

# Mitochondria Control AMPA/Kainate Receptor-Induced Cytoplasmic Calcium Deregulation in Rat Cerebellar Granule Cells

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Although mitochondria mediate the delayed failure of cytoplasmic  $\text{Ca}^{2+}$  homeostasis [delayed  $\text{Ca}^{2+}$  deregulation (DCD)] in rat cerebellar granule cells resulting from chronic activation of NMDA receptors, their role in AMPA/KA-induced DCD remains to be established. The mitochondrial ATP synthase inhibitor oligomycin protected cells against KA- but not NMDA-evoked DCD. In contrast to NMDA-evoked DCD, no additional protection was afforded by the further addition of rotenone. The effects of KA on cytoplasmic  $\text{Ca}^{2+}$  homeostasis, including the protection afforded by oligomycin, could be reproduced by veratridine. KA exposure induced a partial mitochondrial depolarization that was enhanced by oligomycin, indicating ATP

synthase reversal. The nonglycolytic substrates pyruvate and lactate were unable to maintain  $\text{Ca}^{2+}$  homeostasis in the presence of KA. In contrast to NMDA, KA exposure did not cause mitochondrial  $\text{Ca}^{2+}$  loading. The data indicate that  $\text{Na}^+$  entry via noninactivating AMPA/KA receptors or voltage-activated  $\text{Na}^+$  channels compromises mitochondrial function sufficiently to cause ATP synthase reversal. Oligomycin may protect by preventing the consequent mitochondrial drain of cytoplasmic ATP.

*Key words:* calcium; cerebellar granule cells; kainate; mitochondrial membrane potential; NMDA; glutamate excitotoxicity; glutamate receptors

Glutamate excitotoxicity plays a major role in neuronal cell death in the mammalian CNS after ischemia (Greene and Greenamyre, 1996; Henneberry, 1997; Lee et al., 1999). Excessive neuronal  $\text{Ca}^{2+}$  and  $\text{Na}^+$  loading occurs as a consequence of the prolonged elevation of extracellular glutamate and activation of postsynaptic receptors (Choi, 1987). Although much research modeling excitotoxicity in cell culture has focused on a privileged role for the NMDA receptor (Tymianski et al., 1993; Peng and Greenamyre, 1998; Sattler et al., 1998), the nondesensitizing activation of AMPA receptors by KA (Hollmann and Heinemann, 1994) can also induce necrotic (Kato et al., 1991; Brorson et al., 1994; Rajdev and Reynolds, 1994; Carriedo et al., 1998; Kiedrowski, 1998; Leski et al., 1999), apoptotic (Cheung et al., 1998; Larm et al., 1998), or mixed (Cebers et al., 1997) modes of cell death in a variety of primary neuronal cultures.

KA-mediated excitotoxicity is most apparent in those neurons whose AMPA/KA receptors are  $\text{Ca}^{2+}$  permeable as a consequence of the relative absence of glutamate receptor 2 subunits (Hollmann and Heinemann, 1994). These include cerebellar granule cells cultured in elevated KCl (Holopainen et al., 1989, 1990; Puttfarcken et al., 1992; Condorelli et al., 1993; Hack and Balázs, 1995; Savidge and Bristow, 1997, 1998) and the GABAergic subpopulation of cortical neurons (Carriedo et al., 1998, 2000). KA is also toxic toward a variety of neurons in which the increase in

cytoplasmic free  $\text{Ca}^{2+}$  ( $[\text{Ca}^{2+}]_c$ ) is small compared with that with NMDA receptor activation (Hyrz et al., 1997; Stout and Reynolds, 1999). AMPA/KA receptor activation is associated with a large elevation in cytoplasmic free  $\text{Na}^+$  (Courtney et al., 1995; Itoh et al., 1998; Kiedrowski, 1998), and cell death has been proposed to be primarily related to  $\text{Na}^+$  entry and consequent cell swelling (Bindokas and Miller, 1995; Kiedrowski, 1998).

The role of mitochondria in KA-mediated excitotoxicity is unclear. Hoyt et al. (1998) observed no acute effect of KA on  $\Delta\psi_m$  in cultured cortical neurons. Kiedrowski (1998) reported a reversible depolarization, although plasma membrane depolarization may have contributed to the signal (Ward et al., 2000). Mitochondrial  $\text{Ca}^{2+}$  accumulation in response to KA has been reported in striatal (Peng and Greenamyre, 1998), cortical (Hoyt et al., 1998), and spinal motor (Carriedo et al., 2000) neurons. In this paper we examine the role of mitochondria in the acute KA-induced failure of cytoplasmic  $\text{Ca}^{2+}$  homeostasis [delayed  $\text{Ca}^{2+}$  deregulation (DCD)] of cultured rat cerebellar granule cells. In contrast to NMDA receptor-mediated DCD, the cells do not load their mitochondria with  $\text{Ca}^{2+}$  but do undergo a rapid failure of oxidative phosphorylation and are protected against DCD by ATP synthase inhibition, which further depolarizes the mitochondria and decreases the generation of reactive oxygen species. The effects can be mimicked by inhibiting the inactivation of voltage-dependent  $\text{Na}^+$  channels with veratridine, emphasizing the role played by  $\text{Na}^+$  entry into the cell.

## MATERIALS AND METHODS

*Materials.* Fura-2 AM was obtained from TEF Labs (Austin, TX). Tetramethylrhodamine methyl ester (TMRM<sup>+</sup>), rhodamine-123, and dihydroethidium (hydroethidine) were obtained from Molecular Probes (Leiden, The Netherlands). (5*R*,10*S*)-(+)-5-Methyl-10,11-dihydro[*a,d*]-cyclohepten-5,10-imine hydrogen maleate (MK-801) and 2,3-dioxo-6-nitro-1,2,3,4-tetrahydrobenzo[*f*]quinoxaline-7-sulfonamide disodium (NBQX) were obtained from Research Biochemicals (SEMAT, St. Albans, Hertfordshire, UK). Mn(III) tetrakis(4-benzoic acid) porphyrin chloride (MnTBAP) was purchased from Calbiochem (Nottingham,

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UK); a stock solution of 25 mM MnTBAP was prepared in 75 mM NaOH to obtain a final pH of 7.0–7.4. Fetal calf serum and minimal essential medium (MEM) were from Life Technologies (Paisley, Strathclyde, UK). Oligomycin, rotenone, KA, NMDA, glycine, FCCP, lactate, 2-deoxyglucose, and other reagents were from Sigma (Poole, Dorset, UK).

**Culture of cerebellar granule cells.** Preparation of cerebellar granule cells from 6-d-old Wistar rats was performed as described by Courtney et al. (1990). Cells were plated on 1.5% poly-D-lysine-coated glass coverslips (13 mm circle for nonperfusion experiments and 22 mm square for the perfusion experiments) at a density of  $2.10 \times 10^5$  cells/cm<sup>2</sup>. Cells were cultured in MEM containing Earle's salts plus 10% (v/v) fetal calf serum, 25 mM KCl, 30 mM glucose, 2 mM glutamine, 100  $\mu$ g/ml streptomycin, and 100 U/ml penicillin. After 18–24 hr, the medium was changed, and 10  $\mu$ M cytosine arabinoside was added to prevent non-neuronal cell proliferation. Cerebellar granule cells were maintained in a humidified atmosphere of 95% air and 5% CO<sub>2</sub>, at 37°C, and used after 7–8 d *in vitro*.

**Incubation conditions.** Unless otherwise stated, experiments were performed in incubation medium containing (in mM): 120 NaCl, 3.5 KCl, 1.3 CaCl<sub>2</sub>, 1.2 MgCl<sub>2</sub>, 0.4 KH<sub>2</sub>PO<sub>4</sub>, 20 TES buffer, 5 NaHCO<sub>3</sub>, 1.2 Na<sub>2</sub>SO<sub>4</sub>, and 15 glucose, pH 7.4 (adjusted with NaOH), at 37°C. Nifedipine (1  $\mu$ M) was additionally present. In experiments in which NMDA receptor activation was investigated, incubations were performed in Mg<sup>2+</sup>-free incubation medium. In experiments in which the effect of nonglycolytic substrates was analyzed, the cells were incubated in the absence of glucose and in the presence of 2 mM 2-deoxyglucose and 10 mM lactate or 10 mM pyruvate.

**Imaging of single-cell fluorescence.** Single-cell imaging of fluorescent probes was performed in a MiraCal Imaging system (Life Science Resources, Cambridge, UK), using a Nikon Diaphot-TMD inverted epifluorescence microscope equipped with a 40 $\times$  oil immersion objective and Sutter filter wheel. Experiments were performed in a nonperfused thermostated chamber (300  $\mu$ l final volume).

**Cytoplasmic free Ca<sup>2+</sup> and mitochondrial membrane potential.** Intracellular Ca<sup>2+</sup> fluorescence was monitored after loading with 3  $\mu$ M fura-2 AM, for 30 min at 37°C, in incubation medium containing 30  $\mu$ g/ml bovine serum albumin. After rinsing, the fluorescence ratio was determined after excitation at 340 and 380 nm and emission at >505 nm. For combined TMRM<sup>+</sup> and fura-2 cell-imaging fluorescence, the cells were equilibrated with 50 nM TMRM<sup>+</sup> and 3  $\mu$ M fura-2 AM for 30 min (37°C) in incubation medium containing 30  $\mu$ g/ml bovine serum albumin. After rinsing, the cells were maintained in the presence of TMRM<sup>+</sup> and excited at 340, 380 and/or 488 nm, with emission at >515 nm. Although peak TMRM<sup>+</sup> absorption occurs at longer wavelengths (Haugland, 1996), 488 nm excitation has the advantage of limiting photodynamic damage to the cells at the concentrations of TMRM<sup>+</sup> required to observe matrix quenching. Cells were loaded with rhodamine-123 by incubating in the presence of 1.3  $\mu$ M probe for 10 min at room temperature (22°C), followed by 3  $\mu$ M fura-2 AM plus rhodamine-123 for 30 min at 37°C. After washing, the cells were maintained in the presence of 1.3  $\mu$ M rhodamine-123. Combined rhodamine-123 and fura-2 fluorescence was monitored at excitation at 340, 380, and/or 548 nm, and emission was determined via a Chroma technology (Burlington, VT) fura-2–rhodamine beam splitter.

**Data analysis.** Single-cell responses are representative of the indicated number of experiments from independent cell preparations. Ethidium fluorescence data are the means  $\pm$  SEM of the indicated number of individual cell somata from six to eight independent experiments. Statistical analysis was performed by the unpaired two-tailed Student's *t* test.

**Whole-cell fluorescence simulation.** The fluorescence of granule cells loaded with TMRM<sup>+</sup> or rhodamine-123 was simulated using an Excel program exactly as described previously (Ward et al., 2000). The mitochondrial volume was set to 1% of the soma; external probe concentration was 50 nM. For TMRM<sup>+</sup> the quench limit was 15  $\mu$ M and the plasma membrane rate constant was 0.005; for rhodamine-123 the quench limit was 50  $\mu$ M and the rate constant was 0.0005.

## RESULTS

### Delayed Ca<sup>2+</sup> deregulation evoked by activation of NMDA or AMPA/KA receptors

Granule cells have a sufficiently high glycolytic activity to maintain cytoplasmic Ca<sup>2+</sup> homeostasis and viability in the presence of the ATP synthase inhibitor oligomycin (Budd and Nicholls, 1996a). Because the *in situ* mitochondrial membrane potential

$\Delta\psi_m$  is retained and even enhanced in the presence of the inhibitor (Scott and Nicholls, 1980; White and Reynolds, 1996; Ward et al., 2000), the organelles continue to accumulate Ca<sup>2+</sup> and generate reactive oxygen species (ROS). The further addition of a respiratory chain inhibitor such as rotenone allows  $\Delta\psi_m$  to decay, preventing the mitochondria from accumulating Ca<sup>2+</sup>, but without affecting glycolytic ATP production (Budd and Nicholls, 1996a). Previous experiments have shown that the combination of oligomycin plus rotenone, but not oligomycin alone, protects granule cells against the DCD induced by chronic glutamate plus glycine exposure (Budd and Nicholls, 1996b; Castilho et al., 1998).

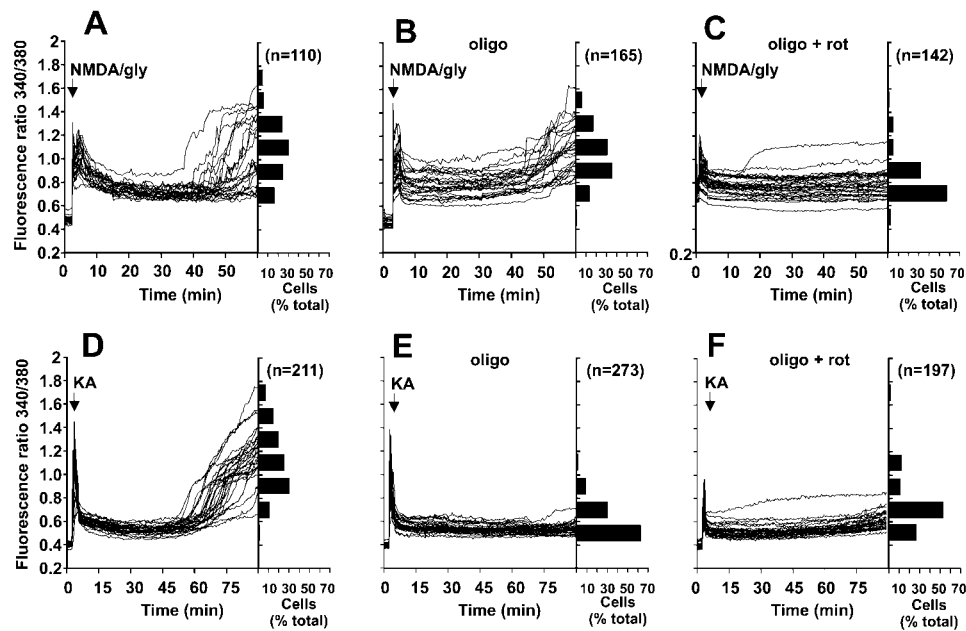
To compare the role of NMDA versus non-NMDA receptors in inducing DCD, the cells were exposed continuously in a nonperfusing chamber to either 100  $\mu$ M NMDA or 10  $\mu$ M glycine in Mg<sup>2+</sup>-free medium in the presence of the non-NMDA receptor antagonist NBQX (Hack and Balázs, 1995) and the L-type Ca<sup>2+</sup> channel inhibitor nifedipine. Alternatively, AMPA/KA receptors were selected for by repeating the experiment with 100  $\mu$ M KA in the presence of 1.2 mM Mg<sup>2+</sup>, MK-801, and nifedipine. DCD was observed after activation of both NMDA and AMPA/KA receptors (Fig. 1*A,D*), although Ca<sup>2+</sup> deregulation induced by KA only occurred after 60–75 min (Fig. 1*D*). KA exposure did not induce the leakage of fura-2 within the time course of our experiments (cf. Kiedrowski, 1998).

Mitochondrial depolarization by rotenone plus oligomycin protected the cells against both NMDA plus glycine-induced and KA-induced DCD (Fig. 1*C,F*). However, a clear distinction between the effects of the two receptor agonists was seen when oligomycin was present alone. Although no significant difference was observed in the time or extent of DCD induced by NMDA plus glycine (Fig. 1, compare *A,B*), oligomycin alone protected against KA-induced DCD (Fig. 1, compare *D,E*). As shown by the horizontal histograms, <15% of 273 cells examined showed a fura-2 ratio >0.8 after 90 min of continuous exposure to KA in the presence of oligomycin, compared with 90% in the absence of the inhibitor.

As in the case of glutamate plus glycine (Budd and Nicholls, 1996b), KA addition to cells inhibited by rotenone in the absence of oligomycin was followed by immediate Ca<sup>2+</sup> deregulation (data not shown). Cells incubated in the absence of agonist and in the presence of 2  $\mu$ M MK-801 or 10  $\mu$ M NBQX maintained a stable baseline [Ca<sup>2+</sup>]<sub>c</sub> for at least 120 min even when the mitochondria were inhibited by oligomycin  $\pm$  rotenone (data not shown).

Stimulation of cerebellar granule cells with KA can activate both KA and AMPA receptors (Hack and Balázs, 1995). However, concanavalin A (10  $\mu$ M), which preferentially inhibits KA receptor desensitization (Partin et al., 1993), did not significantly enhance the KA-induced initial Ca<sup>2+</sup> transient or the extent and timing of DCD (data not shown). Furthermore, 10  $\mu$ M NBQX, which preferentially inhibits AMPA receptors (Sheardown et al., 1990), completely prevented the acute elevation of Ca<sup>2+</sup> induced by KA addition. The KA responses reported here are thus caused by AMPA receptor activation. NBQX prevented DCD when added after KA addition but before the secondary rise in cytoplasmic Ca<sup>2+</sup> (DCD). However it did not reverse KA-induced DCD when added after DCD had been initiated (data not shown).

Ca<sup>2+</sup> entry across the plasma membrane has been shown to be primarily responsible for the subsequent failure of cytoplasmic Ca<sup>2+</sup> homeostasis induced by NMDA receptor activation. Thus



**Figure 1.** Effect of mitochondrial inhibitors on delayed  $\text{Ca}^{2+}$  deregulation induced by NMDA plus glycine or by KA. *A–C*, Cells loaded with fura-2 were incubated in  $\text{Mg}^{2+}$ -free medium containing  $10 \mu\text{M}$  NBQX. Where indicated, the cells were stimulated with  $100 \mu\text{M}$  NMDA plus  $10 \mu\text{M}$  glycine. *D–F*, Cells were incubated in medium containing  $\text{Mg}^{2+}$  and  $2 \mu\text{M}$  MK-801 and were stimulated with  $100 \mu\text{M}$  KA. Experiments were performed in the absence of mitochondrial inhibitors (*A, D*) or in the presence of  $5 \mu\text{g/ml}$  oligomycin (*B, E*) or  $5 \mu\text{g/ml}$  oligomycin plus  $2 \mu\text{M}$  rotenone (*C, F*). The inhibitors were added 5 min before the addition of the receptor agonists. Each figure shows traces from 23 to 34 somata. The frequency histograms indicate the range of fluorescence ratios observed in somata at the end of each experiment (60 min for NMDA and glycine and 90 min for KA). Data shown in each histogram are from at least four independent experiments and the indicated number of cells. *gly*, Glycine; *oligo*, oligomycin; *rot*, rotenone.

increasing external  $\text{Ca}^{2+}$  concentration greatly potentiates the extent of DCD induced by glutamate plus glycine (Tymianski et al., 1993; Castilho et al., 1998). In contrast, elevated external  $\text{Ca}^{2+}$  does not affect KA-induced  $\text{Ca}^{2+}$  homeostasis (Fig. 2). In the presence of oligomycin or oligomycin plus rotenone, cytoplasmic  $\text{Ca}^{2+}$  homeostasis was maintained even in the presence of elevated external  $\text{Ca}^{2+}$  (Fig. 2*D,F*).

#### Delayed $\text{Ca}^{2+}$ deregulation induced by veratridine

The contrast between the high- $\text{Ca}^{2+}$  dependency of NMDA-induced DCD (Castilho et al., 1998) and the relative insensitivity to elevated external  $\text{Ca}^{2+}$  shown in Figure 2 is consistent with the proposal made by Kiedrowski (1998) that the effects of KA might be primarily related to entry of  $\text{Na}^{+}$  rather than  $\text{Ca}^{2+}$ . To investigate this hypothesis, cells were exposed to  $10 \mu\text{M}$  veratridine, an inhibitor of voltage-dependent  $\text{Na}^{+}$  channel desensitization, to induce an independent means of  $\text{Na}^{+}$  entry into the cells. Addition of veratridine to cells incubated in the presence of nifedipine plus antagonists for both NMDA and non-NMDA receptors induced a slight increase in cytoplasmic  $\text{Ca}^{2+}$  (Fig. 3), most likely associated with the activation of non-L-type  $\text{Ca}^{2+}$  channels or reversal of the plasma membrane  $\text{Na}^{+}/\text{Ca}^{2+}$  exchanger. After  $\sim 75$  min, massive DCD occurred in the cell population. As in the case of KA, oligomycin was effective in protecting the cells against this veratridine-induced DCD (Fig. 3*B*). These results demonstrate that  $\text{Na}^{+}$  entry was most likely responsible for the KA-induced DCD and that the protective effect of oligomycin was not dependent on the route of ion entry.

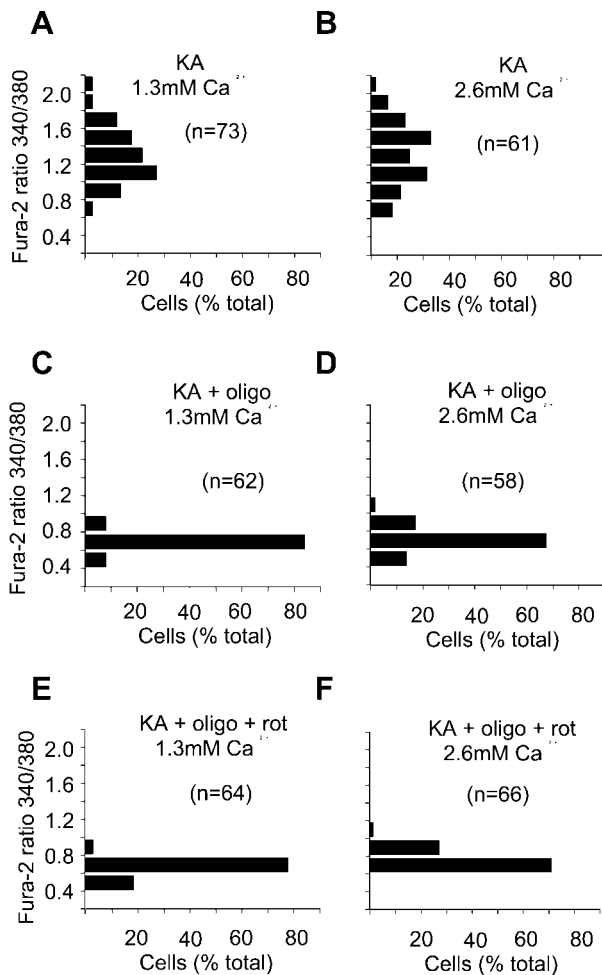
#### Mitochondrial membrane potential changes during KA- and veratridine-induced DCD

TMRM<sup>+</sup> (Ehrenberg et al., 1988) and rhodamine-123 (Johnson et al., 1980) are lipophilic cationic fluorescent probes that distribute across both plasma and mitochondrial membranes in response to the respective membrane potentials. The much greater surface-to-volume ratio of the small, highly invaginated mitochondria compared with the whole cell results in a much faster equilibration of probe across the mitochondrial inner membrane compared with the plasma membrane (Nicholls and Ward, 2000; Ward et al., 2000). Above a critical loading concentration, the

dyes undergo fluorescence quenching in the matrix, resulting in a biphasic whole-cell response to mitochondrial depolarization, namely, an increase in fluorescence as the probe is released from the quenched environment into the cytoplasm followed by a decrease in fluorescence as the excess dye exits from the cell to restore the Nernst equilibrium across the plasma membrane (for review, see Nicholls and Ward, 2000). Rhodamine-123 is less permeant than TMRM<sup>+</sup> (Bunting, 1992). Accordingly the cytoplasm retains rhodamine-123 released from depolarizing mitochondria for longer periods (Nicholls and Ward, 2000). In the subsequent experiments, cells were equilibrated with fura-2 AM and either TMRM<sup>+</sup> or rhodamine-123 to allow simultaneous monitoring of cytoplasmic free  $\text{Ca}^{2+}$  and  $\Delta\psi_m$ .

To separate out effects of KA on  $\Delta\psi_m$  and the plasma membrane potential  $\Delta\psi_p$ , the *in situ* mitochondria were first depolarized by the combination of rotenone and oligomycin (Budd and Nicholls, 1996a) (Fig. 4*A*). The slow mitochondrial depolarization produced by rotenone and oligomycin (Ward et al., 2000) results in an increase in whole-cell fluorescence as the probe is released from the quenched environment of the matrix to the cytoplasm. After the subsequent addition of KA, there is a slow but extensive loss of fluorescence consistent with the redistribution of TMRM<sup>+</sup> out of the cell as a result of plasma membrane depolarization. The fura-2 signal detects the “spike” of free  $\text{Ca}^{2+}$  as the AMPA/KA receptors are activated and then partially desensitize. Note that the presence of oligomycin in this experiment protects the cells against DCD.

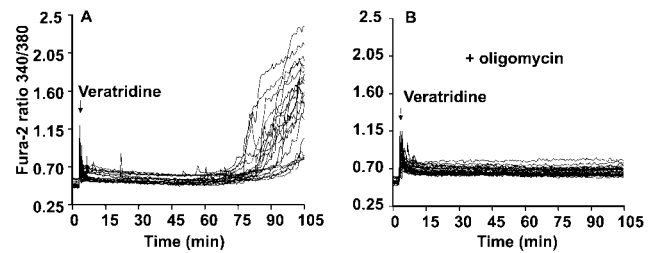
When this experiment is repeated in the absence of the mitochondrial inhibitors (Fig. 4*B*), an initial small increase in fluorescence is observed, similar to that identified previously as the slight mitochondrial depolarization accompanying acute addition of glutamate plus glycine and ascribed to an increased ATP demand on the mitochondrial proton circuit (Ward et al., 2000). The subsequent decrease in fluorescence differs from that seen in the presence of rotenone and oligomycin by being biphasic, with a second decrease associated with the onset of DCD. The interpretation of the TMRM<sup>+</sup> trace in Figure 4*B* is ambiguous, because the biphasic decrease could represent either a two-stage plasma membrane depolarization or a plasma membrane depolarization



**Figure 2.** Elevated external  $\text{Ca}^{2+}$  does not affect KA-induced delayed  $\text{Ca}^{2+}$  deregulation. Cells were loaded with fura-2 and incubated in medium containing 1.2 mM  $\text{Mg}^{2+}$ , 2  $\mu\text{M}$  MK-801, and either 1.3 mM (*A*, *C*, *E*) or 2.6 mM (*B*, *D*, *F*)  $\text{Ca}^{2+}$ . Experiments were performed in the absence of mitochondrial inhibitors (*A*, *B*) or in the presence of 5  $\mu\text{g}/\text{ml}$  oligomycin (*C*, *D*) or oligomycin plus 2  $\mu\text{M}$  rotenone (*E*, *F*). KA (100  $\mu\text{M}$ ) was added at least 5 min after addition of the mitochondrial inhibitors, and the frequency histograms show the 340/380 nm ratios for the indicated number of cell somata after a 90 min exposure to the agonist. Data shown in each histogram are from at least three independent experiments.

on which was superimposed a delayed collapse of mitochondrial potential that was too slow to produce a transient elevation of TMRM<sup>+</sup> fluorescence in the cytoplasm (Ward et al., 2000).

To remove the ambiguity in the interpretation of the potentiometric probe, the KA exposure was repeated with cells equilibrated with the less permeant (Bunting, 1992) rhodamine-123 (Fig. 4*D–F*). This probe allows a slow mitochondrial depolarization to be detected as an increase in whole-cell fluorescence as the probe is released from the quenched environment of the matrix and accumulates in the cytoplasm before its slow equilibration across the plasma membrane (Nicholls and Ward, 2000; Ward et al., 2000). If, in contrast, Figure 4*B* was reporting a continued plasma membrane depolarization, this would result in a further decrease in signal. Figure 4*D–F* shows the response of three typical cells to KA. The increase in whole-cell rhodamine-123 signal may be resolved into two phases; the first small response is synchronous with the recovery of the fura-2 trace from the spike



**Figure 3.** Veratridine induction of oligomycin-sensitive delayed  $\text{Ca}^{2+}$  deregulation. Cells were incubated in medium containing 2  $\mu\text{M}$  MK-801 and 10  $\mu\text{M}$  NBQX, in the absence (*A*) or in the presence (*B*) of 5  $\mu\text{g}/\text{ml}$  oligomycin added 5 min before the addition of 10  $\mu\text{M}$  veratridine. In parallel experiments, KA-induced DCD was apparent by 75 min, and no KA-induced  $\text{Ca}^{2+}$  deregulation was seen in oligomycin-treated cells after 105 min (data not shown). Data are representative of at least 20 somata from five independent experiments.

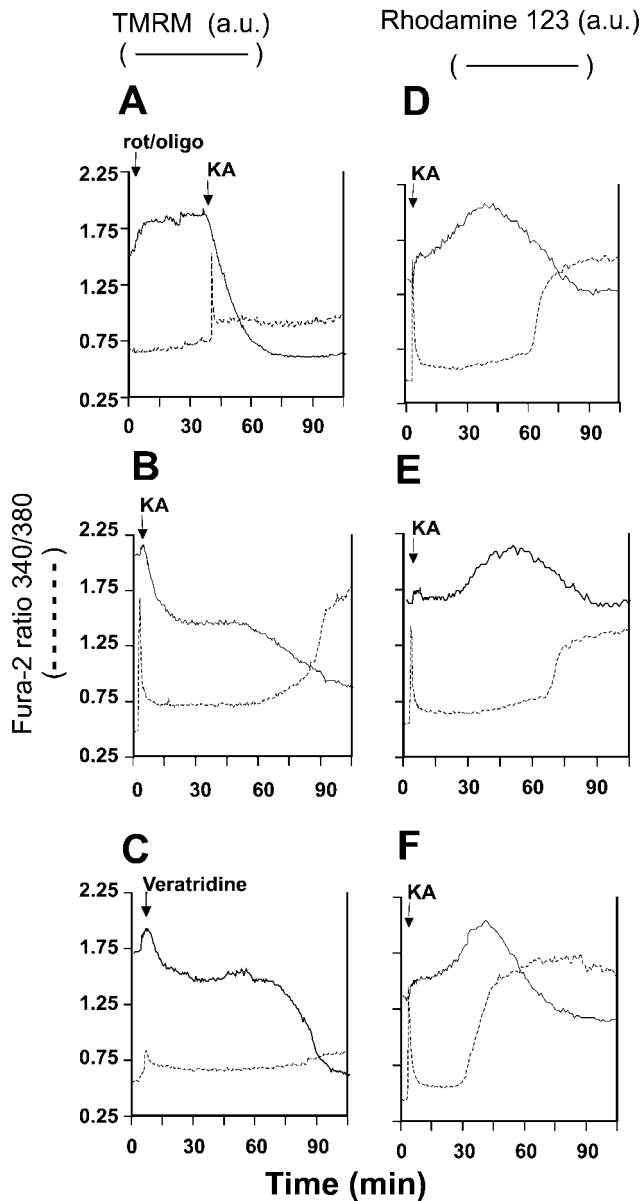
after KA addition and is consistent with the partial mitochondrial depolarization seen after NMDA receptor activation (Ward et al., 2000). This is followed by a second phase of slowly developing mitochondrial depolarization. Importantly, in the majority of cells mitochondrial depolarization can be initiated well before the final increase in  $[\text{Ca}^{2+}]_c$  that is diagnostic of DCD can be detected (Fig. 4*D,E*).

The complex fluorescence signal produced in granule cells by the probes has been analyzed previously in detail by mathematical modeling (Ward et al., 2000). Figure 5, *A* and *B*, shows traces synthesized by the model for fast-responding (TMRM<sup>+</sup>-like) and slow-responding (rhodamine-123-like) probes. A good fit with the experimental traces is obtained if a step plasma membrane depolarization, because of AMPA/KA receptor activation, is combined with a biphasic mitochondrial depolarization. The first phase comprises a slight initial mitochondrial depolarization coincident with agonist addition and attributable to the increased ATP demand on the mitochondrion as a consequence of enhanced  $\text{Ca}^{2+}$  and/or  $\text{Na}^+$  transport during the spike of cytoplasmic  $\text{Ca}^{2+}$  elevation. The second phase is a slowly developing partial mitochondrial depolarization. The “best fit” for both probes is obtained with the model by inputting a depolarization to 100–110 mV over a period of 20–30 min (Fig. 5*A*), although a more extensive depolarization cannot be excluded.

Exposure of a TMRM<sup>+</sup>-equilibrated cell to veratridine (Fig. 4*C*) produces a  $\Delta\psi_m$  response very similar to that seen with KA (Fig. 4*B*), including the initial dequenching attributable to enhanced ATP demand and the delayed secondary depolarization.

#### Oligomycin accelerates mitochondrial depolarization but protects cells against DCD

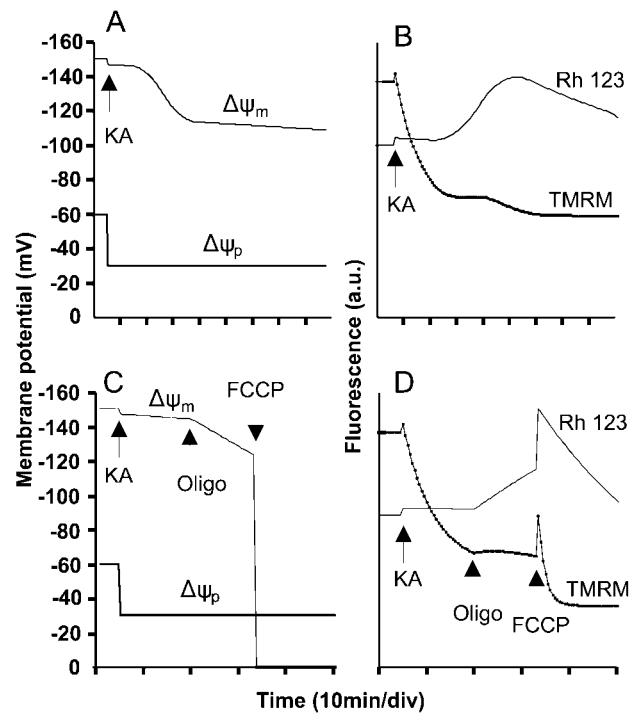
By inhibiting proton transport through the ATP synthase, oligomycin hyperpolarizes “healthy” mitochondria that were generating ATP but depolarizes compromised mitochondria whose suboptimal  $\Delta\psi_m$  was being maintained by ATP synthase reversal and glycolytic ATP hydrolysis (Ward et al., 2000). This simple “null-point” assay can be used to assess the bioenergetic status of *in situ* mitochondria (Ward et al., 2000). When this test was applied to granule cells that had been exposed to KA for 20 min, sufficient for reequilibration of TMRM<sup>+</sup> across the plasma membrane but before the induction of DCD in this cell preparation, 80–90% of cells showed a slow increase in TMRM<sup>+</sup> fluorescence in response to oligomycin (Fig. 6*A*), consistent with a gradual mitochondrial depolarization (Ward et al., 2000). Oligomycin-induced depolarization is seen more clearly in the cells loaded with rhodamine-



**Figure 4.** TMRM<sup>+</sup> and rhodamine-123 fluorescence during KA and veratridine exposure. Cells were loaded with fura-2 and either 50 nM TMRM<sup>+</sup> (A–C) or 1.3 μM rhodamine-123 (D–F) in the presence of 2 μM MK-801. The veratridine experiment additionally contained 10 μM NBQX. Where indicated, 2 μM rotenone plus 5 μg/ml oligomycin, 100 μM KA, or 10 μM veratridine was added. Each pair of traces is from a single cell soma representative of at least 20 cells, from four to six independent experiments. Dashed traces, fura-2; solid traces, TMRM<sup>+</sup> or rhodamine-123 fluorescence. a.u., arbitrary units.

123 (Fig. 6B). The model can simulate this response to oligomycin with an increased rate of mitochondrial depolarization (Fig. 5C,D). This contrasts with the response to oligomycin after NMDA receptor activation (Fig. 6C,D) in which fluorescence quenching, diagnostic of mitochondrial hyperpolarization, is seen in cells that have not yet initiated DCD (Ward et al., 2000). Cytoplasmic Ca<sup>2+</sup> homeostasis in the presence of KA was retained after addition of oligomycin (Fig. 6A,B), consistent with the protection afforded by this inhibitor against DCD (Figs. 1, 2).

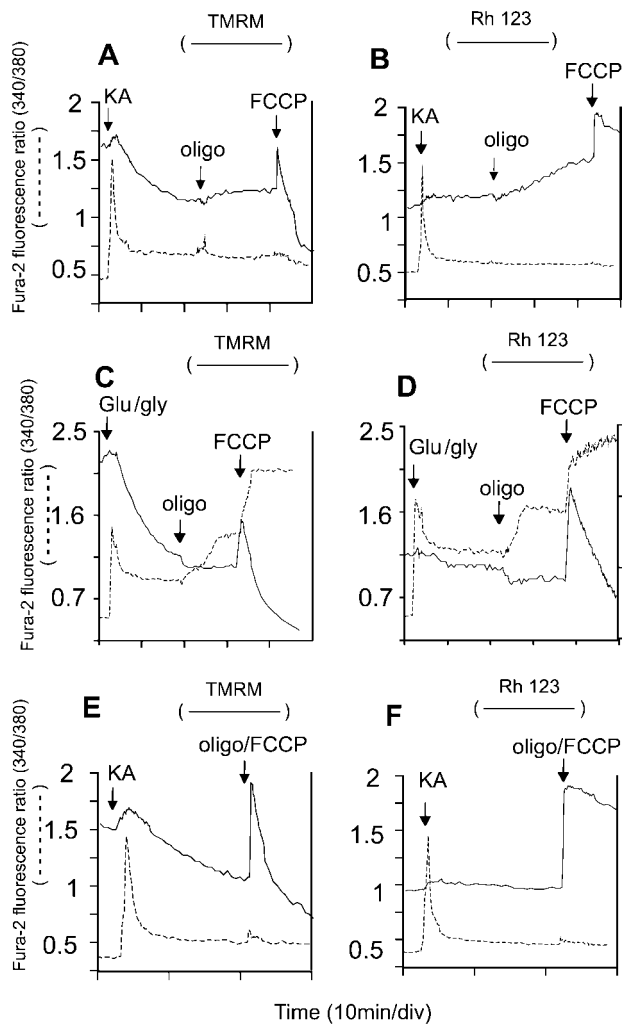
The protonophore FCCP, added to KA-exposed cells 20 min after oligomycin, allows the effects of complete mitochondrial



**Figure 5.** Mathematical simulation of changes in membrane potentials that reproduce the pattern of whole-cell fluorescence in the presence of KA. *A*, Synthetic membrane potential profile for a cell in which KA causes an immediate plasma membrane ( $\Delta\psi_p$ ) depolarization because of AMPA/KA receptor activation followed by an early, partial mitochondrial ( $\Delta\psi_m$ ) depolarization. *B*, Simulated traces for rhodamine-123 and TMRM<sup>+</sup> in the depolarization protocol in *A*. Note the similarity between the TMRM<sup>+</sup> trace and the actual experiment in Figure 4*B* and between the rhodamine-123 trace and the experimental traces in Figure 4*D–F*. *C*, Synthetic membrane potential profile for a cell in which oligomycin initiates a slow depolarization after KA and FCCP collapses  $\Delta\psi_m$ . *D*, Simulated traces for rhodamine-123 and TMRM<sup>+</sup> in the depolarization protocol in *C*. Note the similarity between the TMRM<sup>+</sup> trace and an actual experiment (see Fig. 6*A*) and between the rhodamine-123 trace and an experiment (see Fig. 6*B*). Rh 123, Rhodamine-123.

depolarization to be assessed. The mitochondria retain a significant  $\Delta\psi_m$  until the protonophore is added, as shown by the spike and subsequent efflux of TMRM<sup>+</sup> and rhodamine-123 (Fig. 6*A,B*). Any Ca<sup>2+</sup> that was sequestered within the mitochondrial matrix would be released by this treatment, creating a transient cytoplasmic fura-2 spike (Budd and Nicholls, 1996a; Ward et al., 2000). The absence of such a release in KA-exposed cells indicated that these mitochondria retain little Ca<sup>2+</sup>. Closely similar results were obtained with veratridine (data not shown). This lack of Ca<sup>2+</sup> loading contrasts with the extensive loading seen with glutamate plus glycine exposure, which in these cells was sufficient to prevent the restoration of Ca<sup>2+</sup> homeostasis after addition of protonophore (Fig. 6*C,D*). Note that this preparation of cells showed a partial Ca<sup>2+</sup> deregulation in the presence of glutamate plus glycine after addition of oligomycin, probably as a consequence of ATP limitation (Budd and Nicholls, 1996a).

The spontaneous mitochondrial depolarization seen after exposure to KA (Fig. 4*B,D–F*) implies that the mitochondria will at some stage become thermodynamically incapable of generating ATP and will instead start to hydrolyze glycolytic ATP. Cerebellar granule cells maintained for 7 d *in vitro* have sufficient glycolytic capacity to maintain Ca<sup>2+</sup> homeostasis in the absence of net ATP synthesis, but not if ATP synthase reversal occurs (Budd and

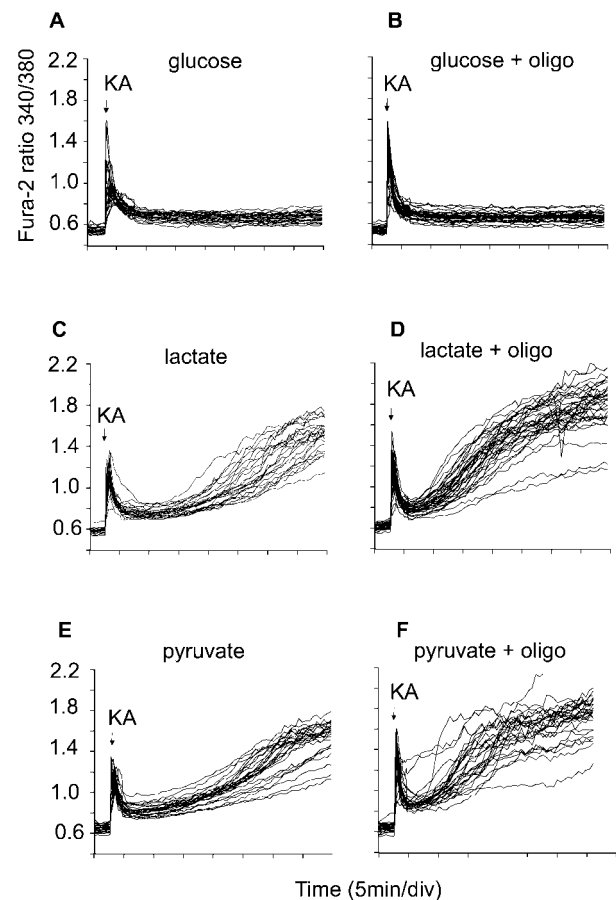


**Figure 6.** The use of oligomycin and FCCP to probe mitochondrial function in KA-exposed cells. Cells were loaded with fura-2 and equilibrated with 50 nM TMRM<sup>+</sup> (*A, C, E*) or 1.3  $\mu$ M rhodamine-123 (*B, D, F*). The media for the KA experiments (*A, B, E, F*) contained additionally 2  $\mu$ M MK-801, whereas *C* and *D* contained 10  $\mu$ M NBQX. Where indicated, additions were made of 100  $\mu$ M KA, 100  $\mu$ M glutamate plus 10  $\mu$ M glycine, 5  $\mu$ g/ml oligomycin, and 2.5  $\mu$ M FCCP. Note that oligomycin induced a slow depolarization of mitochondria in KA-exposed cells (*A, B*) but hyperpolarized mitochondria in NMDA-exposed cells (*C, D*) and that FCCP and oligomycin failed to release Ca<sup>2+</sup> from mitochondria into the cytoplasm (*E, F*). Each pair of traces is from a single soma representative of at least 20 cells from four to six independent experiments.

Nicholls, 1996a,b). The onset of DCD subsequent to the partial mitochondrial depolarization (Fig. 4*D–F*) is consistent with such ATP depletion. By preventing ATP synthase reversal, oligomycin could thus prevent cytoplasmic ATP depletion and the consequent loss of cytoplasmic Ca<sup>2+</sup> homeostasis.

The enhanced rate of mitochondrial depolarization after oligomycin (Fig. 6*A, B*) implies that mitochondrial function is already compromised by 20 min of KA exposure, and even before changes in membrane potential can be detected. In contrast, NMDA-exposed cells contain healthy ATP-synthesizing mitochondria, which hyperpolarize on addition of the inhibitor before DCD (Fig. 6*C, D*).

The implication is that glycolytic ATP is essential for the continued Ca<sup>2+</sup> homeostasis of these KA-exposed cells. Cells maintained in glucose-free medium in the presence of 10 mM



**Figure 7.** Cytoplasmic Ca<sup>2+</sup> homeostasis in cells maintained by non-glycolytic substrates. The cells were loaded with fura-2 and incubated in the presence of 15 mM glucose (*A, B*) or in glucose-free medium containing 2  $\mu$ M MK-801, 2 mM 2-deoxyglucose, and 10 mM L-lactate or 10 mM pyruvate (*C–F*). KA (100  $\mu$ M) was added where indicated. In traces *B, D*, and *F*, 5  $\mu$ g/ml oligomycin was present from the beginning of the experiment. Data are representative of at least 20 somata from four independent experiments.

L-lactate plus 2 mM 2-deoxyglucose are entirely dependent on mitochondrial oxidative phosphorylation for ATP generation and would therefore be predicted to show cytoplasmic Ca<sup>2+</sup> deregulation immediately after mitochondrial ATP synthase activity became compromised. Figure 7 shows that such failure occurs much sooner than in glucose-maintained cells and after a delay similar to that seen in cells supported by lactate or pyruvate in the absence of glycolytic substrate and the presence of oligomycin, i.e., lacking any obvious means of ATP generation. Again this contrasts with chronic NMDA receptor activation, in which mitochondria remain net generators of ATP until the onset of DCD, so that glucose- or lactate-maintained granule cells survive for comparable periods before DCD (Castilho et al., 1998).

## DISCUSSION

Multiple mechanisms can account for cytoplasmic Ca<sup>2+</sup> deregulation in neurons exposed to glutamate agonists, depending on the precise step whose activity becomes limiting. First, immediate Ca<sup>2+</sup> deregulation (ICD) is seen after NMDA receptor activation in granule cells in the presence of a respiratory chain inhibitor (Budd and Nicholls, 1996b; Castilho et al., 1998). ICD can be ascribed to an acute ATP deficit as glycolysis attempts to main-

tain housekeeping functions, extrude  $\text{Na}^+$  and  $\text{Ca}^{2+}$  entering through the receptor, and support  $\Delta\psi_m$  by ATP synthase reversal as  $\text{Ca}^{2+}$  floods into the matrix. ICD is reversible by preventing mitochondrial depletion of ATP by oligomycin.

The second mechanism after 30–60 min of continuous NMDA receptor activation is characterized by a failure of  $\text{Ca}^{2+}$  extrusion from the cell that does not appear to be a consequence of ATP limitation and is partially protected against by antioxidants. This DCD cannot be reversed by NMDA receptor inhibition or addition of additional metabolic substrates. The only mechanism of  $\text{Ca}^{2+}$  extrusion from the cell in the presence of NMDA is the plasma membrane  $\text{Ca}^{2+}$ -ATPase (PMCA), the  $\text{Na}^+/\text{Ca}^{2+}$  exchanger being thermodynamically incompetent because of the elevated cytoplasmic  $\text{Na}^+$ . Because  $\text{Ca}^{2+}$  entry into the cell does not increase (Khodorov et al., 1996; Castilho et al., 1998), the conclusion is that the eventual failure of cytoplasmic  $\text{Ca}^{2+}$  homeostasis is caused by an inhibition of extrusion caused by ROS generated by the  $\text{Ca}^{2+}$ -loaded mitochondria.

The third mechanism becomes apparent during the DCD that follows 60–120 min after transient NMDA receptor activation. The precipitating event here is ATP depletion caused by mitochondrial depolarization, perhaps initiated by oxidative damage and the resulting ATP synthase reversal, because DCD is prevented by oligomycin (Ward et al., 2000). Presumably oxidative damage to the PMCA still occurs but does not become rate limiting because there is only a basal inward  $\text{Ca}^{2+}$  leak after receptor inhibition. KA-induced DCD has similarities with this third mechanism but can be initiated by pathological  $\text{Na}^+$  entry into the cell through AMPA/KA receptors or veratridine-opened  $\text{Na}^+$  channels.

### Mitochondrial control of $[\text{Ca}^{2+}]_c$ in KA-exposed granule cells

Mitochondrial  $\text{Ca}^{2+}$  loading is central to the mechanism by which NMDA receptor activation induces DCD (Budd and Nicholls, 1996b; Castilho et al., 1998, 1999). In contrast, the present study shows that exposure of 7–8 d *in vitro* cells to 100  $\mu\text{M}$  KA does not cause significant mitochondrial  $\text{Ca}^{2+}$  loading, because there is no FCCP-releasable  $[\text{Ca}^{2+}]_c$  spike (Fig. 6), and KA-induced DCD is not facilitated by elevating external  $\text{Ca}^{2+}$  (Fig. 2). Thus  $[\text{Ca}^{2+}]_c$  can be maintained below the threshold “set point” at which mitochondria become net accumulators of  $\text{Ca}^{2+}$  (Nicholls, 1978; Zoccarato and Nicholls, 1982). This is suggested also by the lower fura-2 responses to KA relative to those to NMDA (Figs. 1, 6). However, Peng and Greenamyre (1998) observed a KA-induced increase in free-matrix  $\text{Ca}^{2+}$  monitored by rhod-2 in striatal neurons, whereas KA caused mitochondrial  $\text{Ca}^{2+}$  loading of rat forebrain neurons (Hoyt et al., 1998). The relative importance of  $\text{Ca}^{2+}$  and  $\text{Na}^+$  entry in KA-induced excitotoxicity may thus vary from cell to cell. Certainly a chronically elevated  $[\text{Ca}^{2+}]_c$  that leads to bulk mitochondrial  $\text{Ca}^{2+}$  accumulation would be expected to be excitotoxic; however the present study and others (Kiedrowski et al., 1994a,b; Kiedrowski, 1998) focus on the role of  $\text{Na}^+$ , and it is particularly striking that veratridine, which induces a continuous  $\text{Na}^+$  influx that depolarizes cells, collapses the  $\text{Na}^+$ -electrochemical gradient across the plasma membrane, releases cytoplasmic and vesicular glutamate (McMahon et al., 1990), causes extensive swelling (Churchwell et al., 1996), and initiates an energy-dissipating cycle between the  $\text{Na}^+$  channel and the  $\text{Na}^+/\text{K}^+$ -ATPase (Nicholls and Scott, 1980), is able to mimic the acute excitotoxicity of KA so precisely.

NMDA receptor-mediated DCD in granule cells is unaffected

by oligomycin (Budd and Nicholls, 1996b) and thus cannot be ascribed simply to a failure of mitochondrial ATP synthesis. In contrast, both KA- and veratridine-induced DCD are virtually abolished by oligomycin within the time course of the experiment (Figs. 1E, 3B). These results confirm that  $\text{Na}^+$  entry is central to KA toxicity (Kiedrowski, 1998) and demonstrate that ATP synthase inhibition protects cells against the subsequent effects of the ion.

### KA and $\Delta\psi_m$

Interpretation of changes in whole-cell fluorescence of neurons loaded with cationic probes is complex, particularly if  $\Delta\psi_p$  and  $\Delta\psi_m$  both change (Nicholls and Ward, 2000). Three phases can be resolved (Fig. 5). The first is an immediate slight depolarization of the mitochondria as they transiently accumulate  $\text{Ca}^{2+}$  and/or respond to an increased cellular ATP demand after receptor activation. Modeling this change mathematically (Ward et al., 2000) suggests that this may only amount to 5 mV.

The second phase, a slow decrease in signal, occurs even if  $\Delta\psi_m$  is initially collapsed by rotenone and oligomycin (Fig. 4A) and is caused by plasma membrane depolarization. The third phase monitors the delayed change in  $\Delta\psi_m$  associated with DCD. With continuous NMDA receptor activation the mitochondria remain sufficiently polarized to generate ATP (Ward et al., 2000) until the onset of DCD (see also Fig. 6). In contrast, transient NMDA receptor activation terminated by MK-801 results in a delayed decay of  $\Delta\psi_m$  that precedes DCD, particularly if oligomycin is present to prevent cytoplasmic ATP depletion (Ward et al., 2000).

Mitochondrial depolarization is most clearly visualized with rhodamine-123, because the increase in whole-cell fluorescence is dependent on a temporary accumulation of “excess” probe in the cytoplasm before reequilibration across the plasma membrane (Fig. 4D–F). KA-induced depolarization can most closely be modeled by a slowly developing drop in  $\Delta\psi_m$  of  $\sim 40$  mV (Fig. 5A,B), because a rapid depolarization would produce a spike detectable with either probe (e.g., Fig. 6E,F). The presence of a residual mitochondrial membrane potential after KA-induced DCD is indicated by a small dequenching seen on addition of FCCP at the termination of the experiment (data not shown).

The experiments shown in Figure 4D–F allow the relationships between cytoplasmic free  $\text{Ca}^{2+}$  and  $\Delta\psi_m$  to be addressed. A slow increase in  $[\text{Ca}^{2+}]_c$ , monitored by the fura-2 340/380 nm ratio, can be detected in cells before the final precipitate increase associated with DCD (Figs. 1D, 4B,D,E). Because this slow increase accompanies mitochondrial depolarization (Fig. 4D,E) the most likely explanation is that ATP generation is becoming limiting in these cells as a consequence of impaired oxidative phosphorylation. However DCD itself occurs after the mitochondrial depolarization is well advanced (Fig. 4D–F). The high- $\text{Ca}^{2+}$  uniporter activity of brain mitochondria (Nicholls and Scott, 1980) means that they will be automatically depolarized by elevated  $\text{Ca}^{2+}$ , and this may be the explanation for the mitochondrial depolarization that occurs synchronously with DCD during continuous NMDA receptor activation (Ward et al., 2000). However, the temporal relationship between DCD and  $\Delta\psi_m$  seen here in the presence of KA means that the agonist induces mitochondrial depolarization by a mechanism other than a cytoplasmic  $\text{Ca}^{2+}$  increase.

### KA causes a rapid failure of mitochondrial ATP synthesis

Cells in the absence of glucose can use exogenous pyruvate or lactate to generate ATP by oxidative phosphorylation. Under

these conditions, a failure of mitochondrial ATP synthesis cannot be compensated for by glycolysis, and a rapid loss of cytoplasmic  $\text{Ca}^{2+}$  homeostasis occurs. Pyruvate- or lactate-maintained granule cells exposed to glutamate plus glycine undergo DCD after the same delay as cells maintained on glucose, confirming that mitochondria continue generating ATP under these conditions until a late stage (Castilho et al., 1998). In contrast, KA-exposed cells start to lose cytoplasmic  $\text{Ca}^{2+}$  homeostasis after only 10 min (Fig. 7C,E). Indeed the traces closely resemble those in which oligomycin is additionally present, inhibiting all ATP synthesis (Fig. 7D,F).

In the absence of  $\text{Ca}^{2+}$  loading and in the presence of oligomycin there are a limited number of ways in which a decay in  $\Delta\psi_m$  can occur. Either the mitochondria become increasingly proton permeable, or electron transport and/or substrate supply become limiting. The rhodamine-123 traces (Fig. 4D–F) and the incompetence of nonglycolytic substrates (Fig. 7C,E) both indicate that mitochondrial function is being compromised early on in KA excitotoxicity. A further test is to determine whether the addition of oligomycin after KA results in hyperpolarization (indicating that the mitochondria were generating ATP) or depolarization (indicating that  $\Delta\psi_m$  was in part maintained by ATP synthase reversal and hydrolysis of glycolytic ATP). It is apparent from Figures 5 and 6 that the mitochondria within the large majority of KA-exposed cells start slowly to depolarize after oligomycin. The slow rate and incomplete extent of this depolarization indicate that the mitochondrial inner membrane is still primarily proton impermeable; i.e., no “permeability transition” (Zoratti and Szabo, 1995) has occurred. Indeed, subsequent FCCP addition is required to show the dequenching spike and decay characteristic of a rapid collapse of  $\Delta\psi_m$ . On the other hand we are aware of no studies in the literature that show respiratory inhibition in the presence of KA. Indeed, chronic *in vivo* KA accelerates the respiration of subsequently isolated hippocampal slices (Desagher et al., 1999; Kunz et al., 1999; Martinou et al., 1999). Further studies will be required to determine the mechanism underlying the mitochondrial depolarization.

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