Topical Review

Mitochondria and calcium: from cell signalling to cell death

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(Received 4 July 2000; accepted after revision 25 July 2000)

While a pathway for Ca^{2+} accumulation into mitochondria has long been established, its functional significance is only now becoming clear in relation to cell physiology and pathophysiology. The observation that mitochondria take up Ca^{2+} during physiological Ca^{2+} signalling in a variety of cell types leads to four questions: (i) 'What is the impact of mitochondrial Ca^{2+} uptake on mitochondrial function?' (ii) 'What is the impact of mitochondrial Ca^{2+} uptake on Ca^{2+} signalling?' (iii) 'What are the consequences of impaired mitochondrial Ca^{2+} uptake for cell function?' and finally (iv) 'What are the consequences of pathological $[Ca^{2+}]_c$ signalling for mitochondrial function?' These will be addressed in turn. Thus: (i) accumulation of Ca^{2+} into mitochondria regulates mitochondrial metabolism and causes a transient depolarisation of mitochondrial membrane potential. (ii) Mitochondria may act as a spatial Ca^{2+} buffer in many cells, regulating the local Ca^{2+} concentration in cellular microdomains. This process regulates processes dependent on local cytoplasmic Ca^{2+} concentration ($[Ca^{2+}]_c$), particularly the flux of Ca^{2+} through IP_3-gated channels of the endoplasmic reticulum (ER) and the channels mediating capacitative Ca^{2+} influx through the plasma membrane. Consequently, mitochondrial Ca^{2+} uptake plays a substantial role in shaping $[Ca^{2+}]_c$ signals in many cell types. (iii) Impaired mitochondrial Ca^{2+} uptake alters the spatiotemporal characteristics of cellular $[Ca^{2+}]_c$ signalling and downregulates mitochondrial metabolism. (iv) Under pathological conditions of cellular $[Ca^{2+}]_c$ overload, particularly in association with oxidative stress, mitochondrial Ca^{2+} uptake may trigger pathological states that lead to cell death. In the model of glutamate excitotoxicity, microdomains of $[Ca^{2+}]_c$ are apparently central, as the pathway to cell death seems to require the local activation of neuronal nitric oxide synthase (nNOS), itself held by scaffolding proteins in close association with the NMDA receptor. Mitochondrial Ca^{2+} uptake in combination with NO production triggers the collapse of mitochondrial membrane potential, culminating in delayed cell death.

The existence of a pathway that allows mitochondria to accumulate Ca^{2+} has been firmly established for about 40 years. Nevertheless, only recently has a consensus begun to emerge concerning the physiological significance of the pathway. The central question that has dogged us has been, apparently so simply: 'Do mitochondria take up Ca^{2+} during the normal processes involved in the routine business of $[Ca^{2+}]_c$ signalling?' Alternatively, as long proposed: 'Is the pathway only active under particular conditions or in pathological states?' It is striking that in any contemporary meeting about mitochondrial physiology, a number of presentations will focus significantly on the mechanism, process and impact of mitochondrial Ca^{2+} uptake. At *The Journal of Physiology* Symposium which originated this series of reviews, we have seen the experiments of Rizzuto *et al.* (2000, this issue of *The Journal of Physiology*) using recombinant site-directed aequorins that have gone so far in resolving these questions. The functional importance of this pathway is reflected by the presentation from Maechler & Wollheim (2000, this issue) which focuses on the biochemical and functional consequences of mitochondrial Ca^{2+} uptake in pancreatic β-cells, while that of Hajnóczky *et al.* (2000, this issue) focuses again on the transfer of Ca^{2+} from the ER to mitochondria and on the consequence for cell death when combined with proapoptotic factors, such as ceramide. Crompton (2000, this issue) discusses the mitochondrial permeability transition pore, a pathological consequence of mitochondrial Ca^{2+} accumulation. Inevitably there is a

Presented at The Journal of Physiology Symposium on Mitochondria in Cell Life and Cell Death, San Diego, 18 April 2000.

significant overlap in our various contributions, for which I make no apology, as one of the pleasing aspects of the recent development in this area is the way in which different approaches and different technologies have provided data which (generally) converge towards a consensus view. In this brief review, I propose to discuss the functional consequences of the mitochondrial Ca^{2+} uptake in a variety of models.

Perhaps it is inevitable that many questions remain about the comparative cell physiology of mitochondrial Ca^{2+} handling. Nevertheless, once mitochondrial Ca^{2+} uptake is demonstrable under physiological conditions, this leads directly to a number of interrelated questions. 'What is the impact of Ca^{2+} signalling on mitochondrial function?' Then, conversely: 'What is the impact of mitochondrial Ca^{2+} uptake on $[Ca^{2+}]_c$ signalling?' Both of these questions then have pathophysiological ramifications: 'What is the impact of impaired mitochondrial Ca^{2+} uptake on $[Ca^{2+}]_c$ signalling?' and 'What is the impact of abnormal $[Ca^{2+}]_c$ signalling on mitochondrial function?'

Pathways of mitochondrial Ca^{2+} accumulation

Given the sophistication of our understanding of many ion channels and membrane transporters, our ignorance about the molecular properties of the Ca^{2+} uptake pathway seems remarkable. Mitochondria take up Ca^{2+} primarily through a uniporter, whose molecular nature still eludes us. Some data suggest that this might act like a channel, opening with increased probability once the local $[Ca^{2+}]_c$ rises (for review, see Rizzuto *et al.* 2000, this issue, and Gunter *et al.* 1998). The influx of Ca^{2+} into the matrix by this route is dependent on the electrochemical potential gradient for Ca^{2+}. This is developed and maintained firstly by the process of mitochondrial respiration, which establishes a large potential gradient, the mitochondrial membrane potential ($\Delta\Psi_m$), generally estimated to be in the order of 150–200 mV or so negative to the cytosol, together with a low resting intramitochondrial Ca^{2+} concentration $[Ca^{2+}]_m$, maintained primarily by the mitochondrial Na^+–Ca^{2+} exchanger. The experimental collapse of $\Delta\Psi_m$ using an uncoupler is a simple and much used experimental tool to explore the consequence of preventing mitochondrial Ca^{2+} accumulation. Similarly, the collapse of $\Delta\Psi_m$ as a response to pathological states – during anoxia or as a response to disordered mitochondrial respiration, for example – will also limit mitochondrial Ca^{2+} uptake and may contribute to the emergent cellular pathophysiology. This pathway has often been referred to as the Ruthenium Red (RuR)-sensitive uptake pathway, despite the very poor specificity of RuR, which inhibits Ca^{2+} flux through a variety of different channels. In addition to this uniporter, another pathway has been described recently as the rapid uptake pathway (Sparagna *et al.* 1995), proposed by the authors as a mechanism for the rapid uptake of Ca^{2+} at physiological concentrations. The comparative cell physiology and expression of this mechanism remain to be evaluated. The re-equilibration of mitochondrial Ca^{2+} is largely achieved through the activity of the mitochondrial Na^+–Ca^{2+} exchanger, an exchanger distinct from that found in the plasmalemma, and Na^+ is then exchanged for protons through a rapid Na^+–H^+ exchange. It has been suggested that during hypoxia, mitochondria may become Ca^{2+} loaded by the reversal of the Na^+–Ca^{2+} exchanger (Griffiths *et al.* 1998). This would require that the intracellular Ca^{2+} concentration must be very high, that mitochondria must also be Na^+ loaded and that the rapid Na^+–H^+ exchange be suppressed.

Impact of mitochondrial Ca^{2+} uptake on mitochondrial function

The major targets of the mitochondrial Ca^{2+} import pathway are the dehydrogenases of the TCA cycle, as the rate-limiting enzymes are all upregulated by Ca^{2+}-dependent processes, as discussed by Maechler & Wollheim (2000) and Rizzuto *et al.* (2000) (this issue, and see McCormack *et al.* 1990 for review). Remarkably, it is possible to observe this biochemical activation in single cells by measuring an increase in the autofluorescence of NADH (signalling an increased NADH/NAD^+ ratio) following a rise in $[Ca^{2+}]_c$ in adrenal cortical cells (Pralong *et al.* 1992), in sensory neurons (Duchen, 1992), and in hepatocytes (Hajnóczky *et al.* 1995). A similar process was associated with a measurable increase in oxygen consumption in *Limulus* photoreceptors (Fein & Tsacopoulos, 1988). Therefore it was reasonable, even some 10 years ago, to infer that modest and physiological Ca^{2+} signals must be associated with significant movement of Ca^{2+} into mitochondria, even before that rise in $[Ca^{2+}]_m$ was itself directly demonstrable. The trace shown in Fig. 1*A* illustrates the change in NADH autofluorescence recorded from a single mouse sensory neuron following a very brief (100 ms) depolarisation to initiate voltage-gated Ca^{2+} influx. The change in signal was entirely dependent on external Ca^{2+} and was blocked by microinjection of the cell with Ruthenium Red (Duchen, 1992), demonstrating the dependence of the response on mitochondrial Ca^{2+} uptake. It is perhaps worth noting that the literature concerning mitochondrial Ca^{2+} uptake is currently dominated by studies of $[Ca^{2+}]_c$ signalling in non-excitable cells, and therefore concerns mitochondrial Ca^{2+} uptake following release of Ca^{2+} from ER, while the experiment illustrated in Fig. 1*A* relates to the impact on mitochondrial function of Ca^{2+} influx through voltage-gated Ca^{2+} channels in an excitable cell. It is also noteworthy that the $[Ca^{2+}]_c$ transient associated with this stimulus lasted ~10 s (measured independently, and not shown) while the increased autofluorescence typically lasted minutes, often as long as 5–10 min. The activation of dehydrogenases also stimulates mitochondrial respiration leading to an increase in $\Delta\Psi_m$ (see Robb-Gaspers *et al.* 1998) driving an increase in ATP production (see Rizzuto *et al.* 2000, this issue).

Mitochondrial Ca^{2+} import is an electrogenic process, as the movement of Ca^{2+} is not countered by any other ion exchange and therefore acts like an inward current tending to depolarise the mitochondrial membrane. This is observed as a small and transient depolarisation of the mitochondrial membrane in response to the rising phase of the $[Ca^{2+}]_c$ transient – the period of maximal Ca^{2+} flux (Figs 1*B* and *C*

and 4*A* and see Duchen, 1992; Peuchen *et al.* 1996*b*), which would then be superseded by an increase in potential as the slower enzyme activation takes over. The traces shown in Fig. 1*B* and *C* show the small mitochondrial depolarisation seen in association with a rise in $[Ca^{2+}]_c$ in cortical astrocytes in culture. Here, application of an agonist that mobilised Ca^{2+} from IP_3-sensitive stores (in this case ATP, acting at P_{2u} receptors – see Peuchen *et al.* 1996*a*) raises $[Ca^{2+}]_c$ and, simultaneously, a small transient depolarisation of the mitochondrial membrane is measurable. ATP application can also initiate an intercellular $[Ca^{2+}]_c$ wave that propagates from cell to cell through a network, probably by a combination of IP_3 movement through gap junctions and via the propagated release of ATP from cell to cell (see Guthrie *et al.* 1999; Wang *et al.* 2000). The traces in Fig. 1*C* show the propagation of the signal through a monolayer of cortical astrocytes in which $[Ca^{2+}]_c$ and $\Delta\Psi_m$ were measured simultaneously. Note that the mitochondrial

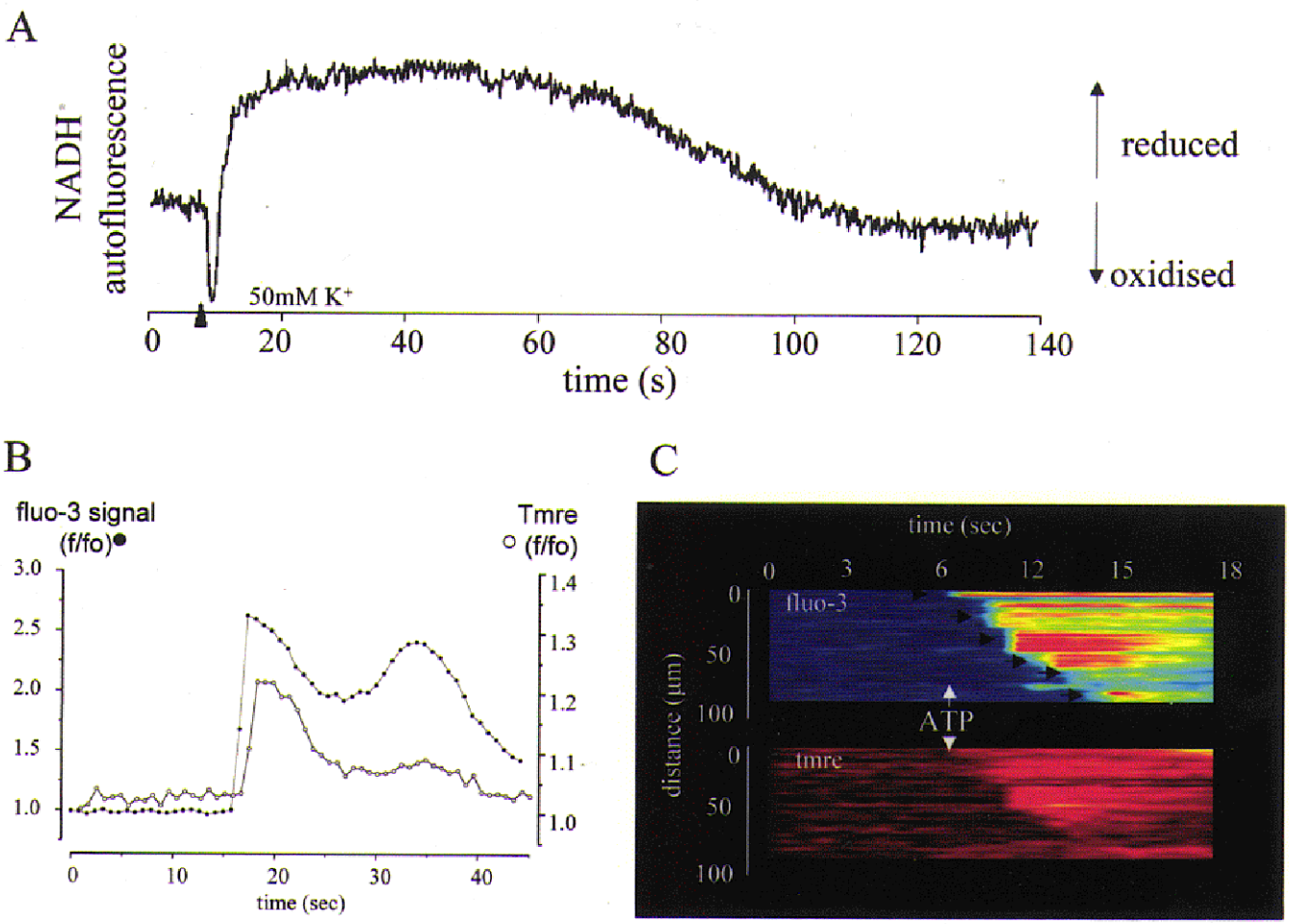

Figure 1. Consequences of mitochondrial Ca^{2+} uptake for mitochondrial function

A, changes in NADH autofluorescence, excited at 350 nm and recorded at 450 nm, are shown following a 100 ms pulse of 50 mM KCl, which was used to depolarise a mouse sensory neuron and thence to raise $[Ca^{2+}]_c$. The autofluorescence initially showed a transient decrease attributable to a transient depolarisation of $\Delta\Psi_m$ that accompanies the Ca^{2+} flux into mitochondria (see below and Fig. 4). This was then superseded by a prolonged increase in signal (increased $NADH/NAD^+$ ratio) which is attributed to activation of the dehydrogenases of the TCA cycle by a high intramitochondrial $[Ca^{2+}]$. The entire response was blocked by microinjection of the cell with Ruthenium Red (not shown). *B*, a rise in $[Ca^{2+}]_c$ causes a transient mitochondrial depolarisation. Rat cortical astrocytes were loaded with tetramethyl-rhodamine ethyl ester (TMRE) and the $[Ca^{2+}]_c$ indicator fluo-3, and imaged simultaneously on a confocal microscope (Zeiss 510CLSM). Application of ATP to a single cell raised $[Ca^{2+}]_c$ (●) by IP_3-dependent mobilisation from ER stores, and caused a small transient mitochondrial depolarisation (○), signalling mitochondrial Ca^{2+} uptake (an increase in TMRE fluorescence signals mitochondrial depolarisation – see Boitier *et al.* 1999). The same phenomenon is seen on a larger scale as $[Ca^{2+}]_c$ waves are propagated through a network of interconnected astrocytes, as shown in *C*. Here, ATP application initiated a wave that propagated from cell to cell, imaged as described above. The steps from cell to cell are indicated by arrows. The simultaneous measurement of $\Delta\Psi_m$ reveals a wave of mitochondrial depolarisation propagating through the network.

depolarisation closely follows the spread of the $[Ca^{2+}]_c$ signal, again demonstrating that the $[Ca^{2+}]_c$ signal must, of necessity, be accompanied by mitochondrial Ca^{2+} uptake.

In addition to the use of mitochondrially targeted aequorin (described by Rizzuto *et al.* 2000, this issue, and see Brini *et al.* 1995), it has recently become possible to measure Ca^{2+} uptake into mitochondria directly using the fluorescent $[Ca^{2+}]$ indicator rhod-2. The AM ester of this dye is positively charged and so partitions between cytosol and mitochondria in response to the mitochondrial potential. Once de-esterified, it is trapped in the compartment, and the localisation is clearly shown when cells are co-loaded with other mitochondrial staining dyes such as mitotracker green or when they are transfected with mitochondrially targeted green fluorescent protein (GFP). In our hands, rhod-2 loading almost always leaves a significant cytosolic signal. It is only really appropriate to assume that the rhod-2 signal from a cell is mitochondrial if positive measures are taken to ensure the loss of the cytosolic component (e.g. by whole-cell patch clamp which dialyses out the cytosolic free dye; Babcock *et al.* 1997). Most measurements of rhod-2 fluorescence in most cell types have shown that $[Ca^{2+}]_c$ signals within a reasonably physiological range are associated with a rise in $[Ca^{2+}]_m$ (Fig. 2*B*). Perhaps this has been most directly demonstrated in the work of David *et al.* (1998) who have shown changes in $[Ca^{2+}]_m$ with trains of action potentials in the nerve terminals of the lizard neuromuscular junction. In most published work, the rise in $[Ca^{2+}]_m$ is significantly slow compared with the rise in $[Ca^{2+}]_c$ and tends also to outlast the cytosolic signal, the exact timing varying apparently between cell types (see Jou *et al.* 1996; Babcock *et al.* 1997; Ricken *et al.* 1998; Boitier *et al.* 1999; Drummond *et al.* 2000). In our experiments, a transient rise in $[Ca^{2+}]_c$ lasting just 20 s following IP_3-mediated release from ER stores in rat astrocytes was followed by a rise in $[Ca^{2+}]_m$ that could last ~10 min (Boitier *et al.* 1999). Similarly, perhaps, the rise in NADH autofluorescence that signals increased dehydrogenase activity could last 5–10 min after a $[Ca^{2+}]_c$ transient lasting only 10 s following voltage-gated Ca^{2+} influx in mouse sensory neurons (Fig. 1, and see Duchen, 1992). Interestingly, it seems likely that a significant proportion of the Ca^{2+} entering mitochondria may not appear as free ionised Ca^{2+} in the matrix, but might rather be present either bound to phosphate or to phospholipids (e.g. see David, 1999), and so measurements of ionised free $[Ca^{2+}]_m$ may usually give an underestimate of total matrix $[Ca^{2+}]$.

It might seem that mitochondrial Ca^{2+} uptake will only occur during a substantial global rise in $[Ca^{2+}]_c$, when the Ca^{2+} concentration throughout the cytosol reaches levels that exceed a high apparent K_d for Ca^{2+} (see, for example, Nicholls & Crompton, 1980, for review). In fact, the work discussed by Rizzuto *et al.* (2000) and by Hajnóczky *et al.* (2000) in this issue demonstrates that mitochondrial Ca^{2+} uptake during global $[Ca^{2+}]_c$ signalling is probably facilitated by structural organisation of the organelles within the cell (see also Rizzuto *et al.* 1998). The proximity of mitochondria to ER Ca^{2+} release sites underlies the generation of microdomains of high $[Ca^{2+}]_c$ close to the IP_3-gated Ca^{2+} release channels of the ER. The same principles might apply to the localisation of mitochondria and Ca^{2+} influx sites in excitable cells (Lawrie *et al.* 1996), although the data here are far less developed. Our own data also suggest similar close contacts between mitochondria and ER Ca^{2+} release sites, revealed in a completely different way, and apparently operating spontaneously even in the absence of global $[Ca^{2+}]_c$ signals. In several cell types, we have observed apparently spontaneous transient depolarisations of $\Delta\Psi_m$ with time constants of the order of 100 ms or so. These events are clearly dependent on the release of Ca^{2+} from internal stores. In cardiomyocytes, for example, they were completely abolished by blockade of the SR Ca^{2+} release channel with ryanodine, or by chelation of Ca^{2+} by loading cells with BAPTA-AM. They were also blocked by inhibition of mitochondrial Ca^{2+} uptake by diaminopentane pentammine cobalt (DAPPAC), a novel inhibitor of the uniporter (Duchen *et al.* 1998 and see Crompton & Andreeva, 1994). Data such as these suggest that mitochondrial Ca^{2+} uptake is far more tightly integrated into the routine business of cell signalling than previously thought, operating at a subtle and continuous level below the threshold of global Ca^{2+} signals.

We need then to ask what is the functional significance of mitochondrial Ca^{2+} uptake. The continuous charging of mitochondria with Ca^{2+} will sustain the activation state of the mitochondrial dehydrogenases, and may be important in sustaining mitochondrial energy production. The failure of this mechanism has been demonstrated in the cardiomyopathic hamster, where a reduction in mitochondrial Ca^{2+} loading is associated with a gradual deterioration of energetic state (Di Lisa *et al.* 1993). While we understand remarkably little about the pathophysiology of the chronic mitochondrial diseases such as the inherited mutations of the respiratory chain, an expected consequence might be a loss of $\Delta\Psi_m$, which will depress mitochondrial Ca^{2+} accumulation (as demonstrated by Brini *et al.* 1999). The consequent downregulation of the TCA cycle might in turn depress mitochondrial ATP generation, ultimately leading to cellular pathophysiology.

Modulation of $[Ca^{2+}]_c$ signals by mitochondrial Ca^{2+} uptake

Mitochondrial Ca^{2+} uptake also plays an intimate role in the fundamental events involved in cellular $[Ca^{2+}]_c$ signalling. The precise features of the interplay between mitochondria and cellular $[Ca^{2+}]_c$ signalling seem to vary between cells. In excitable cells, a fairly consistent pattern has emerged over the past 10 years or so. Thus, in sensory neurons (Werth & Thayer, 1994), central neurons (Wang & Thayer, 1996), sympathetic neurons (Friel & Tsien, 1994), chromaffin cells (Babcock *et al.* 1997) and at the neuromuscular junction (David *et al.* 1998), Ca^{2+} influx through voltage-gated Ca^{2+} channels initiates a rapid rise in $[Ca^{2+}]_c$ which then shows a

biphasic pattern of recovery, consisting of a rapid component with a subsecond time constant followed by a slower component that may take minutes. Inhibition of mitochondrial Ca^{2+} uptake slows the initial rate of recovery of the $[Ca^{2+}]_c$ transient but also removes a slow secondary phase of recovery. This latter component of the $[Ca^{2+}]_c$ response is also blocked by inhibition of the mitochondrial Na^+–Ca^{2+} exchanger (White & Reynolds, 1997). This sequence of responses has been taken to show that mitochondria take up Ca^{2+} during the rapid increase in $[Ca^{2+}]_c$, and, by actively removing Ca^{2+} from the cytosol, accelerate the recovery of the transient. However, the slower re-equilibration of cytosolic and mitochondrial Ca^{2+} pools through the Na^+–Ca^{2+} exchange maintains a slower plateau phase of relatively high $[Ca^{2+}]_c$. This plateau has in turn been implicated in processes such as the post-tetanic potentiation of synaptic transmission, as synaptic vesicle fusion is enhanced in response to renewed Ca^{2+} influx that arrives during the plateau phase (Tang & Zucker, 1997; David *et al.* 1998). Thus what seems to be a simple consequence of the thermodynamic properties of the mitochondrial transport pathways has substantial functional implications for the synaptic circuitry of the organism.

Mitochondria also have a significant impact on the spatiotemporal features of the evolving $[Ca^{2+}]_c$ signal in inexcitable cells, largely reflecting the Ca^{2+} sensitivity of the Ca^{2+}-permeable channels involved. In many cell types, $[Ca^{2+}]_c$ signals following the mobilisation of Ca^{2+} from internal

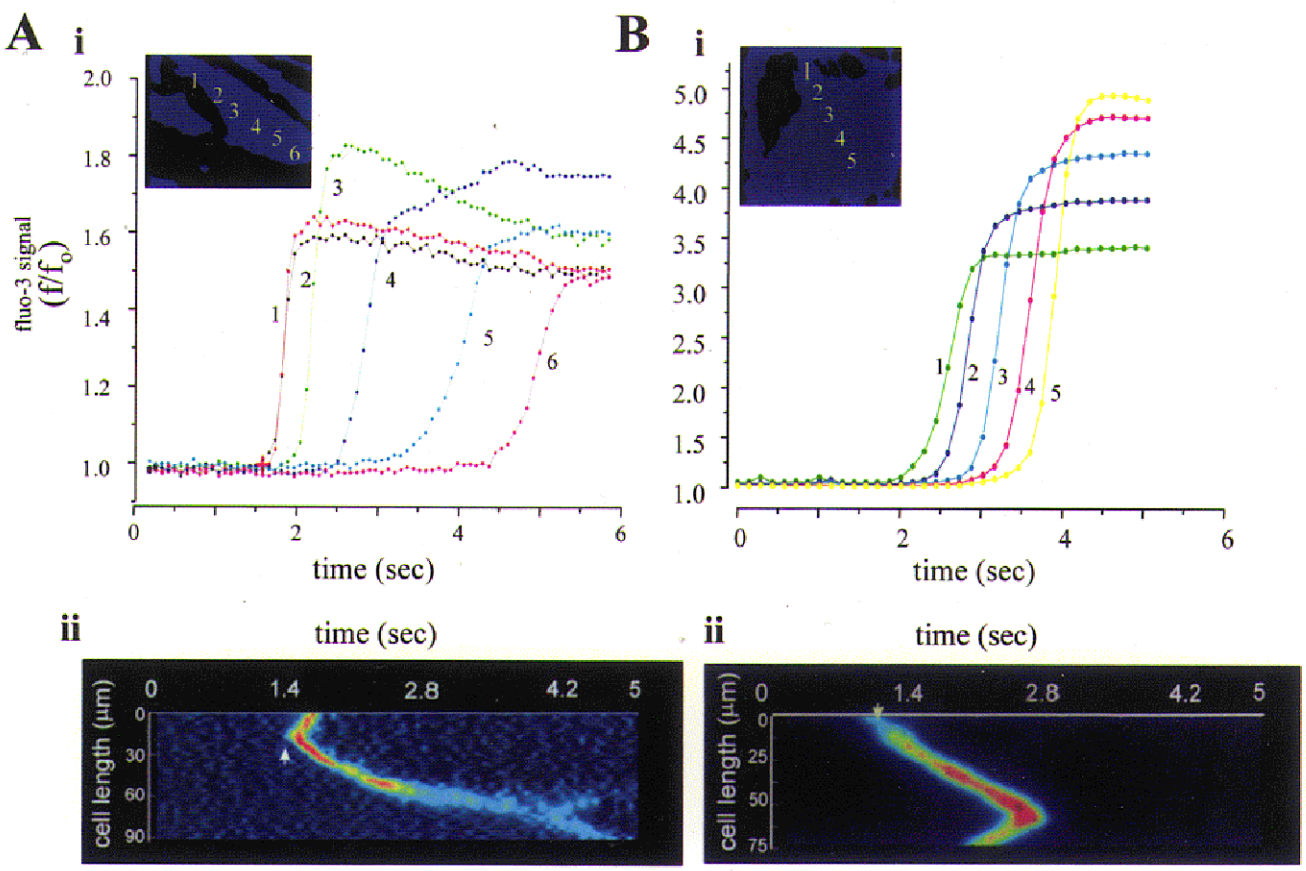

Figure 2. Impact of mitochondrial Ca^{2+} uptake on the propagation of $[Ca^{2+}]_c$ waves in rat cortical astrocytes

A, brief application of a threshold concentration of ATP to an adult astrocyte in culture caused a wave of $[Ca^{2+}]_c$ which propagated across the cell. The cell was loaded with the $[Ca^{2+}]_c$ indicator fluo-3 and imaged using a fast readout cooled CCD camera (Hamamatsu 4880). Plots of intensity with time are shown in *A*i for successive points in space at ~10 μm intervals across the cell as indicated by the inset. It should be clear that both the rate of propagation and the rate of rise of the $[Ca^{2+}]_c$ signal attenuate progressively as the wave progresses along the cell. The mean rate of propagation of the signal was 25 μm s^{-1}. The progress of the wavefront is also illustrated in *A*ii which shows the intensity profile along a line selected along the long axis of the cell with successive image frames from a sequence of just 6 s. The image sequence was first differentiated so that signal is seen only in those pixels in which the signal has changed, and therefore shows the wavefront only. The same principles were applied after depolarisation of $\Delta\Psi_m$ in order to limit mitochondrial Ca^{2+} uptake and data are shown in *B*i and ii. Now the $[Ca^{2+}]_c$ signal clearly propagated faster, with no loss of momentum as it progressed across the cell, and the rate of rise of the signal was sustained throughout the progression of the wave. The mean rate of propagation was now 40 μm s^{-1}.

stores evolve as waves that propagate across the cell from an initiation site, often with oscillatory repeats if the stimulus is maintained. We found that in rat cortical astrocytes the $[Ca^{2+}]_c$ wave generated within single cells in response to a low dose of ATP, which acts at P_{2U} receptors (Peuchen *et al.* 1996*a*) to mobilise IP_3-releasable Ca^{2+}, propagates at an average rate of about 25 μm s^{-1} (Boitier *et al.* 1999). Associated with this was a wave of mitochondrial depolarisation that was dependent on the integrity of the Ca^{2+} store (abolished, for example, by pretreatment with thapsigargin to empty the store), suggesting that there is a flux of Ca^{2+} into mitochondria during the progression of the wave, even though the global rise in $[Ca^{2+}]_m$ detectable with rhod-2 was rather slower and more difficult to identify (see above). When mitochondrial Ca^{2+} uptake is inhibited, by collapsing $\Delta\Psi_m$ while protecting the cell from ATP depletion with oligomycin (which limits mitochondrial ATP consumption by the F_1F_0-ATPase in reverse mode), then the wave propagated almost twice as fast, reaching velocities of 35–40 μm s^{-1}. Also it is very striking that, in control cells, both the rate of rise and the rate of propagation of the wave tended to slow progressively as it travelled across the cell. This gradual decline in propagation disappeared if mitochondrial Ca^{2+} uptake was blocked, and the waves then maintained a more consistent velocity and rate of rise (see Fig. 2*B*), suggesting disclosure of a Ca^{2+}-induced Ca^{2+} release (CICR) process. All these data suggest a mechanism outlined in the cartoon shown in Fig. 3. This scheme proposes that Ca^{2+} uptake by mitochondria close to the ER Ca^{2+} release sites lowers the local $[Ca^{2+}]_c$ into a range below the concentration at which Ca^{2+} sensitises IP_3-mediated Ca^{2+} release. A similar model could explain the observations of Tinel *et al.* (1999), who showed that Ca^{2+} uptake by the mitochondria of pancreatic acinar cells, in which the mitochondria are arranged in a band between the two poles of these polarised cells, tends to restrict the spread of a $[Ca^{2+}]_c$ signal from the apical pole through to the luminal pole. Mitochondria are acting essentially as a spatial buffer in these systems, restricting the propagation of $[Ca^{2+}]_c$ signals by mopping up Ca^{2+} and limiting the concentrations required for (IP_3-dependent) CICR.

Curiously, in *Xenopus* oocytes, mitochondrial Ca^{2+} uptake seemed to have the opposite consequence for the characteristics of the $[Ca^{2+}]_c$ wave (Jouaville *et al.* 1995). In that system, energisation of mitochondria (i.e. increasing mitochondrial membrane potential) by provision of substrate increased the coordination of waves through the oocyte cytosol and slowed the rate of propagation of the wave. It was argued that this could be explained as a consequence of the bell-shaped Ca^{2+} sensitivity of IP_3-dependent Ca^{2+} release. Thus, in the absence of mitochondrial uptake, the local $[Ca^{2+}]_c$ close to the mouth of the IP_3-sensitive Ca^{2+} release channel might rise above the peak of the bell-shaped curve, and so the channel open probability begins to fall. This will tend to reduce the sensitivity of the propagating mechanism and slow the rate of wave propagation. Energisation allows mitochondria to accumulate Ca^{2+}, lowering the local $[Ca^{2+}]_c$ towards the peak of the bell-shaped curve, increasing the sensitivity of the channels to IP_3, and so enhancing the rate of wave propagation. Thus, in one model, mitochondrial uptake slows wave propagation, while in the other, it speeds the process. Why should they be different? One explanation may lie in the different properties of the different classes of IP_3 receptors. Currently, it seems likely that only the IP_3 type I receptor shows the bell-shaped sensitivity to Ca^{2+} described by Iino (1990) and by Bezprozvanny *et al.* (1991), while the type II and III classes of receptor may show a more linear sensitivity to Ca^{2+} (Hagar *et al.* 1998). The astrocytes appear to express predominantly type II receptors (see, for example, Sheppard *et al.* 1997; E. Boitier, S. Brind & M. R. Duchen, unpublished observations) whilst the oocytes express predominantly type I. If this proves to be correct and reproducible in other systems, it implies that the influence of mitochondrial Ca^{2+} uptake on the spatiotemporal features of the $[Ca^{2+}]_c$ may depend on the predominant class of IP_3 receptors expressed by the cells. A similar mechanism can be invoked to account for the mitochondrial regulation of 'capacitative' Ca^{2+} influx in lymphocytes (Hoth *et al.* 1997), where again, local mitochondrial Ca^{2+} buffering seems to keep the local microdomains of $[Ca^{2+}]_c$ close to the Ca^{2+}-sensitive influx channels low enough to keep the channels open and allow sustained influx. Simpson & Russell (1998; a review) have also described amplification sites in the propagation of $[Ca^{2+}]_c$ waves along fine processes in oligodendrocytes, which are enriched in mitochondria and in ER, which co-localise. In that system, though, the impact of removing mitochondrial Ca^{2+} uptake was highly variable, and so the models are rather more difficult to interpret.

Perhaps one of the more surprising recent observations in this field comes from a recent study by Zimmermann (2000). Many cells respond to continuous agonist exposure by generating repetitive oscillatory $[Ca^{2+}]_c$ signals whose oscillatory frequency tends to increase with agonist concentration. Zimmermann (2000) found that in permeabilised cells in which the ER Ca^{2+} content was measured, addition of exogenous IP_3 only generated an oscillatory efflux of Ca^{2+} from the ER when mitochondria were energised. This response could be blocked by collapse of $\Delta\Psi_m$ or by inhibition of the uptake pathway with RuR. That mitochondrial Ca^{2+} uptake should have such a profound impact on the patterning of $[Ca^{2+}]_c$ signals seems remarkable and again demonstrates the tight integration of mitochondria into the process of cellular $[Ca^{2+}]_c$ signalling.

Mitochondrial Ca^{2+} uptake and cell pathology

It seems that, in some circumstances, mitochondrial Ca^{2+} uptake can switch from a useful physiological regulatory mechanism to a potentially harmful process that can initiate the progression towards cell death (e.g. see Ichas & Mazat, 1998). These processes have been mostly attributed to situations arising when mitochondrial Ca^{2+} accumulation is accompanied by some form of oxidative stress. Analogous processes will be dealt with in other models by Hajnóczky *et*

al. (2000, this issue). One such model which we have been exploring is that of glutamate excitotoxicity. It is well established that the excitatory neurotransmitter glutamate may accumulate massively in the CNS during a period of anoxia or ischaemia, largely through reversal of the glutamate transporter in response to the changes in ionic homeostasis that follow ischaemia (Szatkowski & Attwell, 1994). It is also well established that application of glutamate to many classes of neurons in culture may cause cell death; this has long been attributed to 'cellular Ca^{2+} overload' (for review, see Choi, 1994). While the cell death is clearly Ca^{2+} dependent, the downstream processes that couple the rise in $[Ca^{2+}]_c$ to cell death have been less clear. The involvement of mitochondrial Ca^{2+} uptake in this process has been fairly clearly demonstrated by some surprisingly simple experiments that show that preventing mitochondrial Ca^{2+} uptake by depolarising mitochondria with a mitochondrial uncoupler is neuroprotective (Stout *et al.* 1998).

A number of laboratories found that an early feature of the response to toxic glutamate exposure is the depolarisation of mitochondrial membrane potential (see, amongst others, Schinder *et al.* 1996; Khodorov *et al.* 1996; Nieminen *et al.* 1996) and see Fig. 4*A*. Our own contribution focused on the relationship between the change in $[Ca^{2+}]_c$ and the change in $\Delta\Psi_m$ (see Vergun *et al.* 1999 and Keelan *et al.* 1999). One obvious question was whether the change in $\Delta\Psi_m$ is simply an inevitable consequence of an unusually large Ca^{2+} load, or whether there was something else contributing specifically to the glutamate response. It turns out that apparently very similar changes in $[Ca^{2+}]_c$ may be associated with mitochondrial responses that vary enormously in time course and amplitude. For example, 100 μM glutamate exposure for 10 min raises $[Ca^{2+}]_c$ to a similar degree in cells that have been in culture only a short time (7–10 days) and in those that have been in culture for 12–14 days, but in the latter, mitochondria undergo an almost complete collapse of potential and 24 h later ~70% of the cells will be dead,

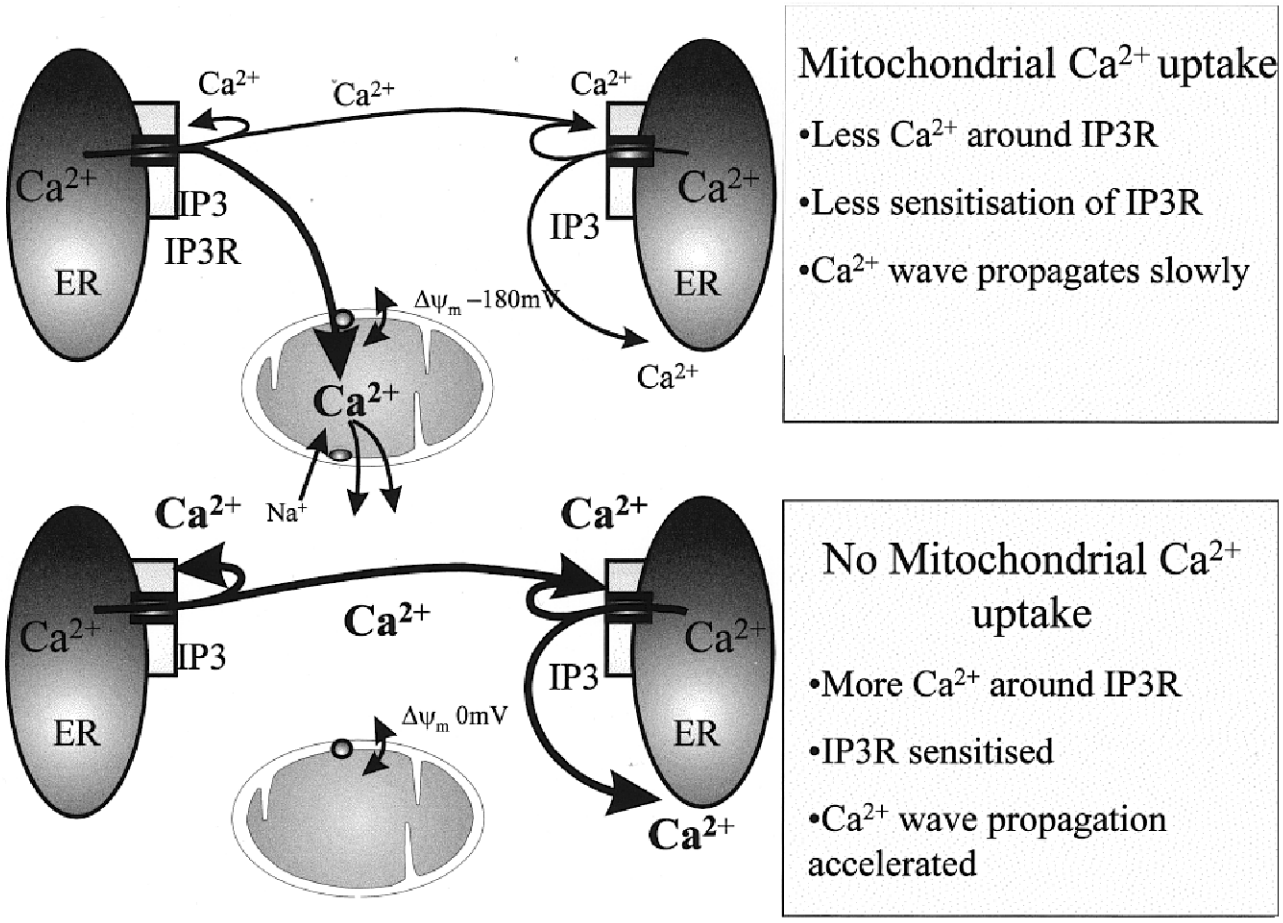

Figure 3. Scheme to illustrate the interplay between mitochondria and ER $[Ca^{2+}]_c$ signalling
These cartoons show a scheme to account for the modulation of $[Ca^{2+}]_c$ signalling by mitochondrial Ca^{2+} uptake in inexcitable cells. The local removal of Ca^{2+} seems able to buffer $[Ca^{2+}]_c$ in local microdomains close to the ER Ca^{2+} release channels. As IP_3-regulated channels are themselves sensitive to $[Ca^{2+}]_c$, this provides a local regulation of their open probability, which serves to regulate the rate and extent of propagation of the signal. When mitochondrial Ca^{2+} uptake is disabled, this modulation is removed, local $[Ca^{2+}]_c$ will be allowed to rise, and will exert feedback regulation on the IP_3 receptor (IP_3R). The precise consequence may vary depending on the class of IP_3 receptor expressed by the cell.

while in the 'younger' cells, the mitochondria undergo only a small reversible loss of potential and the cells do not die. Similarly, although glutamate often causes considerable calcium loading, prolonged depolarisation with high potassium (50 mM) can in some cells raise $[Ca^{2+}]_c$ into the same range as seen with glutamate (when measured with fura-2, but also with $^{45}Ca^{2+}$ – see Sattler *et al.* 1998), and yet we have never seen any substantial mitochondrial depolarisation in response to high potassium alone (Fig. 4*B*), and high potassium does not cause cell death. The correlation between the loss of $\Delta\Psi_m$ and the progression to cell death seems very strong, and so the questions have focused on why mitochondria depolarise in response to glutamate but not to some other similar $[Ca^{2+}]_c$ load. The Ca^{2+} dependence of the loss of $\Delta\Psi_m$ is clear, but in addition to the anomalies set out above, the time course of the $[Ca^{2+}]_c$ response is generally very consistent, but the timing of the profound collapse of $\Delta\Psi_m$ varies enormously between cells. Key to this study is the ability to correlate changes in $[Ca^{2+}]_c$ and $\Delta\Psi_m$ simultaneously on a cell-by-cell basis. These data together suggested that some other 'factor' in addition to Ca^{2+} must play a role in the fate of the mitochondria and thence, perhaps of the cell.

We considered a number of possible agents, including superoxide and nitric oxide, as there is extensive published data suggesting that both antioxidants and nitric oxide synthase (NOS) inhibitors are neuroprotective (e.g. see Kashii *et al.* 1996). In our hands, a number of potent anti-

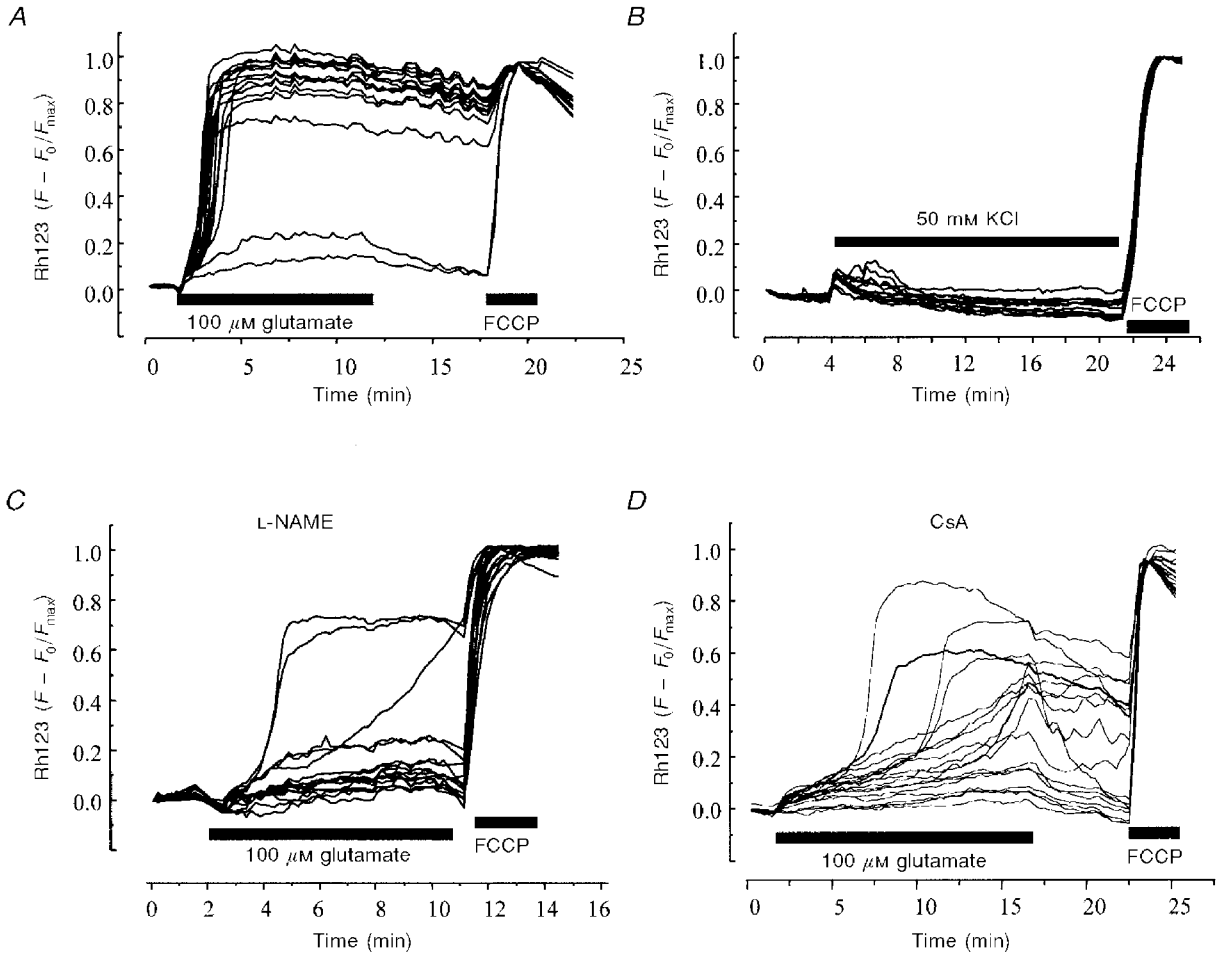

Figure 4. Effects of toxic glutamate exposure on mitochondrial membrane potential in hippocampal neurons

Changes in $\Delta\Psi_m$ in rat hippocampal neurons during changes in $[Ca^{2+}]_c$ caused by the prolonged application of 100 μM glutamate (*A*), 50 mM KCl (*B*), 100 μM glutamate in the presence of the NOS inhibitor L-NAME (100 μM; *C*) and in the presence of cyclosporin A (CsA, 200 nM; *D*). In each case, records were obtained using rat hippocampal neurons in culture. Fluorescence signals were recorded from cells loaded with both rhodamine 123 and fura-2 (or its low affinity equivalent fura-2FF) to measure mitochondrial membrane potential and $[Ca^{2+}]_c$ simultaneously from a field of 20–30 cells. An increase in the rhodamine 123 signal represents a depolarisation of the mitochondrial membrane (see Keelan *et al.* 1999), as indicated by the response to FCCP shown at the end of each experiment. The rhodamine signals were normalised between baseline as 0, and the full depolarisation with FCCP as 1·0. In each case, the early changes in $[Ca^{2+}]_c$ were not significantly different for any of these manipulations.

oxidants failed to make any impression at all on the changes in $[Ca^{2+}]_c$ and the mitochondrial depolarisation, except those that also cause an inhibition of the NMDA-gated conductance (the conductance has redox modulatory sites that may account for these effects and may also account for at least part of the cytoprotection observed with anti-oxidants – see Gozlan & Ben-Ari, 1995 and Vergun *et al.* 2001). However, inhibition of neuronal NOS with L-NAME was significantly protective and also suppressed the mitochondrial depolarisation in a large proportion of cells without reducing the $[Ca^{2+}]_c$ signal (Fig. 4*C*). Conversely, it proved possible to convert an otherwise innocuous $[Ca^{2+}]_c$ signal, in response to K^+-induced depolarisation or in response to glutamate but in 'younger' cells, into a toxic signal associated with a loss of mitochondrial potential if the $[Ca^{2+}]_c$ signal was generated in the presence of exogenous NO at concentrations that had no effect on these variables when applied alone. These data are all consistent with the data published recently by Sattler *et al.* (1999) who demonstrated a possible mechanism for the 'source specificity' of glutamate toxicity. Thus, why should a Ca^{2+}-dependent process of cell death fail to show a direct dependence on $[Ca^{2+}]_c$ and be dependent rather on the route of Ca^{2+} influx? Indeed, if the mechanism involves the additional requirement of NO production, this itself is Ca^{2+} dependent, and so might be expected simply to reflect the Ca^{2+} concentration. This apparent anomaly would seem to have been resolved by the elegant experiments of Sattler *et al.* (1999), who showed that suppression of the expression of the scaffolding protein psd95 by introduction of anti-sense oligonucleotides did not alter the rise in $[Ca^{2+}]_c$ in response to glutamate, but suppressed the production of NO with the result that glutamate toxicity was much reduced. Putting all these data together suggests that Ca^{2+} entering the cell through NMDA-gated channels causes microdomains of high $[Ca^{2+}]_c$ which activate nNOS which is held close to the receptor by the

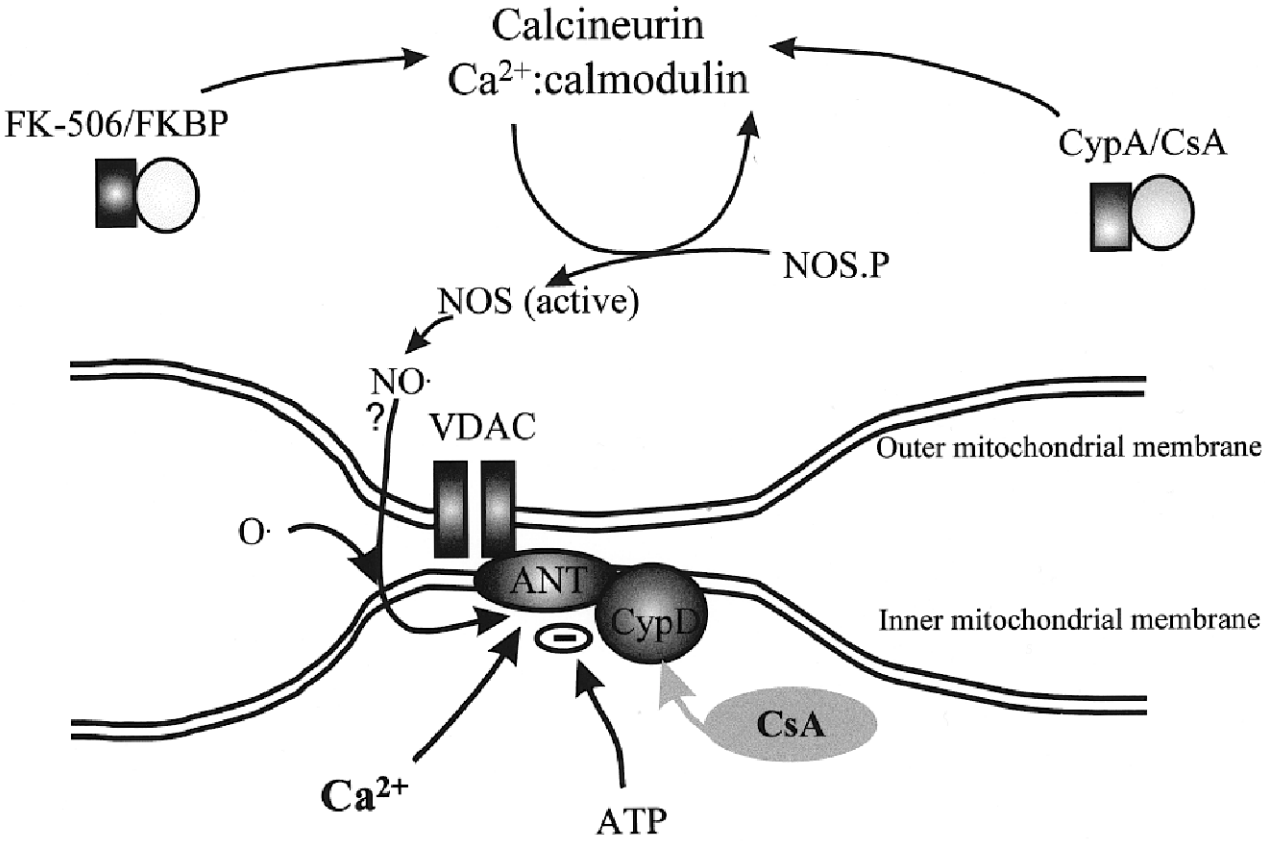

Figure 5. Cartoon to highlight the complex pharmacology of cyclosporin A

Cyclosporin A (CsA) has many actions and cannot be considered specific for the mitochondrial permeability pore. This cartoon illustrates at least two ways in which CsA may interfere with the pathways involved in glutamate excitotoxicity. A local rise in $[Ca^{2+}]_c$ through NMDA receptors activates nNOS whose activity is itself regulated through phosphorylation by calcineurin. CsA (and FKBP, the FK-506 binding protein) inhibit the activation of NOS through calcineurin, and, as NO is required for the glutamate-induced mitochondrial depolarisation, CsA may prevent the collapse of $\Delta\Psi_m$ through mechanisms quite unrelated to the mPTP. The mPTP itself probably consists of the adenine nucleotide translocase (ANT) and possibly also the voltage-dependent anion channel (VDAC), which form a pore under conditions in which SH groups are oxidised and when intramitochondrial $[Ca^{2+}]$ is high. The pore is regulated by cyclophilin D (CypD), which binds CsA. Therefore CsA may also prevent mitochondrial depolarisation by acting to prevent mPTP opening.

scaffolding protein. The combination of NO and high intramitochondrial [Ca^{2+}] then together cause the collapse of the mitochondrial potential, leading ultimately to cell death. It would be misleading to imply that this problem is solved, elegant as it seems. In some hands, it seems that there may be a far more direct and simple relationship between absolute calcium load and cell death (Hyrc *et al.* 1997; Stout & Reynolds, 1999), and it seems clear that there remain elements in the pathway between glutamate exposure and cell death that remain to be clarified.

The other crucial question that remains is the mechanism underlying the collapse of $\Delta\Psi_m$. Two major processes are likely candidates as mechanisms for a loss of $\Delta\Psi_m$ – the opening of the mitochondrial permeability transition pore (mPTP – for review see Crompton, 2000, this issue), or damage to the respiratory chain causing a failure of the very processes that develop and sustain the potential and the gradual loss of potential due to proton leak. With the involvement of NO established, either mechanism seems plausible. NO competes with O_2 at cytochrome oxidase and so may suppress respiration, and may also cause irreversible nitrosylation at complex I (see Clementi *et al.* 1998). A combination of NO and high intramitochondrial Ca^{2+} may also serve to open the mPTP. It has proven surprisingly difficult to resolve this issue, partly because the pharmacological tools available are insufficiently selective to draw unambiguous conclusions. The complexity and ambiguities inherent in this process are illustrated in the cartoon shown in Fig. 5. The mPTP is closed by cyclosporin A (Crompton *et al.* 1988), and the glutamate-induced mitochondrial depolarisation also is suppressed by CsA (Fig. 4*D*; see Nieminen *et al.* 1996; Vergun *et al.* 1999). However, CsA acts by binding to cyclophilins. At the mPTP, the cyclophilin involved is the mitochondrially localised cyclophilin D (CypD). However, CsA will also bind to the cytosolic cyclophilin, CypA, which binds to calcineurin. Calcineurin phosphorylates – and activates – NOS, a phosphorylation inhibited by CsA, and given the involvement of NO in glutamate toxicity, it seems perfectly plausible that the action of CsA is attributable to an interaction with calcineurin. To add spice to this argument, calcineurin is also modulated by the so-called FK binding protein, itself modulated by the drug FK-506, and this has also been shown to be protective in glutamate toxicity (Ankarcrona *et al.* 1996; Keelan *et al.* 1998). Improved specificity may be achieved using the cyclosporin analogue methyl valine Cs (mvCs). This agent binds to all cyclophilins, but the mvCs–CypA complex apparently does not bind to calcineurin. mvCs has been very effective in our experience at suppressing glutamate-induced mitochondrial depolarisation (J. Keelan, O. Vergun & M. R. Duchen, unpublished observations), but had no effect in cerebellar granule cells (Castilho *et al.* 1998). So, while it is tempting to attribute the mitochondrial depolarisation to the mPTP, it is perhaps more appropriate at present to withhold judgement until more unequivocal evidence is available. This is not a purely academic matter, as the hope is obviously that once the mechanism is fully understood, pharamacological tools may be developed which will be significantly neuroprotective during episodes of ischaemia and reperfusion in patients.

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Acknowledgements

I would like to thank *The Journal of Physiology* for organising and funding the symposium which prompted this collection of reviews. I thank the Wellcome Trust, the Royal Society, the BBSRC and the Medical Research Council for financial support. I also thank Eric Boitier, Jake Jacobson, Julie Keelan, Mart Mojet and Olga Vergun who carried out much of the work described, Boris I. Khodorov for lively and provocative discussion, and D. Lilian Patterson for constant technical support in the laboratory for many years.

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