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

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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; Kristian 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 loading is severely depressed, not elevated (Connor and Shuttleworth 2001; Connor et al. 1999; Shuttleworth et al. 2000). Moreover, these functioning neurons show no indication of elevated baseline Ca\(^{2+}\) levels. Thus this important subset of neurons does not fit into a model where cell death results from a canonical increasing Ca\(^{2+}\) load, either static or dynamic.

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 temporalis 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
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 315–320 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 acetoxy 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 used previously to load gerbil CA1 neurons (Connor et al. 1999). Methyl ester form of the indicator, fura-2 AM. The approach follows first described by Regehr and Tank (1991) and has been used previously to load gerbil CA1 neurons (Connor et al. 1999). Individual slices were transferred to the recording chamber and were superfused with warmed (35°C), oxygenated ACSF at 2 ml/min.

**RESULTS**

**Responses to caffeine fura-2-AM–loaded slices**

Figure 1 shows indicator loading, response characteristics of a CA1 region, and the time line of the experimental protocol used. The raw fluorescence picture (Fig. 1A) 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 of damage by the slicing procedure (see Fig. 1B).

Ca2+ measurements were made by ratio imaging of fura-2 (Gryniewicz et al. 1985), using 350/380-nm excitation supplied by a monochromator (T.I.L.L. Photonics), coupled to an upright microscope (Zeiss AxioScope), and >510 nm emission was collected using a cooled, interline transfer CCD-camera system (Imago, T.I.L.L. Photonics). Excitation duration was 50 ms at each wavelength, with frame pairs acquired at 2 Hz. Measurements of Ca2+ are expressed as ratios of the 350/380 excited fluorescence. Uncertainties regarding fura-2 AM remaining in the extracellular space during experiments prevent reliable conversions to Ca2+ concentrations.

Data for analysis were generally taken from a region that included a small number of loaded cell bodies, not from individual somata: Trial examinations showed that it made no significant difference in overall statistics whether one analyzed just multiple cell bodies or a maximally responding region in the flow path of caffeine and 2) this method avoided selection bias in picking among individual somata in a field of view. A comparison of single, maximally responding neurons in the control and postischemic slices is also included as Fig. 3B.
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

FIG. 1.  A: fluorescence image (380-nm excitation) of CA1 pyramidal neuron somata loaded by infusing fura-2-AM into the alveus. Stratum pyramidal runs bottom left to top right. B: ratio image (F350/F380) image of same field before caffeine application. Red arrows mark 2 cells with high prestimulus Ca. Such cells were excluded from analysis in both control and postischemic slices. C: ratio image at the peak response to caffeine applied to the cells. Pipette orifice located toward the bottom left out of field of view. D: time course of Ca change in 0 Ca artificial cerebrospinal fluid (ACSF), expressed as fluorescence ratio, measured in the preceding field within the outlined boundary shown in A–C. Bar above horizontal axis denotes application of caffeine. E: timeline of experimental protocol for caffeine application in 0 Ca and normal ACSF.
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

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**FIG. 2.**  
A: time courses of Ca\(^{2+}\) changes observed in slice from control animal under the stimulus protocol of Fig. 1E. Top and top middle: caffeine application in 0 Ca ACSF. Bottom middle and bottom: caffeine application in normal ACSF. B: identical measurements on slice from a postischemic animal. Note the much finer vertical scale in B. Small bumps in some records are due to individual cells giving a delayed response to the stimulus.
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\textsuperscript{2+}/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\textsuperscript{2+} 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\textsuperscript{2+} 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\textsuperscript{2+} 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) pool. Assuming that constitutive or electrically driven activation of voltage gated calcium channels (VGCCs) is \(\approx 1\) 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 Ca\(^{2+}\) increases in posts ischemic 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 Ca\(^{2+}\) 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 Ca\(^{2+}\) responses to caffeine, especially in 0 Ca\(^{2+}\) ACSF, reflect decreased Ca\(^{2+}\) content of intracellular stores in post ischemic neurons.

Loading of intracellular Ca\(^{2+}\) stores in mature hippocampal pyramidal neurons appears to depend strongly on voltage-dependent Ca\(^{2+}\) influx pathways for filling (Pozzo-Miller et al. 1996). Given that there is little evidence for ER stores-operated Ca\(^{2+}\) 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 Ca\(^{2+}\) influx could lead to a reduction in the Ca content of the ER in posts ischemic neurons as seen here. The fact that stores did not reload as well after reintroduction into regular Ca\(^{2+}\) ACSF is also consistent with a reduction in voltage-dependent Ca\(^{2+}\) entry into posts ischemic 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 posts ischemic slices. In fact, the opposite was seen. There was a significant decrease in Ca\(^{2+}\) release with the second caffeine challenge in posts ischemic 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 posts ischemic 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 proteases, 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; Hara et al. 1997).

The administration window for significant effectiveness of the cysteine proteases 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 Ca2+ influx and the lack of finding any evidence for chronically elevated Ca2+ levels in the posts ischemic neurons (Connor and Shuttleworth 2001; Connor et al. 1999), we proposed depletion of intraluminal Ca as a likely contributing cause of CA1 neuronal degeneration. Ca2+ 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 nonreversible 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 Ca2+ 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 posts ischemic neuronal death in vivo (Kawase et al. 1999; Niwa et al. 2001; Zhu et al. 1998), but there is strong evidence that posts ischemic 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 posts ischemic neuron death (Rao et al. 2002; Sperandio et al. 2000).

We therefore consider the reduced activity–driven Ca2+ 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 Ca2+ levels were substantially elevated in posts ischemic 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 Ca2+ levels appear similar to the control population, and a decrement in voltage-gated Ca2+ 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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