

Interactions between mitochondrial bioenergetics and cytoplasmic calcium in cultured cerebellar granule cells

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Abstract

The mitochondrion has moved to the center stage in the drama of the life and death of the neuron. The mitochondrial membrane potential controls the ability of the organelle to generate ATP, generate reactive oxygen species and sequester Ca^{2+} entering the cell. Each of these processes interact, and their deconvolution is far from trivial. The cultured cerebellar granule cell provides a model in which knowledge gained from studies on isolated mitochondria can be applied to study the role played by the organelles in the maintenance of Ca^{2+} homeostasis in the cell under resting, stimulated and pathophysiological conditions. In particular, mitochondria play a complex role in the response of the neuron to excitotoxic stimulation of NMDA and AMPA-kainate selective glutamate receptors. One goal of research in this area is to provide clues as to possible ways in which modulators of mitochondrial function may be used as neuroprotective agents, since mitochondrial Ca^{2+} accumulation seems to play a key role in glutamate excitotoxicity.

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1. Introduction

Mitochondria integrate their bioenergetics with that of the cell and vary their respiration to match the cellular demand for ATP. The dominant ATP consuming reaction in the neuron is the Na^+/K^+ -ATPase, which maintains the Na^+ electrochemical potential gradient across the enormous surface area of the neuronal plasma membrane. Under conditions of enhanced Na^+ entry into the cell, for example during chronic AMPA, kainate or NMDA receptor activation, or when voltage-activated Na^+ channels are maintained open by veratridine, the Na^+/K^+ -ATPase can utilize almost all the ATP generating capacity of the mitochondrion [1]. While still requiring ATP, maintenance of the Ca^{2+} electrochemical gradient across the plasma membrane appears to be less energy demanding, even under conditions of receptor-mediated Ca^{2+} entry when cytoplasmic free Ca^{2+} , $[\text{Ca}^{2+}]_c$, is elevated, since the maximal activity of the plasma membrane Ca^{2+} -ATPase (PMCA) is considerably less than that of the Na^+/K^+ -ATPase [2]. Fig. 1 shows a highly simplified scheme of the major bioenergetic pathways for a mitochondrion within a neuron.

Many cultured neurons possess sufficiently active glycolysis to supply the cell's ATP requirements in the absence of mitochondrial ATP synthesis even when ATP demand is increased by channel activation. This allows the mitochondrial ATP synthase inhibitor oligomycin to be used as an experimental tool to isolate the cell's ATP-driven processes from any manipulations of mitochondrial function (Fig. 1B and D). As will be seen, oligomycin has proven to be a valuable tool to investigate the role of mitochondrial functions other than ATP synthesis, such as Ca^{2+} sequestration and the generation of reactive oxygen species (ROS).

Since the ATP synthase is reversible, conditions such as respiratory chain inhibition that lower the mitochondrial protonmotive force (Δp) or its major component, the mitochondrial membrane potential ($\Delta\psi_m$) below that required to synthesize ATP can result in the hydrolysis of cytoplasmic ATP by the mitochondrion and an ATP-driven proton extrusion that can maintain a potential that is only slightly lower than that generated by the respiratory chain. Because the native proton conductance of the mitochondrial inner membrane is low, a relatively slow rate of cytoplasmic ATP hydrolysis usually suffices to maintain this potential in the presence of a respiratory chain inhibitor such as rotenone or antimycin A (Fig. 1C). However, addition of a classic uncoupler, or protonophore, greatly increases the membrane proton conductance with a resulting rapid ATP synthase

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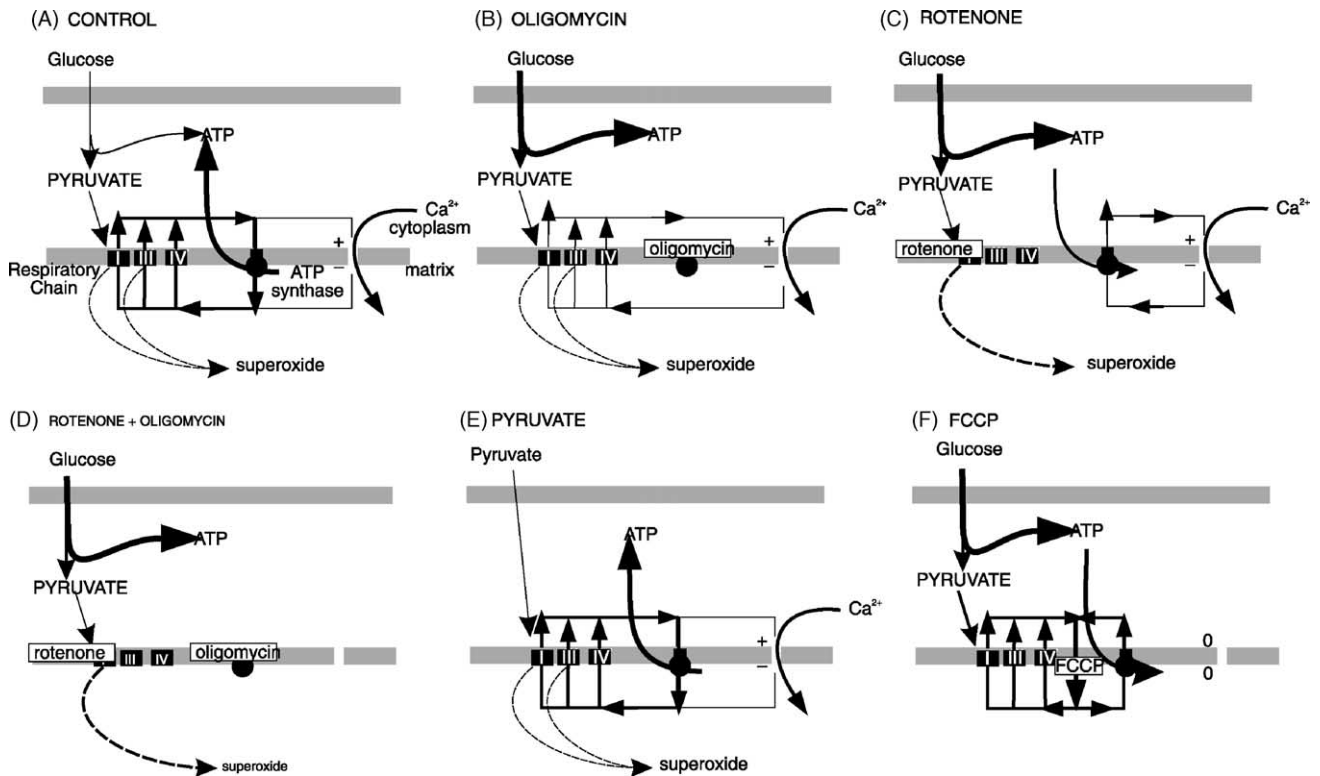


Fig. 1. Simplified schematic of the main neuronal bioenergetic pathways and the influence of mitochondrial inhibitors. (A) The mitochondrial proton circuit is generated by complexes I, III and IV acting in parallel and is utilized primarily by the ATP synthase to generate ATP. When cytoplasmic free Ca^{2+} exceeds a threshold it is accumulated into the matrix, driven by and utilizing the mitochondrial membrane potential. Complexes I and III both contribute to superoxide ($\text{O}_2^{\bullet-}$) generation (B) in the presence of oligomycin, all ATP is produced by glycolysis which accelerates to compensate. Mitochondrial membrane potential ($\Delta\psi_m$) increases slightly, Ca^{2+} can still be accumulated and $\text{O}_2^{\bullet-}$ production continues, or is even enhanced due to the hyperpolarization. (C) Respiratory chain inhibition by rotenone requires that all ATP is generated by glycolysis; however ATP is utilized by the ATP synthase acting in reverse mode to generate a $\Delta\psi_m$ only slightly lower than in control conditions. Ca^{2+} can still be accumulated by the mitochondrion, but the increased demand on glycolytic ATP can lead to ATP depletion. Since rotenone acts downstream of the complex I $\text{O}_2^{\bullet-}$ generating site, $\text{O}_2^{\bullet-}$ production from complex I is increased. (D) The combination of rotenone plus oligomycin allows $\Delta\psi_m$ to decay, preventing Ca^{2+} accumulation. There is no drain on glycolytic ATP, but $\text{O}_2^{\bullet-}$ is still produced. (E) Substituting pyruvate for glucose as substrate means that the cell is entirely dependent on oxidative phosphorylation. (F) Protonophores collapse $\Delta\psi_m$, but potentially deplete cytoplasmic ATP due to the rapid reversal of the ATP synthase. Mitochondrial $\text{O}_2^{\bullet-}$ generation should be decreased due to the increased oxidation state of the respiratory chain.

reversal in a futile attempt to maintain a high Δp (Fig. 1F). Protonophores have been used rather indiscriminately with cultured neurons, usually in the attempt to depolarize the mitochondrion and inhibit Ca^{2+} accumulation, but few neurons are capable of maintaining cytoplasmic ATP levels in the presence of uncouplers when ATP demand is high, and effects ascribed to a block of mitochondrial Ca^{2+} sequestration are frequently merely due to cytoplasmic ATP depletion and consequent failure of the plasma membrane ion pumps. The presence of oligomycin to prevent synthase reversal can prevent this ATP depletion (Fig. 1D).

2. Cytoplasmic Ca^{2+} and mitochondrial Ca^{2+} transport

In addition to their role in ATP generation, in situ mitochondria sequester cytoplasmic Ca^{2+} whenever the concentration of the ion in the vicinity of the organelles rises above a critical 'set-point' at which the activity of the Ca^{2+}

uniporter uptake pathway into the matrix exceeds that of the independent inner membrane $\text{Na}^+/\text{Ca}^{2+}$ exchanger releasing the ion back into the cytoplasm [3]. In the presence of excess cytoplasmic phosphate, the matrix free Ca^{2+} concentration, $[\text{Ca}^{2+}]_m$, is largely independent of the total accumulated Ca^{2+} due to the formation of a calcium phosphate complex and depending on conditions is in the region of 1–5 μM in isolated brain mitochondria (S. Chalmers, unpublished). This buffering means in turn that the activity of the $\text{Na}^+/\text{Ca}^{2+}$ exchanger and hence the value of the set-point, remains constant as the mitochondria load with Ca^{2+} . Experiments with isolated brain mitochondria report a set-point in the region of 0.5 μM [4], and a remarkably similar value seems to be valid also for in situ mitochondria [5,6]. Studies with cultured neurons have validated the proposal we first made in 1983 [7] that a major function of the mitochondrial Ca^{2+} cycle was the reversible sequestration of cytoplasmic Ca^{2+} during periods of elevated $[\text{Ca}^{2+}]_c$ as a consequence of plasma membrane Ca^{2+} entry. It is clearly essential that

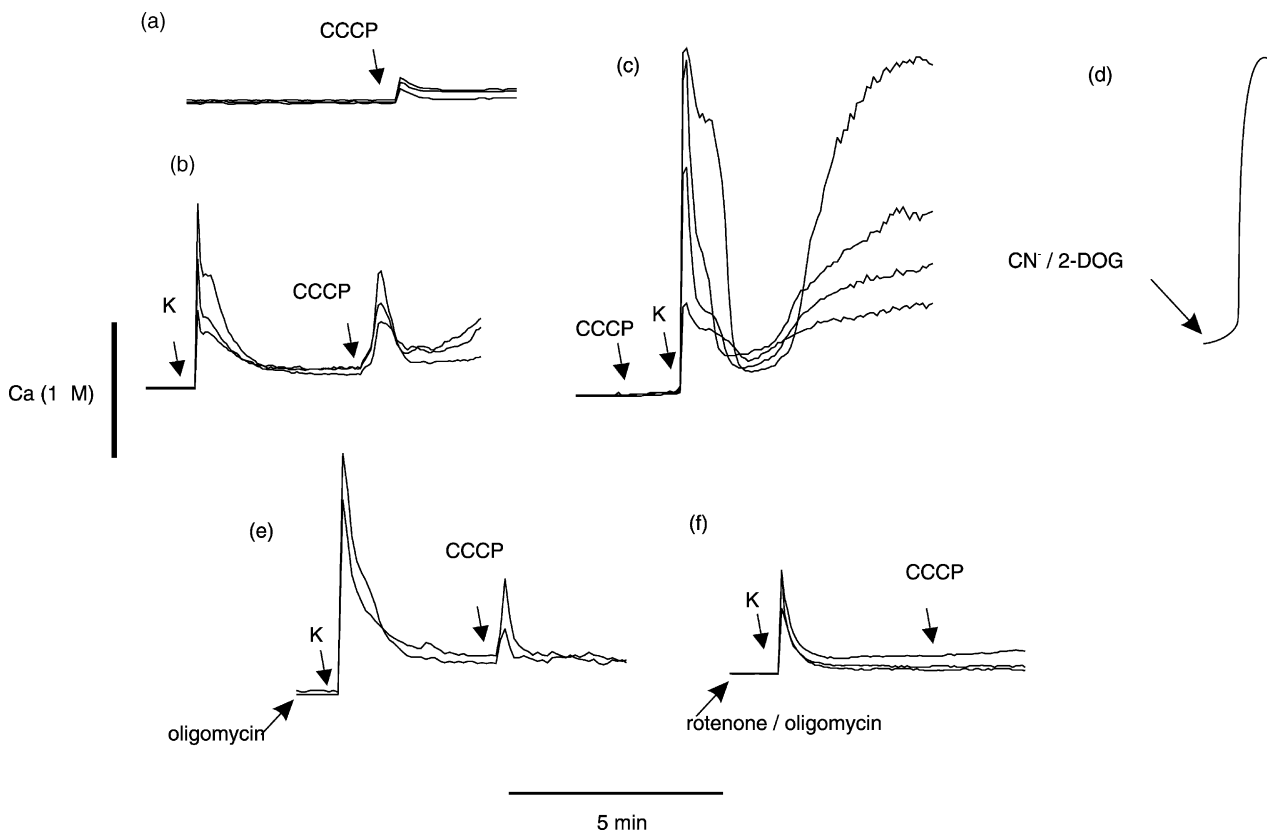


Fig. 2. $[Ca^{2+}]_c$ responses to protonophore addition in cerebellar granule cells. (a) Mitochondria within CGCs suspended in low KCl medium contain little or no protonophore releasable Ca^{2+} . (b) VDCC activation following KCl depolarization of the plasma membrane results in matrix Ca^{2+} loading which can now be released by CCCP; note that some cells fail to maintain subsequent cytoplasmic Ca^{2+} homeostasis. (c) Protonophore added prior to KCl depolarization results in an enhanced transient that may be due to acute ATP depletion, as cells fail to maintain Ca^{2+} homeostasis after release of matrix Ca^{2+} . (d) A similar loss of Ca^{2+} homeostasis is caused by ATP depletion by 2-deoxyglucose in combination with cyanide. (e) Oligomycin does not affect KCl-evoked matrix Ca^{2+} loading, but prevents loss of Ca^{2+} homeostasis subsequent to CCCP addition. (f) Pre-depolarization of mitochondria by rotenone plus oligomycin prevents matrix Ca^{2+} loading and decreases the size of the KCl-evoked $[Ca^{2+}]_c$ spike. Data from [12,80].

the PMCA is able to lower $[Ca^{2+}]_c$ below the set-point in neurons once the influx of Ca^{2+} into the cell decreases to resting values, since otherwise the matrix would inexorably load with the cation. Thus, in resting cells the mitochondrial matrix contains little or no protonophore-releasable Ca^{2+} (Fig. 2a), whereas Ca^{2+} can be released from the matrix in cells which have been Ca^{2+} loaded by voltage-dependent Ca^{2+} channel (VDCC) activation (Fig. 2b).

It would be predicted that specific inhibition of mitochondrial Ca^{2+} uptake would increase the cytoplasmic $[Ca^{2+}]_c$ response to plasma membrane Ca^{2+} entry as the Ca^{2+} would remain in the cytoplasm rather than being sequestered by the mitochondrion. Since no cell-permeant selective inhibitor of the mitochondrial Ca^{2+} uniporter has been described, indirect means (collapse of Δp) must be adopted to inhibit mitochondrial Ca^{2+} transport. As has been discussed above, the presence of oligomycin before and after protonophore addition would prevent a priori changes in cytoplasmic ATP, however protonophores display no membrane selectivity and will additionally equilibrate protons across the plasma and synaptic vesicular membranes. A more discriminating approach is to use oligomycin in combination with a specific

mitochondrial respiratory chain inhibitor such as rotenone or antimycin A, in which case Δp will decay and mitochondrial Ca^{2+} accumulation will be inhibited (see Fig. 1D). A KCl depolarization experiment in the presence of oligomycin is shown in Fig. 2e. The ATP synthase inhibitor prevents the loss of Ca^{2+} homeostasis following CCCP. Significantly, cells preincubated with the combination of rotenone plus oligomycin to depolarize their mitochondria without depleting ATP not only leads to the expected abolition of the CCCP-releasable matrix pool (Fig. 2f) but also decreases rather than increases the size of the KCl transient spike. This paradoxical finding will be discussed below.

Since the kinetics of mitochondrial calcium transport derived from studies with isolated mitochondria strongly suggest that the organelle can serve as a temporary store of Ca^{2+} during periods of neuronal activity, releasing their accumulated Ca^{2+} back into the cytoplasm when the activity is terminated, it would be predicted that mitochondria would blunt peak $[Ca^{2+}]_c$ elevations, and conversely slow recovery to a basal $[Ca^{2+}]_c$ after a period of neuronal activity. This was first demonstrated by Thayer and Miller, who monitored $[Ca^{2+}]_c$ with fura-2 in patched dorsal root

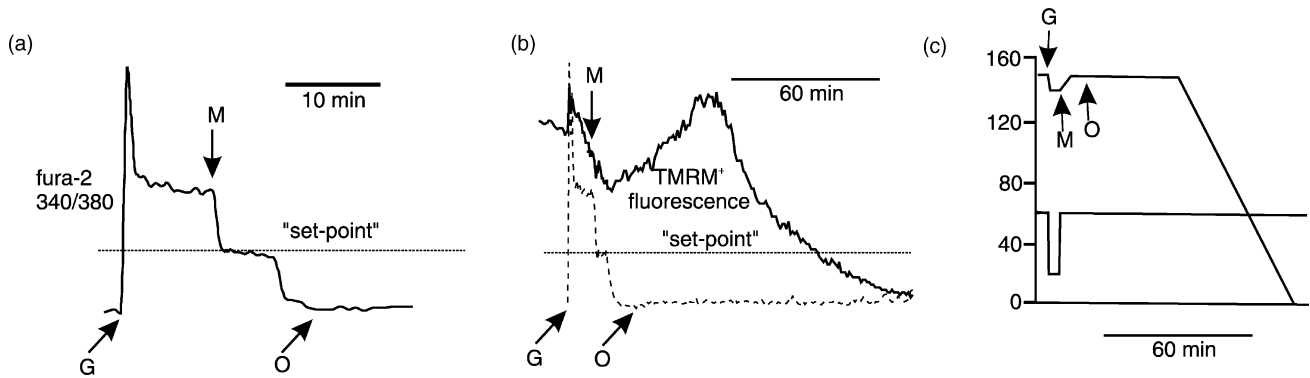


Fig. 3. $[Ca^{2+}]_c$ and TMRM⁺ fluorescence responses to transient NMDA receptor activation. NMDA receptors were activated for 10 min following which they were inhibited by MK801. NBQX was added to inhibit AMPA receptors. (a) Expanded time-scale to show the shoulder in the recovery of $[Ca^{2+}]_c$ equated with the mitochondrial set-point. (b) Prolonged time course showing fluorescence of TMRM⁺. Note that depolarization occurs when $[Ca^{2+}]_c$ is below the set-point. (c) Interpretation of the TMRM⁺ trace in terms of changes in $\Delta\psi_p$ and $\Delta\psi_m$. G, glutamate/glycine; O, oligomycin; M, MK801. Data from [9].

ganglion cells dialyzed with ATP-containing medium [8]. A ruthenium red-sensitive component, identified with mitochondria, was capable of buffering large Ca^{2+} loads imposed by plasma membrane depolarization. In addition these authors showed that recovery to basal free Ca^{2+} on termination of the stimulus was delayed by a plateau at about $0.5 \mu M Ca^{2+}$, consistent with a mitochondrial dumping of Ca^{2+} into the cytoplasm when the PMCA lowered $[Ca^{2+}]_c$ below the set-point. Fig. 3a shows a similar experiment with cerebellar granule cells (CGCs); when 10 min of Ca^{2+} entry through NMDA receptors is terminated by the addition of MK801, $[Ca^{2+}]_c$ recovers, but a shoulder in the recovery phase is seen that can be attributed to the unloading of matrix Ca^{2+} to the cytoplasm at the set-point [9].

Cultured neurons can accumulate substantial amounts of Ca^{2+} through either voltage-dependent Ca^{2+} channels (VDCCs) or Ca^{2+} -permeant ligand-gated ion channels such as the NMDA-selective glutamate receptor. One study using ^{45}Ca estimated that CGCs exposed to glutamate or NMDA were capable of accumulating up to $10 \text{ nmol } ^{45}Ca$ per 10^6 cells before dying [10], corresponding to an averaged internal concentration approaching 20 mM, or 10^4 times higher than the $[Ca^{2+}]_c$ reported by fura-2. A comparable accumulation is shown in the experiment in Fig. 4f. Even allowing for the possibility that the high-affinity Ca^{2+} indicator underestimates the maximal extent of a $[Ca^{2+}]_c$ elevation [11], the great majority of this Ca^{2+} must be sequestered within the mitochondria.

How then does a neuron whose mitochondria are pre-depolarized by the combination of a respiratory chain inhibitor and oligomycin (Fig. 1D) respond to NMDA receptor or VDCC-mediated Ca^{2+} entry? The intuitive response would be an enhanced $[Ca^{2+}]_c$ elevation since the mitochondria are no longer competent to accumulate Ca^{2+} . Such a response can be seen in the presence of protonophore, but this seems to be a consequence of restricted PMCA activity due to ATP depletion (Fig. 2c). However, if precautions are taken to ensure that cytoplasmic ATP remains high, a

decreased fura-2 response to KCl [12] (Fig. 2f), brief glutamate exposure [13,14] (Fig. 4b and c) or kainate (Fig. 4d) can be seen when CGC mitochondria are pre-depolarized by oligomycin in combination with rotenone or antimycin.

A decrease in the plateau $[Ca^{2+}]_c$ elevation can also be seen in glutamate exposed cells in the presence of oligomycin when rotenone is additionally added to allow Δp to decay slowly even though this will be accompanied by an efflux of any Ca^{2+} stored in the mitochondrial matrix (Fig. 4e). At the same time the total glutamate-induced uptake of ^{45}Ca is lower than in cells with polarized mitochondria [13] (Fig. 4f). The implication is that inhibiting mitochondrial Ca^{2+} accumulation causes a decreased Ca^{2+} entry via the channel and/or an enhanced extrusion of the ion via the Ca^{2+} -ATPase. The most feasible way in which inhibited mitochondrial Ca^{2+} uptake can affect plasma membrane Ca^{2+} transport is by increasing the accumulation of Ca^{2+} immediately below the plasma membrane; this could amplify the Ca^{2+} -induced feedback inhibition of the NMDA receptor [15], while at the same time the increased sub-plasma membrane $[Ca^{2+}]_c$ would enhance Ca^{2+} -ATPase activity and its activation by calmodulin. In practice, no decrease in the rate of divalent cation entry through the NMDA receptor could be detected (using Mn^{2+} quenching of matrix fura-2) when the mitochondria were depolarized (Fig. 4g) so the most likely explanation for the lowered Ca^{2+} content and free matrix Ca^{2+} is an enhanced Ca^{2+} extrusion.

In order to reconcile a hypothetical decreased sub-plasma membrane $[Ca^{2+}]_c$ with an observed increase in the averaged $[Ca^{2+}]_c$ reported by fura-2 it is necessary to suppose that the mitochondria have privileged access to Ca^{2+} entering across the plasma membrane. Pivovarova et al. [16] have provided evidence in support of this, by observing that mitochondrial Ca^{2+} accumulation in depolarized frog sympathetic ganglion neurons was graded, with mitochondria close to the plasma membrane showing greater Ca^{2+} accumulation than those more centrally located. Recently,

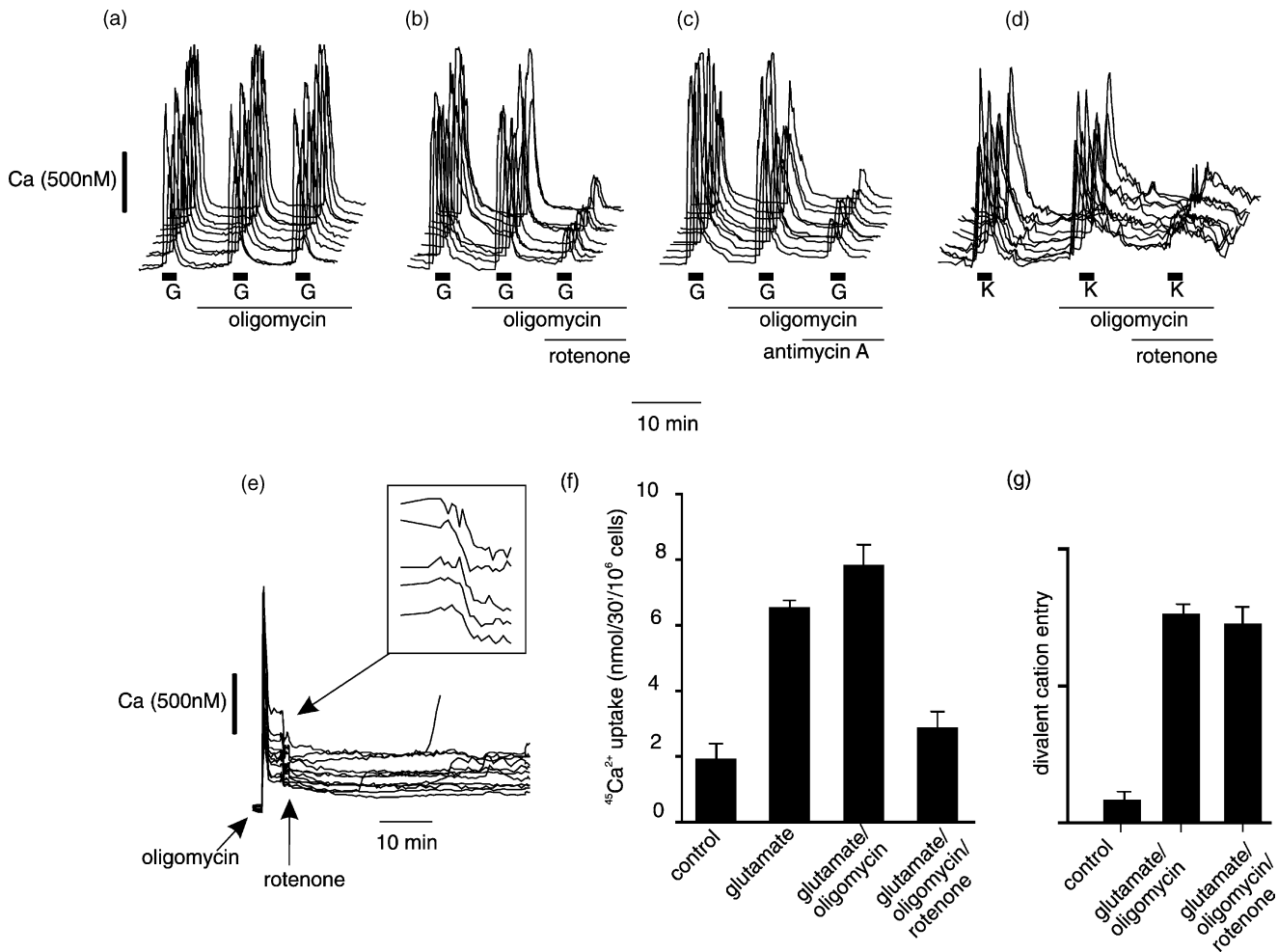


Fig. 4. Mitochondrial depolarization depresses $[Ca^{2+}]_c$ responses to NMDA or AMPA receptor activation. (a–d) $[Ca^{2+}]_c$ responses in the somata of individual CGCs to three 60 s applications of glutamate/glycine (a–c) or kainate (d). Oligomycin was added after the first pulse and rotenone or antimycin A as indicated was added after the second pulse. Note the dramatic inhibition of the $[Ca^{2+}]_c$ response following mitochondrial depolarization. (e) Mitochondrial depolarization during a glutamate-induced plateau lowers $[Ca^{2+}]_c$; the insert shows an expanded scale. (f) Mitochondrial depolarization greatly decreases the ^{45}Ca accumulation in response to NMDA receptor activation. (g) No decrease in the entry of Mn^{2+} (as a Ca^{2+} surrogate) can be detected after mitochondrial depolarization, suggesting enhanced extrusion via the PMCA. Data from [13,14] and unpublished.

Park et al. [17] have reported that mitochondria in different locations within pancreatic acinar cells have distinct functions in regulating Ca^{2+} in response to different stimuli, with sub-plasmalemmal mitochondria selectively accumulating Ca^{2+} entering the cell.

3. Mitochondrial control of cytoplasmic Ca^{2+} by ATP supply

3.1. Protonophores

Maintenance of a low $[Ca^{2+}]_c$ is entirely dependent upon cytoplasmic ATP, whether generated by oxidative phosphorylation or glycolysis. Three factors have to be considered: the rate of Ca^{2+} entry into the cell (and hence the rate of Ca^{2+} extrusion required to maintain homeostasis), the rate of ATP generation and the rate of ATP hydrolysis by com-

peting processes (for example by the Na^+/K^+ -ATPase or by mitochondrial uncoupling). In polarized cells there is only a slow rate of Ca^{2+} entry into the cell and the rate of compensatory Ca^{2+} efflux is correspondingly low. The low ATP requirement means that CGCs can generally maintain cytoplasmic Ca^{2+} homeostasis even in the presence of protonophore when mitochondrial ATP synthase reversal hydrolyzes cytoplasmic ATP (Fig. 2a). However, inhibition of glycolysis and oxidative phosphorylation by the combination of 2-deoxyglucose in a glucose-free medium together with cyanide leads to a spontaneous failure of Ca^{2+} homeostasis (Fig. 2d). An increased transient 'spike' of the fura-2 response on KCl depolarization in the presence of protonophore (cf. Fig. 2b with Fig. 2c) contrasts with the decrease seen in cells after rotenone/oligomycin depolarization of the mitochondria (Fig. 2g) and probably reflects defective Ca^{2+} extrusion, since it is followed by a rapid failure of Ca^{2+} homeostasis. Thus, unless precautions are taken to

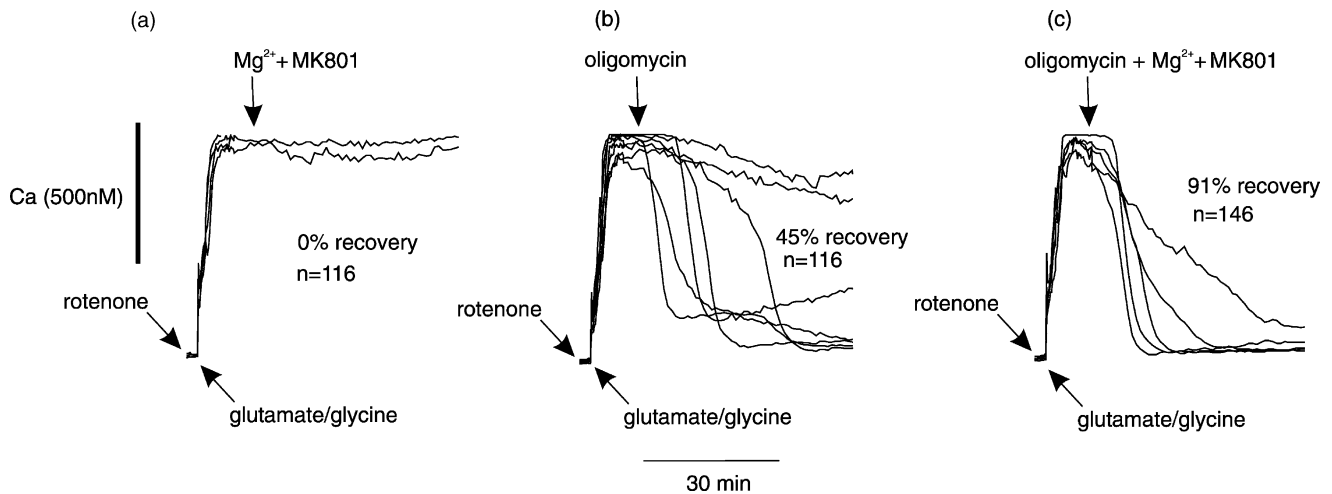


Fig. 5. Immediate Ca^{2+} deregulation due to ATP depletion is acutely reversible. In the presence of rotenone, NMDA receptor activation causes an excessive demand upon glycolytic ATP supply and $[\text{Ca}^{2+}]_c$ rises sufficiently to saturate the fura-2 signal. (a) Inhibition of the receptor by MK801 plus Mg^{2+} is insufficient to allow recovery of Ca^{2+} homeostasis. Oligomycin, either alone (b) or in combination with the receptor inhibitors (c) allows the cells to restore their Ca^{2+} homeostasis. Data from [14].

avoid ATP depletion, an increase in $[\text{Ca}^{2+}]_c$ following any process that might lead to ATP depletion cannot be ascribed unambiguously to release of matrix Ca^{2+} into the cytoplasm.

3.2. Respiratory chain inhibitors and ATP synthase reversal

Addition of a respiratory chain inhibitor such as rotenone or antimycin A imposes a bioenergetic demand upon the cell that is intermediate between that seen with oligomycin (where there is no synthesis or hydrolysis of ATP by the mitochondrion) and protonophore (where there is rapid ATP hydrolysis), Fig. 1. Since the native proton permeability of the inner membrane is low, particularly when $\Delta\psi_m$ is sub-optimal only a slow rate of ATP hydrolysis is required to maintain a $\Delta\psi_m$ that is 10–20 mV lower than that under respiring conditions. This situation however changes dramatically when NMDA receptors are activated. While the receptor shows a selectivity for Ca^{2+} over Na^+ , the far higher concentration of the latter in physiological media means that Na^+ entry will substantially exceed that of Ca^{2+} . The bioenergetic cost of this ion entry is three-fold. First the Na^+/K^+ -ATPase is activated to re-extrude the Na^+ , secondly the PMCA is activated in response to the elevated $[\text{Ca}^{2+}]_c$ and thirdly the net accumulation of Ca^{2+} by the mitochondria utilizes the proton circuit and, in the presence of rotenone, will enhance cytoplasmic ATP hydrolysis (Fig. 1C). The combined effect of these ATP demands is to overwhelm supply and create a rapid failure of cytoplasmic Ca^{2+} homeostasis (Fig. 5) that can be termed ‘immediate Ca^{2+} deregulation’, ICD [14]. Since it can be reversed, ICD does not equate with cell death, in contrast to the excitotoxic ‘delayed Ca^{2+} deregulation’ that will be discussed below. However, the requirements for reversal are interesting. Simple inhibition of the NMDA receptor by MK801 and Mg^{2+} is ineffective (Fig. 5a), inhibiting the ATP depletion result-

ing from the reversed ATP synthase (due to respiratory chain inhibition by the rotenone) is partially effective (Fig. 5b) but the combination of both (Fig. 5c) leads to an almost total restoration of Ca^{2+} homeostasis.

3.3. Pyruvate as substrate

One problem faced by a neuron trying to recover from extensive ATP depletion is that ATP is itself required at two glycolytic steps. This may account for the failure of CGCs to recover from ICD simply by NMDA receptor inhibition (Fig. 5a). As an alternative to glucose, neurons can utilize pyruvate or lactate as effective substrates. ATP generation in this case is purely by oxidative phosphorylation, and does not require ‘priming’ ATP (Fig. 1E). Circulating lactate has been proposed to be of importance in permitting neurons in situ to re-energize following ischemia [18–21]. However, since pyruvate is non-glycolytic, the cell becomes entirely dependent upon oxidative phosphorylation and addition of oligomycin leads to immediate Ca^{2+} deregulation [14]. As will be discussed in the context of glutamate excitotoxicity, these substrates can be used to investigate whether the in situ mitochondria are capable of generating ATP.

4. Ca^{2+} and mitochondrial membrane potential

While established combinations of mitochondrial protonophores and inhibitors can be predicted to cause gross changes in mitochondrial membrane potential ($\Delta\psi_m$), it is of importance to be able to monitor more subtle changes in $\Delta\psi_m$ that occur in neurons in response to physiological or patho-physiological stresses. All approaches rely on the ability of bilayer-permeant synthetic cations to distribute across the inner mitochondrial membrane according to the

Nernst equilibrium, essentially a 10-fold accumulation for each 60 mV of $\Delta\psi_m$, and most attempt to monitor this distribution using fluorescent dyes. Unfortunately, this seemingly simple approach is fraught with potential artifacts (for review see [9]) and a failure to appreciate these has led to considerable confusion in the literature. Three verifiable principles govern the uptake and fluorescent signal from these dyes. First, since they are non-selectively permeable across lipid bilayer regions (and indeed are loaded across the plasma membrane) they will tend to distribute according to the Nernst equilibrium across both the plasma and mitochondrial membranes in response to the respective plasma ($\Delta\psi_p$) and mitochondrial membrane potentials. The

equilibrium probe concentration in both the cytoplasm and matrix will thus always be influenced by $\Delta\psi_p$. However, since the surface-to-volume relationships of the cell and the mitochondrion differ grossly, probes will redistribute across the mitochondrial membrane in response to a change in $\Delta\psi_m$ very much faster than across the plasma membrane. Thus, if the mitochondria are depolarized, probe released from the matrix into the cytoplasm will only slowly redistribute across the plasma membrane to restore the plasma membrane Nernst equilibrium gradient for the dye. Slowly permeant dyes may be retained in the cytoplasm for several minutes before significant loss occurs. Thirdly, at a critical concentration in the matrix the probes will aggregate. The

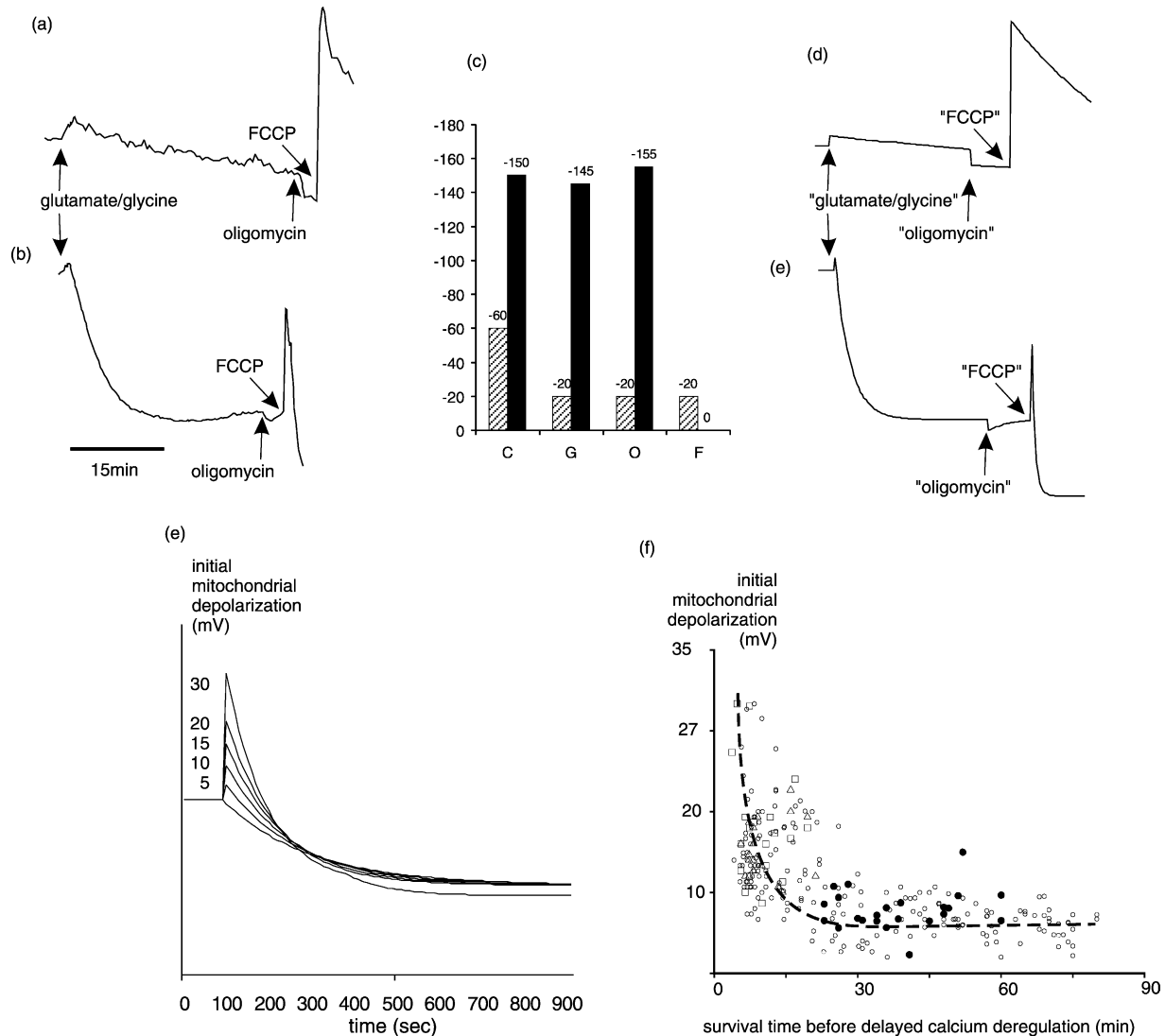


Fig. 6. Glutamate-induced changes in $\Delta\psi_p$ and $\Delta\psi_m$. An experiment is shown in which glutamate/glycine, oligomycin and FCCP are added sequentially to CGCs loaded with (a) rhodamine-123 and (b) TMRM⁺. The 'virtual cell' simulation [9] was used to obtain a best fit to the experimental traces, assuming starting values for $\Delta\psi_p$ and $\Delta\psi_m$ of -60 and 150 mV, respectively, and a plasma membrane permeability for rhodamine-123 that was 5% of that for TMRM⁺ [9]. A good fit (d, e) could be obtained by assuming a 5 mV mitochondrial depolarization coupled to a 40 mV plasma membrane depolarization on activation of the receptor, a 10 mV hyperpolarization of the mitochondria on addition of oligomycin (the null-point test, see text) and a collapse of $\Delta\psi_m$ on addition of FCCP (c). Note that these two cells do not show an excitotoxic response. (e, f) The initial mitochondrial depolarization upon receptor activation predicts the survival time of the neurons prior to delayed Ca²⁺ deregulation; (e) simulation, (f) correlation between mitochondrial depolarization and survival time in 120 individual cell bodies. Data from [9].

fluorescence of most probes is quenched on aggregation, although JC-1 undergoes a green to red shift in its emission spectrum.

It is of crucial importance to control the loading conditions so that it is clear whether the concentration of probe in the matrix is above or below the quench limit. Under quenching conditions mitochondrial depolarization results in a transient increase in whole-cell fluorescence as probe is released from the matrix and diluted into the cytoplasm. Quench conditions can be used at whole cell resolution to monitor changes in $\Delta\psi_m$ occurring during the observation of the cells, but is not appropriate for higher resolution monitoring of single mitochondrial. Furthermore, since the 'excess' cytoplasmic fluorescence decays back to the plasma membrane Nernst equilibrium, quench conditions must never be used to compare populations of cells whose $\Delta\psi_m$ already differ at the time of probe loading (for example in flow cytometry). Using these principles, we have published a simple computer algorithm to interpret fluorescence changes in cells loaded with cationic probes [9].

One complication when studying the mitochondrial response to plasma membrane receptor activation is that there can be synchronous changes in both $\Delta\psi_m$ and $\Delta\psi_p$. The principles discussed above enables these to be distinguished. First as discussed above, under quench conditions depolarization of the two membranes produces opposite responses, and second the plasma membrane responds much more slowly than the mitochondrion to a step change in potential. Fig. 6 shows a matched pair of experiments in which CGCs loaded with either the slowly permeant rhodamine-123 (Fig. 6a) or the more rapidly equilibrating tetramethylrhodamine methyl ester (TMRM⁺) (Fig. 6b) were exposed to glutamate/glycine to activate NMDA receptors. Initial values for $\Delta\psi_p$ and $\Delta\psi_m$ of -60 and 150 mV, respectively, were assumed [9]. Two processes occur synchronously: an extensive plasma membrane depolarization as a consequence of receptor activation, and a small mitochondrial depolarization, primarily as a consequence of Ca^{2+} uptake into the mitochondrial matrix. The former causes a loss of probe from the cytoplasm, at a rate limited by the permeability of the plasma membrane, that is slow for TMRM⁺ (Fig. 6b) and even slower for rhodamine-123 (Fig. 6a). In contrast, the mitochondrial depolarization results in a release from the quenched matrix into the cytoplasm that is rapid for both probes (due to the extremely high surface/volume ratio for the mitochondrial matrix) and results in a small increase in cell body fluorescence due to de-quenching. In the experiments depicted in Fig. 6a and b, the cell simulation can be fitted to the experimental traces assuming about a 5 mV decrease in $\Delta\psi_m$ and a decrease in $\Delta\psi_p$ from -60 to -20 mV (Fig. 6c).

4.1. The oligomycin null-point test

Even with the cell simulation, values for the membrane potential changes remain approximate, and in any case

more important than a numerical value is information as to whether the mitochondria remain competent to generate ATP. Oligomycin can be used diagnostically in this context, since ATP synthesizing mitochondria will slightly hyperpolarize when the ATP synthase inhibitor is added, whereas mitochondria that are damaged and hydrolyzing cytoplasmic ATP will further depolarize on addition of oligomycin [9]. Applying this test to the cells followed in Fig. 6a and b reveals that ATP synthesis continued in the presence of glutamate, since a quenching of whole-cell fluorescence on addition of oligomycin indicates a further uptake of probe from the cytoplasm into the quenched environment of the matrix, interpreted as a 10 mV hyperpolarization. The 'spike' when $\Delta\psi_m$ is collapsed by protonophore confirms that the experiment was performed in quench mode—note the rapid decay of fluorescence as the 'excess' cytoplasmic probe redistributed across the plasma membrane to restore the Nernst equilibrium.

5. Mitochondria–calcium interactions in dying CGCs

5.1. Glutamate excitotoxicity

Glutamate is not only the dominant excitatory neurotransmitter in the mammalian central nervous system, but is also, when released in an uncontrolled fashion in pathological conditions, a potent neurotoxin that is responsible for much of the neuronal cell death that occurs acutely in stroke and chronically in a variety of neurodegenerative diseases such as Parkinson's disease (for review see [22]). The ability of an ubiquitous amino acid such as glutamate to function as a specific neurotransmitter is entirely dependent upon its precise compartmentation. This in turn is controlled by energy dependent transport processes acting both at the synaptic vesicle membrane and plasma membrane. When mitochondrial function is impaired, for example in stroke, the failure of these gradients results in a massive release of glutamate into the synaptic cleft, with a consequent pathological activation of postsynaptic glutamate receptors, i.e. glutamate excitotoxicity [23]. Neurons die rapidly in the ischemic core of an infarct, however the 'penumbra' surrounding an infarct, that is still perfused with blood but is exposed to the pathologically released glutamate diffusing from the core, undergoes a more delayed necrotic death. Much research is centered on the brief 'window of opportunity' following an infarct when it may be possible to restrict the volume of damage in the penumbra. For that reason it is of particular interest to apply the techniques discussed above to the investigation of the mitochondria/ Ca^{2+} interactions during necrotic cell death in response to glutamate receptor activation. Necrosis can be induced in culture by excessive activation of both NMDA and AMPA receptors. Since the mechanisms of cell death differ these will be considered separately.

5.2. NMDA receptor-mediated delayed Ca^{2+} deregulation

Early studies from the laboratories of Thayer and coworkers [24] and Tymianski et al. [25] showed that cultured neurons exposed to glutamate either continuously [25] or for as little as 5 min [24] showed a delayed loss of cytoplasmic Ca^{2+} homeostasis ('delayed Ca^{2+} deregulation', DCD). Our laboratory has investigated the role of mitochondria and mitochondrial Ca^{2+} transport in cerebellar granule cell DCD [9,13,14,26]. After 6–8 days in vitro, rat CGCs respond to continuous NMDA receptor activation with a stochastic DCD after delays in individual cells from a few minutes to an hour. The oligomycin null-point assay (see above) indicates that mitochondria remain net synthesizers of ATP until the onset of DCD [9], thus mitochondrial depolarization is a late event. Furthermore, CGCs with active glycolysis incubated with glutamate in the initial presence of oligomycin (Fig. 7a) undergo DCD after about the same delay as those in the absence of the inhibitor [13]. Thus, DCD cannot be simply attributed to a failure of oxidative phosphorylation, while it is significant that the oligomycin-induced restriction in the cell's maximal ATP generating capacity does not hasten DCD.

There is however a clear relationship between mitochondrial NMDA-receptor dependent Ca^{2+} accumulation and DCD. First, the survival time of individual neurons exposed continuously to glutamate/glycine is inversely related to the extent of the initial mitochondrial depolarization immediately following receptor activation and attributable to Ca^{2+} accumulation into the matrix (Fig. 6e and f). Even more strikingly, cells whose mitochondria are pre-depolarized by the combination of rotenone plus oligomycin show enhanced survival in the presence of glutamate/glycine and restore basal $[Ca^{2+}]_c$ on addition of MK801 (Fig. 7b). In contrast, once under way, DCD cannot be reversed, even by MK801 plus oligomycin (Fig. 7a) and can therefore be distinguished from the ICD caused by an immediate ATP deficit, which is reversed by the combination of these inhibitors (Fig. 5c).

On addition of glutamate to CGC's, most of the net accumulation of ^{45}Ca by the cells, and hence the mitochondria, occurs in the first few minutes [14,27]. Thereafter, cells maintain their Ca^{2+} homeostasis by extruding Ca^{2+} at a rate that closely matches that of the Ca^{2+} influx through the NMDA receptor. The efficiency of Ca^{2+} extrusion by the cell can be assessed by inhibiting the receptor. As just discussed, cells undergoing DCD in the presence of oligomycin cannot restore Ca^{2+} homeostasis on MK801 addition: this suggests a failure of the PMCA, either as a result of some form of damage or as a consequence of ATP restriction. The latter would indicate that an inhibition of glycolysis (the sole source of ATP in the presence of oligomycin) could be causal in DCD. However, this is not an explanation for the ability to see closely comparable DCD in glucose depleted cells supported by the non-glycolytic substrate pyruvate (of necessity in the absence of oligomycin), Fig. 7c.

An alternative explanation for the onset of DCD is a failure of the PMCA as a consequence of damage, either proteolytic or oxidative, to the enzyme. The former is suggested by recent reports that PMCA2 in CGCs can undergo caspase-dependent cleavage after the cells are exposed to glutamate [28], while there is comparable evidence for oxidative damage to the ATPase itself [29–31] or to the calmodulin that amplifies the enzyme's response to elevated Ca^{2+} [32,33].

Recovery of $[Ca^{2+}]_c$ in single cultured neurons to basal levels is delayed following termination of a period of NMDA receptor activation, and this delay increases with the duration of the exposure of the neurons to glutamate [29,34]. If granule cells are exposed to glutamate in the presence of oligomycin for 5 min, following which NMDA receptors are inhibited and matrix Ca^{2+} released as a consequence of mitochondrial depolarization by the simultaneous addition of rotenone, then 100% of cells rapidly restore their basal $[Ca^{2+}]_c$ (Fig. 7d). If however the period of NMDA receptor activation is increased to 30 min prior to addition of MK801/rotenone, then only 50% of cells restore their basal $[Ca^{2+}]_c$ while the remainder of the cells deregulate (Fig. 7e). It is noticeable that the sub-population of cells that fail to extrude their Ca^{2+} already show a statistically increased $[Ca^{2+}]_c$ prior to the addition of the inhibitors. If the 'plateau' $[Ca^{2+}]_c$ in this population remained above the mitochondrial set-point for the duration of the experiment, the mitochondria could continue to load with Ca^{2+} . In combination with the observation that the time to DCD is inversely related to the initial mitochondrial depolarization caused by Ca^{2+} entry into the matrix (Fig. 6f) this raises the possibility that DCD in response to continuous NMDA receptor activation is a consequence of matrix Ca^{2+} overload and the induction of the mitochondrial permeability transition (MPT).

5.3. The controversial role of the mitochondrial permeability transition

Brain mitochondria have been considered to be relatively resistant to the MPT [35,36] or at least only to demonstrate sensitivity under particular conditions [36]. However, when the continuous loading of in situ mitochondria is simulated by the steady infusion of Ca^{2+} into an incubation of isolated brain mitochondria in the presence of adenine nucleotides, a very large effect on the maximal accumulation capacity of the classic MPT inhibitor cyclosporin A is seen (Fig. 8a). Detection of the MPT in intact neurons is not trivial and there is disagreement as to the relationship between the MPT and DCD. Cyclosporin A has limited utility for intact neurons due to the multiple consequences of the resulting inhibition of calcineurin, including decreased NMDA receptor desensitization [37] and enhanced NMDA receptor channel opening [37–41]. With these provisos, 0.5–2 μ M cyclosporin A delays but does not prevent mitochondrial depolarization in NMDA-exposed neurons from hippocampus [34,42] or cortex [43]. The immunosuppressant also decreased the

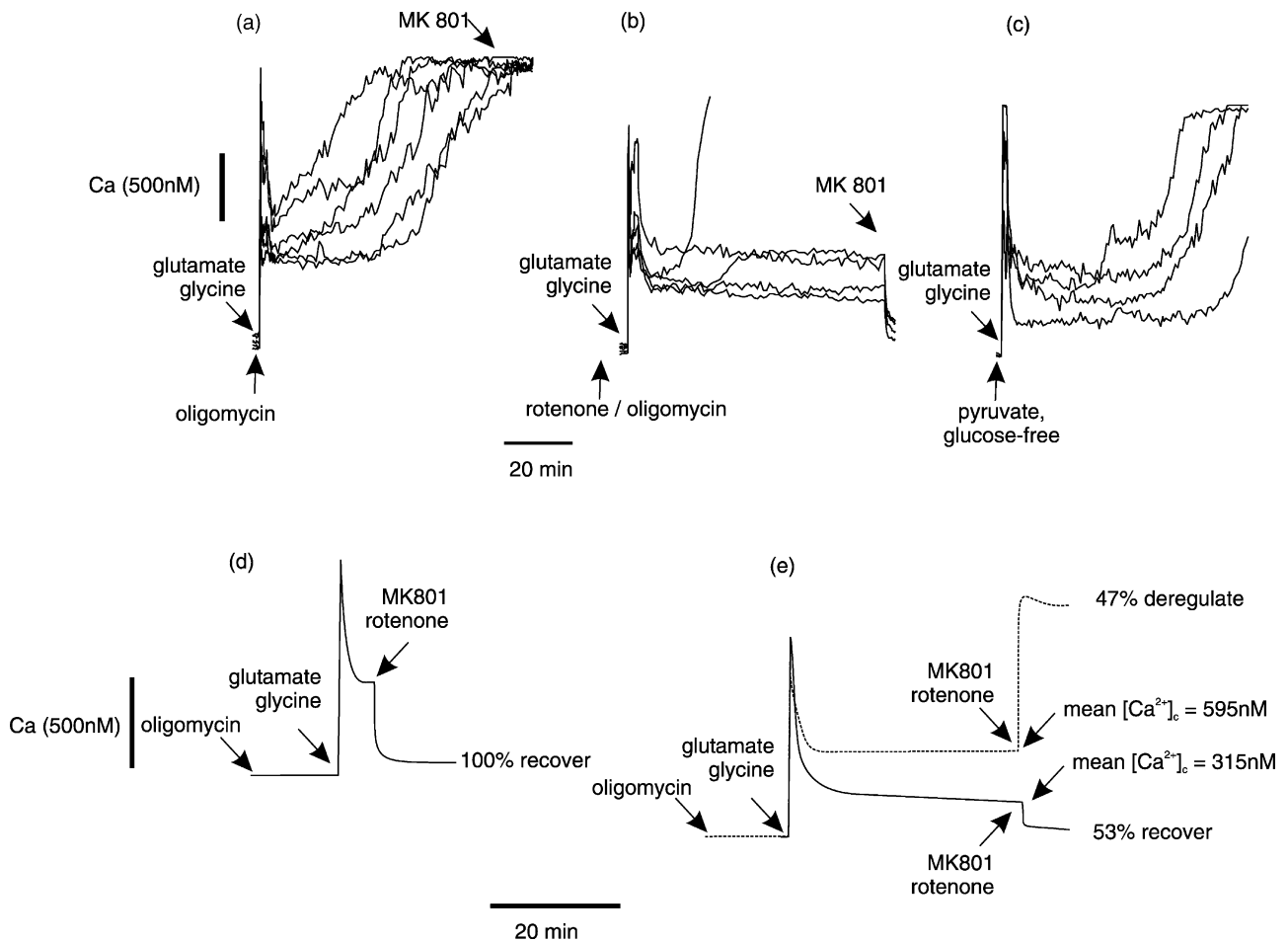


Fig. 7. Mitochondrial pre-depolarization and DCD. (a, b) Cells suspended in elevated Ca^{2+} (2.6 mM) were exposed to glutamate/glycine in the presence of oligomycin (a) or oligomycin plus rotenone (b). High external Ca^{2+} was used to compensate for the decreased $[\text{Ca}^{2+}]_c$ elevation seen when mitochondria are depolarized (see Fig. 4). (c) Cells utilizing pyruvate as substrate undergo DCD. (d, e) The ability of cells to extrude accumulated Ca^{2+} decreases with time of NMDA receptor activation. (d) After 5 min of receptor activation, mitochondria were depolarized and the NMDA receptor inhibited, all cells were able to extrude the Ca^{2+} . (e) After 30 min of receptor activation only 53% of cells could extrude the accumulated Ca^{2+} and 47% deregulated. The latter population had a higher mean $[\text{Ca}^{2+}]_c$ prior to mitochondrial depolarization and may therefore have accumulated more Ca^{2+} . Data from [54].

proportion of mitochondria within hippocampal neurons that changed their morphology from rod-like to a rounded, hollow-appearance [44]. However, FK506, which inhibits calcineurin without affecting the MPT is as effective as CsA in decreasing glutamate excitotoxicity in CGCs [45,46].

N-Methylvaline-4-cyclosporin is generally accepted to be a more selective inhibitor of the MPT. The analogue decreases 24 h cell death following combined oxygen/glucose deprivation [47] and increases the time to DCD in glutamate-exposed striatal neurons [48]. However, in this last study, while DCD was associated with a decrease in the matrix free Ca^{2+} in individual mitochondria as judged by rhod-2 fluorescence, the mitochondria still appeared to retain the dye, suggesting that gross permeabilization of the inner membrane had not occurred [48]. In our studies, neither cyclosporin A nor the analogue altered the onset of DCD (Fig. 8b), contrasting with the large effect of cyclosporin A on the MPT of isolated brain mitochondria (Fig. 8a).

While release of cytochrome *c* into the cytoplasm can occur following the MPT as a consequence of matrix swelling and outer membrane rupture, release of the cytochrome does not prove that the MPT has taken place, since there is abundant evidence that cyt *c* can be released from in situ mitochondria while they retain a high $\Delta\psi_m$ [49,50]. Thus, the ability to detect released cyt *c* in cultured neurons at an early stage following exposure to glutamate [49,51] does not prove that a proportion of mitochondria have undergone the MPT, particularly since $\Delta\psi_m$ is retained until the onset of DCD.

5.4. Transient glutamate exposure

While it is possible to consider that DCD brought on by the continual exposure of neurons to glutamate could be a result of matrix Ca^{2+} overload, it is more difficult to establish the events following transient NMDA receptor activation. It is well established that as little as 5 min exposure to glutamate or NMDA can initiate a Ca^{2+} deregulation that can be

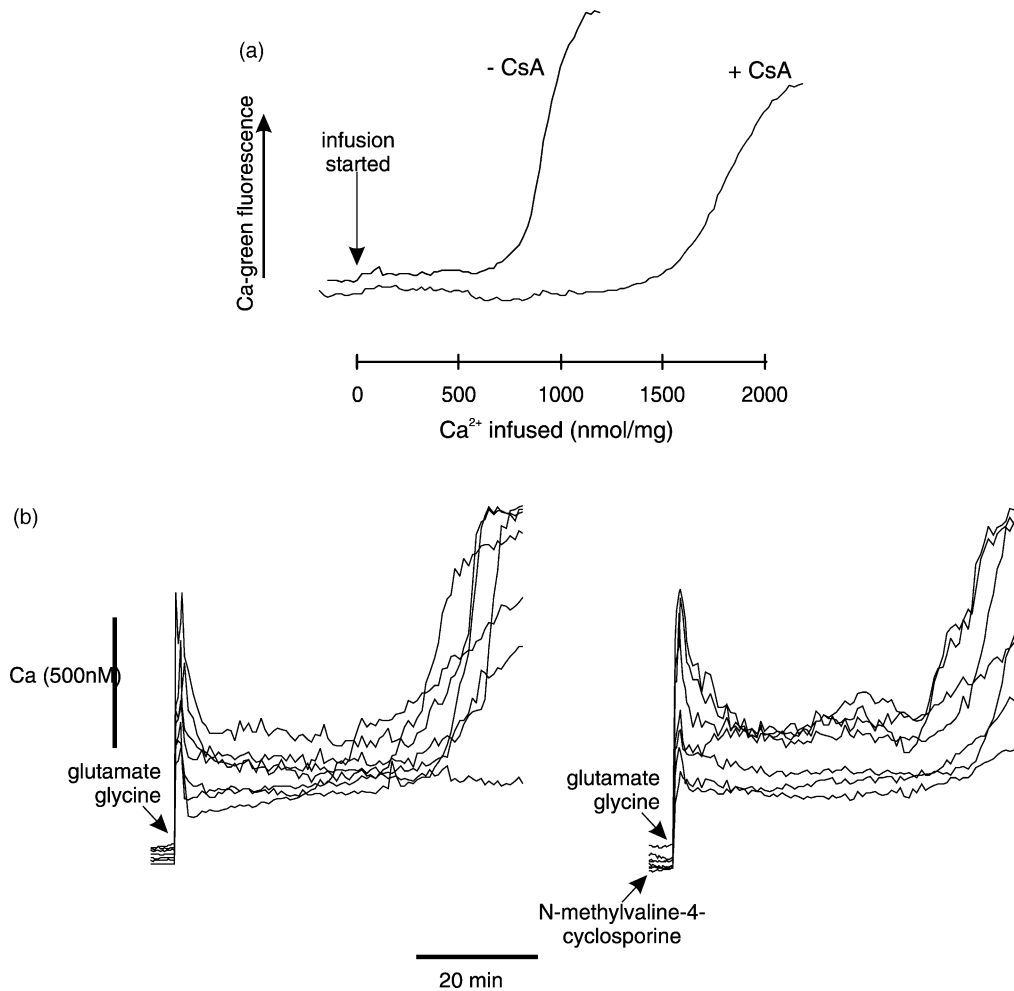


Fig. 8. Mitochondrial permeability transition and DCD. Cyclosporin analogues enhance the capacity of isolated brain mitochondria to accumulate and retain Ca^{2+} but have no effect on the onset of DCD in CGCs. (a) Isolated rat brain mitochondria oxidizing glutamate plus malate were incubated in the presence of 0.5 mM ADP and 1 $\mu\text{g}/\text{ml}$ oligomycin. Ca^{2+} was continuously infused into the cuvette at a rate of 85 nmol/min/mg and external free Ca^{2+} monitored with Calcium Green 5. The onset of the permeability transition is signaled by a rapid rise in external free Ca^{2+} . Cyclosporin A (1 μM) was present where indicated. (b) CGCs were exposed continuously to glutamate/glycine in the presence or absence of 1 μM *N*-methylvaline-4-cyclosporin, cyclosporin A was similarly ineffective. Data from [14].

delayed for 1–3 h following termination of NMDA receptor activation. Under these circumstances, the mitochondria first load with Ca^{2+} and then unload (Fig. 3a), the latter being apparently responsible for the delay or shoulder in the return of the fura-2 trace to a basal value. As in the case of continuous NMDA receptor activation, there is little change in $\Delta\psi_{\text{m}}$, indeed most of the change in TMRM⁺ fluorescence is a consequence of the depolarization and subsequent repolarization of the plasma membrane (Fig. 3b and c). While DCD in the continuous presence of glutamate is consistent with a failure of Ca^{2+} extrusion from the cell, that following transient exposure differs in that mitochondrial depolarization appears to precede changes in cytoplasmic Ca^{2+} homeostasis, even though mitochondria have apparently been restored to their initial state of high $\Delta\psi_{\text{m}}$ and a Ca^{2+} -depleted matrix by the inhibition of the NMDA receptor. Fig. 3b and c shows that, when oligomycin is present to prevent mitochondrial depolarization from depleting

cytoplasmic ATP, the mitochondrial population slowly depolarizes even though $[\text{Ca}^{2+}]_{\text{c}}$ remains stable and below the set-point at which the mitochondria would reload with Ca^{2+} .

How do we reconcile two differing events precipitating DCD in the same cells after continuous or transient glutamate exposure? In the continuous presence of glutamate, a high activity of the PMCA is required to match the influx rate, and thus even a modest loss of activity could be sufficient to cause a failure of Ca^{2+} homeostasis. In contrast, once NMDA receptors are inactivated, only a slow PMCA activity would be required and mitochondrial failure could be brought to the fore. One factor that is sometimes forgotten is that CGCs in lengthy experiments in which the cells are in low KCl medium once NMDA receptor activity is terminated is the possibility that apoptotic events may be proceeding in the background, due to the low KCl, serum-deprived state of the cells. It would perhaps be preferable to carry out such long term experiments in the presence of 25 mM

KCl to depolarize the plasma membrane sufficiently to avoid this. Interestingly, NMDA is much less excitotoxic when added to CGCs that are depolarized by elevated KCl [52], even though the NMDA addition itself will almost immediately depolarize $\Delta\psi_p$. This suggests that the very initial seconds of the glutamate exposure, when $[Ca^{2+}]_c$ is maximal and when the mitochondria first load with Ca^{2+} , are critical for the initiation of the subsequent DCD.

6. Glutamate, Ca^{2+} and reactive oxygen species

A reasonable working hypothesis that a number of groups, including our own, adopted was that NMDA receptor-mediated matrix Ca^{2+} loading increased the production of superoxide by the mitochondria and that this led ultimately to sufficient oxidative damage to the cell to initiate DCD [53,54]. Superoxide, $O_2^{\bullet-}$ is produced by one electron leakage to oxygen at two sites in the respiratory chain, within complex I which may inject $O_2^{\bullet-}$ into the matrix and at the outer UQ-binding site of complex III where $O_2^{\bullet-}$ may be released to the inter-membrane space [55]. With isolated mitochondria the production of $O_2^{\bullet-}$ shows a high dependency on $\Delta\psi_m$ [56]; classically this was thought to be a consequence of a membrane potential dependent occupancy of the outer UQ-binding site by ubiquinone, but current research also favors an increased reduction state of complex I at high $\Delta\psi_m$ increasing leakage to molecular oxygen, particularly in the more closely physiological conditions of NAD-linked substrate oxidation in the absence of respiratory chain inhibitors [57].

Detection of superoxide levels in cells and mitochondria is notoriously difficult, both because of uncertainties concerning the selectivity of the probes for individual reactive oxygen species and the complication that the fluorescence of the oxidized product might be affected by extraneous factors. Thus, H_2O_2 detection by the oxidation of dichlorofluorescein is complicated by the product's sensitivity to cytoplasmic pH, while it has recently been reported that cytochrome *c* released from mitochondria potently catalyzes the oxidation of this supposedly specific probe [58]. The superoxide-mediated oxidation of dihydroethidine to ethidium is complicated by the ability of the latter to act as a mitochondrial membrane permeant cation and thus respond to changes in $\Delta\psi_m$. To a large extent, this latter artifact can be minimized by using sub-micromolar concentrations of hydroethidine, so that the ethidium produced can intercalate into and be fixed by DNA [59]. Finally it should be recognized that probes such as hydroethidine detect steady-state levels of $O_2^{\bullet-}$ rather than its absolute rate of generation, since the probe competes in parallel with other pathways scavenging $O_2^{\bullet-}$ such as superoxide dismutase, peroxynitrite generation and actual $O_2^{\bullet-}$ -mediated oxidative damage to the mitochondrion.

It is instructive to consider three phases of superoxide interaction with CGCs: before glutamate addition, after glutamate but before DCD, and following DCD.

6.1. Prior oxidative stress potentiates DCD

Oxidative stress can reduce the ability of neurons to maintain Ca^{2+} homeostasis in the presence of glutamate, or even lead to a spontaneous mitochondrial depolarization and Ca^{2+} deregulation. Thus, it is a common experience in imaging that singlet oxygen phototoxicity caused by excessive illumination of neurons loaded with a mitochondrial membrane potential indicator can result in rapid mitochondrial depolarization. In addition, superoxide generation by pro-oxidants such as menadione can induce a failure of cytoplasmic Ca^{2+} homeostasis in the absence of glutamate (Fig. 9a), while glutathione depletion by L-buthionine(S,R)-sulfoximine (BSO) decreases the survival of CGCs in response to subsequently added glutamate (Fig. 9b).

6.2. DCD is not caused by glutamate-induced superoxide generation

Despite the above, there is little evidence to support the hypothesis that superoxide generation during acute NMDA receptor activation is responsible for the subsequent DCD. Thus, although glutamate/glycine addition may cause a slight increase in the rate of ethidium production [54], the change in rate seems insufficient to provide the primary life-and-death signal to the glutamate-exposed cell. In addition, the belief that Ca^{2+} loaded mitochondria produce more ROS [60] has come in for re-examination [57]. Although under some circumstances, mitochondrial Ca^{2+} accumulation can activate the citric acid cycle [61], the general response is a slight depolarization as the proton circuit is used to drive uptake of the cation (see Fig. 6), and this seems to run counter to the requirement for high $\Delta\psi_m$ in order to produce superoxide. Indeed, a recent study with isolated brain mitochondria has shown that with physiologically relevant substrates in the absence of inhibitors, Ca^{2+} uptake actually decreases hydrogen peroxide production, perhaps as a consequence of this depolarization [57].

The marginal changes in superoxide levels detected in glutamate-exposed CGCs prior to DCD suggests that the basic hypothesis (NMDA receptor activation leads to mitochondrial Ca^{2+} loading leads to oxidative stress and cell damage) needs re-evaluation (see also [62]). Thus, the potent cell-permeant antioxidant manganese tetrakis (*N*-ethylpyridinium-2yl) porphyrin (MnTe-PyP) [63], fails to delay glutamate-induced Ca^{2+} deregulation (Fig. 9d). Some delay in the onset of DCD is observed with high concentrations of another cell-permeant antioxidant, manganese tetrakis (4-benzoic acid) porphyrin (MnTBAP) [54]; however this may be a consequence of decreased NMDA receptor activity rather than a direct anti-oxidant effect (unpublished observation, see also [64]).

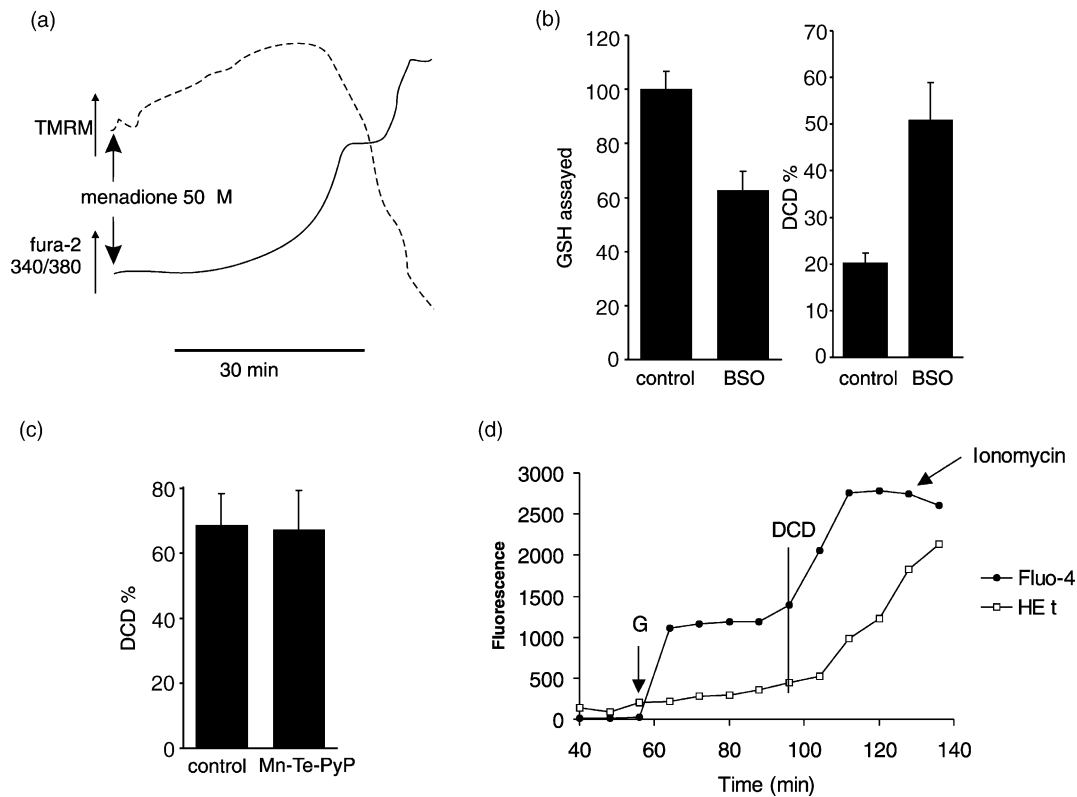


Fig. 9. Superoxide and DCD. Prior oxidative stress enhances subsequent DCD. (a) The pro-oxidant menadione (50 μM) can induce DCD in the absence of glutamate and (b) glutathione depletion by BSO facilitates subsequent glutamate-induced DCD. (c) The superoxide dismutase mimetic MnTE-2-PyP (20 μM, added 30' prior to glutamate) does not affect glutamate-induced DCD despite effectively trapping superoxide [63]. (d) Superoxide levels increase in cells that have undergone DCD; G, glutamate/glycine; DCD, onset of delayed Ca²⁺ deregulation; HEt, hydroethidine oxidized to ethidium; Fluo-4, cytoplasmic free Ca²⁺ indicator.

6.3. Superoxide levels increase subsequent to DCD

When hydroethidine is used to monitor O₂^{•-} levels in individual CGCs exposed to glutamate, a large increase in the rate of oxidation is seen when the cells undergo DCD (Fig. 9c). A similar secondary rise has been reported for hippocampal neurons [64,65]. MnPyP [63] effectively traps much of this superoxide, but since it does not delay DCD (Fig. 9a) this suggests that increased O₂^{•-} levels may be a consequence rather than a cause of DCD. DCD is associated with an increase in [Ca²⁺]_c and a collapse in Δψ_m, and it is of interest to establish if either of these parameters is responsible for increased O₂^{•-} detection. Ionomycin, which mimics the DCD-induced changes in these parameters, increases the rate of ethidium production; however Ca²⁺ elevation without mitochondrial depolarization is ineffective, while protonophore-induced collapse of Δψ_m with no increase in [Ca²⁺]_c does (unpublished observations).

How do we reconcile the apparently contradictory results obtained with isolated mitochondria, where high potentials increase O₂^{•-}, with the situation in intact CGCs where the opposite relationship appears to hold? It is important to remember that a probe such as hydroethidine does not quantify the production of the free radical, but rather its concentration in the cell; this can be raised by a decreased

activity of O₂^{•-} detoxifying reactions in the absence of any increase in generation. In addition it should not be automatically assumed that the O₂^{•-} seen after DCD all originates from the mitochondrion. Ethidium intercalates into DNA with fluorescent enhancement; confocal imaging of CGCs reveals binding to nuclear DNA, with little punctate fluorescence corresponding to mitochondria. While it is possible that the membrane-permeant ethidium leaves the mitochondrion after depolarization, it is also a possibility that superoxide is generated in the cytoplasm. If so, a plausible mechanism should exist to account for the increase being coupled to mitochondrial depolarization.

A major consequence of mitochondrial depolarization is an oxidation of nicotinamide nucleotides, NADH as a result of decreased back-pressure in complex I, and NADPH as a result both of the decreases in NADH concentration for the energy-linked transhydrogenase and the loss of Δψ_m that is responsible for maintaining the redox potential disequilibrium between the NAD and NADP pools. Mitochondrial NADPH is the substrate for glutathione reductase, and mitochondrial depolarization will therefore inhibit glutathione reduction. If disturbance to the glutathione pool is a significant factor in depolarization induced O₂^{•-} production, it would be predicted that glutathione depletion would increase O₂^{•-} levels. In order to reconcile the opposing observations

with isolated mitochondria and intact neurons, it is necessary to postulate that a defective trapping of $O_2^{\bullet-}$ as a consequence of glutathione depletion outweighs any decrease in mitochondrial $O_2^{\bullet-}$ generation itself, particularly since the intact cell will have non-mitochondrial sources of $O_2^{\bullet-}$.

Why is even a modest depletion in glutathione so deleterious to neuronal function? The answer may lay in a combination of thermodynamic and kinetics factors. Firstly, glutathione reductase operates far from equilibrium, since the redox potential of the matrix NADP(H) pool is close to -380 mV, while that of the matrix glutathione pool is in the region of -250 mV [66]. This implies that the redox potential of the glutathione pool is regulated by the kinetics of the reductase, and this would explain why a decrease in NADPH concentration as a result of depolarization could greatly decrease the activity of the enzyme. An additional factor stems from the stoichiometry of the reduction itself. Since two GSH molecules form one GSSG, it follows that the mid-point potential of the GSSG/GSH pool becomes more positive as the pool size is decreased [66]. To maintain a constant redox potential in the face of a decreased total GSx pool requires a disproportionate decrease in the concentration of GSSG. This in turn would impose even more kinetic constraints on glutathione reductase.

6.4. Can controlled uncoupling be neuroprotective?

An initial impetus for the current interest in the possible role of novel mitochondrial uncoupling proteins (UCPs) as neuroprotective agents has come from the studies with isolated mitochondria demonstrating a membrane potential-dependent $O_2^{\bullet-}$ production (see above). While the above discussion concerning glutathione reduction might be thought to throw some doubt on the utility of this mechanism in the intact neuron, it must be born in mind that each $\Delta\psi_m$ -dependent parameter ($O_2^{\bullet-}$ generation, ATP synthesis, proton leak, NADP⁺ reduction and Ca²⁺ accumulation) will have a characteristic dependency on membrane potential. The ideal protective mechanism would lower $\Delta\psi_m$ sufficiently to decrease $O_2^{\bullet-}$ production without compromising maximal ATP generating capacity, glutathione reduction or Ca²⁺ transport.

The original uncoupling protein, now termed UCP1, was characterized by us [67] as a highly specialized mechanism allowing brown adipose tissue to dissipate the proton circuit allowing maximal respiration in the absence of stoichiometric ATP synthesis [68]. At first sight the demands of brown fat seem to be diametrically opposed to those of the brain, where energy conservation seems paramount and where restrictions in mitochondrial ATP generating capacity induced by rotenone or malonate seem to model many aspects of the respective neurodegenerative Parkinson's and Huntington's diseases [69]. A major problem with such an uncoupling hypothesis is the high 'efficiency' of the respiratory chain, which responds to an increased utilization of

the proton gradient with a proportionate increase in the rate of proton pumping (and hence respiration) but with only a slight drop in proton gradient (Fig. 10b). This means that in order to decrease $\Delta\psi_m$ sufficiently to decrease $O_2^{\bullet-}$ generation significantly, a constitutive proton leak would have to dissipate much of the mitochondrion's proton generating capacity (Fig. 10c). In order to assess the consequences of a slight increase in proton conductance on the ability of CGCs to withstand transient NMDA receptor activation, low nanomolar concentrations of the protonophore FCCP were added sufficient to depolarize the mitochondria by less than 10 mV (as estimated by the virtual cell simulation [9]). In every case the survival of the neurons was decreased (Fig. 10a), in other words it was not possible to find an increase in mitochondrial proton conductance that was protective in this acute protocol, since the bioenergetic cost of an increased proton conductance outweighed any supposed beneficial effects of a decreased $O_2^{\bullet-}$ generation.

A major limitation with the above experiment is that protonophores introduce a proton conductance that is uncontrolled and 'Ohmic' [70], i.e. the proton current conducted by the protonophore is linearly related to the driving force ($\Delta\psi_m$ or more strictly the protonmotive force Δp). If novel UCPs are to prove beneficial it would seem essential that they are regulated in order to limit their activity to periods when the ATP demand (and hence respiration) is low and consequently $\Delta\psi_m$ and ROS production are high. One such auto-regulatory mechanism has been suggested by Echtay et al. [71] who propose that UCP1, UCP2 and UCP3 may be capable of being activated by $O_2^{\bullet-}$ itself, perhaps in the process not only allowing proton re-entry to limit $\Delta\psi_m$, but also allowing a pathway for the efflux of $O_2^{\bullet-}$ from the matrix [71]. An alternative possibility is that the rather mysterious endogenous proton leak possessed by all mitochondria [72], but so far unidentified at a molecular level, may possess just the properties required by a self-regulating ROS-limiting proton conductance. The most apparent feature of the endogenous proton leak is that it is highly 'non-Ohmic' (Fig. 10d), i.e. its proton conductance increases greatly above a threshold Δp [73]. This value is set close to the maximal Δp that can be attained by NADH-linked substrate oxidation in state 4 and serves to limit Δp close to 210 mV even if the thermodynamic driving force for proton extrusion is increased further, for example by supplying high concentrations of substrate such as succinate feeding in to complex II and bypassing the thermodynamically 'weaker' complex I [73]. It can be calculated that high concentrations of such a complex II substrate could generate a Δp greater than 250 mV in the absence of such a leak, with presumably catastrophically increased rates of $O_2^{\bullet-}$ generation.

The attraction of the endogenous proton leak is that it almost completely switches off in 'state 3', when ATP generation is maximal, and so does not compromise the maximal ATP generating capacity of the mitochondrion (Fig. 10d). Furthermore, it is significant that recent estimates of the

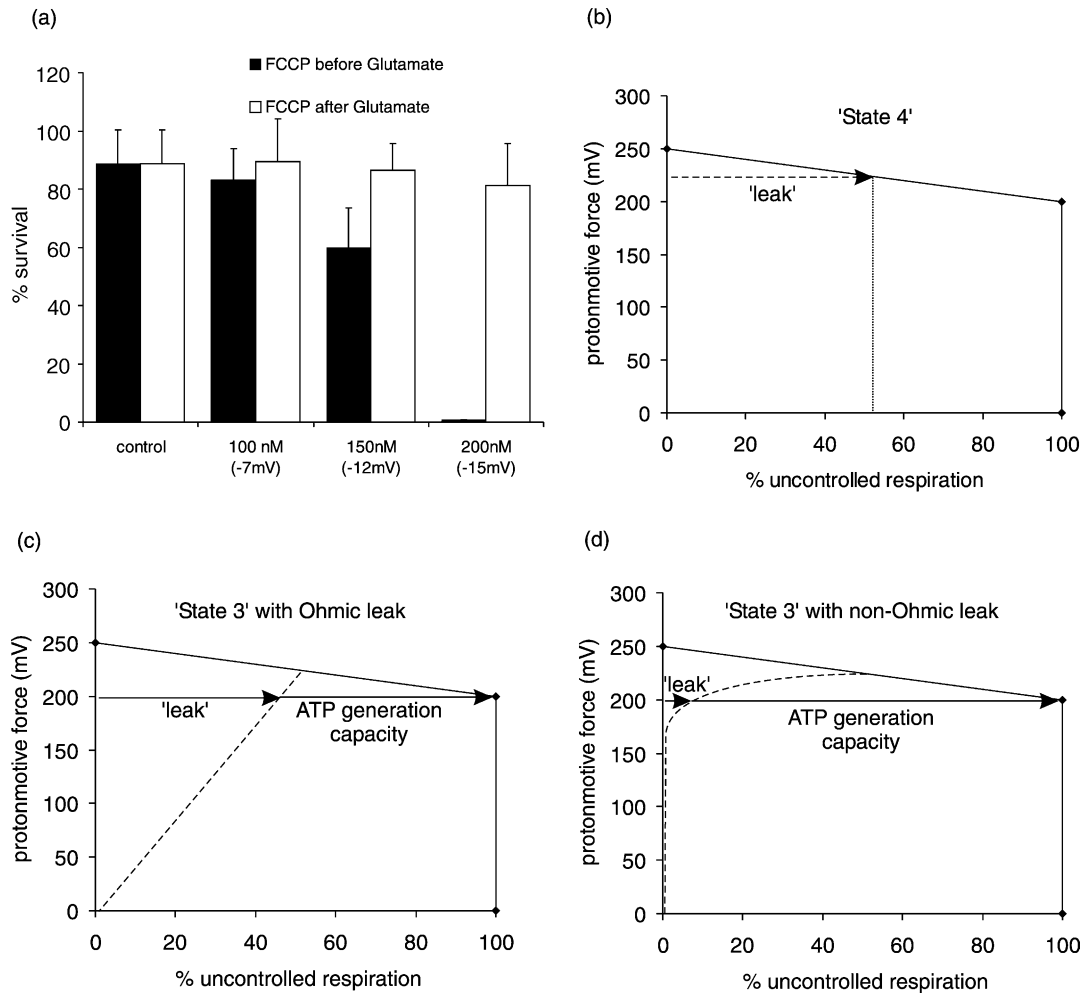


Fig. 10. Proton conductance and DCD. (a) Addition of low concentrations of the protonophore FCCP to lower $\Delta\psi_m$ by defined amounts, as estimated by the virtual cell simulation [9] potentiates DCD when added before glutamate, but has little effect when added after NMDA receptor activation is terminated by MK801 (unpublished data). (b–d) Influence of proton leak on the relationships between respiration, ATP synthesis and protonmotive force, Δp , for a typical mitochondrion. (b) In the (hypothetical) total absence of a proton leak, when state 4 respiration would be zero, a Δp of 250 mV is assumed, while below 200 mV respiration is uncontrolled; the mitochondrion, however, is assumed to have an endogenous proton leak lowering Δp to 225 mV to limit $O_2^{\bullet-}$ production, this would utilize 50% of the respiratory capacity (data adapted from [81]). (c) If the endogenous leak showed an 'Ohmic' conductance (i.e. current proportional to Δp , as in the case of FCCP [72]) then this leak would still use 45% of the respiratory capacity in state 3 (maximal ATP demand) leaving only 55% for ATP synthesis. (d) Since the endogenous proton leak is highly non-Ohmic [70,81] it virtually vanishes at 200 mV, leaving almost all the respiratory capacity for ATP generation.

value of $\Delta\psi_m$ at which $O_2^{\bullet-}$ production sharply increases corresponds almost exactly to that at which the endogenous leak becomes non-Ohmic [56]. If and when the protein responsible for the leak is identified, it should perhaps be designated UCP0. To take a naive analogy, an uncontrolled proton leak is equivalent to a steam locomotive with a deliberate hole in the boiler—preventing a dangerous pressure from building up when the train is waiting in the station, but very wasteful when the locomotive is under way. In contrast a membrane potential regulated leak is precisely analogous to a safety-valve—releasing dangerous pressure, but switching-off when the pressure falls slightly as the train accelerates away. Indeed when we first described the non-Ohmic leak, we proposed just such as safety-valve mechanism [74].

6.5. Kainate receptor-mediated delayed Ca^{2+} deregulation

While most studies of glutamate excitotoxicity with cultured neurons have focused on the NMDA receptor, it must not be forgotten that in vivo the non-desensitizing activation of AMPA receptors by kainate (KA) is highly excitotoxic. Even though the AMPA receptor subunit isoforms found on cultured CGCs permits some Ca^{2+} entry [75], the mechanism of kainate excitotoxicity appears to involve almost exclusively Na^+ . Both NMDA and kainate cause a large increase in cytoplasmic free Na^+ concentration, $[Na^+]_c$ [76–79]. Exposure of CGCs to KA results in DCD after a typical delay of 90 min [26]. In contrast to NMDA receptor activation, the extent and timing of DCD is independent of

external Ca^{2+} and no loading of the mitochondrial matrix with Ca^{2+} can be detected [26]. A further distinction is that, whereas mitochondria in NMDA exposed CGCs remain bioenergetically competent and ATP generating up to the onset of DCD, in the presence of KA an early failure of oxidative phosphorylation can be detected, both by an enhanced mitochondrial depolarization when oligomycin is added after KA for the ‘null-point test’ [9], indicating that the mitochondria were maintaining their $\Delta\psi_m$ by hydrolysis of cytoplasmic ATP, and by an inability of pyruvate to support Ca^{2+} homeostasis in the presence of KA. It is significant however that oligomycin added before KA is protective [26]. This may indicate that the ATP synthase inhibitor, by preventing ATP synthase reversal, protects the cells against ATP depletion. A very similar DCD, including protection by prior oligomycin, can be induced by veratridine, which locks voltage-active Na^+ channels in the open state [26]. The exact reason for the mitochondrial bioenergetic failure remains to be established.

7. Conclusions

Mitochondria play a three-fold role in neurons, as ATP generators, a source and detoxification mechanism for superoxide, and as a temporary Ca^{2+} sequestering compartment. All three of these functions are closely interlocked by their common dependency on the mitochondrial membrane potential and conversely their individual ability to influence $\Delta\psi_m$. However, we still do not fully understand the thresholds that trigger mitochondrial failure in the neuron and their precise causal relationships.

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