Excitotoxic Injury to Mitochondria Isolated from Cultured Neurons*

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Neuronal death in response to excitotoxic levels of glutamate is dependent upon mitochondrial Ca\(^{2+}\) accumulation and is associated with a drop in ATP levels and a loss in ionic homeostasis. Yet the mapping of temporal events in mitochondria subsequent to Ca\(^{2+}\) sequestration is incomplete. By isolating mitochondria from primary cultures, we discovered that glutamate treatment of cortical neurons for 10 min caused 44% inhibition of ADP-stimulated respiration, whereas the maximal rate of ADP-stimulated respiration, which may contribute to decreased cellular ATP and loss of reactive oxygen species and nitric oxide (5–7). As well, excitotoxic death in cultured neurons is preceded by a significant decline in cellular ATP (8, 9).

Recent imaging studies of cultured neurons have demonstrated that mitochondrial Ca\(^{2+}\) loading is in large part responsible for the cell death process that may contribute to decreased cellular ATP and loss of ionic homeostasis that precede commitment to neuronal death.

Glutamate excitotoxicity underlies neuronal loss in ischemic and traumatic brain injury (1) and also likely contributes to dysfunction in chronic forms of neurodegeneration. In particular, the evidence for involvement of excitotoxicity in Huntington disease has been significantly strengthened by recent studies (for a review see Ref. 2). It is well established that glutamate-induced neuronal death depends on entry of extracellular Ca\(^{2+}\) as a result of activation of the NMDA\(^{-}\) subtype of glutamate receptors (3, 4). The description of ensuing events in various types of cultured neurons is extensive (5) and includes an initial transient increase in cytoplasmic Ca\(^{2+}\), followed by a loss in ionic homeostasis (also termed delayed Ca\(^{2+}\) deregulation) (4). The initial increase in cytoplasmic Ca\(^{2+}\) is associated with mitochondrial Ca\(^{2+}\) loading and slight mitochondrial depolarization followed by profound depolarization concurrent with the loss of ionic homeostasis. Other early events may include the release of apoptogenic cytochrome c and generation of reactive oxygen species and nitric oxide (5–7).

EXPERIMENTAL PROCEDURES

Preparation of Cortical Neurons—Primary cultures of cortical neurons were prepared from embryonic day 18 Sprague-Dawley rats. The cerebral cortices were collected and triturated gently (3–4 times) in ice-cold Hibernate E medium (Brain Bits, Southern Illinois University, School of Medicine) plus 1× B27 supplement (Invitrogen), 100 units/ml penicillin, and 100 \(\mu\)g/ml streptomycin. After the tissue settled, the Hibernate E medium was aspirated, and the tissue was triturated for
1 min in 0.1% trypsin in a Ca2+/Mg2+-free phosphate-buffered saline solution supplemented with glucose (1.5 mM), after which trypsin was inactivated by addition of soybean trypsin inhibitor (0.1 mg/ml). The mixture was transferred into Hibernate E medium containing 20 units/ml DNase (Promega) in 0.2X reaction buffer (Promega), and the cells were centrifuged at 200 × g for 1.5 min. The supernatant was quickly aspirated, and the cells were resuspended in 10 ml of Neurobasal (E) medium (Invitrogen) plus glutamate (0.4 μM/g), 0.5 mM L-glutamine, penicillin, and streptomycin (100 units/ml and 100 μg/ml, respectively), 1X B27 supplement, and 5 mM sodium pyruvate. Once in suspension, the cells were diluted into 30 ml of the same medium without pyruvate (initial plating medium), and the number of viable cells was determined by trypsin blue exclusion. Cells were plated on poly-D-lysine-coated 10-cm plates at a concentration of 750,000 cells per ml in a volume of 10 ml per plate and kept at 37 °C in a 5% CO2 incubator. For measurements of cellular ATP and cytoplasmic Ca2+, cells were incubated in BioCoatTM nunc-coated black 96-well plates (BD Biosciences) at 50,000 cells per well. After 4–6 days in vitro, the initial plating medium was diluted with an equal volume of maintenance medium of the same composition lacking glutamate and L-glutamine and supplemented with 1% GlutaMAX-I (Invitrogen). Cultures were fed every 3–4 days with fresh medium. All experiments were performed with cultures that were 15–15 days in vitro. These cultures were used at time zero, an estimate of the starting point, according to the manufacturer’s protocols with anti-neuronal nuclei (Chemicon, mAB377) or anti-neurofilament 200 kDa (Calbiochem, IF06L).

Measurement of Viability of Cortical Neurons Treated with Glutamate—Cell viability was measured in neurons cultured in 96-well plates using a cytotoxicity detection kit (lactate dehydrogenase, LDH) according to the manufacturer’s recommended procedure (Roche Diagnostics, Indianapolis, IN). Data are expressed as percent cell death based upon measurement of LDH activity that was cell-associated versus that which was released to the medium. Cells were treated in HEPES-buffered salt solution (HBSS, containing 137 mM NaCl, 5 mM KCl, 10 mM NaHCO3, 20 mM HEPES, 5.5 mM glucose, 0.6 mM KH2PO4, 1.4 mM CaCl2 0.9 mM MgSO4) with 100 μM glutamate plus 10 μM glycine with or without pretreatment with 1 μM of the NMDA receptor antagonist MK-801. Control cultures were exposed to HBSS without glutamate or glycine. Following a 10-min incubation at 37 °C, an equal volume of maintenance medium was added (supplemented with glutamate plus glycine and/or MK-801 to maintain constant concentration of these effectors). The cells were then placed back in the incubator for 24 h prior to measurement of viability. Alternatively, at the addition of the maintenance medium (20 μM MK-801 was included to block NMDA receptor activity.

Measurement of Neuronal Ca2+—Cytosolic Ca2+ was measured in neurons cultured in the 96-well plates using magfluo-4, a cell permeant low affinity Ca2+ dye (Molecular Probes). A stock solution of magfluo-4 (1 mg/ml) was prepared on the day of the experiment in MeSO and then diluted 1:1000 to a final concentration of 4 μM. The culture medium was carefully aspirated, and cells were loaded with the dye (100 μl of a 4 μM solution per well) for 25 min in the incubator. The loading buffer was then replaced with dye-free HBSS, and the plates were assayed in a fluorescence plate reader (FLIPR, Molecular Devices) at 488 (excitation) and 525 nm (emission).

Measurement of Neuronal ATP—The level of neuronal ATP was determined by using a CellTiter-GloTM luminescent assay kit (Promega). Cells cultured in 96-well plates were treated with glutamate for varying lengths of time (from 10 to 40 min). Control cells were exposed to HBSS without glutamate and glycine for 10 min. At the end of the treatment, CellTiter-Glo™ reagent was added, and the plates were placed on a shaker for 5 min. In some experiments, the glutamate-containing medium was aspirated, and cells were incubated in Ca2+-free HBSS (containing 100 μM EGTA) for 3–6 min prior to the addition of CellTiter-Glo™ reagent. Luminescence was measured in a Polaris membrane plate reader (BMG). ATP levels are expressed as fmol/cell based upon a ATP standard curve, and data are corrected for background luminescent signal. Each set of data was collected from multiple replicate wells (n = 12) and plotted as mean ± S.E.

Preparations of Neuronal Mitochondria—Granule cells were prepared as described previously (18) from 7-day-old postnatal Wistar rats. Cells were plated on poly-D-lysine-coated 10-cm plates at a concentration of 1 × 106 cells per ml in a volume of ~9 ml per plate. Cells were cultured in minimal essential medium containing Earle’s salts (Invitrogen) plus 10% (v/v) fetal calf serum (HyClone), 25 mM KCl, 30 mM glucose, 2 mM glutamine, penicillin, and streptomycin (100 units/ml and 100 μg/ml, respectively). After 24 h, 10 μM cytochrome arabinosine was added to inhibit non-neuronal cell proliferation. Cells were maintained at 37 °C in a 5% CO2 incubator and were used after 7–8 days in vitro.

Isolation of Mitochondria from Control and Glutamate-treated Neuronal Cultures—A method for functional mitochondria from primary neuronal cultures has been described previously (19). We used a modified protocol for rapid preparation of neuronal mitochondria suitable for assessment of bioenergetic parameters and the Ca2+ content. The culture medium was aspirated, and cells (cortical neurons or cerebellar granule cells) were exposed to 100 μM glutamate and 10 μM glycine in HBSS. Control cells were exposed to HBSS without glutamate and glycine. After glutamate addition, cells were returned to the incubator for 10 min. Next, the plates were placed on ice and washed twice with ice-cold Ca2+-free and Mg2+-free phosphate-buffered saline solution (Invitrogen) supplemented with 2 mM EGTA. Cells were rapidly scraped in 1 ml (per plate) of ice-cold mitochondrial isolation buffer containing 210 mM mannitol, 70 mM sucrose, 10 mM HEPES-KOH, pH 7.4, 2 mM EGTA, and 0.1% fatty acid-free bovine serum albumin, and were then homogenized using a Dounce homogenizer (10 passes with a loose pestle and 10 passes with a tight pestle). Cell homogenates were centrifuged for 10 min at 1060 × g for 5 min. The first and second supernatants were pooled together and centrifuged at 14,600 × g for 10 min at 4 °C. The final supernatant was added to the incubating temperature and supplemented with 2 mM EGTA and albumin and centrifuged at 14,600 × g for 10 min. All isolation steps were performed on ice. The protein concentration was measured using the BCA protein assay kit (Pierce). Typically, 10–15 plates of cells were used for one mitochondrial preparation. Note that mitochondria were isolated in the absence of digitonin that is commonly used for disruption of the cholesterol-rich synaposomal membranes to acquire the mitochondria’s respiratory competent state (15). Mitochondrial preparations from cultured neurons appear to be relatively free of synaptosomes as evidenced by the lack of an effect of digitonin on the maximal rate of respiration in the presence of succinate and rotenone (data not shown). Succinate is a mitochondrial substrate that is poorly permeable to the plasma membrane.

Measurement of Mitochondrial Respiration, Membrane Potential, and Mitochondrial Ca2+ Content—Mitochondria—Mitochondrial preparations isolated from cultured cortical and cerebellar neurons were used to determine the respiratory activity of these preparations as measured by the PolarStar Optical Reader (BMG). The light-emitting diode and photodetector permitted changes in mitochondrial light uptake rates and Ca2+ uptake rates.

For measurement of mitochondrial content, mitochondria were incubated at 25 °C in a basal saline medium (125 mM KCl, 5 mM HEPES-KOH, pH 7.4, and 2 mM phosphate) supplemented with 2 mM MgCl2 and either complex I-linked substrates (a mixture of 5 mM glutamate and 5 mM malate) or a complex II substrate (5 mM succinate in the presence of 2 mM rotenone). Protein concentrations were 1–1.5 mg/ml for cortical mitochondria and 0.7–1.3 mg/ml for cerebellar mitochondria. State 3 (phosphorylating) respiration was initiated by addition of 200 μM ADP, after which state 4 (resting) respiration was induced by addition of 100 μM atractylside, an inhibitor of the ADP/ATP antiporter. Finally, to measure uncoupler-stimulated respiration (state 3u), sequential additions of the protonophore carbonyl cyanide (trifluoromethoxy)phenylhyrazide (FCCP) (20–50 nM) were made until the respiration rate reached maximum. The acceptor control ratio was determined as the ratio of state 3 to state 3u. The experiments were completed within 2–3 h after the isolation procedure.

Mitochondrial Ca2+ content was determined in the same basal medium (above) supplemented with glutamate and malate. Protein concentrations were in the same range as for the respiration measurements. After 2–3 min of incubation to allow for uptake of contaminating Ca2+ from the medium, mitochondrial Ca2+ was released into the medium by addition of FCCP (0.5 μM) followed 5 min later by the
addition of the pore-forming peptide alamethicin (40 μg/ml) to ensure complete Ca\textsuperscript{2+} release. In some experiments, A23187 (1.2 mM), a Ca\textsuperscript{2+} ionophore, was also used to release sequestered Ca\textsuperscript{2+}. The amount of Ca\textsuperscript{2+} released from mitochondria was calculated based upon calibration of the Ca\textsuperscript{2+} electrode performed at the end of each experimental day by additions of known concentrations of CaCl\textsubscript{2} to the medium. EGTA (1 mM) was then added to chelate free Ca\textsuperscript{2+} in the medium. According to the Nernst equation, the voltage changes on the electrode are linearly related to the logarithm of Ca\textsuperscript{2+} concentration values in the medium.

The concentration of contaminating Ca\textsuperscript{2+} in the medium was also experimentally determined. It was defined as an independent variable in the regression analysis shown in Equation 1.

\[ \Delta V = a + b \times \log([\text{Ca}^{2+}] + [\text{Ca}^{2+]_0}) \]  

where \( a \) and \( b \) are regression parameters expressed in relative units, and \([\text{Ca}^{2+]_0}\) is the concentration of contaminating Ca\textsuperscript{2+}.

\( R^2 \) values for all calibration curves were no less than 0.995. The best fit parameters were used to calculate Ca\textsuperscript{2+} concentrations from the voltage changes in the experimental runs. The mitochondrial Ca\textsuperscript{2+} load was determined as the difference in Ca\textsuperscript{2+} concentration after addition of the Ca\textsuperscript{2+}-releasing agent (FCCP or alamethicin) minus the contaminating Ca\textsuperscript{2+} in the medium and was normalized to mitochondrial protein concentration.

Mitochondrial Ca\textsuperscript{2+} uptake capacity was measured in the same medium as that used for mitochondrial Ca\textsuperscript{2+} content determination. Energized mitochondria were pulsed with successive additions of Ca\textsuperscript{2+} (30–50 μM) every 2–3 min. The increasing Ca\textsuperscript{2+} load caused a successive decline in the membrane potential and Ca\textsuperscript{2+} uptake rates. Maximal Ca\textsuperscript{2+} capacity was defined as an amount of Ca\textsuperscript{2+} (per mg of protein) required to decrease the Ca\textsuperscript{2+} uptake rate by >90%. Note that because of the duration of the experimental run (25–40 min), most of these measurements were performed in an open chamber to avoid anoxia.

Data are expressed as mean ± S.E., and \( n \) indicates the number of independent experiments. Each mitochondrial preparation was obtained from a separate neuronal harvest (or neuronal preparation).

Statistical Analysis—One-way analysis of variance followed by post hoc Tukey test was performed using SigmaStat. Where differences are noted, analysis of variance detected significant variance at \( p < 0.001 \), and pairwise comparisons are indicated in the figure legends.

Materials—Unless indicated otherwise, all reagents were obtained from Sigma.

RESULTS

Measurements of Cytoplasmic Ca\textsuperscript{2+} in Cortical Cultures—Mitochondrial Ca\textsuperscript{2+} accumulation has been well established in different excitotoxic models (21–24). An intact cell study utilizing a dye with low affinity to Ca\textsuperscript{2+} (Fura-2) has demonstrated that a significant fraction of accumulated Ca\textsuperscript{2+} is retained within the mitochondria during brief glutamate exposures until FCCP is added to induce Ca\textsuperscript{2+} efflux (25). Based on this approach, we performed measurements of cytoplasmic Ca\textsuperscript{2+} in situ with our cortical cultures to demonstrate that the conditions of glutamate treatment used in our model produces results consistent with literature data. Fig. 1 depicts results of a representative experiment in which cortical neurons were challenged with various concentrations of glutamate (in the presence of 10 μM glycine) for 10 min followed by chelation of extracellular Ca\textsuperscript{2+} with EGTA and subsequent addition of FCCP. The responses to glutamate at concentrations between 30 and 300 μM were similar, and the pattern is typified by an initial peak in cytoplasmic Ca\textsuperscript{2+} that is followed relatively rapidly in these cultures by a more sustained and profound rise in cytoplasmic Ca\textsuperscript{2+}. The response to 100 μM glutamate appeared to be maximal with regard to cytoplasmic Ca\textsuperscript{2+} and FCCP-induced release of Ca\textsuperscript{2+}, as noted previously by others (25); therefore, we chose to probe in greater depth the bioenergetic changes occurring at this 10-min time point of glutamate exposure.

Effect of Glutamate Treatment on Cellular ATP—As shown in Fig. 2, 10 min of glutamate exposure results in a significant drop in the ATP level (Fig. 2A). Longer exposures to glutamate (up to 40 min) did not further decrease the content of ATP. Partial ATP depletion could be a direct consequence of acute activation of ATP-requiring efforts by the cell to re-establish ionomic homeostasis during glutamate stimulation, and/or the ATP loss could result from impairment of cellular ATP production. To test whether ATP could be readily recovered if the glutamate and Ca\textsuperscript{2+} challenge were halted, cells were exposed to glutamate for 10 min followed by incubation of the cells in a glutamate- and Ca\textsuperscript{2+}-free buffer. Under these conditions, cytoplasmic Ca\textsuperscript{2+} fairly rapidly returns to a steady state level (see Fig. 1 described above); however, ATP did not recover within this time frame. This result suggests that the compromise in cellular ATP levels reflects a compromise in the ability of the cell to produce ATP at a normal rate.

Effect of Glutamate Treatment on Neuronal Viability—We next tested the effects of glutamate exposure on the viability of the cortical cultures. By using LDH release as a method of assessing plasma membrane permeability, we found that 24 h of continuous exposure of these cultures to 100 μM glutamate resulted in a significant loss in viability that was inhibited by 10 μM of the NMDA receptor antagonist, MK-801 (Fig. 1). Even though, though ionomic homeostasis appears to be lost and mitochondrial Ca\textsuperscript{2+} loading is maximal after 10 min of glutamate exposure (Fig. 1), if this treatment is halted by addition of MK-801 for the ensuing 24 h, the injury is no longer sufficient to induce LDH release substantially above control levels (Fig. 3), at least at this time point. Therefore, cellular events leading up to the 10 min time point may be critical to initiation of the cell death pathway, but these events have not resulted in commitment of the cells to acute cell death.

Quantitative Estimation of Ca\textsuperscript{2+} in Mitochondria from Control and Glutamate-treated Neurons—The ability of mitochondria in glutamate-treated cells to release accumulated Ca\textsuperscript{2+} in response to FCCP argues against pervasive induction of a permeability transition in the mitochondrial population, as this would prevent retention of mitochondrial Ca\textsuperscript{2+}. As shown on Fig. 4A, trace b, addition of FCCP to mitochondria from glutamate-treated cells caused a rapid release of a significant amount of Ca\textsuperscript{2+} presumably via reversal of the Ca\textsuperscript{2+} uniporter as a result of depolarization of the membrane (26). This FCCP-induced release is relatively slow and was still progressing at a
significant rate 5 min after the uncoupler addition (Fig. 4A, trace c; note that the y scale is logarithmic). Therefore, we used a pore-forming peptide alamethicin to quickly attain complete release (Fig. 4A, trace c). Quantitative estimation of mitochondrial Ca\(^{2+}\) (performed as described under “Experimental Procedures”) revealed that the total Ca\(^{2+}\) released in response to FCCP and alamethicin reached 167 ± 19 nmol/mg protein (n = 8). In the 5-min time frame, FCCP-released about half of the total Ca\(^{2+}\) (66 ± 14 nmol/mg protein, n = 8). More importantly, the amount of Ca\(^{2+}\) released from mitochondria isolated from untreated cells was very low (1.9 ± 0.8 nmol/mg protein, n = 5; Fig. 4A, trace b). Note that the amount of contaminating Ca\(^{2+}\) taken up into mitochondria from the incubation medium (2.5 ± 1.6 nmol/mg protein, n = 13) was subtracted from the amount of Ca\(^{2+}\) released into the medium. We do not exclude the possibility that a fraction of damaged mitochondria could be lost during preparation, but there was no apparent difference in the yields of mitochondria isolated from control and glutamate-treated cells. As well, we cannot exclude the possibility that a subfraction of mitochondrial Ca\(^{2+}\) may have undergone the permeability transition and released their accumulated Ca\(^{2+}\) prior to isolation. Both of these possibilities would result in an underestimation of the quantities of retained Ca\(^{2+}\). Although it is possible that mitochondrial Ca\(^{2+}\) could be lost during isolation, the standard conditions for preparation of mitochondria, such as low temperature, the presence of EGTA, and the absence of Na\(^{+}\) in the isolation buffers, minimize changes in mitochondrial Ca\(^{2+}\) during the isolation procedure. This statement is supported by direct Ca\(^{2+}\) measurements in Ca\(^{2+}\)-loaded brain mitochondria after prolonged incubation under conditions that mimic our isolation procedure (27).

Because alamethicin is nonselective, and endoplasmic reticulum is a persistent contaminant of mitochondrial prepara-
Effect of Glutamate Treatment on Mitochondrial Respiration and Membrane Potential—Mitochondria isolated from untreated cortical neurons preserved functional integrity as evidenced by the ability to establish membrane potential (Fig. 6A) and by an acceptor control ratio of 4.8 ± 0.3 (n = 16) in the presence of complex I-linked substrates (Fig. 6B). Exposure of the cells to 100 μM glutamate for 10 min did not compromise the ability of subsequently isolated mitochondria to generate membrane potential (Fig. 6A). During the isolation procedure, mitochondrial Ca\(^{2+}\) transport processes that were taking place following glutamate exposure are inhibited by the low temperature and by permeabilization in medium containing EGTA. Therefore, these conditions are unlike those in the intact cell in which mitochondrial Ca\(^{2+}\) cycling is ongoing. Our observation is in agreement with evidence that mitochondrial depolarization during acute glutamate treatment is reversible upon glutamate removal (5).

Most importantly, glutamate treatment markedly reduced (on average, by 44%) ADP-stimulated respiration (state 3) and decreased the amplitude of ADP-induced depolarization (Fig. 6, A and B). Because state 3 respiration is dependent upon both electron transport and ADP phosphorylation, the observed inhibition could result from injury to either of the two processes. Uncoupler-stimulated respiration (state 3u) is an indicator of the maximal rate of electron transport. As shown in Fig. 6, A and B, state 3u respiration was reduced by ~10%. Under resting conditions, respiration rates (state 4) remained relatively unchanged. The acceptor control ratio in mitochondria from glutamate-treated cells dropped to 2.7 ± 0.2 (n = 15). Therefore, the most significant effect of glutamate treatment on mitochondria from cortical neurons was inhibition of ADP phosphorylation, whereas the effect on the maximal rate of electron transport was less pronounced, and there was no evidence of significant respiratory uncoupling.

Deleterious effects of elevated Ca\(^{2+}\) on neural mitochondria, such as induction of the permeability transition (16, 31–33) or inhibition of respiration (34–37), have been described in the literature. However, the pathophysiological relevance of the amounts of Ca\(^{2+}\) used in many studies remains questionable. We examined the effects of adding exogenous Ca\(^{2+}\) to control mitochondria at a concentration close to the quantity we measured for glutamate-induced Ca\(^{2+}\) loading (Fig. 6B). In these experiments, mitochondria from untreated cortical neurons were exposed to a bolus of 150 μM Ca\(^{2+}\) (100–125 nmol/mg). After sequestration of the added Ca\(^{2+}\) was complete, respira-
Glutamate-induced bioenergetic changes in mitochondria from neurons. A, respiration (traces a and b) and membrane potential (ΔΨ; traces c and d) measured in mitochondria from control (traces a and c) and glutamate-treated (traces b and d) cortical neurons. Glutamate and malate were present as complex I-linked substrates as shown in Fig. 6. Data are means ± S.E. from three independent experiments. Other details are as specified under “Experimental Procedures.”

Reversibility of Glutamate-induced Inhibition of ADP Phosphorylation—Next, we addressed the question of whether glutamate-induced partial inhibition of state 3 respiration can be reversed upon removal of the accumulated Ca²⁺. Addition of the Ca²⁺ ionophore A23187 to mitochondria from glutamate-treated cells induced rapid release of the endogenous Ca²⁺ that was subsequently chelated by EGTA (Fig. 7A). Ruthenium Red, the inhibitor of the Ca²⁺ uniporter, was present in these experiments to prevent Ca²⁺ re-uptake during A23187-induced Ca²⁺ release. Respiration measurements showed that Ca²⁺ depletion partially restored glutamate-inhibited state 3 respiration (Fig. 7B). This observation is consistent with previous studies with Ca²⁺-challenged heart (40) and brain (34) mitochondria, demonstrating that Ca²⁺-induced inhibition of oxidative phosphorylation can be at least partially relieved by treatment with A23187 and EGTA or after Ca²⁺ release via the Ca²⁺/Na⁺ exchanger.

Fig. 6. Glutamate-induced bioenergetic changes in mitochondria from neurons. A, respiration (traces a and b) and membrane potential (ΔΨ; traces c and d) measured in mitochondria from control (traces a and c) and glutamate-treated (traces b and d) cortical neurons. Glutamate and malate were present as complex I-linked substrates as shown in Fig. 6. Data are means ± S.E. from three independent experiments. Other details are as specified under “Experimental Procedures.”

Fig. 7. Effect of Ca²⁺ depletion by A23187 on state 3 respiration measured in mitochondria from glutamate-treated cortical neurons. A, induction of Ca²⁺ release by A23187 (1.2 µM). Ruthenium Red (0.2 µM) was added to prevent Ca²⁺ re-uptake via the Ca²⁺ uniporter. Released Ca²⁺ was then chelated by EGTA (500 µM). The arrows indicate the timing of additions of mitochondria (Mito), Ruthenium Red (RR), A23187, and EGTA. B, restoration of glutamate-inhibited state 3 respiration in Ca²⁺-depleted mitochondria. After removal of the accumulated Ca²⁺ by the combination of A23187 plus EGTA, respiration was measured in the presence of complex I-linked substrates as shown on Fig. 1. Data are means ± S.E. from three independent experiments. * indicates statistically different from state 3 in glutamate (p < 0.01). Other experimental details are specified under “Experimental Procedures.”

tion was measured under the same conditions as in Fig. 6A. As shown on Fig. 6B, which summarizes the data on respiration from multiple experiments, the patterns of respiratory changes induced by 150 µM Ca²⁺ and glutamate treatment were similar, although the added Ca²⁺ caused a more dramatic inhibition of state 3 respiration. The data demonstrate that in this model Ca²⁺ per se mimics the mitochondrial bioenergetic effects of acute glutamate treatment.

Because the extent of Ca²⁺-mediated injury may depend on the type of respiratory substrate (31, 33, 35, 36, 38, 39), we also assessed the effects of glutamate treatment in the presence of the complex II substrate succinate. Mitochondria energized with succinate showed a similar pattern of inhibition of respiration as in the presence of complex I-linked substrates (Fig. 6, C versus A). In the experiment shown in Fig. 6C, succinate-supported state 3 and state 3u respiration were inhibited by 47 and 19%, respectively.
Measurements of Respiration, Ca\(^{2+}\) Content, and Ca\(^{2+}\) Uptake Capacity in Mitochondria from Cerebellar Granule Cells—Little is known about the differences in mitochondria from different neuronal types, although such information is highly desirable in an effort to understand further the varying susceptibility of different neurons to injury in acute and chronic forms of neurodegeneration. Isolated mitochondria from different brain regions have been studied with regard to susceptibility to the permeability transition (41), susceptibility to dysfunction induced by traumatic brain injury (42), ischemic injury (27, 43), and various toxicants (44) with the caveat that these are studies of mixed populations of mitochondria from varied cell types. The current approach allows more appropriate comparison of bioenergetic function between enriched neuronal cultures. A series of experiments was performed to compare the glutamate-induced changes in mitochondria from cortical neurons with those in mitochondria isolated from cerebellar granule cells. Under control conditions, the rates of respiration of mitochondria from the two neuronal cell types were comparable (Fig. 6B and Fig. 8).

With regard to glutamate-induced mitochondrial injury, Atlante et al. (45) have reported progressive inhibition of succinate-supported state 3 respiration measured in homogenates prepared from cerebellar granule cell cultures at different times after glutamate exposure. In agreement with this study, glutamate treatment for 10 min induced 20–30% inhibition of both state 3 and state 3u respiration in the presence of complex I-linked substrates in cerebellar mitochondria (Fig. 8). ADP-stimulated respiration was therefore less inhibited than in cortical mitochondria (Fig. 8 versus Fig. 6). The observation of partial inhibition of ADP-stimulated respiration does not contradict a previous conclusion that cerebellar mitochondria in situ remain capable of producing ATP throughout excitotoxic glutamate exposure (Refs. 5 and 46; see “Discussion”).

The most significant difference between cortical and cerebellar mitochondria was observed in the Ca\(^{2+}\) uptake capacity. The maximal Ca\(^{2+}\) uptake capacity for cerebellar mitochondria was 686 ± 71 nmol/mg protein (n = 4), almost twice as high as for cortical mitochondria. Despite the higher Ca\(^{2+}\) uptake capacity, glutamate-induced increases in the Ca\(^{2+}\) content were similar to those in cortical mitochondria. The Ca\(^{2+}\) pool released by FCCP within 5 min in cerebellar mitochondria was 96 ± 34 nmol/mg protein (n = 7). The total Ca\(^{2+}\) released in response to FCCP plus alamethicin was 173 ± 64 nmol/mg protein (n = 8). These data suggest that in cerebellar granule cells, similar to cortical neurons, glutamate-induced Ca\(^{2+}\) loading does not saturate the mitochondrial Ca\(^{2+}\) uptake capacity, but the extent of loading represents a smaller proportion of the maximal uptake capacity. As with cortical neuronal mitochondria, the alamethicin-releasable pool of Ca\(^{2+}\) in mitochondria from untreated cells was a small fraction of the pool released following glutamate treatment (2.0 ± 1.4% (n = 4)).

**DISCUSSION**

In our cortical neuronal cultures, we identified impairment of ADP phosphorylation as the most dramatic consequence of glutamate exposure to mitochondrial bioenergetics, whereas inhibition of maximal respiratory chain activity was quite modest. The inhibition of oxidative phosphorylation occurred prior to commitment to cell death and therefore did not result from sequelae of the cell death process. The effect of excitotoxin exposure on oxidative phosphorylation was Ca\(^{2+}\)-dependent as we found that it could be mimicked by additions in vitro of the relevant concentrations of Ca\(^{2+}\). Moreover, glutamate-induced inhibition of state 3 respiration could be reversed by rapid depletion of the accumulated Ca\(^{2+}\) using ionophore. We found that the cellular ATP level was compromised within the first minutes of glutamate exposure (Fig. 2A), and it was not readily recovered at 6 min after glutamate/Ca\(^{2+}\) removal (Fig. 2B). The lack of recovery at this time point likely reflects the protracted elevation of matrix Ca\(^{2+}\) following an excitotoxic stimulus because of the relatively slow kinetics of the mitochondrial Ca\(^{2+}\) efflux pathway and continued Ca\(^{2+}\) cycling (23, 25, 47, 48). We propose that the early impairment of ADP phosphorylation contributes to cellular ATP depletion, and we further suggest that this inhibition is likely key to both ensuing neuronal dysfunction and/or cell death.

Certainly, there are other potential modes by which mitochondrial Ca\(^{2+}\) sequestration may trigger neuronal dysfunction and/or death. Mitochondrial Ca\(^{2+}\) overload may result in respiratory inhibition and/or the permeability transition that prevents sufficient mitochondrial ATP production to meet the increased energy demand. Alternatively, Ca\(^{2+}\) sequestration may result in increased oxidative stress that compromises multiple components of cell function including Ca\(^{2+}\) extrusion (5). Nicholls and co-workers (9, 11, 49) have reported that the cause of delayed Ca\(^{2+}\) deregulation, at least in cerebellar granule cells, is a mitochondrial event other than insufficient ATP production. It should be noted, however, that the approaches in these elegant studies, including fluorescence imaging of intact cells and in situ respiration of monolayers, are capable of detecting whether ADP phosphorylation can take place, but they are unable to assess whether significant but incomplete inhibition of oxidative phosphorylation has occurred. This limitation is inherent to studies of mitochondrial bioenergetics using intact cells because of the inability to control ADP and substrate provision to the mitochondria. The isolation technique employed here overcomes this limitation. Jekabsons and Nicholls (49) found that glutamate stimulated the respiration of intact cerebellar granule cells to 40–50% of their maximal electron transport chain capacity. We would suggest that this increase, although stimulated by ATP turnover, is not meeting ATP demand in a manner consistent with continued normal cell function and viability. There are potentially important differences, however, between the Ca\(^{2+}\) responses of our cortical cultures and the cerebellar granule cells studied by Jekabsons and Nicholls (49). Foremost, Ca\(^{2+}\) deregulation is much more delayed in a majority of the granule cells compared with our cortical neurons (Refs. 9, 11, and 49 versus Fig. 1). The inhibition of oxidative phosphorylation, the persistent decline in ATP, and the rapidity of the loss in ionic homeostasis in our cortical neurons are most consistent with death involving insufficient ATP generation. In fact, the pattern of cytoplasmic Ca\(^{2+}\) responses to glutamate in cortical neurons appears sim-
ilar to those in cerebellar granule cells when both respiration and mitochondrial ATP synthesis have been chemically blocked (11). As a result, we propose that mechanisms of injury are common in different neurons, but the events most critical to loss in ionic homeostasis and cell death may vary in different cultures and may depend on multiple factors, including the glycolytic capacity to produce ATP, reactive oxygen species-detoxifying systems, and the proportion of the mitochondria of the cells that sequesters excessive loads of Ca
details.

More importantly, the fact that our cortical cultures do not undergo cell death if the stimulus has been halted at 10 min (Fig. 3), despite the fact that they have undergone Ca2+-deregulation (Fig. 1), clearly implies that additional event(s) critical to the commitment to cell death occur after this profound increase in cytoplasmic Ca2+. In fact, others have found that although antioxidants have no effect on the onset of delayed Ca2+-deregulation, they protect against cell death measured 24 h later (50), suggesting that an oxidative event critical to death occurs after the loss of ionic homeostasis. Furthermore, our data suggest that inhibition of oxidative phosphorylation may be a consequence of glutamate exposure that, if brief, may result in a compromise in normal neuronal function but not necessarily result in neuronal death. This may have profound implications for the chronic dysfunction of neurons in diseases such as Huntington disease, in which expansion of the huntingtin protein may lead to NMDA receptor activation, exaggerated cytoplasmic Ca2+ signaling via inositol 1,4,5-trisphosphate-mediated mechanisms, and thus enhanced mitochondrial Ca2+ loading (2, 51).

Inhibition of respiration and/or ADP phosphorylation by elevated Ca2+ is not a unique feature of neuronal mitochondria. A similar injury was observed in earlier studies with mitochondria from tumor (38, 52) and transformed neural cells,2 brain (34, 35), and heart (40). The precise mechanisms underlying this injury remain to be elucidated. The maximal rate of respiration (FCCP-stimulated respiration) is mildly decreased by glutamate treatment in both cortical and cerebellar mitochondria in our hands. Inhibition of maximal respiration in response to glutamate has been observed in cerebellar granule cells (45, 49) and cortical neurons (53), although Atlante et al. (45) and Almeida et al. (53) did not correct for losses in cell viability and measured respiration at later time points. The effect that we observe is independent of the type of respiratory substrate (complex I versus complex II substrates) and therefore could be explained by a partial loss of cytochrome c (33, 54, 55). ADP phosphorylation (state 3 respiration) was significantly more inhibited than FCCP-stimulated respiration (state 3u respiration) in our cortical cultures, indicating that components other than the respiratory chain are affected, e.g. the ATP synthase and/or availability of adenine nucleotides and phosphate. As one potential mechanism, Ca2+ has been proposed to bind the adenine nucleotides in the mitochondrial matrix causing inhibition of state 3 respiration and ATP synthase activity (34, 56).

This study estimates that the glutamate-induced mitochondrial Ca2+ loading for both cortical and cerebellar neurons is ~170 nmol/mg protein. This amount was determined by releasing the cation into the medium with the pore-forming agent alamethicin. We found that alamethicin was more effective than FCCP at rapidly releasing Ca2+-, likely because of the fact that the bulk of Ca2+ in mitochondria is stored in the form of the poorly soluble Ca2+-phosphate complex (16, 57–60). The rate of solubilization of this complex may be slower in response to uncoupler as opposed to when alamethicin-induced pores allow dilution of the matrix contents. As noted earlier, one of the shortcomings of our current approach is that mitochondrial Ca2+ can potentially be lost during the isolation. Although there is evidence against this (27), it must be stated that the actual mitochondrial Ca2+ load may be even higher than our estimates. A second reason for this statement is that nonmitochondrial protein typically contaminates mitochondrial preparations. As judged by Western blot analysis (data not shown), the degree of cytosolic and membranous contaminations of our cortical preparation was comparable with that of brain mitochondria isolated according to a published protocol (15), and therefore, the Ca2+ loads represent reasonable estimates for comparison to isolated mitochondria models.

The glutamate-induced mitochondrial Ca2+ load is significantly below the maximal Ca2+ uptake capacity that we measured in vitro. There are a number of potential explanations for this observation, including that the quantity of available phosphate (the necessary counter-ion in Ca2+ transport) could limit glutamate-induced mitochondrial Ca2+ uptake (61), that the activity of the mitochondrial Na+/Ca2+ exchanger is enhanced in response to cytosolic Na+ (23, 48), or that there is loss of loaded Ca2+ because of the permeability transition or the isolation procedure. Furthermore, mitochondrial Ca2+ uptake may be altered by glutamate-induced generation of reactive oxygen species or nitric oxide (for reviews see Refs. 7 and 21). An equally tenable explanation for the apparent “intermediate” Ca2+ loading in situ is that only a subpopulation of mitochondria may be sequestering Ca2+ in intact neurons, for which there is strong evidence (62, 63). Mitochondria within cells display functional and morphological heterogeneity that, among other factors, reflects their proximity to Ca2+ sources (63–65). One can hypothesize that heterogeneous Ca2+ loading causes more dramatic respiratory injury to a subpopulation of mitochondria that are in close proximity to NMDA receptors. Inhibition of ATP production to a critical subset of mitochondria may produce a localized inability to re-establish ionic homeostasis and membrane potential at the plasma membrane that ultimately perpetuates a collapse of ionic homeostasis.

The difference between the glutamate-induced Ca2+ load and the maximal Ca2+ uptake capacity is more dramatic in mitochondria from cerebellar granule cells. The higher Ca2+ uptake capacity of cerebellar mitochondria (as compared with cortical mitochondria) correlates with the relative resistance of this brain region in excitotoxic and ischemia/reperfusion models (66, 67). Mitochondria isolated from cerebellar tissue are less susceptible to a Ca2+-induced injury than mitochondria isolated from cortex and hippocampus (41) potentially because of higher adenine nucleotide content (38, 41). Therefore, these data lead us to suggest that the proportional load of mitochondrial Ca2+ resulting from glutamate exposure compared with the maximal Ca2+ uptake capacity may determine susceptibility to excitotoxic neuronal death.

Our data do not support that glutamate exposure induces pervasive stimulation of the mitochondrial permeability transition in cortical neurons or cerebellar granule cells. If the permeability pore was open at the 10-min time point that we began our isolation, the EGTA in the buffer would sequester the matrix Ca2+ (68). We observe substantial alamethicin-induced Ca2+ release from the mitochondria, and therefore a significant portion of mitochondria was impermeable to EGTA at the time of isolation. However, we cannot eliminate the possibility that a subpopulation of mitochondria has undergone the permeability transition, which would lead to an underestimation of the glutamate-induced mitochondrial Ca2+ load. However, if the permeability transition occurs in a subpopulation of mitochondria during a 10-min glutamate exposure, this

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2 A. Y. Andreyev, S. E. Wiley, Y. E. Kushnareva, and A. N. Murphy, unpublished observations.
event is insufficient to induce significant neuronal death (Fig. 3). Neuronal mitochondria were also resistant to a permeability transition when they were loaded with Ca\(^{2+}\) in vitro. At Ca\(^{2+}\) concentrations sufficient to saturate Ca\(^{2+}\) uptake capacity, no permeability changes were detected (Fig. 5). Although others have found indications of increased mitochondrial permeability in intact neurons (24, 69–74), the lack of consistent protective effects of permeability transition inhibitors in excitotoxicity models (11, 70, 74–76) renders the literature data controversial. Recently, it has been suggested that the permeability transition pore in brain mitochondria has altered sensitivity to conventional pore inhibitors (77). Regardless, the extent of permeability pore contribution to excitotoxic neuronal death requires further investigation.

In conclusion, these results have revealed that glutamate exposure of cortical neurons results in early inhibition of oxidative phosphorylation, in the absence of profound respiratory inhibition, or changes that might be the downstream result of commitment to cell death. Although the approach taken here has caveats, it revealed a type of Ca\(^{2+}\)-induced injury that fluorescence imaging or whole cell respiration experiments are unable to detect. We propose that Ca\(^{2+}\)-mediated inhibition of oxidative phosphorylation may have pathological relevance to acute as well as chronic excitotoxic injury.

REFERENCES

1. Choi, D. W. (1994) Ann. N. Y. Acad. Sci. 747, 162–171
2. Bezprozvanny, I., and Hayden, M. R. (2004) Biochem. Biophys. Res. Commun. 322, 1310–1317
3. Choi, D. W. (1988) Neuron 1, 623–634
4. Tymianski, M., Charlton, M. P., Carlen, P. L., and Tator, C. H. (1993) J. Neurosci. 13, 2085–2104
5. Nicholls, D. G. (2004) Curr. Mol. Med. 4, 149–177
6. Khodorov, B. I. (2002) Membr. Cell Biol. 21, 149–162
7. Krieger, C., and Duchen, M. (2002) J. Biol. Chem. 277, 177–188
8. Tsuji, K., Nakamura, Y., Ogata, T., Shibata, T., and Kataoka, K. (1994) J. Neurosci. Res. 40, 292–299
9. Budd, S. L., and Nicholls, D. G. (1996) J. Neurochem. 67, 2282–2291
10. Stout, A. K., Raphael, H. M., Kantorzewicz, B. I., Klann, E., and Reynolds, I. J. (1998) Nat. Neurosci. 1, 366–373
11. Castillo, R. F., Hanson, O., Ward, M. B., Ward, S. L., and Nicholls, D. G. (1998) J. Neurosci. 18, 10277–10286
12. Urasuhiyi, M., Nakamoto, T., Inoue, R., Sawada, H., Kihara, T., Honda, K., Akaike, A., and Shimohama, S. (2000) J. Neurosci. Res. 60, 377–387
13. Lai, J. C., and Clark, J. B. (1979) Biochim. Biophys. Acta 573, 1–9
14. Chatfield, R. F., and Bygrave, F. L. (1974) Nature 249, 255–262
15. Rosenthal, R. E., Hamud, F., Fiskum, G., Varghese, P. J., and Sharpe, S. (1998) J. Neurochem. 32, 1085–1094
16. Andreyev, A. Y., Fahy, B., and Fiskum, G. (1998) FEBS Lett. 439, 373–376
17. Brustovetsky, N., Memmert, R., and Dubinsky, S. (2002) Mem. Cell Biol. 23, 167–172
18. Anderegg, A. Y., Fahy, B., and Fiskum, G. (1998) FEBS Lett. 439, 373–376
19. Brustovetsky, N., Memmert, R., and Dubinsky, S. (2002) Membr. Cell Biol. 23, 321–326
20. Courtenay, M. J., Lambert, J. J., and Nicholls, D. G. (1999) J. Neurosci. 19, 3873–3879
21. Almeida, A., and Medina, J. M. (1998) Brain Res. Brain Res. Protocols 2, 209–214
22. Kamo, N., Muratsugu, M., Hongoh, R., and Kobayate, Y. (1979) J. Membr. Biol. 40, 105–121
23. Peng, T. I., and Greenamyre, J. T. (1998) Mol. Pharmacol. 53, 974–980
24. Nicholls, D., and Буд, S. (2000) Physiol. Rev. 80, 315–360
25. Wang, G. J., and Thayer, S. A. (2002) J. Neurophysiol. 87, 740–749
26. Alano, C. C., Beutner, G., Kirskien, R., and Sheu, S. (2002) J. Neurochem. 80, 531–538
27. Breard, J. B., Tassetto, M., and Reynolds, I. J. (2001) J. Physiol. (Lond.) 531, 795–805
28. Jurkowitz, M. S., Geisbuhler, T., Jung, D. W., and Brierley, G. P. (1983) Arch. Biochem. Biophys. 223, 120–128
29. Toker, E., and Sims, N. R. (1994) J. Neurochem. 63, 1812–1819
30. Hunter, D. R., and Haworth, R. A. (1979) Arch. Biochem. Biophys. 195, 453–459
31. Lapinov, R. G., and Sokolove, P. M. (1994) J. Biol. Chem. 269, 18932–18936
32. Rottenberg, H., and Marbach, M. (1990) Biochim. Biophys. Acta 1016, 77–86