

# Rapid $\text{Ca}^{2+}$ Entry through $\text{Ca}^{2+}$ -Permeable AMPA/Kainate Channels Triggers Marked Intracellular $\text{Ca}^{2+}$ Rises and Consequent Oxygen Radical Production

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The widespread neuronal injury that results after brief activation of highly  $\text{Ca}^{2+}$ -permeable NMDA channels may, in large part, reflect mitochondrial  $\text{Ca}^{2+}$  overload and the consequent production of injurious oxygen radicals. In contrast, AMPA/kainate receptor activation generally causes slower toxicity, and most studies have not found evidence of comparable oxygen radical production. Subsets of central neurons, composed mainly of GABAergic inhibitory interneurons, express AMPA/kainate channels that are directly permeable to  $\text{Ca}^{2+}$  ions. Microfluorometric techniques were performed by using the oxidation-sensitive dye hydroethidine (HET) to determine whether the relatively rapid  $\text{Ca}^{2+}$  flux through AMPA/kainate channels expressed on GABAergic neurons results in oxygen radical production comparable to that triggered by NMDA. Consistent with previous studies, NMDA exposures triggered increases in fluorescence in most cultured cortical neurons, whereas high  $\text{K}^+$  (50 mM) exposures (causing depolarization-induced  $\text{Ca}^{2+}$  influx through voltage-sensitive  $\text{Ca}^{2+}$  channels) caused little fluorescence change. In contrast, kainate exposure caused fluores-

cence increases in a distinct subpopulation of neurons; immunostaining for glutamate decarboxylase revealed the responding neurons to constitute mainly the GABAergic population. The effect of NMDA, kainate, and high  $\text{K}^+$  exposures on oxygen radical production paralleled the effect of these exposures on intracellular  $\text{Ca}^{2+}$  levels when they were monitored with the low-affinity  $\text{Ca}^{2+}$ -sensitive dye fura-2FF, but not with the high-affinity dye fura-2. Inhibition of mitochondrial electron transport with  $\text{CN}^-$  or rotenone almost completely blocked kainate-triggered oxygen radical production. Furthermore, antioxidants attenuated neuronal injury resulting from brief exposures of NMDA or kainate. Thus, as with NMDA receptor activation, rapid  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels also may result in mitochondrial  $\text{Ca}^{2+}$  overload and consequent injurious oxygen radical production.

*Key words:* cell culture; glutamate; AMPA; kainate; NMDA; cobalt; hydroethidine; calcium imaging; fura-2; fura-2FF; free radicals; superoxide; tetramethylrhodamine ethylester

Agonist-triggered  $\text{Ca}^{2+}$  influx may constitute a key link between glutamate receptor activation and subsequent neurodegeneration. In cortical culture, brief periods of activation of NMDA channels, which are highly  $\text{Ca}^{2+}$ -permeable (MacDermott, 1986), are capable of triggering widespread neurodegeneration. In contrast, much more prolonged periods of activation of AMPA/kainate receptor-gated channels are required before comparable neurotoxicity develops (Koh et al., 1990; Choi, 1992). This may reflect the fact that most AMPA/kainate channels are poorly permeable to  $\text{Ca}^{2+}$  and likely cause secondary  $\text{Ca}^{2+}$  influx via the depolarization and activation of voltage-sensitive  $\text{Ca}^{2+}$  channels (VSCCs; Murphy and Miller, 1989; Weiss et al., 1990a). Multiple factors have been hypothesized to contribute to the differences in toxicity that result from NMDA and AMPA/kainate receptor activation. First, observations that NMDA and kainate exposures induce comparable elevations in intracellular  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_i$ ) (Tymianski et al., 1993; Rajdev and Reynolds, 1994; Dugan et al., 1995) have led to the hypothesis that excito-

toxic injury is  $\text{Ca}^{2+}$  source-dependent (Tymianski et al., 1993), with NMDA receptors most closely linked to injury-initiating machinery (Tymianski et al., 1993; Rajdev and Reynolds, 1994). However, the observation that brief NMDA exposures cause much more  $^{45}\text{Ca}^{2+}$  influx than kainate exposures has lent support to an alternative (but not exclusive) hypothesis that the amount of  $\text{Ca}^{2+}$  entry is a critical determinant of neuronal injury (Hartley et al., 1993; Eimerl and Schramm, 1994; Lu et al., 1996).

Oxygen radicals are likely mediators of neuronal injury resulting from glutamate exposure (Coyle and Puttfarcken, 1993). Evidence is particularly compelling in the case of NMDA receptor activation, which triggers rapid oxygen radical production (Lafon-Cazal et al., 1993; Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996) and injury, against which antioxidants are partially protective (Monyer et al., 1990; Chow et al., 1994). Mitochondria buffer the large amounts of  $\text{Ca}^{2+}$  that accumulate intracellularly in response to NMDA receptor activation (Werth and Thayer, 1994; White and Reynolds, 1995, 1997; Wang and Thayer, 1996) and may be the primary source of NMDA-triggered oxygen radical production (Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996). The role of oxygen radicals in AMPA/kainate receptor-mediated injury is less clear. Although oxygen radical scavengers and inhibitors of oxygen radical production have been protective against kainate-induced excitotoxicity (Dykens et al., 1987; Patel et al., 1996), most (Lafon-Cazal et al., 1993; Dugan et al., 1995; Reynolds and

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Hastings, 1995) but not all (Bindokas et al., 1996) studies that used spin-trapping agents or oxidation-sensitive fluorescent dyes have failed to detect oxygen radical production after kainate exposure.

The discovery of subpopulations of central neurons that express  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels (Iino et al., 1990) and are unusually vulnerable to AMPA/kainate receptor-mediated injury (Brorson et al., 1994; Turetsky et al., 1994; Yin et al., 1994a) provides an opportunity to evaluate injury mechanisms initiated by the activation of a distinct agonist-activated  $\text{Ca}^{2+}$  entry route. Recent electrophysiological and histological studies indicate that nearly all GABAergic forebrain neurons express  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels (Bochet et al., 1994; Jonas et al., 1994; Yin et al., 1994a) and suggest that GABAergic neurons may be the primary population of central neurons expressing large numbers of these channels. The purpose of this project was twofold. We first sought to determine whether kainate activation of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels on GABAergic cortical neurons can trigger acute oxygen radical production. Second, we used  $\text{Ca}^{2+}$ -sensitive fluorescent dyes to examine the relationship between intracellular  $\text{Ca}^{2+}$  levels achieved on the activation of different  $\text{Ca}^{2+}$  entry routes (NMDA channels,  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels, and VSCCs) and the resultant oxygen radical production.

## MATERIALS AND METHODS

**Chemicals and reagents.** Hydroethidine (HET), tetramethylrhodamine ethylester (TMRE), and fura-2 were purchased from Molecular Probes (Eugene, OR). Fura-2FF was purchased from Texas Fluorescence Lab (Austin, TX). MK-801 was purchased from Research Biochemicals (Natick, MA). Tissue culture media and serum were obtained from Life Technologies (Grand Island, NY). 2,3-Dihydroxy-6-nitro-7-sulfamoylbenzo(F)quinoxaline (NBQX) was kindly provided by Novo Nordisk (Malov, Denmark). NMDA, kainate, rotenone, cyanide, trolox, and carbonyl cyanide *p*-(trifluoromethoxy)phenyl hydrazone (FCCP) were obtained from Sigma (St. Louis, MO). U74500 was kindly provided by Upjohn (Kalamazoo, MI). All other chemicals and reagents were obtained from common commercial sources.

**Cortical cultures.** Cultures were prepared mainly as described previously (Yin et al., 1994a). Briefly, dissociated mixed neocortical cell suspensions were prepared from 14- to 16-d-old embryonic Swiss-Webster mice and plated ( $1-2 \times 10^5$  cells/cm<sup>2</sup>) on a previously established layer of cortical astrocytes. Initial plating medium consisted of Eagle's Minimum Essential Medium (EMEM; Earle's salts prepared glutamine-free) supplemented with 10% heat-inactivated horse serum, 10% fetal bovine serum, 2 mM glutamine, and 25 mM glucose. Cultures were kept in a 37°C/5% CO<sub>2</sub> incubator. After 4–6 d *in vitro* (DIV), non-neuronal cell division was halted by exposure to  $10^{-5}$  cytosine arabinoside for 24 hr. Then the cells were switched to an identical maintenance medium lacking fetal serum. Subsequent media replacement occurred twice a week, and cells were studied after 14–16 DIV.

The same procedure was used to prepare glial cultures, except that tissue was obtained from early postnatal (1–3 d) mice, plating media were supplemented with epidermal growth factor (10 ng/ml), and cell suspensions were plated directly on Primaria tissue culture-treated multiwell plates (Falcon, Franklin Lake, NJ) or poly-L-lysine-coated glass.

**Immunocytochemistry.** GABAergic cortical neurons were labeled with glutamic acid decarboxylase (GAD; Developmental Studies Hybridoma Bank at the University of Iowa, Iowa City, IA) immunohistochemistry. Cultures were fixed for 40 min in 4% paraformaldehyde, washed three times with PBS, and incubated for 30 min with "blocking solution" (10% horse serum in PBS) to minimize background staining. Primary antibody exposures (1:500) occurred for 72 hr at 4°C. Biotinylated horse anti-mouse (Vector Laboratories, Burlingame, CA), ABC solution (Vector Laboratories), and 3-amino-9-ethyl-carbazole (AEC; Sigma) were used to visualize stained cells.

**$\text{Co}^{2+}$  labeling.**  $\text{Co}^{2+}$  labeling was performed generally as previously described (Pruss et al., 1991) with minor modifications (Yin et al., 1994a). Cultures were  $\text{Co}^{2+}$ -loaded by exposure to kainate (100  $\mu\text{M}$ ) with  $\text{Co}^{2+}$  (2.5 mM) in an uptake buffer [containing (in mM) 139 sucrose, 57.5 NaCl,

5 KCl, 2 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 12 glucose, and 10 HEPES, pH 7.6] for 15 min. Then the cultures were washed in uptake buffer with 3 mM EDTA to remove extracellular  $\text{Co}^{2+}$  and were incubated in 0.05% (NH<sub>4</sub>)<sub>2</sub>S for 5 min to precipitate intracellular  $\text{Co}^{2+}$ , followed by an uptake buffer wash and fixation (4% paraformaldehyde for 40 min). For silver enhancement the cultures were washed in development buffer [containing (in mM) 292 sucrose, 15.5 hydroquinone, and 42 citric acid] and incubated in 0.1% AgNO<sub>3</sub> in development buffer at 50–55°C. This solution was changed at 15 min intervals while enhancement was monitored periodically via microscopic observation. When enhancement was complete (usually after 30 min), the reaction was terminated by being washed three times in warm development buffer.

**Neurotoxicity experiments.** Toxic exposures to either NMDA (100  $\mu\text{M}$  + 10  $\mu\text{M}$  NBQX for 10 min) or to kainate (100  $\mu\text{M}$  + 10  $\mu\text{M}$  MK-801 for 20 min) were performed in room air (25°C), using a HEPES-buffered salt solution (HSS) with the following composition (in mM): 130 Na<sup>+</sup>, 5.4 K<sup>+</sup>, 0.8 Mg<sup>2+</sup>, 1.8 Ca<sup>2+</sup>, 130 Cl<sup>-</sup>, 20 HEPES, pH 7.4 at 25°C, and 15 glucose. Exposures were terminated by replacing the exposure solution with MEM plus glucose along with 10  $\mu\text{M}$  MK-801/10  $\mu\text{M}$  NBQX and returning the cultures to the incubator. To assess neuroprotection from antioxidants (20  $\mu\text{M}$  U74500 or 3 mM trolox), we exposed the cultures to the antioxidant for 1 hr before and during and for 20–24 hr after the toxic exposure. Overall neuronal injury was assessed 20–24 hr after the start of the exposures by morphological examination and was assessed quantitatively by measurement of lactate dehydrogenase (LDH) in the bathing medium, an index proportional to the total number of damaged neurons (Koh and Choi, 1987). LDH values are scaled to the near-maximal mean value found in sister cultures exposed to 300  $\mu\text{M}$  kainate for 24 hr (equal to 100% cell loss). Kainate-induced damage to the GAD(+) subpopulation was assessed by direct cell counts of intact GAD(+) neurons in kainate-exposed cultures, in comparison to numbers present in sister cultures exposed to sham wash alone.

**Imaging studies.** Cultures were plated on glass-bottomed dishes (Mattek Cultureware, Ashland, MA) and mounted to a stage adapter on an inverted microscope (Nikon Diaphot, Tokyo, Japan). All agonist exposures were performed at room temperature (25°C) in a 1.5 ml static HSS bath. High K<sup>+</sup> exposures were in identical buffer except for the substitution of 50 mM K<sup>+</sup> for equimolar Na<sup>+</sup>. Preselected fields were illuminated by a xenon light source and a Nikon 40 $\times$  magnification, 1.3 numerical aperture, epifluorescence oil immersion objective. Emitted fluorescence was imaged with a Hamamatsu intensified charge-coupled device camera (Hamamatsu City, Japan). In all experiments the electronic background signals (obtained with the camera shutter closed) were obtained at each wavelength for subtraction from measured signals, and baseline fluorescence measurements were obtained for 10 min before the addition of agonists. To analyze experiments, we outlined neuronal somata and gathered data on an 80486-based computer, using Image-1/Fluor software from Universal Imaging (West Chester, PA).

For [ $\text{Ca}^{2+}$ ]<sub>i</sub> measurements the cultures were loaded in the dark, with a 5  $\mu\text{M}$  concentration of either fura-2 AM or fura-2FF AM in HSS containing 0.2% pluronic acid and 0.4% dimethyl sulfoxide (DMSO) for 30 min at 25°C. Then the cultures were washed in HSS (three times) and kept in the dark for an additional 30 min to allow for complete dye deesterification. Cells were illuminated alternately at 340 and 380 nm, and fluorescence was monitored at 510 nm. For fura-2, [ $\text{Ca}^{2+}$ ]<sub>i</sub> levels were expressed as the ratio of emitted fluorescence on excitation at 340 and 380 nm. For fura-2FF, [ $\text{Ca}^{2+}$ ]<sub>i</sub> was determined by the equation:

$$[\text{Ca}^{2+}]_i = K_D(F_{\min}/F_{\max})[(R - R_{\min})/(R_{\max} - R)],$$

where  $R$  is the observed 340:380 fluorescence ratio (Grynkiewicz et al., 1985).  $R_{\min}$  is the 340:380 fluorescence ratio value determined in cortical neurons exposed to  $\text{Ca}^{2+}$ -free HSS in the presence of a 2 mM concentration of the  $\text{Ca}^{2+}$  chelator EGTA and a 10  $\mu\text{M}$  concentration of the  $\text{Ca}^{2+}$  ionophore ionomycin.  $R_{\max}$  is the 340:380 fluorescence ratio value determined in the same neurons exposed to ionomycin in the presence of 10 mM  $\text{Ca}^{2+}$ .  $F_{\min}$  indicates 380 nm fluorescence at  $R_{\min}$ , and  $F_{\max}$  indicates 380 nm fluorescence at  $R_{\max}$ . For Fura-2FF, the  $K_D$  used was 35  $\mu\text{M}$  (Golovina and Blaustein, 1997). The system was recalibrated after any adjustments to the apparatus. Oxygen radical production was monitored with the oxidation-sensitive dye HET. Stock HET (1 mg/ml) was prepared as previously described (Bindokas et al., 1996) in dry DMSO and stored in frozen aliquots for use within 8 weeks. Cultures were loaded in the dark with 5  $\mu\text{M}$  HET in HSS (for 45 min at 25°C), and the same concentration of HET was maintained in the bath throughout each

experiment. To minimize dye photo oxidation, we decreased light illumination to 2.5% of initial output, using UV grade neutral density filters (Omega Optical, Brattleboro, VT) and images (16 frame samples) obtained at 1–2 min intervals. Under these conditions, little fluorescence increase occurred within 50 min (see Fig. 3). Cells were excited at 510–560 nm, and emission was monitored at  $>590$  nm. Camera gain was adjusted to give baseline maximal fluorescence levels of 40–60 (arbitrary units) of a maximal eight-bit signal output of 256. Fluorescence measurements for each cell ( $F_x$ ) were normalized to the average fluorescence intensity for that cell during the 10 min baseline period ( $F_0$ ). Because fluorescence is cumulative, oxygen radical production rate was assessed as the rate of increase (or slope) of the  $F_x/F_0$  curves over time, and net oxygen radical production was assessed as the increase in  $F_x/F_0$  over baseline. Control experiments using 1  $\mu\text{M}$  HET were performed exactly as those above (using 5  $\mu\text{M}$  HET), except for the concentration of HET in the bath.

For dual imaging of oxygen radicals and  $[\text{Ca}^{2+}]_i$ , cultures were loaded first with fura-2FF as described. During the deesterification of the fura-2FF, cultures were loaded with HET as described. For data acquisition the fura-2FF images were obtained immediately before HET images, each with appropriate excitation and emission filters, at 2 min intervals. Data were analyzed separately, including separate background subtractions, for each dye as described. Control experiments, which used excitation and emission settings for one dye in the presence of the other dye only, revealed no cross fluorescence.

For assessment of changes in mitochondrial potential ( $\Delta\Psi$ ), neurons were incubated for 30 min with TMRE (0.1  $\mu\text{M}$ ) at 37°C, and the same concentration of TMRE was maintained in all bathing solutions throughout the experiments (performed at 25°C; Farkas et al., 1989). Cells were excited at 495 nm, emission was monitored at  $\geq 530$  nm, and images were acquired every 60 sec. To avoid photobleaching, we attenuated the fluorescence intensity with neutral density filters (Omega Optical). Camera gain was adjusted to give baseline maximal fluorescence levels of 150–200 (arbitrary units) of a maximal eight-bit signal output of 256. Fluorescence changes were quantified by selecting a cytoplasmic region of each cell that was strongly fluorescent at baseline (indicating that it was “mitochondria-rich”) and normalizing subsequent fluorescence measurements to the baseline fluorescence ( $F_0$ ), which was assessed as the emitted fluorescence of the initial image taken during the experiment.

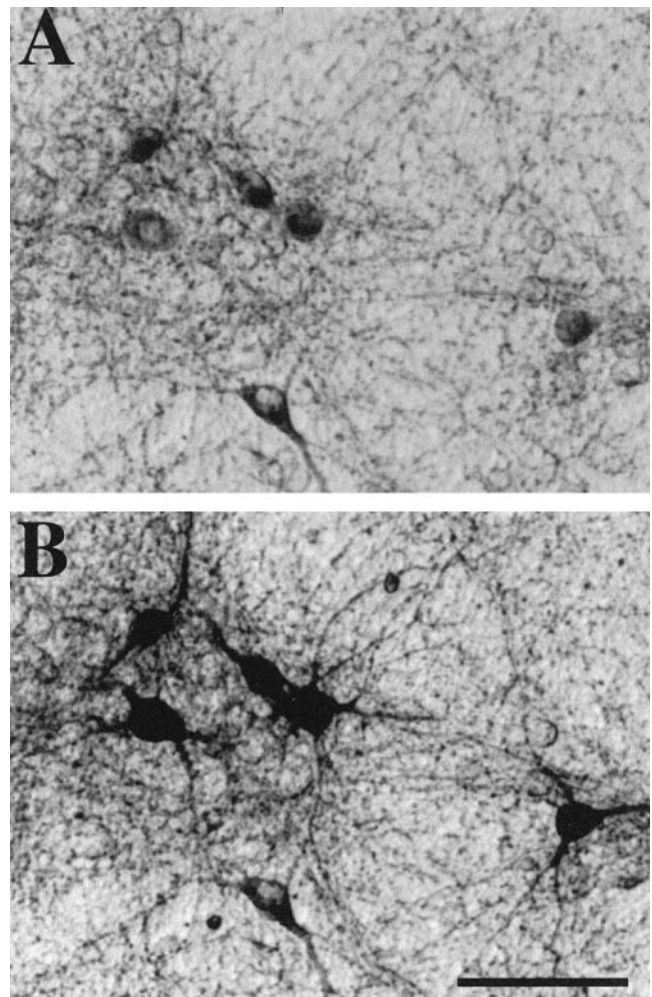
For experiments involving cyanide ( $\text{CN}^-$ ; 3 mM), cultures were preexposed to  $\text{CN}^-$  for 15 min before the addition of agonists. For rotenone, cultures were preexposed (10  $\mu\text{M}$ ) for a 40 min period that ran concurrently with HET or TMRE loading. For experiments involving  $\text{CN}^-$  or rotenone, glutamate antagonists (10  $\mu\text{M}$  NBQX or 10  $\mu\text{M}$  MK-801) were added as appropriate both before and during agonist exposures to minimize the effects attributable to endogenous glutamate release and to isolate measured effects to the desired channel type.

**Experiment replication.** All reported experiments represent at least three independent replications. All imaging studies represent at least 15 GAD(+) neurons and 100 GAD(-) cortical neurons.

## RESULTS

### Kainate-triggered oxygen radical production is dependent on extracellular $\text{Ca}^{2+}$

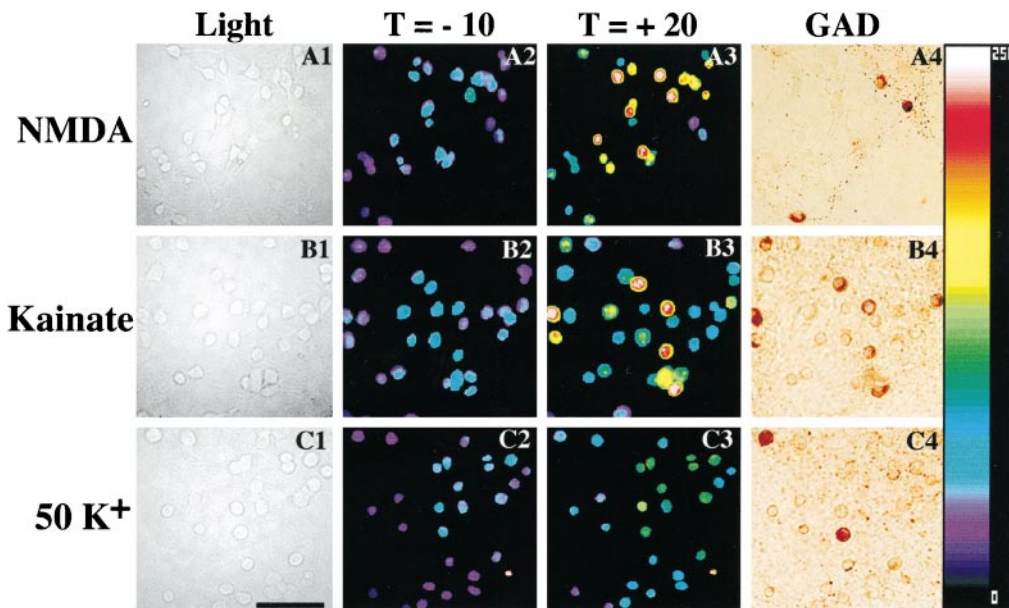
Neurons possessing  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels (Iino et al., 1990) can be identified by a histochemical technique that is based on kainate-triggered uptake of  $\text{Co}^{2+}$  ions [ $\text{Co}^{2+}(+)$  neurons; Pruss et al., 1991]. The specificity of this stain is indicated by the inability of NMDA or high  $\text{K}^+$  to substitute for kainate in triggering  $\text{Co}^{2+}$  ion entry. Consistent with electrophysiological studies (Bochet et al., 1994; Jonas et al., 1994), we have reported previously that nearly all (90%) of GABAergic cortical neurons are  $\text{Co}^{2+}(+)$  (Yin et al., 1994a) (Fig. 1). We have chosen to focus the present studies of effects of  $\text{Ca}^{2+}$  entry through  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels on the GABAergic population for two reasons. First, whereas the overall  $\text{Co}^{2+}(+)$  population contains heterogeneous neuronal cell types, the GABAergic population [identified via glutamic acid decarboxylase immunohistochemistry; GAD(+) neurons] constitutes a well defined and physiologically important subset of cortical neurons. In addition, because  $\text{Co}^{2+}$  labeling depends on the normal



**Figure 1.** Glutamic acid decarboxylase (GAD)-immunoreactive cortical neurons exhibit kainate-stimulated  $\text{Co}^{2+}$  uptake. Cultures were subjected to kainate-stimulated  $\text{Co}^{2+}$  loading (see Materials and Methods), followed by processing for GAD immunocytochemistry. Then selected immunostained fields were photographed (400 $\times$  magnification) before (*A*) and again after (*B*) development of the  $\text{Co}^{2+}$  stain.  $\text{Co}^{2+}(+)$  neurons can be identified by a darkening in the cell body and processes. Scale bar, 50  $\mu\text{m}$ .

functioning of AMPA/kainate channels, labeling after toxic agonist exposures may fail in a portion of the neurons with  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels.

Because both  $\text{Ca}^{2+}$ -permeable AMPA/kainate and NMDA channels may permit rapid  $\text{Ca}^{2+}$  influx (Lu et al., 1996), we sought first to determine whether the activation of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels can cause oxygen radical production similar to that observed on NMDA receptor activation (Lafon-Cazal et al., 1993; Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996). Intracellular oxygen radical production was monitored by measuring changes in the fluorescence of cells loaded with HET, a dye that readily permeates living cells and is reported to be oxidized selectively by superoxide radicals into a highly fluorescent compound, ethidium (Bindokas et al., 1996; Satoh et al., 1998). HET has certain advantages over other frequently used oxidation-sensitive dyes for the present purposes. Dihydrorhodamine gives a speckled signal of mitochondrial origin that is best appreciated under high-power confocal microscopy (Dugan et al., 1995) and is poorly suited for



**Figure 2.** NMDA, kainate, and high  $K^+$  exposures produce distinct patterns of oxygen radical generation. Cortical cultures were exposed to NMDA ( $200 \mu\text{M} + 10 \mu\text{M}$  NBQX) (*A*), kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) (*B*), or high  $K^+$  ( $50 \text{ mM} + 10 \mu\text{M}$  MK-801/NBQX) (*C*). In each experiment the images were obtained under visible light before exposure (*column 1*), under fluorescence for oxidized hydroethidine (HET; see Materials and Methods) 10 min before (*column 2*) and 20 min after (*column 3*) drug application, and again after immunostaining for GAD (*column 4*). Note the widespread fluorescence increases after NMDA exposure and the relatively selective increases in GAD(+) neurons after kainate exposure. As indicated by the pseudocolor scale bar, HET images represent fluorescent intensity on an eight-bit/0–256 scale. Scale bar,  $50 \mu\text{m}$ .

comparing responses in large fields of neurons. Dichlorofluorescein fluorescence is potently blocked by intracellular acidification (Reynolds and Hastings, 1995) and is thus not suitable, because agonist-triggered intracellular acidification is highly dependent on the influx of extracellular  $\text{Ca}^{2+}$  (Irwin et al., 1994) and may differ markedly between cells expressing large numbers of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels (which permit rapid  $\text{Ca}^{2+}$  influx) and cells lacking these channels. An advantage of HET is that ethidium intercalates within nuclear DNA where the fluorescence intensity of the dye increases greatly (LePecq and Paoletti, 1967), providing high sensitivity. Also, the relative resistance of HET to auto-oxidation and photo-oxidation (in comparison to other oxidation-sensitive fluorescent dyes) (Dugan et al., 1995; Reynolds and Hastings, 1995) permits the prolonged periods of fluorescence monitoring needed for the present studies (see Materials and Methods). Because the oxidized dye accumulates within the cell, the oxygen radical production rate was assessed as the rate of fluorescence increase over time, and net oxygen radical production was assessed as the increase in fluorescence over baseline. To compare oxygen radical production across experiments, we normalized all fluorescence readings for a given cell ( $F_x$ ) to the average fluorescence that was seen in the 10 min baseline period ( $F_0$ ) for that cell.

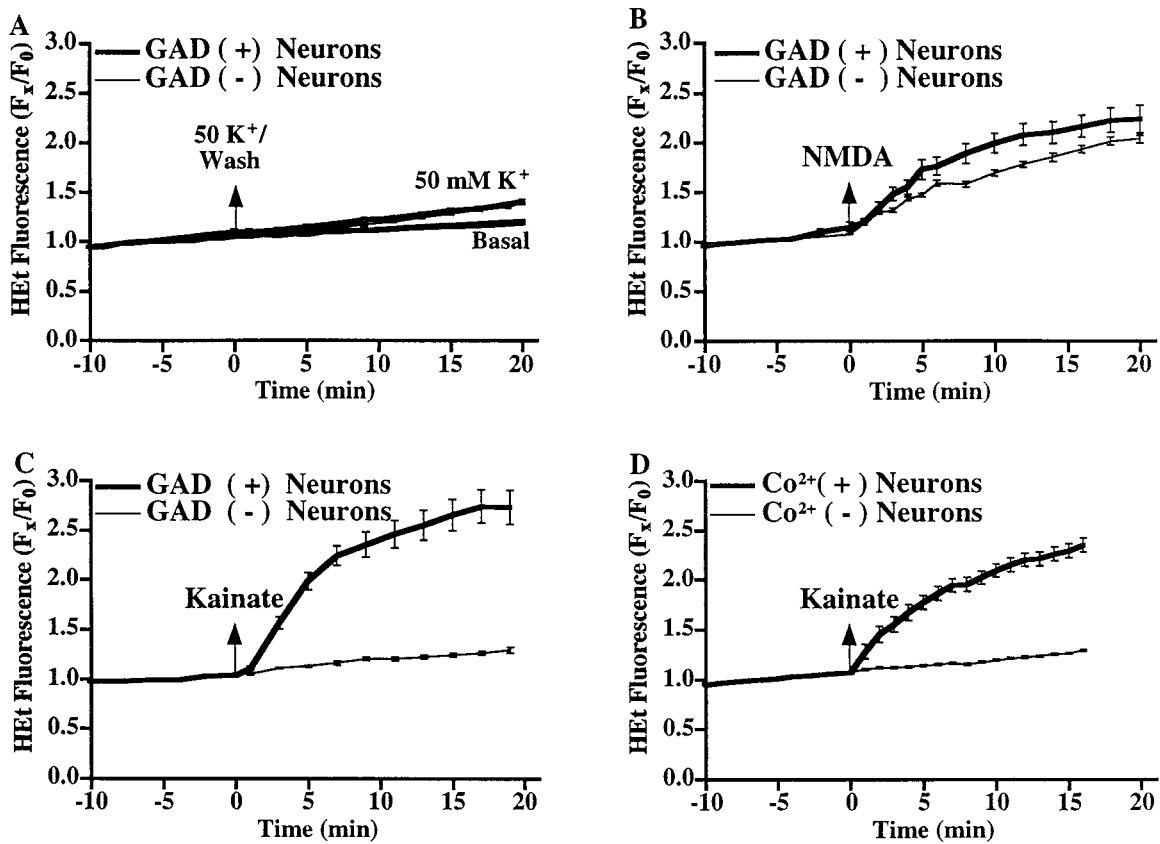
In cultures loaded with HET (see Materials and Methods), a low rate of basal oxygen radical production was evidenced by stable, slow increases in fluorescence in neurons, but not in the underlying glia (see Fig. 3*A*). Baseline fluorescence readings were acquired for 10 min before the addition of NMDA ( $200 \mu\text{M} + 10 \mu\text{M}$  NBQX), kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801), or high  $K^+$  ( $50 \text{ mM} + 10 \mu\text{M}$  NBQX/MK-801) for an additional 20 min. On exposure to NMDA, noticeable increases in fluorescence were seen within 2 min, with the majority of neurons showing substantial increases in fluorescence (normalized fluorescence increase of  $1.23 \pm 0.14$  over baseline) by the end of the 20 min exposure

(Figs. 2, 3*B*). In contrast, on exposure to high  $K^+$  ( $50 \text{ mM}$ ; a concentration sufficient to cause significant neuronal depolarization) the fluorescent increases ( $0.4 \pm 0.01$ ) were not significantly greater than those observed in cultures exposed to buffer alone ( $0.23 \pm 0.1$ ) (Figs. 2, 3*A*). Kainate exposures produced more selective increases in fluorescence. Consistent with previous studies (Dugan et al., 1995; Reynolds and Hastings, 1995), kainate exposure had little effect on the rate of oxygen radical production in the majority of cortical neurons ( $0.29 \pm 0.03$ ). However, a subset of cortical neurons, consisting predominantly of the GAD(+) neuronal population, showed rapid fluorescence increases ( $1.68 \pm 0.17$ ) similar to those seen with NMDA (Figs. 2, 3*C*). As expected, we found that kainate triggered a similar selective increase ( $1.28 \pm 0.07$ ) in fluorescence in the overall  $\text{Co}^{2+}$ (+) neuronal population (Fig. 3*D*).

To examine the role of extracellular  $\text{Ca}^{2+}$  in kainate-triggered oxygen radical production in GAD(+) neurons, we exposed cultures to kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) in consecutive extracellular  $\text{Ca}^{2+}$  concentrations of 0, 1.8, and 10 mM (Fig. 4). Kainate exposures in the absence of extracellular  $\text{Ca}^{2+}$  failed to trigger significant increases in fluorescence. Almost immediately on the return of extracellular  $\text{Ca}^{2+}$  to physiological levels, rapid increases in fluorescence were observed in GAD(+) neurons, but not in GAD(–) neurons. Only when extracellular  $\text{Ca}^{2+}$  levels were raised to 10 mM did the GAD(–) neurons begin to show fluorescence increases.

#### Relationship between agonist-triggered oxygen radical production and intracellular $\text{Ca}^{2+}$ elevations

To assess more directly the role of  $\text{Ca}^{2+}$  influx via different routes in oxygen radical production, we set out in subsequent experiments to measure intracellular free  $\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) in GAD(+) and GAD(–) neurons on exposures to NMDA, kainate, or high  $K^+$ . Initial experiments used the high-affinity ratio-



**Figure 3.** Time course of oxygen radical generation after high  $K^+$ , NMDA, and kainate exposures. HET-loaded cultures were imaged for 10 min before and 20 min after drug application. For each cell the HET fluorescence at each time point ( $F_x$ ) was normalized to the mean fluorescence for that cell during the 10 min baseline period ( $F_0$ ). Cultures were exposed to either normal HSS alone or high  $K^+$  modified HSS (+ 10  $\mu$ M MK-801/NBQX) (A), to NMDA (200  $\mu$ M + 10  $\mu$ M NBQX) (B), or to kainate (200  $\mu$ M + 10  $\mu$ M MK-801) (C, D). Immediately after imaging the cultures were processed for GAD immunocytochemistry (A–C) or for kainate-stimulated  $Co^{2+}$  labeling (D). All traces represent the means  $\pm$  SEM of 15–30 GAD(+)/ $Co^{2+}$ (+) and 100–200 GAD(-)/ $Co^{2+}$ (-) neurons, derived from at least four experiments.

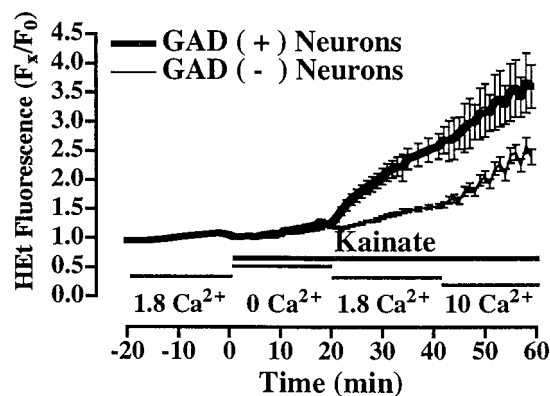
metric  $Ca^{2+}$ -sensitive dye fura-2 ( $K_D \sim 224$  nM). After loading cells with the dye and establishing the 10 min baseline recording (see Materials and Methods), we exposed the cultures to NMDA (200  $\mu$ M + 10  $\mu$ M NBQX), kainate (200  $\mu$ M + 10  $\mu$ M MK-801), or high  $K^+$  (50 mM + 10  $\mu$ M NBQX/MK-801) for 15 min. Immediately after the addition of each of these exposure solutions, sharp increases in  $[Ca^{2+}]_i$  were seen in both GAD(+) (Fig. 5A) and GAD(-) neurons (Fig. 5C). Consistent with previous studies that used this dye (Tymianski et al., 1993; Rajdev and Reynolds, 1994; Dugan et al., 1995), measured  $[Ca^{2+}]_i$  rises on each of these exposures were similar.

Because fura-2 has been reported as underestimating maximal agonist-triggered  $[Ca^{2+}]_i$  elevations (Regehr and Tank, 1992; Petrozzino et al., 1995; Hyrc et al., 1997), the above experiments were repeated by using a low-affinity  $Ca^{2+}$ -sensitive dye. Because mag-fura-5 has some sensitivity to  $[Mg^{2+}]_i$  that could interfere with the accuracy of calibrated measurements and the AM ester of the  $Mg^{2+}$ -insensitive  $Ca^{2+}$  dye, benzothiazole coumarin (BTC), loads poorly (Hyrc et al., 1997), we chose to use a newly available ratiometric dye, fura-2FF ( $K_D \sim 35$   $\mu$ M) (Golovina and Blaustein, 1997), which has similar spectral properties to fura-2 and is completely insensitive to  $Mg^{2+}$ . Unlike fura-2, fura-2FF showed distinct differences in the  $[Ca^{2+}]_i$  elevations triggered by NMDA, kainate, and high  $K^+$  exposures (Fig. 5B,D). Although NMDA triggered large and persistent increases in  $[Ca^{2+}]_i$  levels in all neurons, high  $K^+$  exposures produced a sharp but transient

increase in  $[Ca^{2+}]_i$  levels, which returned to basal levels within 5 min. As in the case of oxygen radical production, the effects of kainate on  $[Ca^{2+}]_i$  levels differed markedly between GAD(+) and GAD(-) neurons, with the former population showing a sharp and persistent increase in  $[Ca^{2+}]_i$  (Fig. 5B), whereas  $[Ca^{2+}]_i$  rises seen in GAD(-) neurons (Fig. 5D) were similar to those seen during high  $K^+$  exposures. The direct relationship between  $[Ca^{2+}]_i$  levels and HET fluorescence is illustrated further by simultaneous double imaging with fura-2FF and HET; neurons with the greatest increases in HET fluorescence also showed the highest  $[Ca^{2+}]_i$  responses on activation of either NMDA or AMPA/kainate receptors (see Fig. 6A,B). Qualitatively similar results were obtained by using mag-fura-5 (data not shown).

#### Mitochondria are a major source of agonist-triggered oxygen radical production

Because previous studies have found that mitochondria appear to be a significant source of NMDA-triggered oxygen radical production (Dugan et al., 1995; Reynolds and Hastings, 1995; Bindokas et al., 1996), subsequent experiments used the mitochondrial electron transport inhibitors cyanide ( $CN^-$ ; 3 mM) and rotenone (10  $\mu$ M) to examine the role of mitochondria in oxygen radical production in our culture system. In initial experiments the HET-loaded cultures were exposed to 3 mM  $CN^-$  for 15 min (which caused a variable slight increase in the rate of basal



**Figure 4.** Kainate-triggered oxygen radical production is  $\text{Ca}^{2+}$ -dependent. After a 20 min baseline recording the HET-loaded cultures were exposed to kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) in the presence of the indicated extracellular  $\text{Ca}^{2+}$  concentration. The traces represent the means  $\pm$  SEM of  $>30$  GAD(+) and 100 GAD(-) neurons from four experiments.

fluorescence change), followed by the addition of NMDA ( $200 \mu\text{M} + 10 \mu\text{M}$  NBQX) or kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) in the continuing presence of  $\text{CN}^-$ . NMDA exposures caused only a brief increase in fluorescence in the presence of  $\text{CN}^-$  in comparison to the much greater fluorescence increase triggered in sister cultures identically exposed in the absence of  $\text{CN}^-$  (Fig. 7A). Kainate-triggered fluorescence increases in GAD(+) neurons were eliminated almost completely by  $\text{CN}^-$  preexposure (Fig. 7B). To control for possible nonspecific effects of high concentrations of  $\text{CN}^-$ , we examined in further experiments whether rotenone, which has been shown previously to block NMDA receptor-mediated mitochondrial oxygen radical production (Dugan et al., 1995), also could block kainate-triggered oxygen radical production. As with  $\text{CN}^-$ , cultures exposed to rotenone for 40 min before and during exposure to kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) showed almost complete inhibition of oxygen radical production (Fig. 7C).

Studies of NMDA receptor-mediated excitotoxicity have demonstrated the occurrence of rapid  $\text{Ca}^{2+}$ -dependent loss of mitochondrial membrane potential ( $\Delta\Psi$ ), which may be integral to the disruption of mitochondrial functioning (Schinder et al., 1996; White and Reynolds, 1996). Such loss of  $\Delta\Psi$  also may occur in the case of rapid  $\text{Ca}^{2+}$  influx through  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels. Thus, the recent report by Budd et al. (1997), suggesting that loss of  $\Delta\Psi$  per se (triggered by the addition of the protonophore FCCP) might cause the voltage-dependent release of oxidized ethidium from mitochondria, compels control studies to examine the degree to which observed kainate-triggered HET signals reflect oxygen radical production. Initial control studies examined HET signal in cultures loaded with only  $1 \mu\text{M}$  HET, a concentration at which Budd et al. (1997) found ethidium to remain bound within mitochondrial DNA on FCCP-triggered loss of  $\Delta\Psi$ . Indeed, consistent with their findings, no FCCP-triggered HET signal was seen under these conditions (data not shown). However, on exposure to kainate, increases in HET fluorescence were seen in GAD(+) neurons that were significantly greater than those seen in GAD(-) neurons [normalized increase of  $0.49 \pm 0.06$  in GAD(+) neurons vs  $0.11 \pm 0.01$  in GAD(-) neurons;  $n \geq 50$  cells from three experiments]. Although these absolute fluorescence increases were less than those seen when  $5 \mu\text{M}$  HET was used, the relative increases were similar,

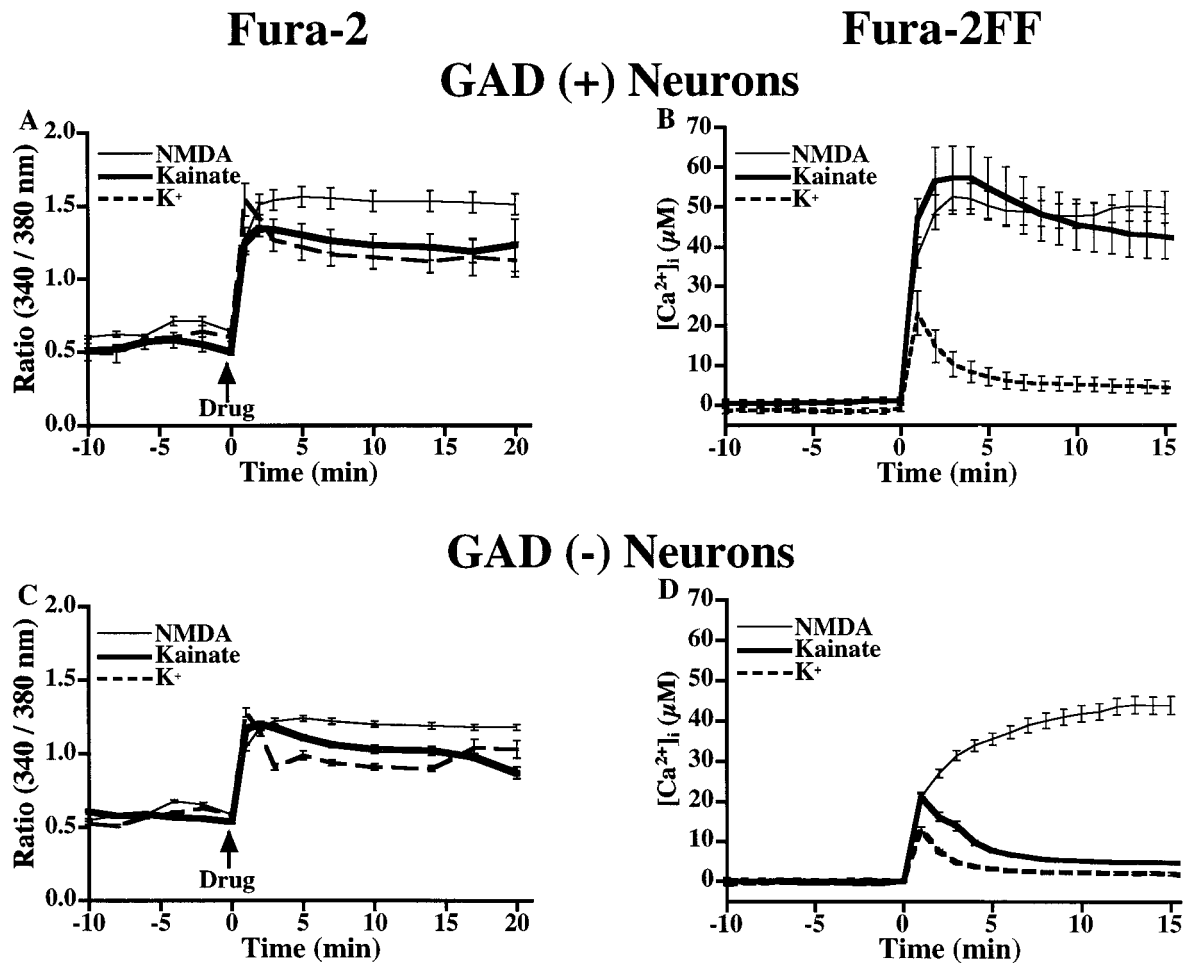
in both cases being approximately five to six times greater in GAD(+) neurons than in GAD(-) neurons [with  $5 \mu\text{M}$  HET, the increase was  $1.68 \pm 0.17$  in GAD(+) neurons vs  $0.29 \pm 0.03$  in GAD(-) neurons].

Further experiments used the potential-sensitive dye TMRE to examine the effects of NMDA and kainate exposures on  $\Delta\Psi$  in our system. This dye rapidly equilibrates between cellular compartments as a function of potential differences; the rapid loss of fluorescence from cellular domains rich in mitochondria is indicative of the loss of  $\Delta\Psi$  (Farkas et al., 1989). After the cultures were loaded with TMRE, baseline fluorescence readings were acquired for 10 min before the addition of NMDA ( $200 \mu\text{M} + 10 \mu\text{M}$  NBQX) or kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801) for an additional 20 min. On the addition of NMDA a rapid and sharp increase in fluorescence was seen (possibly reflecting an increase in  $\Delta\Psi$  relative to the depolarized cytosol) (Farkas et al., 1989), followed by a rapid loss of fluorescence in virtually all neurons (Fig. 8A), which reflects redistribution of the dye from depolarized mitochondria (Farkas et al., 1989; Schinder et al., 1996). On kainate exposure, an increase in fluorescence was seen in most neurons that was similar to (but somewhat smaller than) that seen with NMDA. However, although most neurons showed a slow decline in fluorescence toward baseline levels, the GAD(+) neuronal population showed a much more pronounced decrease in fluorescence (although still somewhat less than that seen with NMDA exposures; Fig. 8B). Indeed, this comparison between GAD(+) and GAD(-) neurons provides a useful internal control indicating that the rapid fall in signal is attributable to the loss of  $\Delta\Psi$  and not simply to cytosolic depolarization, because kainate certainly triggers effective  $\text{Na}^+$ -dependent neuronal depolarization in virtually all neurons.

To address further the concern that the kainate-triggered HET signal could partially reflect the loss of  $\Delta\Psi$ , we next examined the effects of rotenone ( $10 \mu\text{M}$ ) on kainate-triggered changes in TMRE fluorescence. In cultures that were exposed to rotenone for 40 min before and during exposure to kainate ( $200 \mu\text{M} + 10 \mu\text{M}$  MK-801), changes in  $\Delta\Psi$  in GAD(+) neurons (assessed as the fall in TMRE fluorescence) were indistinguishable from those seen in the absence of rotenone (Fig. 8C). Thus, because rotenone blocks the kainate-triggered HET signal in GAD(+) neurons without affecting kainate-induced changes in  $\Delta\Psi$ , the HET signal blocked by rotenone most likely reflects oxygen radical production.

#### Oxygen radicals contribute to NMDA or kainate receptor-mediated injury to cortical neurons

Although the above experiments demonstrate that the NMDA or kainate exposures do trigger oxygen radical production, the relevance of this oxygen radical production to resultant neurotoxicity was examined by using two antioxidants: U74500 (a 21 amino steroid; Monyer et al., 1990) and trolox (a soluble vitamin E derivative; Chow et al., 1994; Ciani et al., 1996). Toxic exposures were calibrated to trigger  $\sim 75\%$  injury either to the entire neuronal population ( $100 \mu\text{M}$  NMDA; 10 min) or to only the GAD(+) neuronal population ( $100 \mu\text{M}$  kainate; 20 min). To assess protection by anti-oxidants, we added either  $20 \mu\text{M}$  U74500 or  $3 \text{ mM}$  trolox to the culture media for 1 hr before and during and for the 20–24 hr period after the toxic agonist exposure until injury was assessed the next day (see Materials and Methods). Both antioxidants significantly attenuated overall neuronal injury resulting from NMDA exposures and the selective GAD(+) neuronal loss resulting from kainate exposures (Fig. 9).



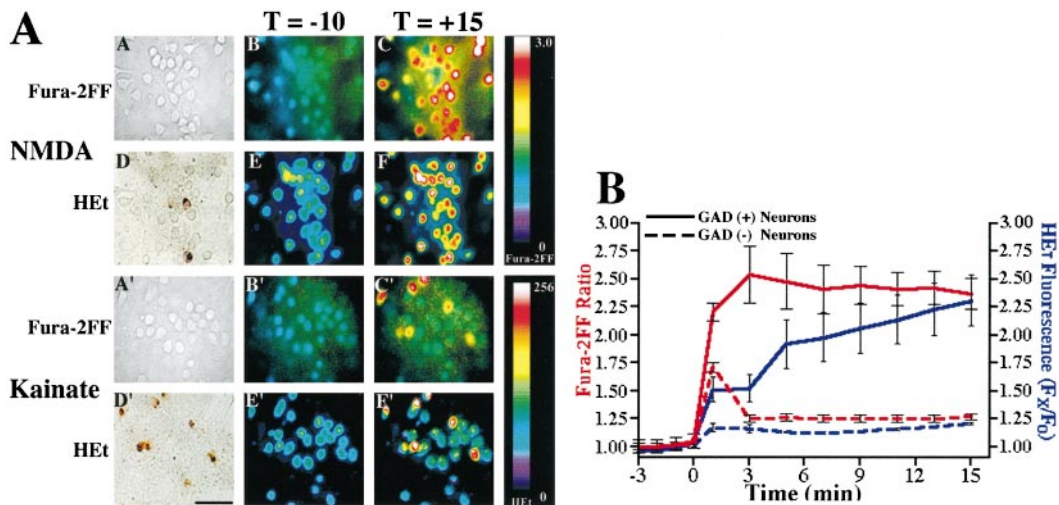
**Figure 5.** Measured  $[Ca^{2+}]_i$  rises induced by exposure to NMDA, kainate, or high  $K^+$  vary markedly, depending on the affinity of the fluorescent  $Ca^{2+}$  indicator that was used. Cultures were loaded with either fura-2 (*A, C*) or fura-2FF (*B, D*), as described (see Materials and Methods). Fura-2  $[Ca^{2+}]_i$  levels were expressed as fluorescence ratios, whereas fura-2FF  $[Ca^{2+}]_i$  levels were expressed as calibrated values. After baseline imaging the cultures were exposed for 15 min (starting at time 0) to NMDA (200  $\mu M$  + 10  $\mu M$  NBQX), kainate (200  $\mu M$  + 10  $\mu M$  MK-801), or high  $K^+$  (50 mM + 10  $\mu M$  MK-801/NBQX), as indicated, and were processed for GAD immunocytochemistry. Traces show the means  $\pm$  SEM of  $[Ca^{2+}]_i$  responses in GAD(+) (*A, B*) and in GAD(-) neurons (*C, D*) [15–25 GAD(+) neurons and >150 GAD(-) neurons in three experiments].

## DISCUSSION

The present study attempts to compare  $[Ca^{2+}]_i$  responses, oxygen radical production, and resultant neurotoxicity after the activation of the three primary routes of  $Ca^{2+}$  entry: NMDA channels,  $Ca^{2+}$ -permeable AMPA/kainate channels, and VSCCs. Using the oxidation-sensitive fluorescent dye HET, we find that NMDA exposures, but not high  $K^+$ -induced neuronal depolarization, caused increased signal in most cortical neurons. Kainate exposures caused selective  $Ca^{2+}$ -dependent signal increases in GABAergic neurons, a subpopulation of cortical neurons known to express large numbers of  $Ca^{2+}$ -permeable AMPA/kainate channels. The HET signal correlated with agonist-triggered elevations in  $[Ca^{2+}]_i$  and was attenuated by the inhibition of mitochondrial electron transport, suggesting a mitochondrial origin. Control experiments showing that electron transport inhibitors blocked kainate-triggered HET signal without affecting kainate-triggered loss of  $\Delta\Psi$  provide strong evidence that the kainate-triggered HET signal in GAD(+) neurons reflects oxygen radical generation. Indicating that the oxygen radicals contribute to neurodegeneration, antioxidants decreased injury resulting from toxic kainate and NMDA exposures.

## Relationship between intracellular $Ca^{2+}$ and neurodegeneration

Although excitotoxic injury is dependent on extracellular  $Ca^{2+}$ , the relationship between agonist-induced  $Ca^{2+}$  load and neuronal injury has been in question. As discussed in the introductory remarks, the lack of a clear relationship between agonist-induced  $[Ca^{2+}]_i$  elevations and neuronal survival (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991; Randall and Thayer, 1992; Dubinsky, 1993; Tymianski et al., 1993; Dugan et al., 1995) led to the proposal that the specific route of  $Ca^{2+}$  entry may be a critical determinant of neuronal injury ("source specificity hypothesis"; Tymianski et al., 1993), whereas the strong correlation between rapid NMDA-triggered  $^{45}Ca^{2+}$  influx and subsequent neurodegeneration (Hartley et al., 1993; Eimerl and Schramm, 1994) lent support to the alternative hypothesis that net intracellular  $Ca^{2+}$  accumulation is critical (" $Ca^{2+}$  load hypothesis"). The characterization of neurons expressing large numbers of  $Ca^{2+}$ -permeable AMPA/kainate channels (Iino et al., 1990; Brorson et al., 1992; Turetsky et al., 1994) provides new opportunity to examine the relationship between agonist-induced  $Ca^{2+}$  entry and neuronal injury. Indeed, we can now compare the



**Figure 6.** NMDA- or kainate-triggered  $[Ca^{2+}]_i$  elevations that were assessed by fura-2FF predict oxygen radical production. *Part A, Pseudocolor images.* Cultures were loaded with both fura-2FF and HET, and visible light images were obtained (*A, A'*). Fluorescent fura-2FF (*B, C* and *B', C'*), and HET (*E, F* and *E', F'*) images were obtained 10 min before (*B, B'* and *E, E'*) and 15 min after (*C, C'* and *F, F'*) exposure to NMDA (200  $\mu$ M + 10  $\mu$ M NBQX) (*top*) or kainate (200  $\mu$ M + 10  $\mu$ M MK-801) (*bottom*). Then the cultures were immunostained for GAD (*D, D'*). Note that, although NMDA triggers strong HET and fura-2FF fluorescence increases in nearly all neurons, with kainate exposures the fluorescence of both dyes was increased selectively in the GAD(+) neurons. As indicated by the scale bars, fura-2FF images represent fluorescence ratios, and HET images represent fluorescent intensity on an eight-bit/0–256 scale. Scale bar, 50  $\mu$ m. *Part B, Time course.* Cultures were loaded with both fura-2FF and HET as above. After baseline imaging the cultures were exposed to kainate (200  $\mu$ M + 10  $\mu$ M MK-801), and changes in  $[Ca^{2+}]_i$  levels (*left axis, red lines*) and oxygen radical production (*right axis, blue lines*) were monitored for an additional 15 min (see Materials and Methods). Traces show the means  $\pm$  SEM of 7 GAD(+) and 25 GAD(–) neurons from one experiment, which is representative of three.

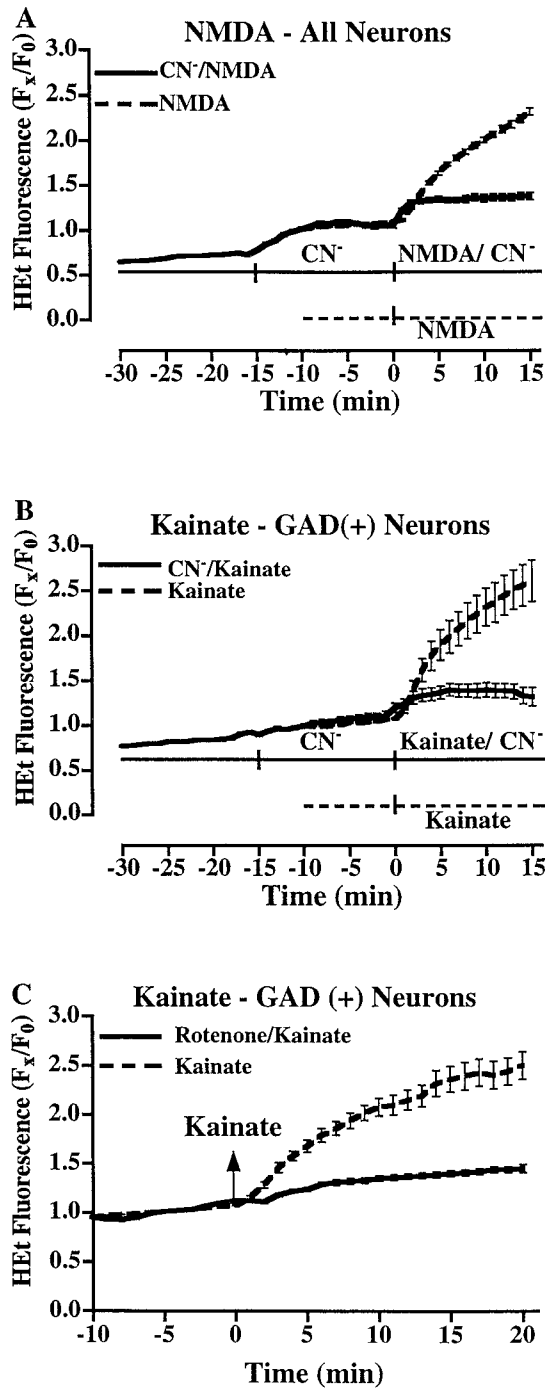
effects of  $Ca^{2+}$  entry through NMDA and  $Ca^{2+}$ -permeable AMPA/kainate channels (which likely share similar dendritic localization; Bekkers and Stevens, 1989; Gu et al., 1996) and  $Ca^{2+}$  entry through VSCCs and  $Ca^{2+}$ -permeable AMPA/kainate channels (both of which are activated by AMPA/kainate receptor stimulation but differ markedly in cellular distribution).

Recent studies have provided some support for the  $Ca^{2+}$  load hypothesis. First, despite the lack of a clear difference in fura-2-measured  $[Ca^{2+}]_i$  responses on the activation of the three routes of  $Ca^{2+}$  entry, we recently found, using  $^{45}Ca^{2+}$  accumulation measurements, that  $Ca^{2+}$ -permeable AMPA/kainate channels appeared to permit high rates of  $Ca^{2+}$  influx (comparable to those triggered by NMDA). Furthermore, the estimated  $Ca^{2+}$  influx rate on the activation of any of the  $Ca^{2+}$  entry routes generally predicted the degree of resultant injury (Lu et al., 1996). Also, a recent study that used a low-affinity  $Ca^{2+}$ -sensitive dye found NMDA-triggered  $[Ca^{2+}]_i$  responses to be much greater than those to kainate (Hyrz et al., 1997), suggesting that the lack of relationship between  $[Ca^{2+}]_i$  and injury seen in previous studies (Michaels and Rothman, 1990; Dubinsky and Rothman, 1991; Randall and Thayer, 1992; Dubinsky, 1993; Tymianski et al., 1993; Dugan et al., 1995; Lu et al., 1996) might reflect technical limitations of the measurements. Specifically, high-affinity dyes like fura-2 and indo-1 may be unable to resolve micromolar  $Ca^{2+}$  responses accurately (Tsien, 1988; Petrozzino et al., 1995; Hyrc et al., 1997) and at high concentrations actually may buffer intracellular  $Ca^{2+}$  (Regehr and Tank, 1992; Tatsumi and Katayama, 1994; Petrozzino et al., 1995). The present observation that the low-affinity dye fura-2FF, but not fura-2, distinguishes kainate-triggered  $[Ca^{2+}]_i$  responses in GABAergic neurons from those in other neurons extends the findings of Hyrc et al. (1997) by suggesting that, as with NMDA, the activation of  $Ca^{2+}$ -permeable AMPA/kainate channels can cause high  $[Ca^{2+}]_i$  responses that predict high toxicity.

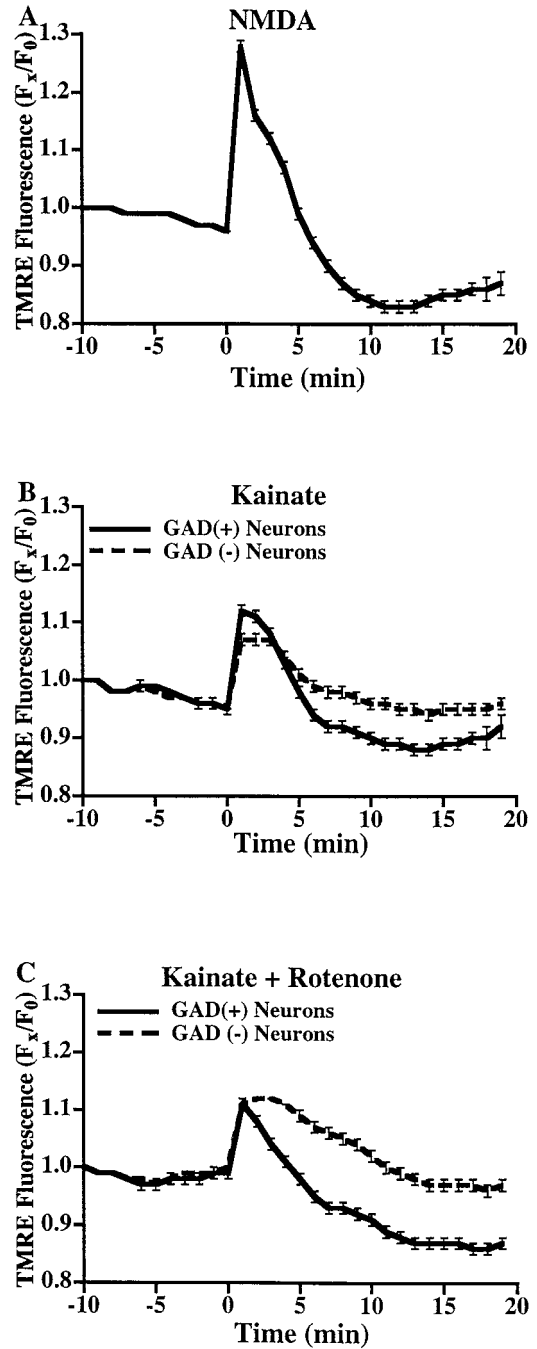
#### Oxygen radical production in excitotoxic injury: relationship to $Ca^{2+}$ load

To compare NMDA and  $Ca^{2+}$ -permeable AMPA/kainate receptor-mediated injury mechanisms, we find that it is necessary to examine not only  $[Ca^{2+}]_i$  responses but also the processes downstream from  $Ca^{2+}$  entry that may contribute to the injury. Imaging and toxicity studies have provided considerable evidence that  $Ca^{2+}$ -dependent oxygen radical production contributes critically to NMDA receptor-mediated injury (Monyer et al., 1990; Lafon-Cazal et al., 1993; Chow et al., 1994; Dugan et al., 1995; Reynolds and Hastings, 1995). However, the finding of most previous studies (Lafon-Cazal et al., 1993; Dugan et al., 1995; Reynolds and Hastings, 1995) that AMPA/kainate receptor activation failed to trigger evident oxygen radical production lent support to the source specificity hypothesis by suggesting that NMDA and AMPA/kainate receptor-mediated neurotoxicities are fundamentally different (Tymianski et al., 1993; Rajdev and Reynolds, 1994). In contrast to the present study, a previous study failed to find evidence of kainate-triggered oxygen radical production in cortical neurons, even those expressing  $Ca^{2+}$ -permeable AMPA/kainate channels (Canzoniero et al., 1994). Possible differences include the use of dihydorhodamine from the previous study, which may be less sensitive to oxygen radical production than HET, and their coloaded with fura-2 (to pre-identify neurons with  $Ca^{2+}$ -permeable AMPA/kainate channels), which could have buffered and limited the availability of  $[Ca^{2+}]_i$  (Regehr and Tank, 1992; Tatsumi and Katayama, 1994; Petrozzino et al., 1995). Indeed, a study that used HET did report kainate to trigger oxygen radical production in certain hippocampal neurons but did not characterize the responding neuronal population (Bindokas et al., 1996).

Present findings that kainate exposures can trigger rapid and relatively selective oxygen radical production in GABAergic neu-

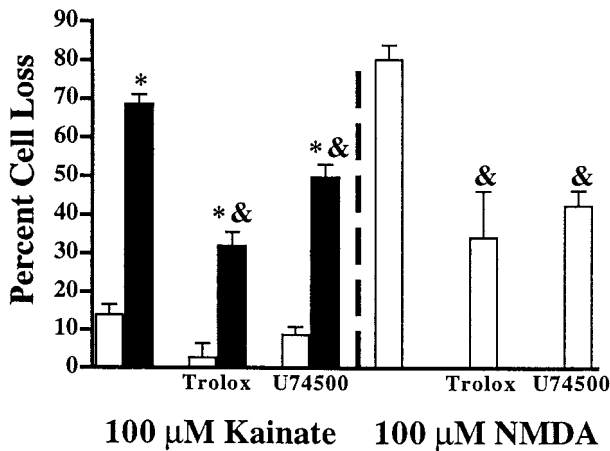


**Figure 7.** Electron transport inhibitors attenuate NMDA- or kainate-triggered oxygen radical production. After baseline recordings the cultures were exposed to 3 mM CN<sup>-</sup> for 15 min before NMDA (200  $\mu$ M + 10  $\mu$ M NBQX) (*A*) or kainate (200  $\mu$ M + 10  $\mu$ M MK-801) (*B*) was added in the continuing presence of CN<sup>-</sup> (*solid traces*). For a comparison of agonist effects between CN<sup>-</sup>-treated and normal conditions, fluorescence intensities for each neuron were normalized to its average fluorescence during the 10 min immediately before agonist addition. Other HET-loaded cultures were preexposed to rotenone (10  $\mu$ M) for 40 min before kainate (200  $\mu$ M + 10  $\mu$ M MK-801) was added in the continued presence of rotenone (*solid traces*, *C*). For the purposes of comparison, in each experiment the sister cultures were exposed identically in the absence of the electron transport inhibitor (*broken traces*). Note that, because kainate caused little oxygen radical production in GAD(-) neurons, *B* and *C* illustrate the effect of electron transport inhibitors on kainate-triggered oxygen radical production only on GAD(+) neurons.



**Figure 8.** Kainate causes mitochondrial depolarization in GAD(+) neurons, but not in GAD(-) neurons. Cultures were loaded with 0.1  $\mu$ M TMRE for 30 min (see Materials and Methods). After baseline recordings ( $F_0$ ) the cultures were exposed to NMDA (200  $\mu$ M + 10  $\mu$ M NBQX; *A*), to kainate (200  $\mu$ M + 10  $\mu$ M MK-801; *B*), or to kainate (200  $\mu$ M + 10  $\mu$ M MK-801) in the presence of rotenone (10  $\mu$ M; *C*) (see Materials and Methods); the fluorescence was monitored for 20 min more. Traces show the means  $\pm$  SEM of TMRE fluorescence in all neurons (*A*) or in GAD(+) neurons (*B*, *C*, *solid traces*) and in GAD(-) neurons (*B*, *C*, *broken traces*) [30–70 GAD(+) neurons and >150 GAD(-) neurons, more than or equal to three experiments].

rons and that oxygen radical scavengers attenuate the resultant injury provide strong evidence that rapid Ca<sup>2+</sup> flux through Ca<sup>2+</sup>-permeable AMPA/kainate channels is capable of causing injurious oxygen radical production. Thus, present data lend



**Figure 9.** Antioxidants are neuroprotective against NMDA- or kainate-induced neuronal injury. Cultures were exposed to kainate (100  $\mu$ M for 20 min) or NMDA (100  $\mu$ M for 10 min) in the presence or absence of an antioxidant (3 mM trolox or 20  $\mu$ M U74500; see Materials and Methods), and injury to the overall neuronal population (*open bars*) and to the GAD(+) neuronal population (*filled bars*) was assessed the next day. Values represent the means  $\pm$  SEM compiled from at least four experiments;  $n = 12$ –20 cultures per condition. An *ampersand* indicates neuronal loss significantly different from that caused by agonist alone; an *asterisk* indicates GAD(+) neuronal loss significantly different from total neuronal loss ( $p < 0.01$  by two-tailed  $t$  test).

support to the  $\text{Ca}^{2+}$  load hypothesis by suggesting that similar mechanisms underlie NMDA receptor-mediated injury and the selective injury triggered by strong activation of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels. The importance of  $\text{Ca}^{2+}$  load, however, need not exclude the importance of  $\text{Ca}^{2+}$  source effects. Of note,  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels and NMDA channels likely are concentrated in postsynaptic regions of dendrites (Bekkers and Stevens, 1989; Perkel et al., 1993; Malinow et al., 1994; Murphy et al., 1994; Gu et al., 1996).  $\text{Ca}^{2+}$  entering through either of these channels often may be constrained spatially and thus could cause similar effects related to the particularly high local  $\text{Ca}^{2+}$  concentrations that are achieved (Petrozzino et al., 1995). In contrast, the more uniform distribution of VSCCs throughout the plasma membrane may lead to a dilution of  $\text{Ca}^{2+}$  entering through these channels, possibly resulting in less toxicity.

#### Oxygen radical production is of mitochondrial origin

Consistent with previous studies (Dugan et al., 1995; Bindokas et al., 1996), we find that the inhibition of electron transport markedly attenuated NMDA or kainate-triggered oxygen radical production, suggesting that it is of mitochondrial origin. Indeed, present results suggest that mitochondria may play a similar role in injury caused by the activation of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels, as in that caused by NMDA receptor activation where they clearly have been demonstrated to buffer large  $\text{Ca}^{2+}$  loads, with consequent oxygen radical production (Werth and Thayer, 1994; White and Reynolds, 1995, 1997; Wang and Thayer, 1996).

Recent studies have begun to clarify the deleterious effects that may result from mitochondrial  $\text{Ca}^{2+}$  overload. Rapid  $\text{Ca}^{2+}$  uptake causes direct mitochondrial depolarization (Schinder et al., 1996; White and Reynolds, 1996), impairment of energy metabolism (Wang et al., 1994), and uncoupling of electron transport

from ATP production (Beatrice et al., 1980; Gunter and Pfeiffer, 1990), with the resultant release of oxygen radicals (perhaps particularly superoxide) from the electron transport chain (Turrens et al., 1985; Coyle and Puttfarcken, 1993). Superoxide, to which HET appears to be particularly sensitive (Bindokas et al., 1996), may be a critical intermediate in acute excitotoxic injury (Lafon-Cazal et al., 1993; Patel et al., 1996) via its conversion into more reactive oxygen species such as  $\text{OH}^-$  or  $\text{ONOO}^-$  (Coyle and Puttfarcken, 1993; Packer et al., 1996). Thus, mitochondria may be a key cellular organelle responsible for converting rapid  $\text{Ca}^{2+}$  entry caused by the activation of NMDA channels or  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels into neuronal injury. Localization of mitochondria at dendritic sites of  $\text{Ca}^{2+}$  entry through NMDA and  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels may be linked to rapid localized oxygen radical production and the resultant initiation of injury in dendrites (Bindokas and Miller, 1995).

#### Disease relevance

Both glutamate-mediated excitotoxicity and oxidative stress have been implicated in acute neuronal injury such as occurs in ischemia or trauma, and present results are consistent with a role of AMPA/kainate receptor-mediated entry in those conditions (Sheardown et al., 1990; Wrathall et al., 1994). Present findings also may be relevant to the selective neurodegeneration seen in Alzheimer's disease and amyotrophic lateral sclerosis, diseases in which many of the neurons that preferentially degenerate are highly vulnerable to AMPA/kainate receptor-mediated injury (Hugon et al., 1989; Beal et al., 1991; Page et al., 1991; Rothstein et al., 1993), likely reflecting the expression of large numbers of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels (Weiss et al., 1990b; Pruss et al., 1991; Yin et al., 1994b; Burke et al., 1995; Carriedo et al., 1996). Low-level activation of  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels and consequent oxygen radical production could cause slowly cumulative oxidative damage to mitochondrial DNA (Mecocci et al., 1993) or cell membranes (Coyle and Puttfarcken, 1993), eventually contributing to the enhanced tissue oxidation and impairment of energy metabolism noted in these diseases (Beal, 1995; Bergeron, 1995; Good et al., 1996; Markesbery, 1997). Indeed, neurons expressing  $\text{Ca}^{2+}$ -permeable AMPA/kainate channels might be particularly prone to cumulative excitotoxic injury because these channels (unlike NMDA channels, which are blocked by  $\text{Mg}^{2+}$  ions at resting potentials) permit unimpeded high ion flux whenever activated, regardless of membrane potential.

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