Seizure-Induced Cell Death Produced by Repeated Tetanic Stimulation In Vitro: Possible Role of Endoplasmic Reticulum Calcium Stores

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[1]*Bloorview Epilepsy Research Laboratory,* [2]*Playfair Neuroscience Unit,* [3]*Department of Medicine (Neurology) and*
[4]*Department of Physiology, University of Toronto, Toronto, Ontario M5T 2S8, Canada*

Pelletier, Marc R., Jehangir S. Wadia, Linda R. Mills, and Peter L. Carlen. Seizure-induced cell death produced by repeated tetanic stimulation in vitro: possible role of endoplasmic reticulum calcium stores. *J. Neurophysiol.* 81: 3054–3064, 1999. Seizures may cause brain damage due to mechanisms initiated by excessive excitatory synaptic transmission. One such mechanism is the activation of death-promoting intracellular cascades by the influx and the perturbed homeostasis of Ca^{2+}. The neuroprotective effects of preventing the entry of Ca^{2+} from voltage-dependent Ca^{2+} channels, NMDA receptors, and non-NMDA receptors, is well known. Less clear is the contribution to excitotoxicity of Ca^{2+} released from endoplasmic reticulum (ER) stores. We produced epileptiform discharges in combined entorhinal cortex/hippocampus slices using repeated tetanic stimulation of the Schaffer collaterals and assessed cell death after 1, 3, or 12–14 h with gel electrophoresis of genomic DNA and immunohistologically using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5′-triphosphate (dUTP) nick end labeling (TUNEL) staining. We manipulated ER Ca^{2+} stores using two conventional drugs, dantrolene, which blocks the Ca^{2+} release channel, and thapsigargin, which blocks sarco-endoplasmic reticulum Ca^{2+}-ATPases resulting in depletion of ER Ca^{2+} stores. To monitor epileptogenesis, and to assess effects attributable to dantrolene and thapsigargin on normal synaptic transmission, extracellular potentials were recorded in stratum pyramidale of the CA1 region. Repeated tetanic stimulation reliably produced primary afterdischarge and spontaneous epileptiform discharges, which persisted for 14 h, the longest time recorded. We did not observe indications of cell death attributable to seizures with either method when assessed after 1 or 3 h; however, qualitatively more degraded DNA always was observed in tetanized slices from the 12- to 14-h group compared with time-matched controls. Consistent with these data was a significant, four-fold, increase in the percentage of TUNEL-positive cells in CA3, CA1, and entorhinal cortex in tetanized slices from the 12- to 14-h group (16.5 ± 4.4, 33.7 ± 7.1, 11.6 ± 2.1, respectively; means $\pm$ SE; $n = 7$) compared with the appropriate time-matched control (4.1 ± 2.2, 7.3 ± 2.0, 2.8 ± 0.9, respectively; $n = 6$). Dantrolene (30 μM; $n = 5$) and thapsigargin (1 μM; $n = 4$) did not affect significantly normal synaptic transmission, assessed by the amplitude of the population spike after 30 min of exposure. Dantrolene and thapsigargin also were without effect on the induction or the persistence of epileptiform discharges, but both drugs prevented seizure-induced cell death when assessed with gel electrophoresis. We suggest that Ca^{2+} entering a cell from the outside, in addition to the Ca^{2+} contributed from ryanodine-sensitive stores (i.e., Ca^{2+}-induced Ca^{2+} release), may be necessary for seizure-induced cell death.

INTRODUCTION

Pathology of the brain attributable to epileptic seizures has been reported as a consequence of prolonged febrile seizures (Aicardi and Chevrie 1970; Verity et al. 1993), complex partial seizures (Babb et al. 1984; Duncan and Sagar 1987), and status epilepticus (Corsellis and Bruton 1983; Lothman 1990). Seizure-induced brain damage results in death of susceptible cell types and fibrillary gliosis, which occurs typically in hippocampus but also in other structures including dentate gyrus, cerebellum, amygdala, and neocortex (reviewed in Honavar and Meldrum 1997). Consistent with these clinical observations are studies describing brain damage produced by experimentally induced seizures (Ben-Ari 1985; Cavazos et al. 1994; Lothman and Collins 1981; Meldrum et al. 1973; Sloviter and Damiano 1981; Thompson et al. 1996; Vicedomini and Nadler 1990).

Substantial evidence implicates cellular cascades initiated by the influx and the perturbed intracellular homeostasis of Ca^{2+} in excitotoxic cell death (Choi 1988; Meldrum and Garthwaite 1990; Nicotera and Orrenius 1992). The neuroprotective effects of preventing the entry of Ca^{2+} from a variety of sources including voltage-dependent Ca^{2+} channels (VDCC) (Siesjo 1990; Siesjo and Bengtsson 1989), *N*-methyl-D-aspartate (NMDA) receptors (Clifford et al. 1989; Garthwaite and Garthwaite 1989), and non-NMDA receptors (Brorson et al. 1994; Penix and Wasterlain 1994) is well known. Less clear is the contribution to cell death of Ca^{2+} released from endoplasmic reticulum (ER) stores.

Neuronal Ca^{2+} signaling mediated by stores associated with the ER regulates a wide variety of processes including excitability, synaptic plasticity, and gene transcription (reviewed in Alkon et al. 1998; Berridge 1998). Two types of intracellular stores identified in neurons and associated with the ER are the inositol 1,4,5-trisphosphate (IP_3)-and the ryanodine-sensitive stores (Ehrlich et al. 1994; Henzi and MacDermott 1992; McPherson et al. 1991). The two major classes of receptor/channel complexes that mediate Ca^{2+} release from ER stores share considerable structural and molecular similarity (Coronado et al. 1994; McPherson et al. 1991; Mignery et al. 1990). Thus far, four IP_3 receptor (IP_3R_{1-4}) and three ryanodine receptor ($RYR_{I–III}$) isoforms have been identified (reviewed in Berridge 1993; Simpson et al. 1995). The molecular diversity is due to the existence of separate genes, and for type 1 IP_3Rs, alternative splicing.

The refilling state of the stores determines whether they

function as a sink or a source of Ca^{2+}. When stores are replete, Ca^{2+} entry can promote the release of Ca^{2+} from ryanodine-sensitive stores, referred to as Ca^{2+}-induced Ca^{2+} release (CICR), which amplifies and prolongs the cytosolic Ca^{2+} transient (Clapham 1995; Irving et al. 1992; Simpson et al. 1995). Prolonged depolarization effectively activates CICR as observed in sympathetic ganglion neurons (Kuba et al. 1992) and cerebellar purkinje cells (Llano et al. 1994) and with epileptiform discharges in both hippocampal slices (Albowitz et al. 1997) and cultured hippocampal neurons (Segal and Manor 1992).

We produced epileptiform discharges, similar to stimulus train-induced bursting (STIB) (Stasheff et al. 1985), with repeated tetanic stimulation in entorhinal cortex/hippocampus slices and manipulated ER Ca^{2+} stores using two conventional drugs, dantrolene and thapsigargin. Dantrolene blocks the Ca^{2+} release channel and is cytoprotective in hepatoma 1C1C7 cells (Dypbukt et al. 1990), cultured cortical neurons (Frandsen and Schousboe 1991), and CA1 pyramidal neurons (Wei and Perry 1996). Thapsigargin inhibits the sarco-ER Ca^{2+}-ATPase (SERCA) pumps that participate in refilling the stores resulting in an increase in cytosolic Ca^{2+}, which persists for several minutes (Thastrup et al. 1990; Thomas and Hanley 1994; Treiman et al. 1998). Both apoptosis of thymocytes (Jiang et al. 1994; Waring and Beaver 1996) and neuroprotection of sympathetic neurons (Lampe et al. 1992) and cultured cerebellar granule cells (Levick et al. 1995; Lin et al. 1997) have been reported for thapsigargin. In this paper, we demonstrate seizure-induced cell death in vitro, which was prevented by both dantrolene and thapsigargin.

METHODS

Slice preparation

Male Wistar rats (30–65 days of age) were anesthetized with 2-Bromo-2-chloro-1,1,1-trifluoroethane (Halothane; Halocarbon Laboratories, River Edge, NJ) and then decapitated. The brain was removed rapidly and placed for $\approx$1 min in ice-cold, oxygenated (95% O_2-5% CO_2) sucrose-based artificial cerebrospinal fluid (ACSF) containing (in mM): 210 sucrose, 26 $NaHCO_3$, 3.5 KCl, 1 $CaCl_2$, 4 $MgCl_2$, 1.25 NaH_2PO_4, and 10 glucose. Because of the long duration of these experiments, to promote slice viability we used a sucrose-based, high Mg^{2+}-containing ACSF during the preparation of slices to reduce dissection-induced damage and Na^+-dependent excitotoxicity (Rafiq et al. 1993; Rasmussen and Aghajanian 1990). The brain was hemisected with a midsagittal cut, then the cerebellum and the forebrain were removed. Finally, the dorsal cortex was cut parallel to the longitudinal axis, and the remaining block of brain was glued dorsal surface down, caudal end facing the blade, to an aluminum chuck, which was secured at a 12° angle. To ensure reliability of the cutting angle, a line subtending a 12° angle from the front corner of the chuck was scored on the side of the chuck. Brain slices (500 μm) comprising both the entorhinal cortex and the hippocampus then were prepared using a Vibratome. We employed this method of slice preparation because sustained epileptiform discharges are provoked reliably in slices prepared with this orientation (Jones and Heinemann 1988; Rafiq et al. 1993; Walther et al. 1986), which might be attributable to intrinsic reverberatory circuitry in the entorhinal cortex and the frequency-dependent transfer of this neuronal activity to the hippocampus via the dentate gyrus (Iijima et al. 1996). The ACSF for the incubation period and the recording of electrophysiological responses contained (in mM) 125 NaCl, 26 $NaHCO_3$, 5 KCl, 2 $CaCl_2$, 0.5 $MgCl_2$, 1.25 NaH_2PO_4, and 10 glucose. After a minimum of 1 h, slices were transferred to an interface-type chamber and perfused continuously (2–4 ml/min) with oxygenated ACSF. Time-matched, untetanized control slices were included in the recording chamber with slices receiving tetanic stimulation. Therefore the only difference in the treatment of the slices was the application or the absence of tetanic stimulation. A maximum of four slices (2 tetanized/2 control) were used from an individual rat.

Electrophysiology

To monitor epileptogenesis in the slice, extracellular responses were recorded in the stratum pyramidale of CA1 with NaCl-filled (150 mM; 2–4 MΩ) microelectrodes. Orthodromic responses were evoked via a bipolar stimulating electrode (enamel-insulated nichrome wire; 125 μm diam) placed in the stratum radiatum. Signals were recorded, amplified, and filtered (3 kHz) with an Axoclamp 2A amplifier in bridge mode. Input/output relations were determined by varying the amplitude of 100-μs pulses and acquired using the CLAMPEX program of pClamp version 6.0 software (Axon Instruments, Foster City, CA). The slice then was tetanized once every 10 min: 100-Hz, 2-s train duration at twice the threshold intensity for evoking a population spike of 1 mV, total of 10 episodes. To permit full recovery of synaptic function after evoking a primary afterdischarge (PAD), orthodromic responses were not evoked between episodes of tetanic stimulation (Anderson et al. 1990). Experiments also were recorded on video tape (Instrutech Corporation; VR-10) and digitized using software (WCP V1.2) provided by Dr. John Dempster, University of Strathclyde. Seizure-induced cell death was assessed at three time points; 1, 3, and 12–14 h after the 10th tetanization. Orthodromic responses were evoked at these time points, and only slices where orthodromic responses could be evoked were included in the analysis. Experiments were conducted at 34°C.

Gel electrophoresis

The integrity of DNA from tetanized and time-matched control slices was determined by size fractionation in agarose gels. Slices designated for gel electrophoresis were stored at −80°C. Genomic DNA was isolated from slices using a conventional protocol (Easy-DNA Kit, Invitrogen, San Diego, CA). Two slices, from the same rat and in the same condition, were pooled. Briefly, cells were lysed with lysis buffer then proteins and lipids were precipitated. DNA was extracted with Tris-saturated phenol/chloroform (1:1) and centrifuged at 13,000 g for 20 min followed by reextraction with chloroform only and further centrifuging. The DNA then was precipitated with ethanol, resuspended in tris ethylenediamine tetraacetic acid (TE; 10:1, pH 8.0) buffer, then treated with DNase-free RNase (40 μg/ml). The amount of DNA in each sample (10–25 μg) was determined with a spectrophotometer (Perkin Elmer Lambda 3B). Samples were heated at 65°C for 10 min, centrifuged for 2 min, then put on ice for 1 min before loading. Approximately 5 μg of DNA from each sample was electrophoresed through 2.0% agarose in Tris borate buffer. DNA was visualized by ethidium bromide staining and photographed under UV illumination. For a positive control, organotypic hippocampal slice cultures were prepared as described previously (Perez Velazquez et al. 1997; Stoppini et al. 1991) and exposed for 3 or 6 h to actinomycin D (5 μg/ml), a potent inducer of apoptosis in a variety of cells (Martin et al. 1990).

Immunohistochemistry

Slices designated for immunohistochemistry were fixed in 10% buffered formaldehyde, embedded in paraffin, then resectioned (5 μm). Sections were taken $\geq$100 μm below the slice surface then mounted on glass slides. In situ labeling of new 3′-OH DNA ends generated by DNA fragmentation was performed using terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5′-triphos-

phate (dUTP) nick end labeling (TUNEL; in situ cell death detection kit, Boehringer Mannheim). Briefly, sections were deparaffinized, rehydrated through a graded series of ethanols, then washed in phosphate-buffered saline (PBS). Sections then were treated with Proteinase K (20 μg/ml; Boehringer Mannheim) for 30 min at room temperature and then washed with PBS. Endogenous peroxidase activity was blocked with 0.3% hydrogen peroxide in methanol for 30 min at room temperature. Positive control sections were treated with DNaseI (1 μg/ml; Pharmacia Biotech) for 10 min at room temperature. DNaseI was dissolved by gentle inversion in DN reaction buffer that contained 50 mM Tris-HCl (pH 7.5), 10 mM $MgCl_2$, and 50 μg/ml BSA. Sections were washed with PBS and TdT-fluorescein was added to the sections, which then were covered with Parafilm and incubated in a humidified chamber for 60 min at 37°C. Negative control sections were incubated without TdT. After washing with PBS, fluorescein-labeled DNA strand breaks were analyzed under a confocal microscope (Biorad MRC600; excitation wavelength, 488 nm). If fluorescent cells were observed, an antifluorescein antibody FAB fragment conjugated with horse-radish peroxidase was applied to the slice, covered with Parafilm, and incubated in a humidified chamber for 30 min at 37°C. Labeling was then visualized with metal-enhanced diaminobenzidine (Boehringer Mannheim). Some sections were counterstained lightly with cresyl violet and coverslipped for assessment of morphology of TUNEL-positive cells.

TUNEL-positive cells were counted either visually or with the use of Imagetool version 1.28 software (University of Texas Health Science Center in San Antonio) by scorers that were blind to the treatment condition. The two methods of scoring produced close to identical results and the interrater reliability was $r = 0.92$. Scoring was done in five regions of the section and were defined as follows: DG, both superior and inferior blades of the granule cell layer; hilus (H), area lying within the two blades of the DG and delimited by a line connecting the ends of the blades of the DG; CA3, stratum pyramidale commencing from a line connecting the blades of the DG to a line level with the superior blade of the DG; CA1, stratum pyramidale (excluding CA2) extending to the subiculum; and entorhinal cortex (EC), a region the width of the DG extending from the hippocampal fissure to the peripheral limit of the section. Cells were counted and expressed as a percentage of the average number of cells per region determined from slices ($n = 4$) fixed and stained with hematoxylin/eosin immediately after sectioning with the Vibrotome.

Drugs

Dantrolene sodium (30 μM; Sigma) and thapsigargin (1 μM; Sigma) were dissolved in dimethyl sulfoxide (DMSO). Actinomycin D-mannitol (5 μg/ml; Sigma) was dissolved in purified water. All drugs were stored frozen as aliquots in stock solutions. Final concentration of DMSO was 0.05%.

Statistics

Differences in the percentage of TUNEL-positive cells were determined using a 5 (region) $\times$ 2 (tetanized/control) factorial ANOVA (SPSS version 8.0). Post hoc analyses were conducted with the Scheffé test. To assess the effect of dantrolene and thapsigargin on normal synaptic transmission, population spikes were evoked at a frequency of 0.05 Hz and after a stable baseline had been achieved (typically 15 min), the drugs were applied. Three responses, at the end of the baseline period and after 30 min of drug application, were averaged and the population spike amplitude was measured using the CLAMPFIT program of pClamp version 6.0 software (Axon Instruments). Differences in population spike amplitude attributable to the application of drugs were determined using a paired t-test. Significance was determined at $P < 0.05$. Results are presented as the means $\pm$ SE.

RESULTS

Electrophysiology

PADs were produced reliably by tetanic stimulation in all experiments. As illustrated in Fig. 1, PAD increased progressively in duration and amplitude with repeated tetanization. The duration of PAD after the 10th tetanic stimulation ranged from 40 to 140 s. In contrast to previous reports using this preparation (Rafiq et al. 1993, 1995), where secondary afterdischarge (SAD) was observed in 85% of experiments, we observed SAD in only 29% of experiments. Consistent with intense, prolonged epileptiform activity, we observed episodes of spreading depression (SD) after tetanic stimulation in 46% of experiments, which consisted of a negative DC potential shift (10–25 mV) during which time the slice was synaptically quiescent. Spontaneous epileptiform discharges typically emerged during the recovery from SD. A representative record of SD is presented in Fig. 1B.

Input/output relations included four to five stimulation intensities: weak stimulation produced a small-amplitude, positive-deflecting, postsynaptic potential (PSP) that increased in amplitude with increasing stimulation intensity. As seen in Fig. 2A, before tetanization, suprathreshold stimulation for evoking a population spike produced a single population spike. After the 10th tetanization, responses evoked at all intensities were of greater amplitude and longer duration compared with control responses, with multiple population spikes, characteristic of epileptiform responses (Fig. 2B). We could record epileptiform responses in tetanized slices for ≤14 h, the longest time
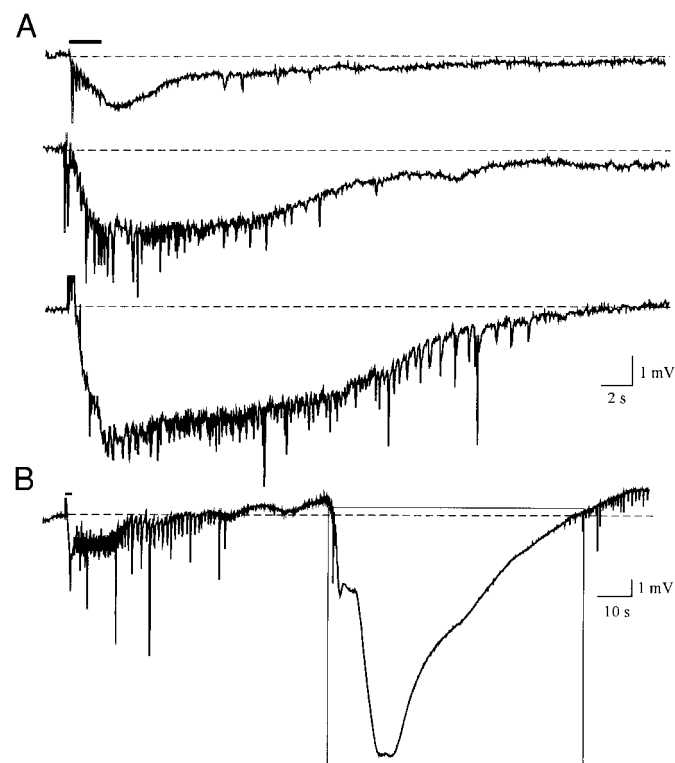

FIG. 1. Repeated tetanization reliably evokes primary afterdischarge (PAD). *A, top*: after the 1st tetanic stimulation, the PAD is of small amplitude and of brief duration but increases progressively in amplitude and duration as illustrated after the 5th (*middle*) and 10th (*bottom*) tetanic stimulation (same experiment as in *top*). *B*: representative record of PAD followed by an episode of spreading depression (enclosed in box).
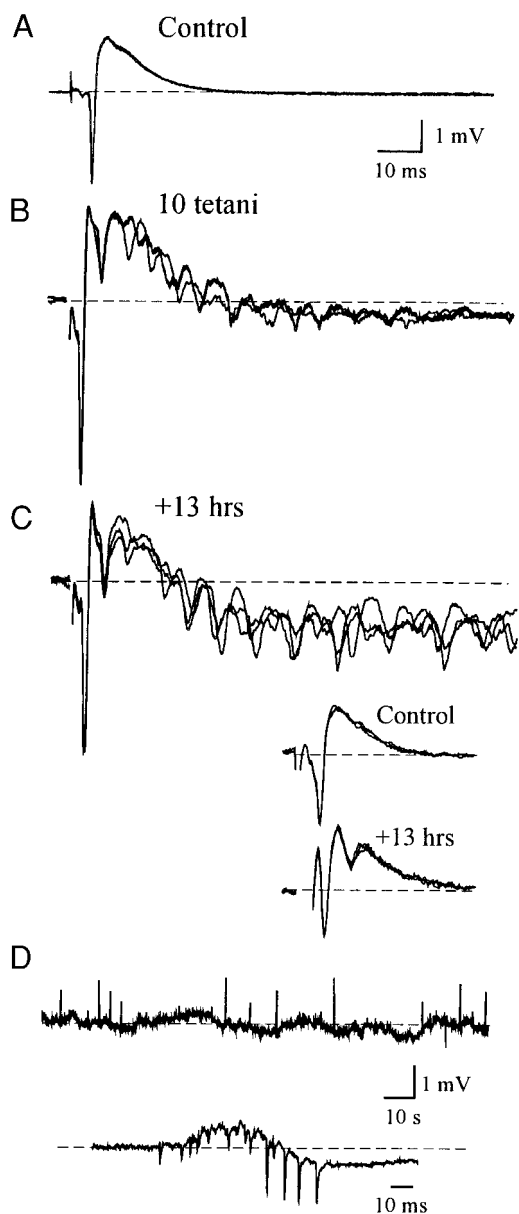
FIG. 2. Epileptiform discharges persist during long-duration experiments. *A*: responses evoked with maximum stimulation intensity, before tetanization, possess a single population spike (3 consecutive responses). *B*: after 10 tetani, the responses evoked with the same stimulation intensity as in *A* are longer in duration and possess multiple population spikes, characteristic of epileptiform responses. *C*: epileptiform responses persist for 13 h after the 10th tetanization. *Inset*: responses from a time-matched control slice at the beginning (*top*) and end (*bottom*) of the experiment. *D, top*: continuous record of spontaneous epileptiform discharges. *Bottom*: single discharge from the record *above* is presented at a faster sweep speed.

assessed. Representative responses from a long-duration recording are presented in Fig. 2*C*. We also recorded spontaneous epileptiform discharges from slices that received tetanic stimulation. Spontaneous epileptiform discharges were observed as early as after the second tetanization but typically were observed after the fifth tetanization and persisted for the duration of the experiment ($\leq$14 h). A representative record of spontaneous epileptiform discharges is presented in Fig. 2*D*.

In contrast to the responses evoked in slices receiving tetanic stimulation, responses evoked from time-matched slices not

receiving tetanic stimulation were not epileptiform and were similar to the responses evoked at the beginning of the experiment. In the record presented in the *inset* in Fig. 2*C,* a small-amplitude, second population spike is seen after 13 h. Additionally, spontaneous epileptiform discharges were never recorded in control slices. These experiments demonstrate evoked and spontaneous epileptiform discharges were produced reliably in entorhinal cortex/hippocampus slices with repeated tetanic stimulation, responses evoked in time-matched controls not receiving tetanic stimulation were not epileptiform, and both epileptiform responses from tetanized slices and physiological responses from time-matched control slices, could be recorded for $\leq$14 h after the tetanization protocol, the longest time we measured.

Gel electrophoresis

The integrity of DNA was preserved from slices assessed at the early time points, 1 h (tetanized, $n = 4$; time-matched control, $n = 4$) and 3 h (tetanized, $n = 4$; time-matched control, $n = 4$) after the tetanization protocol (data not shown). In contrast, we observed indications of internucleosomal DNA degradation in 9/11 slices receiving repeated tetanic stimulation and assessed 12–14 h later. Because endogenous Ca^{2+}-dependent endonucleases cleave DNA in regular 180- to 200-bp pieces, the classical profile of apoptosis in gel electrophoresis is a regular pattern of banding referred to as ''DNA laddering'' (Wyllie et al. 1980). The results of the DNA fragmentation assay we performed did not produce the typical banding pattern consistent with apoptosis but one more representative of necrosis. The results of the DNA fragmentation assay we employed may have been obscured by the overwhelming presence of intact DNA from normal cells. The appearance of degraded DNA in our study ranged from a smear of low-molecular-weight fragments (<100–400 bp) to a more diffuse smear and a few discernable bands. We observed no degradation (8/11), or qualitatively less (3/11), degradation of DNA isolated from 12 to 14 h time-matched control slices compared with tetanized slices. A representative example of the degradation in the DNA isolated from tetanized slices after 12–14 h is presented in Fig. 3. Interestingly, in the long-duration experiments where DNA degradation was not observed (2/11), repeated tetanic stimulation potentiated greatly the amplitude of the population spike but did not produce epileptiform responses, suggesting that the damage we observed was a direct result of the sustained epileptiform discharges and not attributable to the persistent presence of the stimulating electrode.

Immunohistochemistry

To determine whether the degraded DNA we observed with electrophoresis was expressed as region-specific cell death, as reported previously for the CA3 region after intraamygdaloid infusion of kainate (Pollard et al. 1994), we assessed the percentage of TUNEL-positive cells in each of the five regions described above. TUNEL-positive cells were not observed in sections prepared from slices 1 and 3 h after the tetanization protocol (data not shown); this is consistent with the absence of degraded DNA we observed after gel electrophoresis in slices from these groups. However, when the percentage of TUNEL-positive cells was quantified from sections prepared from the late time point group (12–14 h), there was a significantly
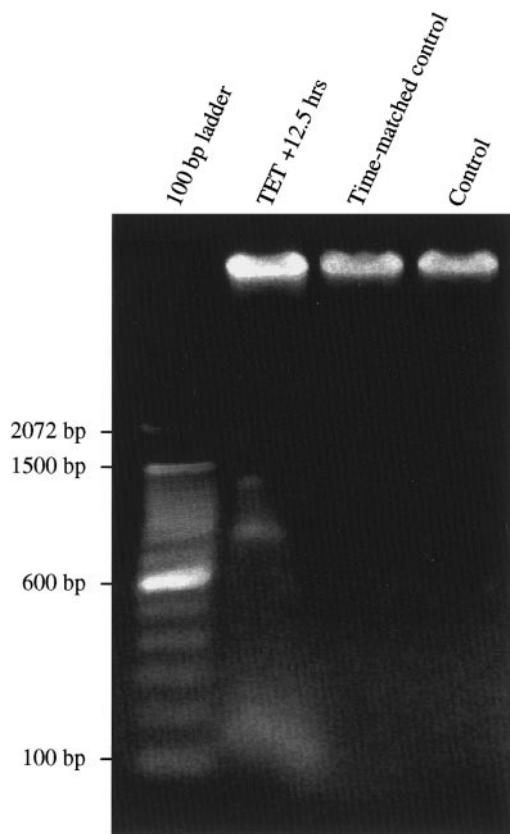
FIG. 3.   Electrophoresis of genomic DNA isolated from tetanized slices (12-to 14-h group) demonstrates degradation, which is indicative of cell death. *A, lane 1*: 100-bp marker; *lane 2*: tetanized slice + 12.5 h. Note, degraded DNA appears as a diffuse smear and bands at 800 and 1.3 kb. *Lane 3*: untetanized time-matched control. *Lane 4*: control refers to DNA isolated from slices immediately after preparation with the Vibratome.

greater percentage of TUNEL-positive cells in CA3, CA1, and EC when the tetanized group (16.5 ± 4.4, 33.7 ± 7.1, 11.6 ± 2.1, respectively; $n = 7$) was compared with the appropriate time-matched control group (4.1 ± 2.2, 7.3 ± 2.0, 2.8 ± 0.9, respectively; $n = 6$). Although there was an increase in TUNEL-positive cells in both the DG and the H in tetanized slices compared with time-matched controls, due to variability, the differences failed to reach significance in these regions. Some of the morphological features we observed in the TUNEL-positive cells were characteristic of apoptosis, including well-delimited chromatin aggregates located peripherally on the nuclear membrane and cell shrinkage; however, we did not observe membrane blebbing or apoptotic bodies. TUNEL-positive cells were observed in small clusters or individually. TUNEL-positive cells were observed typically, but not exclusively, in the principal cell body layer. For example, in the DG, TUNEL-positive cells ranged from the infragranular layer to the most distal border of the granule cell layer. Representative examples of TUNEL-positive cells are presented in Fig. 4*A*. A summary of the percentage of TUNEL-positive cells in each of the five regions of the section assessed after 12–14 h is presented in Fig. 4*B*.

Pharmacological manipulation of ER Ca^{2+} stores

Having demonstrated seizure-induced cell death in entorhinal cortex/hippocampus slices, we then were interested in

testing the hypothesis that Ca^{2+} released from ER stores contributes to seizure-induced cell death. We, therefore, repeated the long-duration (12–14 h) experiments and applied two conventional drugs known to modify the functioning of ER Ca^{2+} stores, dantrolene and thapsigargin. Dantrolene or thapsigargin was applied for 30 min before the commencement of tetanic stimulation to enable the assessment of their effect on normal synaptic transmission, which was determined by the amplitude of the population spike evoked with maximal stimulation. The drugs were present for the duration of the experiments. Dantrolene (30 μM) was without significant effect on normal synaptic transmission, something that has been reported previously (Obenaus et al. 1989; O'Mara et al. 1995). The amplitude of the population spike after 30 min of dantrolene exposure was $104.3 \pm 9.8\%$ of control ($n = 5$). Dantrolene also was without effect on the induction or the maintenance of epileptiform discharges. That is, there were no differences compared with experiments where no drugs were applied in any feature of epileptiform discharges, e.g., PAD, epileptiform responses evoked with single 100-μs shocks, spontaneous epileptiform discharges. The amplitude of the population spike, when measured at the end of the 30 min application of thapsigargin (1 μM) was $133.2 \pm 31.7\%$ of control ($n = 4$), which failed to reach significance due to the variability. Thapsigargin also had no effect on the induction or the maintenance of epileptiform discharges. Electrophysiological responses evoked in the presence of dantrolene and thapsigargin are presented in Fig. 5, *A* and *B,* respectively.

Gel electrophoresis

DNA isolated from slices that had been tetanized and exposed to dantrolene ($n = 5$) or thapsigargin ($n = 4$) demonstrated no indications of degradation. This was true also for time-matched control slices. To rule out the possibility that our observation of the absence of degraded DNA in slices exposed to dantrolene and thapsigargin was a false negative, we simultaneously electrophoresed DNA isolated from organotypic hippocampal slice cultures exposed to actinomycin-D (5 μg/ml), a potent inducer of apoptosis in a variety of cells (Martin et al. 1990). A representative example of the gel electrophoresis of DNA from these experiments is presented in Fig. 6.

DISCUSSION

Repeated tetanic stimulation in entorhinal cortex/hippocampus slices produced both evoked and spontaneous epileptiform activity, which we could record for $\leq$14 h. We assessed seizure-induced cell death using gel electrophoresis of genomic DNA and immunohistologically by counting TUNEL-positive cells. Indications of cell death by either method were not observed when assessed at 1 and 3 h after the tetanization protocol. In contrast, we observed degraded DNA isolated from tetanized slices in the 12- to 14-h group, which ranged in appearance from a smear of low-molecular-weight DNA fragments ($<100-400$ bp) to a more diffuse smear accompanied by a few discernable bands. The amount of degraded DNA from time-matched controls was either below the threshold detection limit or qualitatively less compared with tetanized slices. Consistent with the observation of degraded DNA from tetanized slices in the 12–14 h group was the presence of
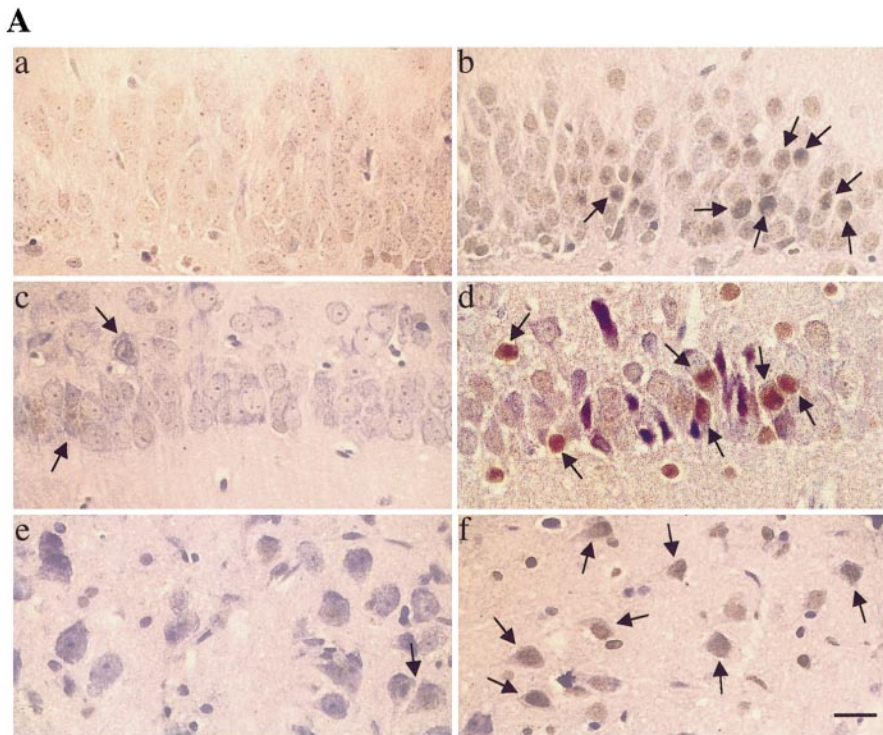
A

B

FIG. 4. Epileptiform discharges evoked by repeated tetanic stimulation increase significantly the percentage of terminal deoxynucleotidyl transferase (TdT)-mediated deoxyuridine 5′-triphosphate (dUTP) nick end labeling (TUNEL)-positive cells. *A, a, c,* and *e*: time-matched control, superior blade of dentate gyrus (DG), CA1, and entorhinal cortex (EC), respectively. Same regions from tetanized slices (12–14 h) appear in *b, d,* and *f.* Examples of representative TUNEL-positive cells are denoted (→. Scale bar is 20 μm. *B*: summary of percentage of TUNEL-positive cells by region in sections prepared from tetanized slices (12–14 h) compared with time-matched controls. *, significantly different ($P < 0.05$) from appropriate time-matched control.
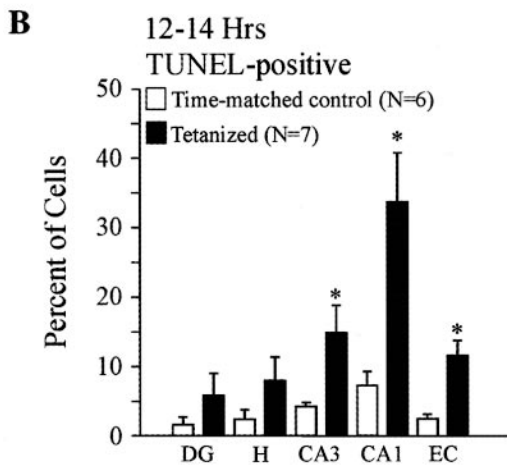
TUNEL-positive cells in sections prepared from this group. We observed a significant fourfold increase in the percentage of TUNEL-positive cells in CA3, CA1, and EC from tetanized slices compared with the respective time-matched control.

Cell death

There are two fundamental and discernable patterns of cell death, necrosis and apoptosis (reviewed in Buja et al. 1993; Robbins 1994). Necrosis occurs after an exogenous insult and manifests as cell swelling, or rupture, denaturation, and coagulation of cytoplasmic proteins, breakdown of cell organelles, and a significant inflammatory response. Apoptosis, described first by Kerr, Wyllie, and Currie (1972), is a feature of normal development in both the central and the peripheral nervous system, may require de novo gene transcription, and drives the elimination of cells during embryogenesis (Stewart 1994; White 1996). In addition to its physiological role, apoptosis also has been observed in a variety of pathological conditions (Thompson 1995).

There is a complex relation between experimentally induced seizures and cell death. Cell death produced by limbic motor seizures induced by intraamygdaloid injection of kainate has been described previously to have features of both necrosis and apoptosis (Charriaut-Marlangue et al. 1996; Pollard et al. 1994; Represa et al. 1995). Conversely, status epilepticus produced by systemic injection of pilocarpine produced cell death that was described as being solely necrotic (Fujikawa 1996). Additionally, kindling can give rise to both apoptosis and proliferation of DG neurons (Bengzon et al. 1997). Because both necrosis and apoptosis often are described in pathology and share some features, distinguishing between the type of cell death sometimes can be difficult. Convergent evidence from different methods of analysis is required to differentiate between apoptosis and necrosis (Charriaut-Marlangue and Ben-Ari 1995). Additionally, differences in

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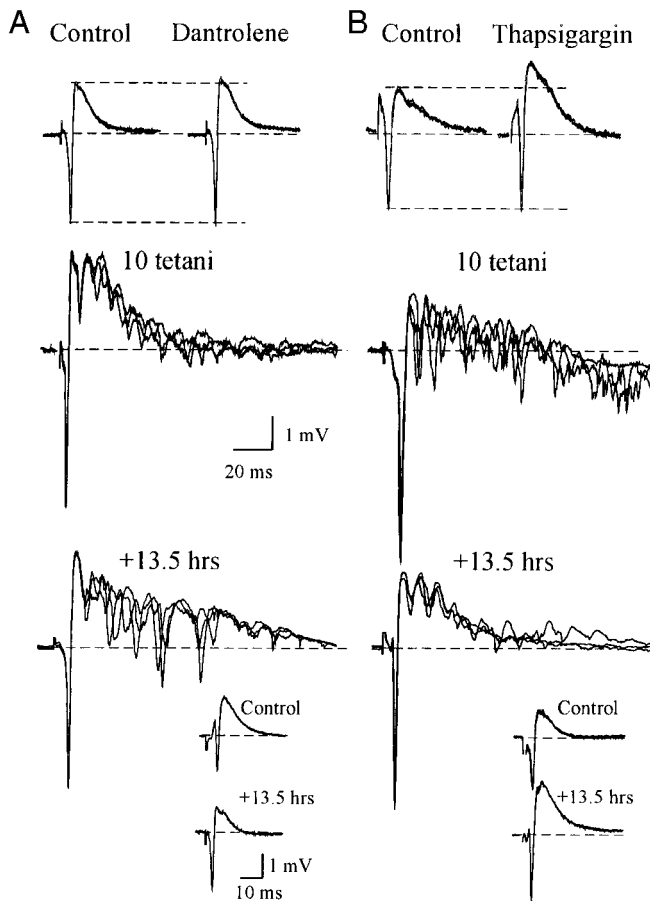

FIG. 5.   Dantrolene and thapsigargin have no significant effect on normal synaptic transmission, the induction, or the maintenance of epileptiform discharges. *A*: dantrolene. *Top, left*: responses evoked by maximum stimulation intensity during control period (5 superimposed records). *Right*: responses evoked after 30 min application of dantrolene (30 μM). *Middle*: responses evoked after 10 tetani are epileptiform. *Bottom*: epileptiform discharges persist after >13 h. *Inset*: time-matched control responses. Dantrolene was present for the duration of the experiment. *B*: thapsigargin. *Top, left*: responses evoked by maximum stimulation intensity during control period (5 superimposed records). *Right*: population spike amplitude is increased after 30 min application of thapsigargin (1 μM), but due to variability the mean increase failed to reach significance. *Middle*: responses evoked after 10 tetani are epileptiform. *Bottom*: epileptiform discharges persist after >13 h. *Inset*: time-matched control responses. Thapsigargin was present for the duration of the experiment.

the type of cell death produced, and other features such as regional susceptibility, might be attributable to the method employed for inducing the seizures.

Once we established seizure-induced cell death in vitro, characterization between necrosis and apoptosis was not the focus of this investigation, and on the basis of these data, we cannot with confidence discriminate which form of cell death predominated. Additionally, although we observed that the majority of TUNEL-positive were located in the principal cell body layers, because we did not use immunohistochemical methods to differentiate between neurons and glia, we cannot address the differential susceptibility of neurons compared with nonneuronal cells. Therefore we use the term ''cell death.'' The results of the gel electrophoresis were more reminiscent of the random DNA cleavage that occurs in necrosis. Nevertheless, some of the morphological features we observed in the TUNEL-positive cells are characteristic of apoptosis,

including well-delimited chromatin aggregates located peripherally on the nuclear membrane and cell shrinkage. TUNEL-positive cells were present in small clusters or individually, typically, but not exclusively, in principal cell body layers. However, membrane blebbing or apoptotic bodies were not seen.

Mesial temporal sclerosis associated with complex partial seizures typically involves the CA1 region (Honavar and Meldrum 1997). We observed the greatest percentage of TUNEL-positive cells in the CA1 region. We may have observed DNA laddering if the DNA from the CA1 region alone was electrophoresed. This would require pooling the CA1 region from several slices to yield a sufficient quantity of DNA. If these cells are dying due to apoptosis and if this process is dependent on the synthesis of macromolecules (Wyllie et al. 1984), blockade of this synthesis might be of therapeutic relevance. Cleavage of DNA by endogenous nucleases is an event that occurs well after the cell has committed itself to death (Mesner et al. 1995). Therefore we suggest that assessment of earlier occurring apoptotic events, such as release of cytochrome C (Yang et al. 1996), caspace activation (Miura et al. 1993), or translocation of plasma membrane phosphatidylserine (Martin 1995), between 3 and 12 h, is necessary for a more refined temporal assessment of the induction of seizure-induced apoptosis in vitro.

Mechanisms of seizure-induced cell death

Seizures are characterized cellularly by synchronized synaptic activity, which results in excessive glutamate release.
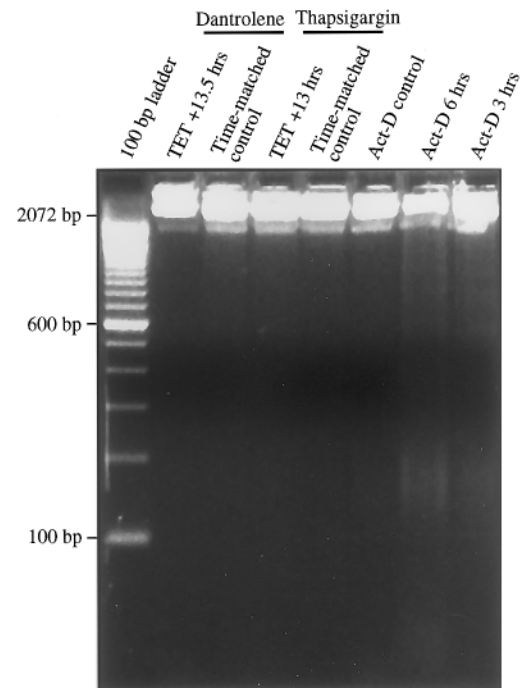

FIG. 6.   Dantrolene and thapsigargin prevented seizure-induced cell death. Electrophoresis of DNA isolated from tetanized slices (12- to 14-h group) demonstrates no degradation. *Lane 1*: 100-bp marker; *lane 2*: tetanized slice + dantrolene; *lane 3*: time-matched control + dantrolene; *lane 4*: tetanized slice + thapsigargin; *lane 5*: time-matched control + thapsigargin; *lane 6*: control organotypic hippocampal slice culture; *lane 7*: hippocampal slice culture exposed to Act-D (6 h); and *lane 8*: hippocampal slice culture exposed to Act-D (3 h). Note laddering in *lanes 7* and *8*.

Prolonged overactivation of postsynaptic glutamate receptors is neurotoxic, and this form of cell death is referred to as ''excitotoxicity'' (Olney and Sharpe 1969).

Illustrative of this relation is the recent report that homozygous mice deficient in the glutamate transporter GLT-1 presented with lethal spontaneous seizures and an exacerbation of the seizure-induced cell death compared with wild type (Tanaka et al. 1997). Excitotoxicity is a biphasic process and includes both a rapid and a delayed component (Meyer 1989; Randall and Thayer 1992). The early damage is the result of an influx of Na^+, Cl^-, and H_2O with osmolysis. The delayed cell death is secondary to an increase in intracellular Ca^{2+}, which is thought to initiate a variety of deleterious consequences including activation of phospholipase A2, which leads to the production of arachidonic acid and free radicals, endonuclease fragmentation of DNA, and production of nitric oxide, which contributes to inhibition of mitochondrial oxidative phosphorylation (Choi 1988; Dugan and Choi 1994; Meldrum and Garthwaite 1990).

Role of ER Ca^{2+} stores

Ca^{2+} released from ER stores participates in a variety of physiological processes including gene transcription, protein synthesis, cell differentiation, and synaptic plasticity (Berridge 1998; Henzi and MacDermott 1992). This source of Ca^{2+} also is recognized as being important for pathological events. Blockade of Ca^{2+} release from ER stores with dantrolene has been reported previously to not significantly affect normal synaptic transmission (Obenaus et al. 1989; O'Mara et al. 1995); this is consistent with what we observed. The relation of ER Ca^{2+} stores to long-term potentiation (LTP) is more complex. The induction of LTP has been reported to be blocked by both thapsigargin (Harvey and Collingridge 1992) and dantrolene (Obenaus et al. 1989). Conversely, O'Mara et al. (1995) reported that dantrolene inhibited long-term depression and depotentiation but was without effect on LTP. This discrepancy is likely attributable to differences in experimental conditions such as the concentration of dantrolene (20 or 50 μM), the duration of the perfusion period (180 or 20 min), or the synapses that were tetanized (Schaffer collateral to CA1 vs. medial perforant path to DG). Although we did not systematically assess the effect of dantrolene and thapsigargin on LTP, we did not observe any differences in the epileptiform activity induced by repeated tetanic stimulation attributable to these drugs. Our observation that dantrolene prevented seizure-induced cell death is consistent with the reports of many others; however, the neuroprotection we observed for thapsigargin was somewhat unexpected. Thapsigargin has been reported to produce multiple effects including inhibition of protein synthesis, cell proliferation, tumor promotion, and at low concentrations (i.e., nanomolar), apoptosis (Treiman et al. 1998). In our hands, thapsigargin did not produce apoptosis in control slices and did not exacerbate seizure-induced cell death in tetanized slices. Indeed, thapsigargin prevented seizure-induced cell death (compare Fig. 3 with Fig. 6).

Maintenance of the filling state of intracellular stores is attributable to the dynamic interplay between the leak of Ca^{2+} out of the store via the leak channel, SERCAs, and capacitative Ca^{2+} influx (Thomas and Hanley 1994). Blockade of SERCAs by thapsigargin produces a biphasic elevation of cytosolic

Ca^{2+} influx (Treiman et al. 1998). The initial increase (15–120 s) is due to release of Ca^{2+} from stores via the leak channel (Thomas and Hanley 1994), but its persistence (minutes) is attributable to capacitative Ca^{2+} influx (Putney 1986), which is thought to be carried by the Ca^{2+}-release activated Ca^{2+} current (I_{CRAC}) (Hoth and Penner 1992; Penner et al. 1993). Before tetanization we did observe a thapsigargin-induced increase in population spike amplitude; this might be attributable to the initial passive leak of Ca^{2+}. We applied thapsigargin at a concentration of 1 μM. At micromolar concentrations, thapsigargin should maximally discharge the contents of the store (Thomas and Hanley 1994) but also might block capacitative Ca^{2+} influx (Mason et al. 1991), Ca^{2+} channels (Shmigol et al. 1995) and protein synthesis (Paschen et al. 1996). Additionally, chronic exposure to thapsigargin may have increased the capacity of endogenous Ca^{2+} buffers (Petersen et al. 1993).

The maximum probability of opening for the IP_3-sensitive store occurs at 200 nM free Ca^{2+}, with sharp decreases on either side of the maximum. In contrast, the ryanodine-sensitive store is maximally responsive to concentrations of Ca^{2+} ranging from 1 to 100 μM (Bezprozvanny et al. 1991). Because IP_3-sensitive stores are inhibited by concentrations of free intracellular Ca^{2+} only slightly higher than physiological levels, the most likely candidate for the source of ER Ca^{2+} that would participate in Ca^{2+}-dependent cell death cascades is the ryanodine-sensitive store; however, cross-talk between the two stores has been described whereby Ca^{2+} released from IP_3-sensitive stores can induce CICR (Simpson et al. 1995).

Type I RYRs, or skeletal muscle RYRs, are located on the sarcoplasmic reticulum, and proximal to dihydropyridine-sensitive voltage-operated Ca^{2+} channels on T-tubule infoldings of the plasma membrane, which together form the functional unit underlying excitation-contraction coupling. Type I RYRs also are present in cerebellar Purkinje neurons. Type II, or cardiac muscle RYRs, are ubiquitous in the brain, whereas type III RYRs are restricted to specific brain regions such as CA1 stratum pyramidale, caudate, and dorsal thalamus (Furuichi et al. 1994). Although RYR isoforms are expressed differentially in a variety of tissues, more than one isoform may be coexpressed in both neurons (Furuichi et al. 1994) and in nonneuronal cells (reviewed in Sutko and Airey 1996). Functional differences between receptor isoforms are understood only poorly; however, differential regulation of types 1 and 3 IP_3R by cytosolic Ca^{2+} has been described recently (Cardy et al. 1997). Although only speculative, types II and III RYRs appear to be the most likely candidates to participate in the seizure-induced, CICR-dependent cell death we observed.

We did not employ the use of blockers of glutamatergic transmission or VDCCs, and neither dantrolene nor thapsigargin affected the induction or maintenance of epileptiform discharges. Therefore the entry of extracellular Ca^{2+} into the cell due to the prolonged epileptiform depolarizations was unperturbed. We interpret our results as being consistent with the hypothesis that Ca^{2+}-dependent excitotoxicity requires both the entry of Ca^{2+} and the release of Ca^{2+} from ER stores, resulting in the initiation, the amplification, or the persistent functioning of Ca^{2+}-dependent death cascades. A direct test of this hypothesis would require the comparison of $[Ca^{2+}]_I$ during epileptiform discharges (e.g., PAD) in the presence and the absence of CIRC blockers followed by an assessment of cell

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death. Our hypothesis is consistent with Frandsen and Schousboe (1991, 1993), who postulate that Ca^{2+} derived from internal stores participates in NMDA-receptor-mediated excitotoxicity they observed in cultured cortical neurons. Additionally, Ca^{2+}-dependent excitotoxicity might be dependent on the source of Ca^{2+} influx (Tymianski et al. 1993). The source specificity hypothesis requires the spatial constraint of proximity of Ca^{2+}-dependent cell death nanomachinery and NMDA receptors. Intracellular Ca^{2+} stores associated with the ER are distributed throughout neurons (Sharp et al. 1993). In the dendrites of CA1 pyramidal neurons, the ER terminates in various forms in the dendritic spines, is referred to as the spine apparatus (Spacek and Harris 1997; Svoboda et al. 1996), and sometimes comes into contact with the postsynaptic density (Spacek and Harris 1997). Additionally, in CA1 pyramidal neurons, where we observed the greatest percentage of TUNEL-positive cells, there is a greater density of ryanodine receptors compared with IP$_3$ receptors (Sharp et al. 1993). The smaller volume of dendrites compared with soma might promote the interaction between ryanodine-sensitive ER stores, NMDA receptors, and the Ca^{2+}-dependent nanomachinery required for excitotoxicity.

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