\) after staurosporine treatment or oxygen-glucose deprivation reduces the degree of apoptosis that occurs in primary cultures of cortical neurons (Canzoniero et al. 2004).

The apoptotic pathway has been proposed as a mechanism in postischemic neuronal death in vivo (Kawase et al. 1999; Niwa et al. 2001; Zhu et al. 1998), but there is strong evidence that postischemic cell death does not conform to all the strict requirements of apoptosis (Colbourne et al. 1999; Petito et al. 1997). More recently, Bredesen and colleagues have proposed an alternative form of programmed cell death that also is coupled to ER stress, which must also be given consideration as a mechanism of delayed postischemic neuron death (Rao et al. 2002; Sperandio et al. 2000).

We therefore consider the reduced activity-driven Ca\(^{2+}\) influx and reduced stores content as being intermediate events in neuron degeneration, and as such, problems that could possibly be overcome before irreversible damage occurs. The findings here are not necessarily at variance with other studies, indicating that cytosolic Ca\(^{2+}\) levels were substantially elevated in postischemic CA1 neurons (Tsubokawa et al. 1992, 1994). The latter studies were performed at a later time after the insult when the neurons should have been considerably nearer their endpoint. In studies at 2 days (≤3 days with unilateral occlusions), we have found similar resting potentials and active and passive membrane properties (Connor et al. 1999). At these times during the progression of delayed cell death, resting Ca\(^{2+}\) levels appear similar to the control population, and a decrement in voltage-gated Ca\(^{2+}\) influx is the main difference. Given all these findings, it is reasonable to propose that elevated Ca levels represent a later, highly compromised state resulting from derivative, not primary, factors (see also Bonnekoh et al. 1992).

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REFERENCES


COMPROMISED INTRACELLULAR Ca\(^{2+}\) STORES FOLLOWING ISCHEMIA


