Mitochondria are extraordinary structures. With a bacterial evolutionary origin they have become perhaps the ultimate symbiont, maintaining their own DNA while also deriving many important proteins from the nuclear DNA of the host cell. While they may maintain a modicum of independence from the host cell in some respects, they nevertheless lie at the heart of the life of almost all eukaryotic cells. In this special issue of Cell Calcium, the focus is clearly on the integration of mitochondria into cellular \([\text{Ca}^{2+}]_c\) signalling, but we should not forget that the primary function of the mitochondrion remains the business of oxidative phosphorylation and ATP supply, and, at the end of the day, this is the function upon which cellular integrity depends.

The oxidative phosphorylation of ADP to generate ATP follows upon the import of carbon chain substrates – e.g. pyruvate from glycolysis – into the mitochondrial matrix. The TCA cycle metabolizes the substrates and sustains the provision of reducing equivalents – as NADH and FADH2 – to the respiratory chain. In the progressive transfer of electrons ultimately to molecular oxygen, the respiratory chain also translocates protons across the inner mitochondrial membrane. This process creates and sustains the mitochondrial inner membrane potential \((\Delta \Psi_m)\) of some 150mV negative to the cytosol that provides the energy required to drive the phosphorylation of ADP to ATP. These processes together provide the machinery which defines almost everything that mitochondria can do, and are all ultimately interdependent.

There is now a risk that all contributing authors to a special issue such as this will write essentially the same things, within our various remits. This would not have been true some years ago, and itself reflects the radical change in our view of the relationship of mitochondria to \([\text{Ca}^{2+}]_c\) signalling that has taken place in recent years. There seems now to be an emerging consensus that mitochondria will routinely take up \([\text{Ca}^{2+}]_c\) during physiological \([\text{Ca}^{2+}]_c\) signalling. The major concerns must be with the mechanisms of \([\text{Ca}^{2+}]_c\) handling (see Gunter, this...
issue) and then with the functional consequences for cell physiology. Perhaps the most significant functional consequence of mitochondrial Ca\(^{2+}\) uptake in terms of day-to-day cell and tissue physiology is the regulation of mitochondrial metabolism itself (for reviews see [10,27]). However, mitochondrial Ca\(^{2+}\) uptake may also exert subtle effects on the spatiotemporal characteristics of the [Ca\(^{2+}\)]\(_i\) signal by regulating local [Ca\(^{2+}\)]\(_m\) in microdomains through the cell (see [4], and Lechleiter et al. this issue) and see below. The basic objective of this essay is to draw attention to the functional consequences of mitochondrial Ca\(^{2+}\) uptake and particularly to draw attention to some observations that may have interesting and even important consequences in terms of both cell physiology and pathophysiology. Some of these issues have also been reviewed recently (see [11]).

**MITOCHONDRIA AND CELLULAR [Ca\(^{2+}\)]\(_c\) SIGNALLING**

During cellular [Ca\(^{2+}\)]\(_c\) signalling, in almost every cell studied, it seems that mitochondria will take up Ca\(^{2+}\). The change in [Ca\(^{2+}\)]\(_m\) may be measured directly, using site directed expression of aequorin [31] or using fluorescent indicators, exemplified by rhod-2, although there is still debate about the quantities of Ca\(^{2+}\) accumulated. Mitochondrial Ca\(^{2+}\) uptake via the uniporter is electrogenic, just as Ca\(^{2+}\) influx through voltage-gated Ca\(^{2+}\) channels across the plasma membrane causes an inward current and a membrane depolarization — indeed, it seems likely that the uniporter is a channel, although it remains to be identified at the molecular level. Thus, Ca\(^{2+}\) uptake may be detected as a transient depolarization of the mitochondrial membrane, reflecting the period of maximal Ca\(^{2+}\) flux. It is important to emphasize that this is not a measure of [Ca\(^{2+}\)]\(_m\), which may remain high long after repolarization of the membrane, as [Ca\(^{2+}\)]\(_m\) re-equilibrates relatively slowly by a Na\(^+\)/Ca\(^{2+}\) exchanger. Imaging of the mitochondrial membrane potential in single cells has shown that [Ca\(^{2+}\)]\(_c\) signals are frequently associated with mitochondrial depolarization (see [8,9,30,26]). In such conditions, spontaneous localized transient depolarizations may occur in the absence of global [Ca\(^{2+}\)]\(_c\) signals, strongly suggesting that even subtle local changes in [Ca\(^{2+}\)]\(_c\) may suffice to induce uptake of Ca\(^{2+}\) by nearby mitochondria (see [8]). The ability of mitochondria to accumulate Ca\(^{2+}\) locally then allows mitochondria to act as a local spatial buffering system and so to influence local [Ca\(^{2+}\)]\(_c\). This becomes particularly important as so many intracellular processes are Ca\(^{2+}\) sensitive, most clearly illustrated in relation to the spatiotemporal features of [Ca\(^{2+}\)]\(_c\) signalling. This reflects the [Ca\(^{2+}\)] sensitivity of the various Ca\(^{2+}\) permeable channels involved in [Ca\(^{2+}\)]\(_c\) signalling, notably the conductance that regulates capacitative influx (see [18]) and IP\(_3\)-gated Ca\(^{2+}\)-release channels of the endoplasmic reticulum (ER). The open probability of the latter shows a complex [Ca\(^{2+}\)] dependence, as a modest increase in [Ca\(^{2+}\)]\(_c\) enhances their open probability in the presence of a constant IP\(_3\) concentration. The open probability of the Type I receptor is then suppressed as the Ca\(^{2+}\) concentration rises above the micromolar range [3], while that of the Type II and III is probably continually enhanced with rising [Ca\(^{2+}\)]\(_c\) [32]. Thus, buffering of [Ca\(^{2+}\)]\(_c\) by mitochondrial uptake, by regulating the local rise in [Ca\(^{2+}\)]\(_c\), may act either to enhance or to restrict the evolution of [Ca\(^{2+}\)]\(_c\) signals. In astrocytes, which express predominantly Type III IP\(_3\) receptors, we found that mitochondrial Ca\(^{2+}\) uptake limits the rate of propagation of a [Ca\(^{2+}\)]\(_c\) wave. Thus, disabling mitochondrial Ca\(^{2+}\) uptake almost doubled the rate of propagation of [Ca\(^{2+}\)]\(_c\) waves from about 25 mm/s to about 40 mm/s [4]. A fairly conventional description of a propagating [Ca\(^{2+}\)]\(_c\) wave requires that Ca\(^{2+}\) is released locally by IP\(_3\) acting at the ER Ca\(^{2+}\) release channel [35]. As [Ca\(^{2+}\)]\(_c\) rises, adjacent ER pools become sensitized to IP\(_3\) and the [Ca\(^{2+}\)]\(_c\) signal propagates effectively as a process of IP\(_3\) sensitive Ca\(^{2+}\) induced Ca\(^{2+}\) release (CICR). Not only was the mean velocity of propagation slowed, but the rate of propagation slowed progressively as the wave progressed across the cell, sometimes dying away before reaching the other end of a long cell (Figs 1Ai & iii). Similarly, the rate of rise of the [Ca\(^{2+}\)]\(_c\) signal fell and the amplitude of the response became progressively smaller as the wave crossed the cell (Figs 1Ai & ii). Remarkably, once mitochondrial Ca\(^{2+}\) uptake was inhibited, the rate of propagation across the cell remained fairly constant, and the rate of rise of the response and amplitude if anything tended to increase as one might expect for a full process of CICR (Fig. 1B). A similar process has been described by Tinel et al. [39] in pancreatic acinar cells, in which a ribbon of mitochondria through the cell centre seems to act as a ‘fire-wall’ between apical and basal poles and restricts the propagation of [Ca\(^{2+}\)]\(_c\) signals from one to the other. It seems that the mitochondrial control of local [Ca\(^{2+}\)]\(_c\) may even be sufficient to modulate the oscillatory pattern of ECR Ca\(^{2+}\) release in response to a constant level of IP\(_3\) [42].

These observations may have important clinical significance. A surprisingly large number of clinical disorders have been associated with mitochondrial dysfunction, perhaps most obviously the inherited mitochondrial genetic defects, but also Parkinson's, Huntington's and Alzheimer's diseases, and possibly some of the myopathies. Most studies have placed an emphasis on [ATP] depletion in the development of symptomatology.
However, it seems quite plausible that subtle alterations in mitochondrial function could be sufficient to disturb the fine tuning of the integration of mitochondrial function with \([\text{Ca}^{2+}]_c\) signalling, leading to abnormal patterning of \([\text{Ca}^{2+}]_c\) signals. The spatiotemporal features of \([\text{Ca}^{2+}]_c\) signals are vital for normal cell function, and so alterations could readily lead to tissue dysfunction, especially in the CNS, without any gross metabolic defect. Indications of such mechanisms have been implied by the measurements of \([\text{Ca}^{2+}]_c\) signals in cybrids with abnormal mitochondria \([14,36]\), and there is great scope for development of such ideas.

**OXIDATIVE STRESS MAY SWITCH MITOCHONDRIAL \([\text{Ca}^{2+}]_c\) UPTAKE FROM A PHYSIOLOGICAL REGULATOR TO A DEATH SIGNAL**

The mitochondrial permeability transition pore

Isolated mitochondria will accumulate \([\text{Ca}^{2+}]_c\) with impunity in the presence of adenine nucleotides. However, if adenine nucleotides are depleted, and in the presence of pro-oxidants, \([\text{Ca}^{2+}]_c\) uptake may trigger opening of a large conductance pathway in the mitochondrial inner membrane usually referred to as the mitochondrial permeability transition pore. This pore can allow large influx of \([\text{Ca}^{2+}]_c\) leading to cell death. Here, oxidative stress may switch \([\text{Ca}^{2+}]_c\) uptake from a physiological regulator to a death signal.
permeability transition pore (mPTP). This process was described in a remarkable series of papers by Haworth and Hunter in the 1970s (e.g. see [17]), but was largely neglected until very recently. It now seems clear that the pore is produced by the association of several proteins concentrated at contact sites between inner and outer mitochondrial membranes. These are the outer membrane voltage-dependent anion channel (VDAC), the inner membrane adenine nucleotide translocase (ANT), and the cyclophilin A (CsA) binding protein, cyclophilin D or (CypD). The involvement of the ANT confers sensitivity of the pore to agents which modulate adenine nucleotide transport (bongkrekic acid and atractyloside) while that of CypD confers sensitivity to CsA, which tends to prevent pore opening (see [5] for review). This is a minimal model, illustrated schematically in Figure 2, and it is likely that there are many other components which serve to modulate pore opening, most notably the pro- and anti-apoptotic family proteins bid, bax and Bcl-2. The pore has a conductance of \( -1 \) nS and a likely diameter of \( -2.4 \) nm, allowing passage to compounds of about 1500 Daltons (for a recent review see [5]).

A question almost invariably asked by physiologists is ‘what is the normal function of this pore?’ It is not at all clear that the pore has a ‘normal’ function. Some have argued that transient pore opening may act as a safety valve to release accumulated \( [Ca^{2+}]_{\text{im}} \) (e.g. [1]) or that it may even be involved in the process of \([Ca^{2+}]_{\text{c}}\) signalling and wave propagation [20,21]. Others have suggested that opening of the pore may promote cytochrome c release in pathways to programmed cell death. The evidence for both of these roles in any except rather unusual circumstances seems to be controversial. A role of the mPTP in the normal processes of programmed cell death now seems unlikely, although if the pore opens, cytochrome c release is likely to follow (for a recent review, see [7]). Nevertheless, it now seems that most models of apoptosis do not require pore opening to cause cytochrome c release [16]. It seems rather that the formation and opening of the pore reflects a malfunction of a number of proteins which themselves have major roles in mitochondrial biology, when exposed to a pathological environment. Most strikingly, the major factors that promote pore opening are pathological – a high intramitochondrial \([Ca^{2+}]_{\text{im}}\), adenine nucleotide depletion, oxidative stress and a high inorganic phosphate. Pore opening is particularly governed by the oxidation state of thiol groups (see [2] for a recent review). Perhaps the models in which pore opening seems most convincingly involved are in reperfusion injury in the heart and in glutamate excitotoxicity in the CNS.

During ischaemia in the heart, \([Ca^{2+}]_{\text{im}}\) rises and adenine nucleotides are depleted, but mitochondria will tend to be depolarized limiting \( [Ca^{2+}]_{\text{c}} \) accumulation.
However, having withstood the period of anoxia, a number of cells will paradoxically die upon reperfusion. The prevailing conditions at reperfusion are almost exactly those that favour mPTP opening in isolated mitochondria – oxidative stress, a high intramitochondrial \([\text{Ca}^{2+}]\) (the repolarizing mitochondria will accumulate \([\text{Ca}^{2+}]\) from the cytosol), adenine nucleotide depletion and high \(\text{Pi}\) [12]. Both isolated cells and the intact heart are protected from anoxia/reperfusion injury by CsA ([29] and [15] respectively) suggesting involvement of the mPTP in reperfusion induced cell death. Nevertheless, it has still proved difficult to demonstrate mPTP opening directly.

We have devised a simple method that allows induction of mPTP opening in single cells so that the consequences can be studied directly [25]. This involves using an intramitochondrial potentiometric indicator such as tetramethylrhodamine ethyl or methyl ester (TMRE) to act both as a photosensitizer to generate reactive oxygen species (ROS) from within the mitochondria and to report mitochondrial membrane potential. The production of ROS can be directly demonstrated using the fluorescent dye, dihydro-dichloro-carboxyfluorescein (H\(_2\)DCF) to measure the rate of ROS production with or without TMRE loading (Fig. 3A). In the heart, a role for reactive oxygen species (ROS) during reperfusion injury is widely accepted, so much so that the antioxidant allopurinol is routinely used in clinical practice after cardiac bypass surgery. It is widely recognized that the mitochondria themselves represent a major source of ROS which is generated by a damaged mitochondria.

Fig. 3 A model for inducing mPTP opening in cardiomyocytes: illumination of intramitochondrial TMRE causes of ROS generation, loss of mitochondrial potential, and ATP depletion. (A) The rate of ROS production in single dissociated adult rat cardiomyocytes was assessed by using the fluorescent indicator 6-carboxy 2',7'-dichlorodihydrocarboxyfluorescein (H\(_2\)DCF, used at 10 \(\mu\)M for 30 min), a non-fluorescent fluorescein derivative which is oxidized to a fluorescent product by free radicals, probably predominantly by \(\text{H}_2\text{O}_2\). In cells loaded with this indicator alone, and exposed to minimal illumination consistent with a reasonable signal/noise ratio, a slow increase in fluorescence was seen (mean slope \(\pm\) 0.45 fluorescent units/s, \(n=4\)). This partly indicates a resting rate of radical formation, and probably partly reflects the auto-oxidation of the dye. However, when cells were dual loaded with H\(_2\)DCF and tetramethyl rhodamine ethyl ester (TMRE; loaded with 3 \(\mu\)M for 15 min and then washed), the rate of oxidation of the H\(_2\)DCF was increased some six- to eightfold, (mean slope 31 \(\pm\) 16 fl u/s, \(n=6\)) even though the optical arrangements for such experiments were of necessity sub-optimal for the excitation of the TMRE (excitation at 490 nm rather than the optimal 530 nm was used in order to excite the H\(_2\)DCF) and will, therefore, underestimate the rate of ROS generation by the illumination of TMRE at an equivalent intensity at 530 nm. That this was not an artefactual increase in signal due to dye cross talk is confirmed by the spectra shown in ii. The full emission spectrum of the signal from a single cell was obtained using a Hamamatsu spectrograph using H\(_2\)DCF alone and with the addition of TMRE. Note that in this example, the H\(_2\)DCF peak signal after 200 s of illumination of DCF alone was reached in 90 s when cells were co-loaded with TMRE, without any change in the spectral profile of the H\(_2\)DCF. (B) Illumination of TMRE loaded cells caused collapse of mitochondrial potential (under these loading conditions, mitochondrial depolarization increases the TMRE signal). This was followed by cell shortening, or rigor, which signals ATP depletion. The rate of onset of both mitochondrial depolarization and of rigor (C) were dependent on the intensity of illumination. At 1% of excitation light, cells did not shorten even after 50 min of illumination. For each bar, \(n=6\).
respiratory chain, and so this seems an attractive model that allows us to examine directly the consequences of intramitochondrial ROS production for both cell and mitochondrial function. A similar model has been used to study pore opening at the level of single isolated mitochondria [19], whereby illumination of intramitochondrial TMRM was able to induce abrupt transitions in potential.

In cells loaded with TMRE, continuous exposure to increased illumination intensity causes a stereotypical sequence of changes in $\Delta \Psi_m$ measured with the TMRE fluorescence itself. Initially, brief localized flashes of signal are seen, which we have previously shown to be dependent on local SR Ca\textsuperscript{2+} release ([8] and see above). With increased and sustained illumination, however, the frequency of these events increases progressively culminating in a global mitochondrial depolarization (Fig. 3B). This collapse of $\Delta \Psi_m$ is invariably closely followed by cell shortening to a rigor contracture that signals ATP depletion (Figs 3B & 4). The time taken for the onset of mitochondrial depolarization and rigor can be substantially increased by reducing the light intensity (Fig. 3C), and so it is possible to regulate the rate of onset of the pathology experimentally by controlling light intensity. The rate of progression to rigor is clearly defined largely by the rate of ROS production: it is slowed by using antioxidants, or by removal of oxygen, and the process is accelerated if the cells are supplied with excess oxygen (Fig. 4A). The process seems to depend not only on ROS generation, but also shows a dependence on intracellular Ca\textsuperscript{2+} stores. Thus, the progression to rigor is independent of external Ca\textsuperscript{2+}, but can be substantially prolonged by depletion of internal SR Ca\textsuperscript{2+} stores using a combination of caffeine to empty the SR with thapsigargin to inhibit

**Fig. 4**  A model for inducing mPTP opening in cardiomyocytes: ATP depletion following intramitochondrial ROS generation is caused by mPTP opening, is dependent on SR Ca\textsuperscript{2+} release and is driven by mitochondrial ATP consumption. (A) The rate of onset of rigor was accelerated by excess oxygen delivery to the cell, and was significantly prolonged by anoxia, and by a mixture of the free radical scavengers (labelled 'frs': trolox (1 mM), ascorbate (1 mM) and catalase (250 $\mu$M)), it was also delayed by exposure to CsA (200 nM) and to trifluoperazine (tfp; 10 $\mu$M) both of which inhibit mPTP opening. Depletion of SR Ca\textsuperscript{2+} stores using caffeine (10 mM) to promote store emptying in the presence of thapsigargin (100 nM, to inhibit store refilling; labelled 'thaps') also prolonged the time to rigor, suggesting that mPTP opening and consequent mitochondrial depolarization, are potentiated by SR Ca\textsuperscript{2+} release. The time to cell shortening was also greatly prolonged by oligomycin, although the time course of mitochondrial depolarization was unaltered (see panel B), suggesting that oligomycin had no effect on the underlying process causing the loss of potential, but suppressed ATP consumption by the mitochondrial ATPase. For all data sets, the number of cells was at least 6 and in many groups it was $>20$. In the 'line images' shown in B, images of TMRE signal in a cardiomyocyte were acquired at 10 s intervals. A line was then selected along the length of the TMRE loaded myocyte, and the intensity profile along that line is illustrated for each image frame from the sequence. Mitochondrial depolarization increases the TMRE signal. In the control, (i) a profound global mitochondrial depolarization progressed across the cell as a slow wave, followed shortly afterwards by cell shortening (arrow) after about 20 min of illumination. (ii) After inhibition of the F$_{0}$-ATPase with 2.5 $\mu$g/ml oligomycin, the mitochondrial depolarization still evolved at about 20 min of illumination, but the cell did not shorten and thereafter only the slow loss of dye from the cell is seen over the course of about 60 min.

the Ca\textsuperscript{2+} ATPase and thus prevent refilling (Fig. 4A). These observations suggest that neither ROS nor Ca\textsuperscript{2+} are sufficient alone, and that both are required for the process. That the loss of mitochondrial potential is due to opening of the mPTP is supported by the effect of CsA and by trifluoperazine (which also suppresses mPTP opening) which both prolong the time to rigor (Fig. 4A).

It is important to recognize that when the mitochondrial potential is dissipated and cannot be maintained, mitochondria may become major ATP consumers. The F\textsubscript{1}F\textsubscript{0}-ATP synthase is a proton pumping ATPase that operates in reverse by virtue of the proton gradient. Once that gradient is lost, it acts as an ATPase and may hydrolyse the full complement of cellular ATP in seconds (see for example [24]). While this may help to maintain mitochondrial volume, it will ultimately lead to ATP depletion, energetic collapse and cell death. Cardiomyocytes signal ATP depletion by shortening to a rigor contraction. The ATPase is inhibited by oligomycin. Oligomycin did not alter the progression of light-induced mitochondrial depolarization in TMRE-loaded cells, but profoundly prolonged the time to rigor, demonstrating a role of the mitochondrial ATPase in the consumption of ATP and the progression to rigor (Figs 4A & B). Thus, in this model, we would suggest that a combination of ROS and mitochondrial Ca\textsuperscript{2+} uptake within the cells promotes mPTP opening, reversal of the mitochondrial ATPase and ATP depletion largely by mitochondrial ATP consumption, followed by rigor and thence to cell death. Therefore, in this state of oxidative stress, mitochondrial Ca\textsuperscript{2+} uptake switches from a ‘useful’, physiological mechanism to a switch that opens the mPTP and hastens (necrotic) cell death.

The increase in the frequency of the ‘flickering’ (see Fig. 5) mitochondrial depolarizations with illumination is also interesting. We have previously shown that the local depolarizations require local SR Ca\textsuperscript{2+} release and mitochondrial Ca\textsuperscript{2+} uptake (see above). However, the fact that the frequency of such events increases with light exposure does raise questions about whether these are truly physiological events, or whether they are due to the sensitization of the mitochondria by illumination of TMRE, a question that will require a different technology to answer: most fluorophores will generate ROS, and so the act of looking may induce the events, and so we need to find a way to monitor their occurrence without

**Fig. 5** A model for inducing mPTP opening in cardiomyocytes: continued illumination first increases the frequency of localized transient mitochondrial depolarizations, followed later by global collapse of mitochondrial potential. Panel A shows plots of intensity over two small areas in a single TMRE loaded myocyte, sampled at 1 Hz at time 0, after 10 min of illumination and after 20 min of illumination. In the initial period, only sporadic small mitochondrial depolarizations are seen. Note that these are quite different in each area of the cell sampled, and seem quite independent in time and space. This is further illustrated in the panels below (B) which show line images (see legend to Fig. 4) taken from another cell – the intensity profile along a line selected along the axis of the cardiomyocyte is plotted for each frame from a sequence of 50 images, showing the localization of the events in space and time. Events are initially highly localized and sporadic, and may be physiological. Before the onset of global mitochondrial depolarization, sustained illumination causes the increased appearance of these events which increase in frequency and distribution, and become increasingly chaotic, evolving into local waves of mitochondrial depolarization (10 min) which culminate in global depolarization (at 20 min) and ATP depletion. As the transient events are dependent on SR Ca\textsuperscript{2+} release, this suggests that illumination may sensitize the SR release channel as well as the mitochondria.
looking at them! Surely, this is the Heisenberg uncertainty principle applied to fluorescence microscopy? Nevertheless, why should continued illumination cause a progressive increase in the frequency of these Ca\(^{2+}\) dependent events? One possibility may lie in the modulation of the SR Ca\(^{2+}\)-release channel open probability by ROS. There is a significant literature that suggests that the probability of Ca\(^{2+}\) release from the SR is increased by oxidation of key sulphhydril groups on the SR release channel [41,38]. Thus, we raise a hypothesis that proposes that the response to mitochondrial oxidative stress involves the initiation of a positive feedback cycle dependent on the properties of SH groups in both the SR and in mitochondrial membranes and on the proximity of the mitochondria to the SR. ROS generation in mitochondria may increase the open probability of nearby SR Ca\(^{2+}\)-release channels, increasing the probability of spontaneous local Ca\(^{2+}\) release. This, in turn, will increase the mitochondrial Ca\(^{2+}\) loading which increases mitochondrial ROS generation [13], further increasing SR Ca\(^{2+}\) release probability etc., until the mitochondrial Ca\(^{2+}\) and oxidative loads are sufficient to fully open the mPTP. It has to be said that this remains speculative, and we are still trying to establish experimental evidence for this model, but it does suggest that the specific properties of Ca\(^{2+}\) release channels and mitochondrial proteins and their sensitivity to Ca\(^{2+}\) on the one hand and ROS on the other, may together conspire to generate an interesting and possibly even important pathological state.

[Ca\(^{2+}\)]\(_c\) AND ROS IN GLUTAMATE NEUROTOXICITY

At first glance, it seems that a rather similar model may operate in the central nervous system during glutamate neurotoxicity (see Reynolds, this issue). The excitatory neurotransmitter, glutamate, accumulates in the intercellular space during periods of cerebral ischaemia and is responsible for a significant proportion of the ensuing cell death in the penumbra beyond the immediate ischaemic focus. It has long been clear that glutamate-induced cell death is Ca\(^{2+}\) dependent, a consequence of the high Ca\(^{2+}\) permeability of the NMDA-gated glutamate receptor, and it has recently been shown that mitochondrial Ca\(^{2+}\) uptake plays a central role in the pathway to cell death [23,37]. Exposure of neurons to toxic levels of glutamate to hippocampal neurons in culture is, in most models, associated with a collapse of mitochondrial potential which is sensitive to CsA (Fig. 6 and see [28,34,40]). The loss of mitochondrial potential is dependent on the rise in [Ca\(^{2+}\)]\(_m\) (although this relationship is not a simple function of the change in [Ca\(^{2+}\)]\(_c\), see [22]), and also apparently on the generation of NO (Fig. 6C) through the association of neuronal nitric oxide synthase (nNOS) with the NMDA receptor (see [6,33] for review). In addition accumulated evidence suggests that glutamate application causes an increase in the production of ROS. The simple explanation of the underlying mechanisms would seem to be that a rise in intramitochondrial [Ca\(^{2+}\)]\(_m\) increases ROS production [13], the combination triggers opening of the mPTP, this leads to ATP depletion and the cells die, much as described above. A closer scrutiny of the detailed cellular events in experimental models of glutamate toxicity suggest that it may not be so simple.

Firstly, the sensitivity of the mitochondrial depolarization to CsA is not straightforward. The sensitivity of a mitochondrial depolarization to CsA is often equated with mPTP opening. However, the pharmacology of CsA is complex (see Fig. 2). CsA acts by binding to cyclophilins, of which at least nine have so far been identified, mostly with unknown functions. One cytosolic cyclophilin, CypA, interacts with calcineurin when bound to CsA, preventing its kinase activity. Calcineurin modulates the activity of nNOS decreasing NO production (see Fig. 2; and see [6]). And so the action of CsA may be attributable not to an involvement of the mPTP but rather to the involvement of nNOS (and NO) in the excitotoxic process. This is not to say definitively that the mPTP is not somehow involved, but simply to be cautious in interpreting evidence which is based on a pharmacology which is not sufficiently selective.

Secondly, a role of ROS in glutamate induced collapse of mitochondrial potential has often been assumed, but not demonstrated. Indeed, a careful scrutiny of the available literature reveals that by far the bulk of evidence for the role of ROS in excitotoxicity comes from the neuro-protective actions of antioxidants – cells are exposed to glutamate in the presence of an antioxidant and the number of dead cells assayed. In only a few studies has ROS production been measured directly. Of these, a spin trap was used in one study to document an increase in superoxide which appeared after a latency of 15–20 min following glutamate application – too late to contribute to the glutamate induced collapse of mitochondrial potential. We have found that antioxidants that are clearly able to scavenge high doses of exogenous superoxide (applied using xanthine/xanthine oxidase) have no significant effect on the glutamate induced collapse of \(\Delta\Psi_{\text{mito}}\) while mixtures of antioxidants that did affect the mitochondrial response in fact also suppressed the NMDA induced current, probably by interaction with redox sites on the NMDA receptor (Vergun et al., in press). Therefore, many of the neuroprotective actions of antioxidants may simply be attributable to a decrease in the [Ca\(^{2+}\)]\(_c\) load imposed by glutamate, quite independently of any role for ROS production. Thus, our data...
suggest that NO, but not other ROS are involved, together with Ca²⁺, in the glutamate induced loss of ΔΨₘ.

It should be clear that our perceptions of both the integration of mitochondria into intracellular signalling processes, and of their role in cellular dysfunction and death in disease, have undergone a radical shift in recent years. It should also be clear that, despite this apparent progress, there remain a number of issues, especially perhaps in the roles of mitochondria in cell death, that remain confused and that still require clarification.

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Fig. 6 [Ca²⁺]c overload caused by glutamate application to central neurons causes a collapse of mitochondrial potential sensitive to CsA but also to L-NAME. (A) In a hippocampal neuron in culture co-loaded with the low-affinity Ca²⁺ indicator Fura-2 FF and the mitochondrial potentiometric indicator rhodamine 123 (Rh123) application of 100 μM glutamate for 10 min causes a rise in [Ca²⁺]c, which is characteristically associated first with a slow mitochondrial depolarization followed by a complete collapse of ΔΨₘ. The mitochondrial depolarization is in turn associated with a secondary rise in [Ca²⁺]c to a new plateau level. This combination of events is a predictor of delayed cell death. The collapse of mitochondrial potential can be greatly delayed using cyclosporin A (CsA; 200 nM) (B), but is also largely suppressed following inhibition of nNOS using L-NAME (100 μM) (C) suggesting converging effects of both Ca²⁺ and NO. Furthermore, the specificity of action of CsA as an inhibitor of mPTP opening is uncertain, as it inhibits nNOS activity as well as suppressing mPTP opening (see Fig. 2).


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