

Topical Review

Contributions of mitochondria to animal physiology: from homeostatic sensor to calcium signalling and cell death

Michael R. Duchen

*Department of Physiology, University College London, Gower Street,
London WC1E 6BT, UK*

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Over recent years, it has become clear that mitochondria play a central role in many key aspects of animal physiology and pathophysiology. Their central and ubiquitous task is clearly the production of ATP. Nevertheless, they also play subtle roles in glucose homeostasis, acting as the sensor for substrate supply in the transduction pathway that promotes insulin secretion by the pancreatic β -cell and that modulates the excitability of the hypothalamic glucose-sensitive neurons involved in appetite control. Mitochondria may also act as sensors of availability of oxygen, the other major mitochondrial substrate, in the regulation of respiration. Mitochondria take up calcium, and the high capacity mitochondrial calcium uptake pathway provides a mechanism that couples energy demand to increased ATP production through the calcium-dependent upregulation of mitochondrial enzyme activity. Mitochondrial calcium accumulation may also have a substantial impact on the spatiotemporal dynamics of cellular calcium signals, with subtle differences of detail in different cell types. Recent work has also revealed the centrality of mitochondrial dysfunction as an irreversible step in the pathway to both necrotic and apoptotic cell death. This review looks at recent developments in these rapidly evolving areas of cell physiology in an attempt to draw together disparate areas of research into a common theme.

Since the award of the 1978 Nobel prize for Chemistry to Peter Mitchell for his formulation of the chemiosmotic theory of oxidative phosphorylation (Mitchell & Moyle, 1967), most physiologists have regarded mitochondria as 'solved'. They have been perceived as divorced from those aspects of physiology that are deemed exciting and significant, and dismissed as boring structures whose sole function is to manufacture ATP unobtrusively and on demand, subservient to the energetic demands of the cell. This essay was unwittingly prompted by a colleague who sat down next to me at coffee one morning, after giving an undergraduate lecture on basic cell biology and commented 'You work on mitochondria, don't you? Why are they interesting? What should I tell the students?' Over the last few years, mitochondria have re-emerged into the spotlight of scientific scrutiny after many years in the wings, as unfamiliar roles are attributed to them that even Peter Mitchell would probably never have imagined (see also Miller, 1998).

'The biggest picture?'

Oxygen is necessary for life – but only because our mitochondria use it to generate ATP. Thought to be derived from prokaryotic micro-organisms which evolved a symbiotic

relationship with their eukaryotic hosts (for review, see Margulis, 1996 and see Gray, 1998), mitochondria are our primary consumers of oxygen, and it could be argued that the mitochondrial requirement for oxygen delivery has driven the evolution of the respiratory and cardiovascular systems. Mitochondria, despite all that follows here, are primarily ATP generators. This is far from trivial: ATP is the major currency in the cellular economy, required to drive by far the majority of energy-requiring reactions in all living things, from bacteria and plants to man. It is necessary for the phosphorylation reactions that modulate so many fundamental cellular processes. It may be stored and used as a (neuro)transmitter, and it controls the activity of several classes of ion channel (such as the ATP-sensitive K^+ (K_{ATP}) channel, the calcium release channel of sarcoplasmic reticulum and voltage-gated calcium channels).

Mitochondria also take up calcium, and are functionally tightly integrated into mechanisms of cellular calcium signalling, and this will provide a major focal point below. It is also often forgotten that mitochondria house a number of critically important biochemical processes quite distinct and separate from energy metabolism – key enzymes involved in haemoglobin and steroid synthesis, for example, and even the carbonic anhydrase needed to make gastric acid.

Mitochondrial energy production may also be harnessed in different ways, diverted from the business of ATP synthesis into the generation of heat through the expression of catecholamine-regulated uncoupling proteins in brown fat in neonates and in hibernating mammals (Palou *et al.* 1998). Recent developments suggest other more subtle, if speculative, actions of these proteins in cell physiology, perhaps in regulating the production of free radical species by the respiratory chain (see Boss *et al.* 1998 for review).

Given that mitochondria play such a major role in the life of the cell, perhaps it is not so surprising to find that mitochondrial dysfunction plays a crucial role in disease and cell death. Acute mitochondrial dysfunction causes acute energy failure and necrotic cell death, for example during ischaemia or anoxia. More subtle, insidious forms of mitochondrial dysfunction may contribute to the pathophysiology of neurodegenerative disorders such as Parkinson's disease (for review, see Schapira *et al.* 1998), and motoneuron disease (or amyotrophic lateral sclerosis; see Kong & Xu, 1998), while the debilitating mitochondrial encephalomyopathies result from a range of genetic disorders of mitochondrial enzyme pathways (for reviews, see Morgan-Hughes *et al.* 1990; Graeber & Muller, 1998). Heritable disorders of the haem synthetic pathway cause the porphyrias, and the mitochondrially generated protoporphyrin IX accounts for the photosensitivity that is so disabling in porphyria cutanea tarda, a phenomenon exploited in the design of photodynamic therapies for cancer. Most recently, it has become clear that mitochondria play a crucial role in apoptosis (for recent reviews see Kroemer *et al.* 1998; Green & Reed, 1998; and see below), which plays so fundamental a role both in shaping organ systems during development and as a route to delayed cell death following subacute injury or reperfusion after ischaemia (Kerr *et al.* 1972). Indeed, the role of mitochondria in apoptotic cell death may well underpin the pathological changes seen in the neurodegenerative disorders listed above.

A little background bioenergetics: some basic principles and definitions

A reasonable understanding of mitochondrial physiology requires a little background knowledge of basic biophysics and an appreciation of the actions of a few pharmacological agents. These are revisited in Fig. 1A, which shows a cartoon representing the key elements of oxidative phosphorylation (see figure legend, and Nicholls & Ferguson, 1992, for a comprehensive account and see also Noji *et al.* 1997). The fundamental principles outlined here govern mitochondrial function and inevitably dictate the approach adopted to investigate mitochondrial function in cells.

Several points shown in this scheme may be manipulated pharmacologically (a few are indicated in blue in Fig. 1A). Agents are available that block respiration at each of the complexes of the respiratory chain, others inhibit the ATP synthase, while protonophores (such as carbonyl cyanide

m-chlorophenyl hydrazone (CCCP) and carbonyl cyanide *p*-trifluoromethoxy-phenylhydrazone (FCCP)) short circuit the electrochemical potential for protons (primarily expressed as a mitochondrial membrane potential ($\Delta\Psi_m$), as the ion concentration gradient is small) and uncouple respiration from phosphorylation. As the functional consequences for each is distinct, careful use of these agents permits a systematic dissection of mitochondrial function under many conditions. The scheme also introduces several variables which are accessible for measurement and which can be used to inform us about the behaviour of mitochondria in cells, most particularly mitochondrial potential and redox state (Fig. 1B and D).

Apart from ATP synthesis, mitochondrial Ca^{2+} uptake represents a major function of mitochondria which is also driven by chemiosmotic principles. The pathway is provided by a uniporter, an electrogenic mechanism, quite possibly a channel opened when $[\text{Ca}^{2+}]_e$ is high, which is blocked by Ruthenium Red (RuR) and various RuR derivatives and analogues (e.g. see Crompton & Andreeva, 1994; Matlib *et al.* 1998). Calcium uptake is driven by the electrochemical potential gradient generated by the combination of the mitochondrial inner membrane potential and the low concentration of Ca^{2+} in the matrix. The latter is maintained by efflux pathways – carriers which exchange Ca^{2+} for protons or Na^+ ions. More recently, evidence has emerged suggesting that a rapid higher-affinity uptake pathway may also be expressed in heart and, with some subtle differences, in liver, which may contribute specifically to the mitochondrial responses to brief physiological stimuli (see Gunter *et al.* 1998).

The mitochondrial permeability transition (MPT) or megachannel. This has recently been recognized as a central participant in several important pathological and possibly physiological processes, and needs to be introduced. This is a pore with a large maximal conductance (~ 1200 pS), but with multiple subconductance states, and an estimated pore diameter of ~ 2 nm (Crompton & Costi, 1990), apparently concentrated at contact sites between the mitochondrial inner and outer membranes (Fig. 1A). First discovered by the observation of Ca^{2+} -induced mitochondrial swelling (Haworth & Hunter, 1979*a, b, c*), this phenomenon was intensively studied for many years in a small number of laboratories before leaping into prominence recently through the proposal that it may play a role in apoptosis (reviewed by Kroemer *et al.* 1998 and see below). The MPT is triggered by a host of different conditions (see the extensive review by Zoratti & Szabo, 1995), but most notably by a rise in intramitochondrial $[\text{Ca}^{2+}]$ ($[\text{Ca}^{2+}]_m$), accompanied by a fall in ATP/ADP ratio, a rise in inorganic P_i , a state of oxidative stress (excessive generation of reactive oxygen species coupled with depletion of cellular antioxidant defences) and resulting oxidation of critical thiol groups that appear to govern the channel properties (Petronilli *et al.* 1994). The channel is thought to arise from an alternate function of the adenine nucleotide translocase,

which in isolation, can show channel-like properties in the presence of high $[Ca^{2+}]$ (Brustovetsky & Klingenberg, 1996). Perhaps most significantly, the channel is closed by cyclosporin A (CsA; Crompton *et al.* 1988), which binds to cyclophilin D (cyp D), a mitochondrial protein that mediates channel opening (Fig. 1A). The pharmacology is unsatisfactory as CsA has many sites of action, interacting with a whole family of cyclophilins whose properties remain poorly characterized. Recently, methyl-valine CsA was made available with a more selective action on the channel. A detailed review of the channel is out of place here, and the reader is referred to a number of recent reviews (Zoratti & Szabo, 1995; Ichas & Mazat, 1998), while its possible physiological and pathophysiological roles will be discussed below.

Causes and consequences of mitochondrial depolarization; mitochondria as ATP consumers. It is perhaps important here to emphasise that mitochondrial depolarization is not specific to any single process. Influx of cations such as Ca^{2+} or K^+ will carry a current and depolarize the membrane, just as an inward current depolarizes the plasma membrane. Opening of a channel such as the large conductance pathway provided by the MPT, or the action of the protonophoric uncouplers, such as FCCP or CCCP, which shuttle protons across the membrane, short circuits $\Delta\Psi_m$. The intact respiratory chain responds to the depolarization by consuming oxygen at a maximal rate. Under these conditions, there is no driving force for ATP synthesis, and ATP may be consumed as the F_1F_0 -ATP synthase runs in reverse mode: the equilibrium of the ATP synthase is determined both by $\Delta\Psi_m$ and by the ATP/ADP. P_i ratio. Indeed, the enzyme may be regarded simply as an ATPase which is driven as an ATP synthase by virtue of the mitochondrial potential. In response to mitochondrial depolarization, the enzyme activity reverts to its ATPase activity, i.e. it now consumes ATP and pumps protons outwards, in a futile, energy-consuming cycle. Indeed, ATP may be consumed in minutes in response to an uncoupler (e.g. see Leyssens *et al.* 1996). Removal of substrate – either of oxygen or of carbon sources – or exposure to inhibitors of the respiratory chain stops respiration, the process that sustains the potential. The consequence then depends on the leakiness of the inner mitochondrial membrane and on the activity of the ATPase in reverse mode, which, by acting as a proton pump, can sustain the potential in some cells until ATP is depleted (e.g. see Duchon *et al.* 1993a). Is it not remarkable that the ‘powerhouse of the cell’ may provide a primary mechanism of ATP depletion and may precipitate cell death when function is compromised? This activity can be ascribed to the relative independence of mitochondria: it is useful for the mitochondrion to consume ATP and conserve $\Delta\Psi_m$ as this prevents mitochondrial swelling. However, it is not in the best interests of the cell, and many cells have evolved a process to impose their own interests over and above that of the mitochondrial pool, and express a protein known as IF1 (the inhibitory protein – see Walker, 1994), a component of

the ATP synthase complex, which inhibits mitochondrial ATP consumption. Under these conditions, the power play between the interests of mitochondrial and cell requirements is revealed, perhaps giving a reminder of their evolutionary origins.

Studies of mitochondrial function in single cells

Until recently, almost all the literature concerning mitochondrial function was derived from studies of isolated mitochondria in suspension. Typically, this means brain, heart or liver. It was very difficult to gather data about the specific properties of mitochondria in discrete cell types – in cerebellar granule cells as opposed to Purkinje cells or hippocampal pyramidal neurons for example. The growth of our knowledge about the behaviour of mitochondria in living cells owes much to the development of fluorescence measurement and imaging technologies (see Fig. 1B–D). Measurements of mitochondrial potential, of intra-mitochondrial $[Ca^{2+}]$ ($[Ca^{2+}]_m$) and of mitochondrial redox state in single cells has made it possible to explore the properties of mitochondria within identifiable cell types and their responses to physiological and pathophysiological stimuli in great detail, although it has to be said that these measurements are not without pitfalls, as interpretation of the fluorescence signals from some of the indicators used remains somewhat ambiguous. Presumably there is still much to be learned about the specializations of mitochondrial function in different cell types.

Mitochondrial contributions to ‘macrophysiology’: mitochondria play a central role in sensing glucose and oxygen

The primary energy supply for mitochondrial metabolism is derived from glucose and oxygen. It might, therefore, seem appropriate that mitochondria are used as substrate sensors in the major biological systems involved in the homeostatic regulation of these fundamental variables – in the pancreatic β -cell (Ashcroft & Gribble, 1998), in the glucose-sensing neurons of the hypothalamus (Ashford *et al.* 1990) and in the oxygen-sensing carotid body (Duchen & Biscoe, 1992a, b).

Role of mitochondria in glucose homeostasis. In both the pancreatic β -cell and the glucose-sensing neurons of the hypothalamus, delivery of substrate stimulates mitochondrial respiration, and the resulting increase in ATP/ADP. P_i ratio closes a class of K^+ channels in the plasmalemma (K_{ATP} channels). The cell membrane potential depolarizes, opening voltage-gated Ca^{2+} channels, raising $[Ca^{2+}]_i$. In the β -cell, this promotes insulin secretion, the insulin lowers plasma [glucose] and closes the feedback loop. In the hypothalamus, regulation of neuronal excitability by ATP-dependent K^+ channels also appears to play a role in the control of total body glucose through the regulation of appetite. Indeed, the situation is even more complex: two cell types have been described, some expressing a K_{ATP} channel and depolarizing in response to an increased supply of glucose, while another class of cells express an ATP-activated K^+ channel, and

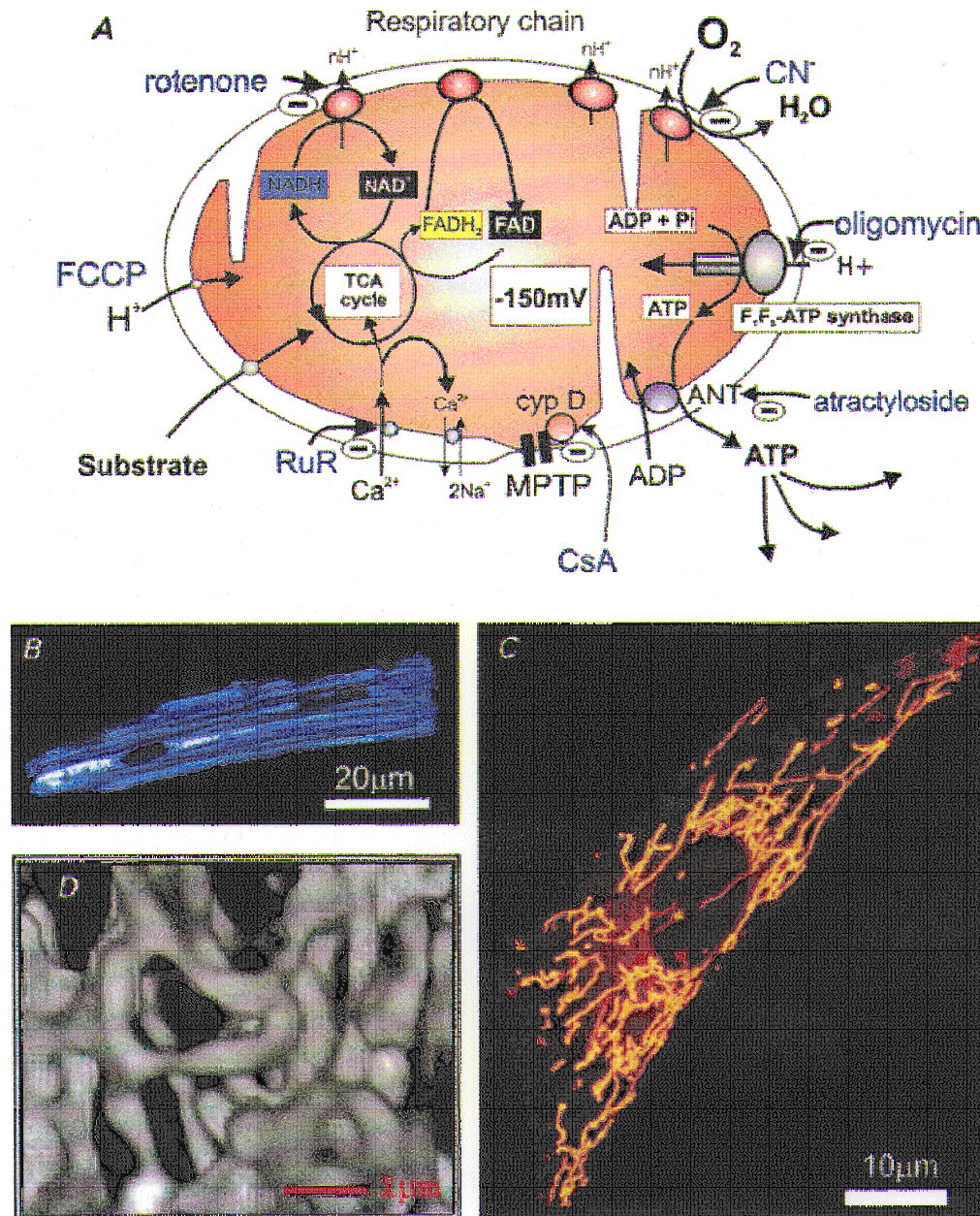


Figure 1. Imaging mitochondrial function

A, cartoon of a mitochondrion, illustrating the basic principles of the chemiosmotic basis for oxidative phosphorylation. The supply of substrate (only glucose is shown, but other substrates – ketones and fatty acids – are also used by some cells) to the tricarboxylic acid cycle (TCA or Krebs' cycle) promotes the reduction of NAD⁺ to NADH and of FAD to FADH₂. As these are re-oxidized, they supply electrons to the respiratory chain. Those electrons are transferred through the enzyme complexes of the respiratory chain to oxygen, with the production of water. In the process, protons are translocated across the inner mitochondrial membrane, generating a potential gradient of approximately -150 to -200 mV (referred to as $\Delta\Psi_m$). ATP synthesis takes place at a separate site, the ATP synthase, which consists of a proton channel (the F_0 subunit) and an ATP synthase (the F_1 subunit). The enzyme is driven by the downhill movement of protons through the F_0 channel and phosphorylates ADP, producing ATP, which is transported out of the mitochondria by the adenine nucleotide translocase (ANT, which also imports ADP). The ANT forms part of the mitochondrial permeability transition pore (MPTP) but has been shown separately for clarity. The sites of action of a number of commonly used reagents are indicated in blue. *B–D*, images of living mitochondria within single cells. *B* shows a confocal image to illustrate the distribution of endogenous or 'auto'fluorescence derived from NADH in a single rat cardiomyocyte illuminated with UV light (at 350 nm). The mitochondria within these cells run longitudinally with the axis of the cell (M. R. Duchen, unpublished observation). In *C*, the mitochondria of a rat cortical astrocyte

therefore hyperpolarize on the supply of glucose (Rowe *et al.* 1996). The modulation of these conductances by leptin, the modulator of appetite, (Spanswick *et al.* 1997), and abnormalities in the genetically obese mouse seem to place their role firmly in the realm of appetite control (and see Friedman & Halaas, 1998).

In the β -cell, the supply of glucose to the cell increases substrate supply to the TCA cycle, increases the provision of NADH and FADH₂ to the respiratory chain, and increases respiratory rate. This drives the hyperpolarization of the mitochondrial inner membrane which is the basis for the increased rate of ATP production (Duchen *et al.* 1993*b*; for review, see Ashcroft & Rorsman, 1989). This sequence results in a high [Ca²⁺]_c in the presence of hyperpolarized mitochondria, encouraging mitochondrial Ca²⁺ accumulation. A most surprising story to emerge recently in the β -cell literature suggests that, following mitochondrial Ca²⁺ uptake, and given an appropriate carbon substrate for the TCA cycle, mitochondria release a (still unidentified) factor which facilitates insulin secretion at a permissive [Ca²⁺]_c (Maechler *et al.* 1997). These authors have also suggested that the modulation of mitochondrial Ca²⁺ uptake may be involved in the desensitization of insulin secretion in response to repeated exposures to metabolic substrate: a dissection of the metabolic pathway (Maechler *et al.* 1998) revealed that ATP production was equivalent for each exposure, but the key difference between responses was a decrease in associated mitochondrial Ca²⁺ accumulation. These authors suggested that a reduction in the mitochondrial [Ca²⁺] load could account for the reduction in insulin secretion, although the mechanism for this process remains unknown. While this factor remains mysterious, the observations emphasize the pivotal role of mitochondria in the regulation of insulin secretion (see also, Kennedy & Wollheim, 1998). Adding a little flavour of integrative physiology to the story, the β -cell K_{ATP} channels (at least, in an insulin-secreting cell line, Harvey *et al.* 1997) also are regulated by leptin, and so appetite control and glucose homeostasis appear closely linked. The central role of mitochondria in the transduction pathway has led to suggestions that at least some forms of late onset diabetes mellitus, a disorder responsible for such widespread morbidity and mortality, may involve mitochondrial dysfunction.

Role of the mitochondria in oxygen sensing. The role of the mitochondria in oxygen sensing is far more contentious. As I have a personal interest in this story, I will rehearse it, if only briefly, but stress that this mechanism is by no

means widely accepted (for counter views, see Acker, 1994; Lopez Barneo, 1996; Buckler, 1997). The carotid body senses a fall in arterial oxygen tension. Excitation increases the frequency of action potentials conveyed by the sinus nerve to the respiratory centres. It is the excitation of the carotid body that makes you feel breathless in the rarefied atmosphere at high altitude and that contributes to the sense of breathlessness of the chronic bronchitic. In response to a fall in oxygen tension, the type I (or 'glomus') cells of the structure depolarize, [Ca²⁺]_i rises and catecholamines are secreted. The basis for the transduction mechanism is still troublesome – how do the cells sense subtle changes in oxygen tension that would be insignificant for most other cells? There is evidence for a specialized NADPH oxidase in the plasma membrane (Acker, 1994), for K⁺ channels that directly sense oxygen (Lopez-Barneo, 1996) and for modulation of Ca²⁺-dependent K⁺ channels (Peers, 1997; but see also Lahiri *et al.* 1998 and Donnelly, 1997). Nevertheless, one of the remarkable features of the carotid body is that it is excited by all agents known to impair mitochondrial oxidative phosphorylation, agents that have no structural pharmacokinetic features in common (Anichkov & Belen'kii, 1963; Biscoe & Duchen, 1990; Duchen & Biscoe, 1992*a, b*; see also Buckler & Vaughan-Jones, 1998). T. Biscoe and I found that mitochondrial membrane potential and redox state of the Type I cells show a unique sensitivity to moderately low oxygen tensions, changing at P_{O₂} values of 20–30 mmHg, which have no effect on mitochondrial function in any other cell that we have ever studied. The jury is still out, but there is a case to be addressed for a specialized role of mitochondria in these cells coupled with evidence for expression of a specialized cytochrome aa3 (Mills & Jöbsis, 1970, 1972; see also Lahiri *et al.* 1996), and it is tempting to speculate that this is another example in which mitochondrial substrate supply plays a central role in a cellular transduction process. There is an attractive symmetry in a scheme whereby the organelles that require oxygen and glucose are also responsible for ensuring the homeostasis of those very substrates. Even local oxygen delivery to tissues is regulated by the energy-transducing K_{ATP} channels expressed in vascular smooth muscle cells (see Quayle *et al.* 1997), although these are probably mainly regulated by transmitter (rather than metabolic) activity.

Mitochondrial calcium uptake and physiological calcium signalling

Apart from the production of ATP, the major contribution of mitochondria to cell physiology must lie in their impact on Ca²⁺ signalling. Isolated mitochondria will accumulate

have been stained with the potentiometric dye, tetramethyl rhodamine ethyl ester (TMRE), which partitions between the mitochondria and cytosol in response to $\Delta\Psi_m$ by virtue of the positive charge on the dye. The image was obtained on a confocal system and shows a reconstruction of a series of confocal slices through the 'z' plane (M. R. Duchen, unpublished observations). *D* shows a 3-dimensional reconstruction of digital fluorescent images of mitochondria expressing targeted green fluorescent protein (GFP) in a HeLa cell, in which the mitochondria appear to form a contiguous but labile network (reproduced from Rizzuto *et al.* 1998, with permission).

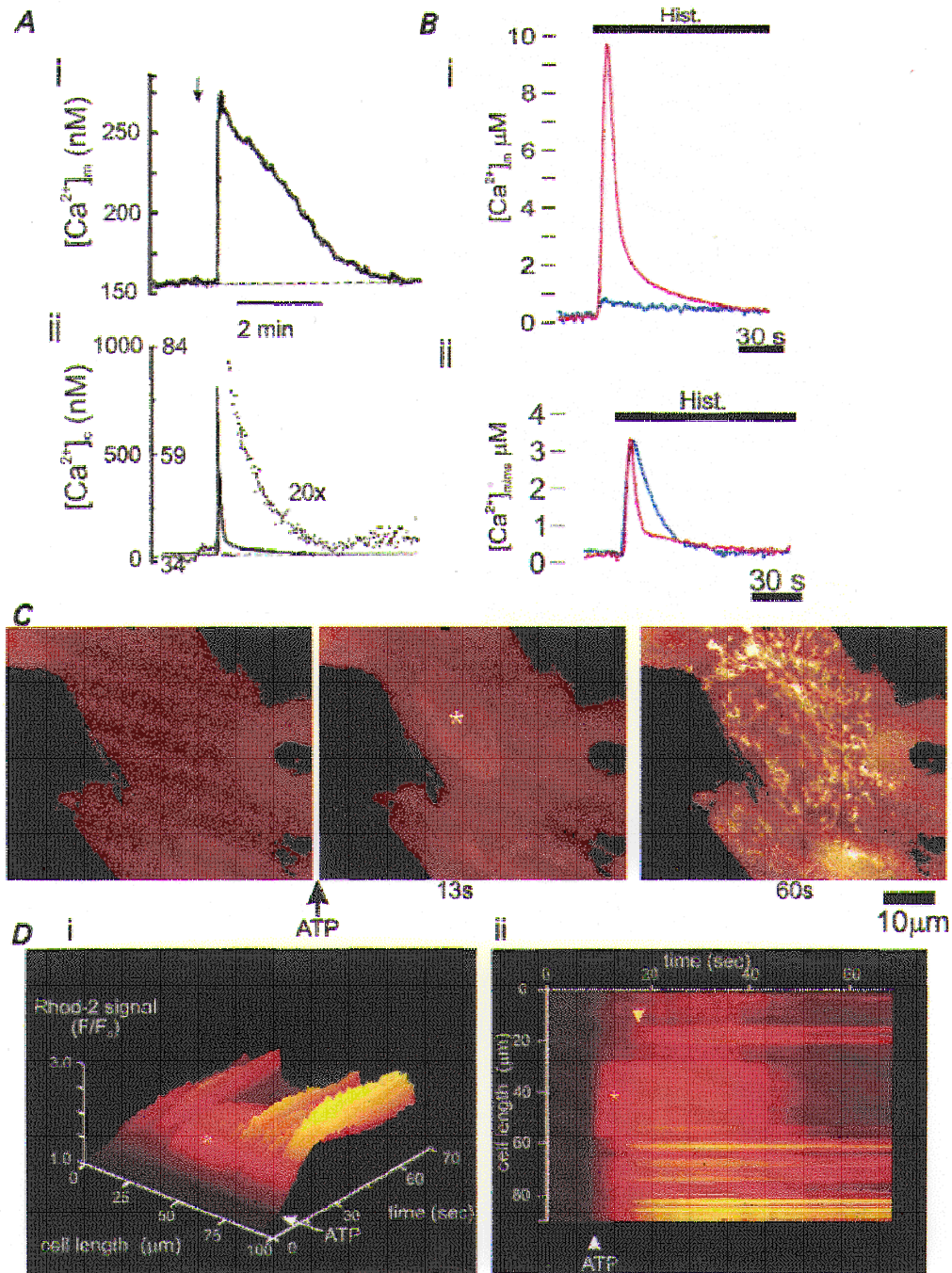


Figure 2. Direct measurements of mitochondrial Ca^{2+} uptake

A, measurements of mitochondrial $[\text{Ca}^{2+}]_m$ (i) and cytosolic $[\text{Ca}^{2+}]_i$ (ii) in a rat adrenal chromaffin cell following depolarization of the plasma membrane and Ca^{2+} influx through voltage-gated channels. The brisk $[\text{Ca}^{2+}]_i$ increase and early recovery (ii) were followed by a prolonged slower decay phase (seen as the dotted and enlarged trace) which coincided with the time course of the slow decline in $[\text{Ca}^{2+}]_m$ revealed with rhod-2 fluorescence (i) (modified from Babcock *et al.* 1997, with permission). *B*: (i) stimulation of a HeLa cell with histamine mobilizes Ca^{2+} from IP_3 -sensitive ER stores. The red trace shows the rise in $[\text{Ca}^{2+}]_m$ revealed with aequorin targeted to the mitochondrial matrix. Note the very large increase in $[\text{Ca}^{2+}]_m$ which was abolished by pretreatment with FCCP (blue trace), preventing mitochondrial Ca^{2+} uptake. (ii), an aequorin construct targeted to the mitochondrial intermembrane space (mims) to sense local changes in $[\text{Ca}^{2+}]_i$ showed a brisk but smaller rise in $[\text{Ca}^{2+}]_i$ in response to the same stimulus. Pretreatment with FCCP had no significant effect on the amplitude of the response but significantly slowed the decay phase, consistent with a role of mitochondrial uptake in removing $[\text{Ca}^{2+}]_i$. (These data were obtained from populations of cells; from Rizzuto *et al.* 1998, with permission.) *C* shows digital fluorescence images of rat

huge amounts of Ca^{2+} in the presence of inorganic phosphate. Ca^{2+} influx is driven primarily by the mitochondrial inner membrane potential. Thus, the driving force for Ca^{2+} movement will favour uptake into the matrix once the concentration of Ca^{2+} close to the mitochondria rises into the range of the affinity of the uniporter, typically described as a low affinity ($K_d \approx 1\text{--}5 \mu\text{M}$), high capacity pathway (see Rizzuto *et al.* 1994; Gunter *et al.* 1998), although it must be said that the concept of a K_d may be misleading, and net Ca^{2+} uptake is rather a kinetic balance between uniporter activity and the efflux pathways (Fig. 1A). Until recently, two major questions remained: firstly, is mitochondrial Ca^{2+} uptake significant during physiological Ca^{2+} signalling? Secondly, a more teleological question – what is that uptake for, or more objectively, what is its functional significance? More recently, these questions have been extended to ask about the impact of mitochondrial Ca^{2+} uptake on the quantitative and spatiotemporal characteristics of Ca^{2+} signals (see also recent reviews by Miller, 1998; Babcock & Hille, 1998; Simpson & Russell, 1998).

A substantial impetus was given to attempts to address these issues by the discovery that the key rate-limiting enzymes of the citric acid cycle are upregulated by Ca^{2+} , either directly or through the regulation of phosphorylation reactions (for review, see McCormack *et al.* 1990). There is now clear evidence that this process is significant during physiological $[\text{Ca}^{2+}]_c$ signalling. The first direct study in intact cells was probably that of Fein & Tsacopoulos (1988), who showed that a rise of $[\text{Ca}^{2+}]_c$ following a flash of light increased oxygen consumption in *Limulus* photoreceptors. In sensory neurons, a rise in $[\text{Ca}^{2+}]_c$ in response to voltage-gated Ca^{2+} influx was associated with a modest transient mitochondrial depolarization and a prolonged increase in NADH autofluorescence (an increase in NADH/NAD⁺ ratio, consistent with upregulation of the TCA cycle) which was blocked by inhibition of the mitochondrial uniporter by microinjection of RuR (Duchen, 1992). More recent studies have extended these observations to explore the metabolic consequences of Ca^{2+} mobilization from IP₃-dependent internal stores (Pralong *et al.* 1994; Hajnoczky *et al.* 1995; Robb-Gaspers *et al.* 1998). Most intriguingly, there appear to be subtle differences in the responses of different cell types. Thus, IP₃-dependent oscillations of $[\text{Ca}^{2+}]_c$ in hepatocytes, although communicated to mitochondria as oscillatory changes in $[\text{Ca}^{2+}]_m$ (Hajnoczky *et al.* 1995), were translated as graded but sustained changes in the reduction of mitochondrial NAD⁺ to NADH (Pralong *et al.* 1994;

Hajnoczky *et al.* 1995). In the hepatocytes, the Ca^{2+} -dependent metabolic activation clearly outlasted the $[\text{Ca}^{2+}]_m$ signal, thereby acting as an integrator of oscillatory $[\text{Ca}^{2+}]_c$ -signalling activity. The rise in $[\text{Ca}^{2+}]_m$ was shown to upregulate pyruvate dehydrogenase, increase NADH autofluorescence and then to initiate a slower increase in mitochondrial potential (Robb-Gaspers *et al.* 1998), which would then drive an increase in ATP production. In contrast, $[\text{Ca}^{2+}]_c$ oscillations in pancreatic β -cells and adrenal glomerulosa cells were associated with oscillatory changes in NADH (Pralong *et al.* 1994). These apparent differences in signal need to be explored further to establish the degree and origin of variability in mitochondrial function between different cell types.

It now seems likely that the major consequence of the Ca^{2+} uptake pathway in terms of mitochondrial function is the upregulation of energy metabolism. This makes good sense – oxidative phosphorylation is modulated in relation to energy demand by the relayed Ca^{2+} signal, as increased energy demand is almost always accompanied by a rise in $[\text{Ca}^{2+}]_c$ – for secretion or contraction for example. The functional importance of this mechanism is illustrated nicely by studies of the cardiomyopathic hamster (Di Lisa *et al.* 1993), in which an impaired $[\text{Ca}^{2+}]_c$ signal in cardiac muscle is associated with impaired energy metabolism.

Not only has this body of work demonstrated the functional significance of mitochondrial Ca^{2+} uptake, but it also showed that physiological Ca^{2+} signalling is associated with significant mitochondrial Ca^{2+} uptake. Indeed, it is now clear that mitochondria do indeed take up Ca^{2+} during physiological signalling in most cells that have been studied. The technology required to answer these questions has developed largely through improvements in fluorescence microscopy and imaging techniques, especially through improvements in confocal microscopy, and, perhaps most dramatically, through the introduction of genetically engineered, site-directed expression of the Ca^{2+} -sensitive photoprotein, aequorin, that permits specific measurement of $[\text{Ca}^{2+}]$ within unambiguously identifiable compartments (see Brini *et al.* 1995 and Fig. 2) or using mitochondrially localized fluorescent indicators, such as rhod-2 (e.g. see Hajnoczky *et al.* 1995; Babcock *et al.* 1997; Robb-Gaspers *et al.* 1998; Fig. 2).

The kinetics of mitochondrial Ca^{2+} uptake and release appear to differ between cell types (Fig. 2), perhaps reflecting differences in $[\text{Na}^+]_i$ availability for the mitochondrial

cortical astrocytes loaded with rhod-2, normalized with respect to the first image to reveal relative changes in signal. Stimulation with ATP (100 μM) to mobilize Ca^{2+} from ER stores raised $[\text{Ca}^{2+}]_c$ first, best seen over the nucleus (*, at 13 s into the sequence), followed later by a large and sustained increase over the mitochondria. This is illustrated further by the images in *D*, which show the evolution of the signal with time, measured as an intensity profile along a line selected along the axis of the cell and displayed as a surface plot in (i) and as a 'line image', seen as if from above in (ii). The relatively fast and transient increase over the nucleus (*) contrasts with the slower but sustained increase in signal over the mitochondria (downward-pointing arrowhead) (M. R. Duchen & E. Boitier, unpublished observations).

$\text{Na}^+-\text{Ca}^{2+}$ exchanger. Almost all reports to date describe a rapid uptake followed by much slower re-equilibration, with the exception of the measurements by Rizzuto *et al.* (1992, 1994) in HeLa cells, using site-directed aequorin and by Robb-Gaspers *et al.* (1998) in hepatocytes, using rhod-2. In both these studies, recovery of $[\text{Ca}^{2+}]_m$ appeared relatively fast despite a sustained rise in $[\text{Ca}^{2+}]_c$. Our own imaging study of mitochondrial Ca^{2+} uptake in adult rat cortical astrocytes using the mitochondrially localized Ca^{2+} indicator rhod-2 suggests that a brief transient rise in $[\text{Ca}^{2+}]_c$ was followed by a sustained increase in $[\text{Ca}^{2+}]_m$ that could last for many minutes (Boitier *et al.* 1999; see Fig. 2C and D), although Jou *et al.* (1996) described oscillations of $[\text{Ca}^{2+}]_m$ associated with $[\text{Ca}^{2+}]_c$ oscillations in a neonatal astrocytic cell line. It is not yet clear whether these are real differences in $[\text{Ca}^{2+}]_m$ homeostasis between cell types, or technical issues related to the properties (consumption, affinity, loading conditions etc.) of the indicators used. However, the kinetics are important and have a number of fascinating functional consequences. At nerve terminals, for example, the slow release of the Ca^{2+} that accumulates within mitochondria during periods of rapid repeated stimulation maintains an elevated $[\text{Ca}^{2+}]_c$ in the terminal for a substantial period of time. Further stimulation during that period therefore raises $[\text{Ca}^{2+}]_c$ more than would otherwise be expected and potentiates transmitter release – in other words, the temporal buffering of $[\text{Ca}^{2+}]_c$ by mitochondrial Ca^{2+} uptake plays a central role in post-tetanic potentiation (Tang & Zucker, 1997; David *et al.* 1998; Melamed-Book & Rahamimoff, 1998).

Most obviously, if mitochondria take up Ca^{2+} , they may act as a fixed buffering system. The impact that this has on cellular $[\text{Ca}^{2+}]_c$ signalling will depend on a number of factors, not least the balance of Ca^{2+} -regulating systems expressed by the cell, the relative volume of mitochondria in the cell and the mitochondrial membrane potential. The consequences of mitochondrial Ca^{2+} uptake for $[\text{Ca}^{2+}]_c$ signalling has now been widely studied, revealing profound alterations in shaping global $[\text{Ca}^{2+}]_c$ responses to depolarization in adrenal chromaffin cells, sensory neurons and central neurons when mitochondrial Ca^{2+} uptake was disabled, achieved most simply by dissipating $\Delta\Psi_m$ using an uncoupler (Thayer & Miller, 1990; Werth & Thayer, 1994; Friel & Tsien, 1994; White & Reynolds, 1995; Herrington *et al.* 1996; Khodorov *et al.* 1996; Stout *et al.* 1998). On this point it is crucial to differentiate between changes in $[\text{Ca}^{2+}]_c$ signal due specifically to reduced mitochondrial Ca^{2+} uptake, and changes due to ATP depletion that may follow a loss of $\Delta\Psi_m$ (see also Landolfi *et al.* 1998), as depletion of ATP will clearly alter the ability of cells to extrude or sequester a Ca^{2+} load quite independently of mitochondrial Ca^{2+} uptake. In many excitable cells, depolarization causes Ca^{2+} influx through voltage gated channels and a fast $[\text{Ca}^{2+}]_c$ transient which shows a biphasic recovery; a rapid early phase followed by a long slow phase that may last for minutes (Fig. 3A and B). Inhibition of mitochondrial Ca^{2+} uptake generally increases the amplitude of the transient response

and removes the slow recovery phase (Fig. 3A and B; Werth & Thayer, 1994; Herrington *et al.* 1996). Mitochondrial depolarization during the slow recovery phase causes a large $[\text{Ca}^{2+}]_c$ transient (Fig. 3B), suggesting Ca^{2+} release from Ca^{2+} -loaded mitochondria. The slow phase is also impaired by inhibition of the mitochondrial $\text{Na}^+-\text{Ca}^{2+}$ exchange by agents such as diltiazem or CGP-37157 (White & Reynolds, 1997). This mechanism thus shapes the global $[\text{Ca}^{2+}]_c$ transient following periods of depolarization, promoting rapid recovery from a peak of high $[\text{Ca}^{2+}]_c$ followed by a sustained plateau of raised $[\text{Ca}^{2+}]_c$ as the mitochondrial Ca^{2+} homeostatic systems re-equilibrate.

In some other models, the functional consequences of mitochondrial Ca^{2+} uptake are rather more complex and subtle. For example, during measurements of capacitative Ca^{2+} signalling in populations of T lymphocytes (Hoth *et al.* 1997), the plateau phase of sustained Ca^{2+} influx seen in measurements from cell populations was impaired by the uncoupler CCCP (Fig. 3C). High $[\text{Ca}^{2+}]_c$ reduces the open probability of the channel that carries the Ca^{2+} flux in response to emptying of ER stores (I_{CRAC} – the calcium release-activated calcium current). These authors therefore proposed that the spatial buffering of Ca^{2+} by mitochondrial Ca^{2+} uptake limits the rise in $[\text{Ca}^{2+}]_c$ close to the channel mouth, the I_{CRAC} channels stay open, the total Ca^{2+} flux is greater and ER store refilling is more effective. Remove mitochondrial Ca^{2+} uptake, and the local accumulation of $[\text{Ca}^{2+}]_c$ close to the channel mouth will close the channels, and therefore limits store refilling. Mitochondrial Ca^{2+} export through a $\text{Na}^+-\text{Ca}^{2+}$ exchange mechanism in turn apparently maintains the high plateau *global* $[\text{Ca}^{2+}]_c$ signal during the activation of the Ca^{2+} influx pathway. This whole process is perhaps more complex than it initially seems – it must of necessity require some spatial separation of the mitochondrial efflux pathway from the focal high $[\text{Ca}^{2+}]_c$ near the channel mouth responsible for channel inactivation, or the model could not work – mitochondrial efflux would simply sustain the high local $[\text{Ca}^{2+}]_c$ and cause channel inactivation. These considerations might prompt speculation about the spatial organization of mitochondrial structures: the observations of Rizzuto *et al.* (1998) in HeLa cells transfected with a mitochondrially localized green fluorescent protein (GFP) suggested that, at least in these cells, mitochondria may form a contiguous network (Fig. 1D) and even the more conventional confocal reconstruction shown in Fig. 1C shows a substantial network structure. Does this mean that Ca^{2+} could be taken up in one part of the cell and released at some distant site from another part of the same mitochondrial network – a notion of Ca^{2+} tunnelling through an organelle structure akin to that described for the ER network of pancreatic acinar cells (Mogami *et al.* 1997)?

Modulation of spatiotemporal $[\text{Ca}^{2+}]_c$ signals by mitochondria

The elegant experiments of Rizzuto *et al.* (1998) using site-directed transfection of the Ca^{2+} -sensitive photoprotein aequorin, more recently combined with GFP targeted to ER

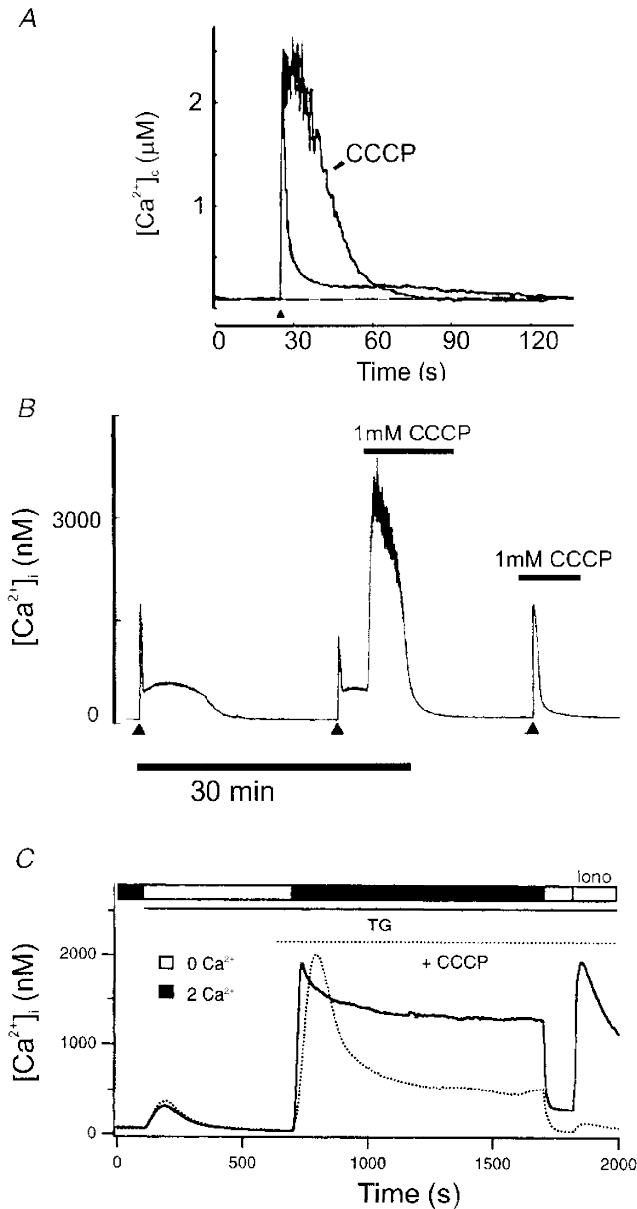


Figure 3. Impact of mitochondrial Ca^{2+} uptake on global $[Ca^{2+}]_c$ signalling in a variety of cell types

A, in a bovine adrenal chromaffin cell, a brief depolarization caused a transient rise and rapid recovery of $[Ca^{2+}]_c$ which was followed by a prolonged 'tail' phase of slow recovery (cf. Fig. 2*A*). Preventing mitochondrial Ca^{2+} uptake by prior depolarization of $\Delta\Psi_m$ with CCCP: (i) increased the amplitude of the response; (ii) greatly slowed recovery; and (iii) abolished the slow recovery phase (modified from Herrington *et al.* 1996, with permission). *B*, a similar pattern of $[Ca^{2+}]_c$ signal was seen in sensory neurons: a large $[Ca^{2+}]_c$ load following prolonged depolarization showed a biphasic recovery – a rapid phase followed by a prolonged secondary plateau. Depolarization of $\Delta\Psi_m$ by the application of CCCP during the plateau caused a massive increase in $[Ca^{2+}]_c$, suggesting Ca^{2+} release from Ca^{2+} -loaded mitochondria. Prior application of CCCP abolished the plateau phase completely (modified from Werth & Thayer, 1994, with permission). *C*, in a population of T lymphocytes, capacitative Ca^{2+} influx was studied thus: internal stores were emptied by inhibition of the ER Ca^{2+} ATPase with thapsigargin (TG) in the absence of external Ca^{2+} . Reintroduction of $[Ca^{2+}]_o$ caused a large sustained $[Ca^{2+}]_c$ signal. If Ca^{2+} was then again removed, a challenge with ionomycin revealed that an internal store, probably mitochondrial, had now been filled. With mitochondrial uptake disabled with CCCP (dotted line), the sustained component of the capacitative influx signal was much reduced, and the final ionomycin challenge confirmed the lack of mitochondrial Ca^{2+} accumulation. This, along with other data from the study, showed that mitochondrial uptake helps to define the characteristics of capacitative Ca^{2+} influx (modified from Hoth *et al.* 1997, with permission).

membranes and a separate aequorin construct targeted to the outer leaflet of the inner mitochondrial membrane (Rizzuto *et al.* 1998), have revealed a tight apposition of mitochondria with ER membranes and a functional exchange of ER Ca^{2+} release and mitochondrial Ca^{2+} uptake. This means that in cells that depend primarily on IP_3 -mediated Ca^{2+} signalling from ER (or perhaps, ryanodine-sensitive Ca^{2+} -induced Ca^{2+} release from SR), the anatomical organization of mitochondria results in the exposure of mitochondria to microdomains of high $[\text{Ca}^{2+}]_c$ close to ER Ca^{2+} release sites (Fig. 2B). This is important, as it implies that mitochondria within the cell may be exposed in some sites to a local $[\text{Ca}^{2+}]_c$ far higher than the averaged $[\text{Ca}^{2+}]_c$ measured across the cell. Thus attempts to extrapolate from studies of Ca^{2+} handling by isolated mitochondria in order to predict the functional consequences of mitochondrial Ca^{2+} uptake must consider the local not the global Ca^{2+} concentrations. Observations from our own laboratory also point to a tight local coupling of ER/SR Ca^{2+} release and Ca^{2+} uptake by nearby mitochondria. We have measured local changes in mitochondrial potential in cardiomyocytes and, more recently, in astrocytes in culture, and noted highly localized transient mitochondrial depolarizations which were dependent on local Ca^{2+} release from internal stores and subsequent mitochondrial Ca^{2+} uptake (Fig. 4A; Duchen *et al.* 1998; Jacobson & Duchen, 1998). Ultra-structural studies also suggest that the cellular organization of mitochondria may be arranged in relation to cell function: EM studies described by Lawrie *et al.* (1996) suggest that while mitochondria may be juxtaposed to ER in inexcitable cells, they are more likely to lie close to the plasmalemma in excitable cells. As far as I am aware, there are no clues to tell us how this organization is controlled.

The functional consequences of the local juxtaposition of mitochondria and ER/SR for $[\text{Ca}^{2+}]_c$ signalling are not so easy to predict. Intuitively, two quite opposing consequences suggest themselves: (i) mitochondria take up Ca^{2+} as it is released and therefore limit the spatial spread of the $[\text{Ca}^{2+}]_c$ signal, i.e. acting as a spatial buffer; (ii) the regulation of local $[\text{Ca}^{2+}]_c$ by strategically placed mitochondria may regulate local Ca^{2+} flux through Ca^{2+} -sensitive Ca^{2+} release channels and thus modulate $[\text{Ca}^{2+}]_c$ signalling, much as described above for I_{CRAC} . For example, the IP_3 (type 1) Ca^{2+} release channel is sensitive to $[\text{Ca}^{2+}]_c$, enhanced at low $[\text{Ca}^{2+}]_c$ ($< \sim 500$ nM), but inhibited as the Ca^{2+} concentration rises into the micromolar range, giving rise to a bell-shaped $[\text{Ca}^{2+}]_c/\text{Ca}^{2+}$ release curve (Iino, 1990; Bezprozvanny *et al.* 1991). Thus the local buffering of $[\text{Ca}^{2+}]_c$ by mitochondria may have a major impact on ER Ca^{2+} release. The peak of the Ca^{2+} release/ $[\text{Ca}^{2+}]_c$ curve lies around 400–500 nM, the value at which mitochondrial Ca^{2+} uptake becomes significant. As $[\text{Ca}^{2+}]_c$ rises to levels closer to 1 μM , channel opening is inhibited. Removal of local Ca^{2+} by mitochondria may thus serve to keep the channels open and therefore help sustain ER Ca^{2+} efflux, while the spatial orientation of mitochondria may support the dispersal of the Ca^{2+} in the cytosol away from the mouth of open Ca^{2+} -sensitive IP_3 -sensitive

channels. This is the mechanism proposed by Jouaville *et al.* (1995) to account for observations in *Xenopus* oocytes, in which the rate of propagation of $[\text{Ca}^{2+}]_c$ waves was modulated by varying mitochondrial potential – the larger the mitochondrial potential, the faster and more co-ordinated the wave (Fig. 4A). This might seem counter-intuitive – with a larger potential, mitochondrial Ca^{2+} uptake is more efficient and should serve as a more powerful buffer. However, by buffering microdomains of $[\text{Ca}^{2+}]_c$, mitochondria could regulate the rate of efflux from the ER Ca^{2+} release channels, pulling the $[\text{Ca}^{2+}]_c$ into a region in which the $[\text{Ca}^{2+}]_c$ dependence of Ca^{2+} release lay at the rising phase of the bell-shaped curve.

We have recently examined the role of mitochondrial Ca^{2+} uptake as a determinant of the rate of Ca^{2+} wave propagation in mammalian astrocytes, and have found the opposite – $[\text{Ca}^{2+}]_c$ wave propagation was clearly significantly faster when mitochondrial Ca^{2+} uptake was impaired (Boitier *et al.* 1999; Fig. 4B). Why should these similar experimental models yield different answers? One possibility is simply that, in the astrocytes, local $[\text{Ca}^{2+}]_c$ may not have reached levels at which ER Ca^{2+} release is impaired. Alternatively, the answer may lie in the properties of different classes of IP_3 receptors. Astrocytes predominantly express type 2 IP_3 -dependent ER Ca^{2+} release channels (Shepherd *et al.* 1997; E. Boitier, S. Brind & M. R. Duchen, unpublished observations) which do not show the same bell-shaped $[\text{Ca}^{2+}]_c$ sensitivity as the type 1 receptors (Ramos-Franco *et al.* 1998). Indeed, type 3 receptors also show a monotonic $[\text{Ca}^{2+}]_c$ sensitivity (Hagar *et al.* 1998). Thus, the consequence of this focal interaction between organelles may vary between cells, depending on the relative expression of these proteins. In the astrocyte, with type 2 receptors dominant, mitochondria act effectively as a spatial buffer, limiting the rate and extent of spread of a $[\text{Ca}^{2+}]_c$ signal. This may have significant implications for the propagation of Ca^{2+} signals during periods of anoxia/ischaemia in the CNS, during which mitochondrial potential will dissipate, and becomes particularly interesting in the light of recent demonstration that ‘death’ signals may also propagate through cellular networks (Lin *et al.* 1998).

A most intriguing contribution to this story has evolved recently, growing out of a number of related observations. It has been suggested that mitochondria may participate in the active propagation of a $[\text{Ca}^{2+}]_c$ signal across the cell through a process of Ca^{2+} -induced Ca^{2+} release (CICR; see Ichas & Mazat, 1998, for review). The mechanism that underpins this story is the mitochondrial permeability transition, or MPT (see above). While most of the relevant literature deals with pathological consequences of the MPT – with proposed roles for initiation of apoptosis, ATP depletion and reperfusion injury – Ichas *et al.* (1997) suggested that the MPT may open transiently at a low conductance state during normal $[\text{Ca}^{2+}]_c$ signalling. It is proposed that a rise in $[\text{Ca}^{2+}]_m$ and a resultant small fall in intramitochondrial pH might suffice to trigger transient

flickering openings of the channel in a low conductance state; once open, the accumulated Ca^{2+} is released, causing a local microdomain of high $[\text{Ca}^{2+}]_c$. This may trigger local CICR from ER or may be taken up by the next bank of quiescent mitochondria which will in turn release the Ca^{2+} . The authors were able to create a propagating, CsA-sensitive $[\text{Ca}^{2+}]$ wave through a 'lawn' of isolated mitochondria seeded

onto a coverslip, simply in response to a microdomain of $[\text{Ca}^{2+}]_c$ generated at one corner of the lawn, i.e. a wave of Ca^{2+} through the mitochondrial lawn was generated in the absence of any other components of the cytosol. This was, of course, a wave of $[\text{Ca}^{2+}]_m$, not the propagation of a cytosolic wave. Evidence for the role of this mechanism in cellular $[\text{Ca}^{2+}]_c$ signalling was based on the observations that the

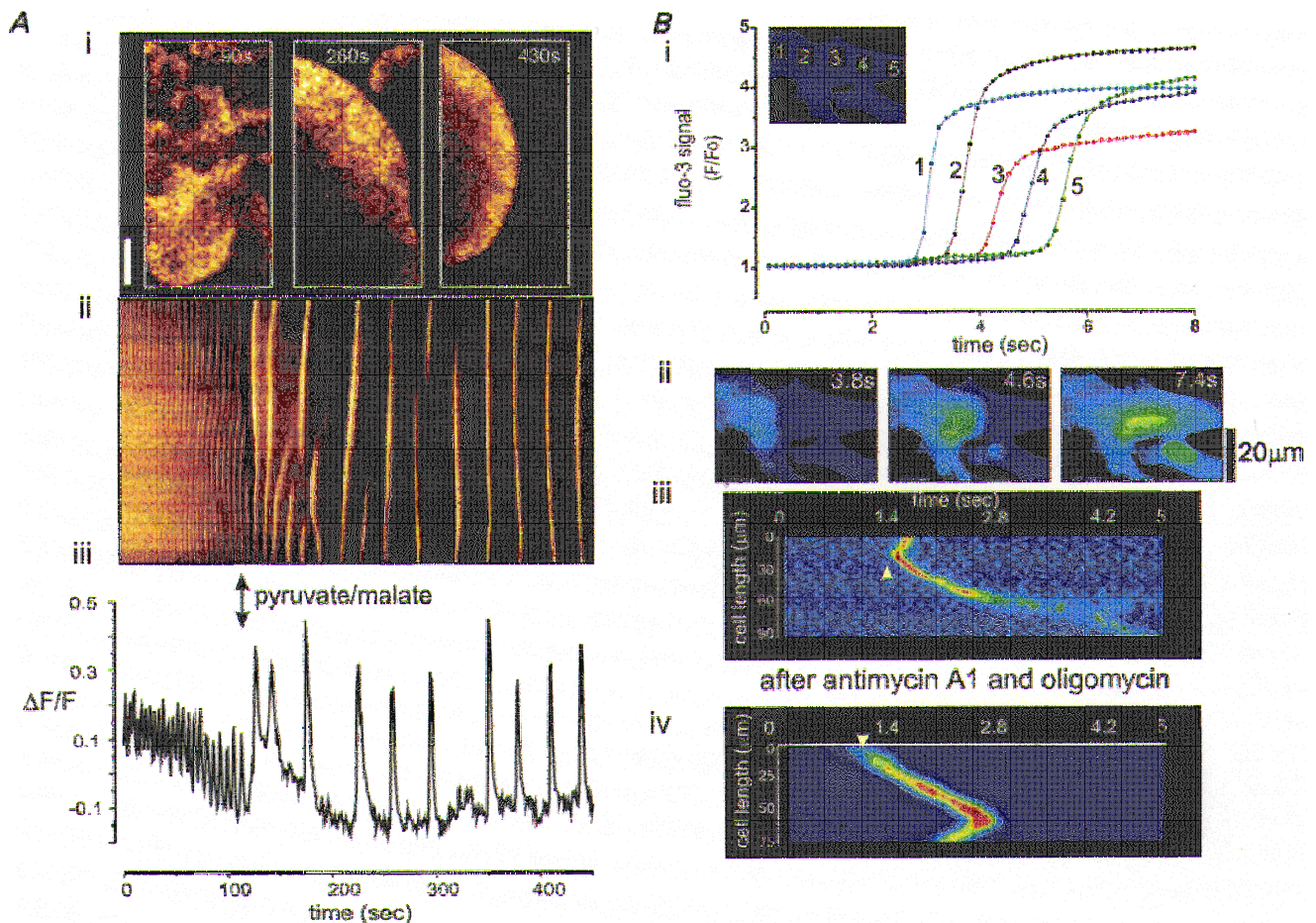


Figure 4. Impact of mitochondrial Ca^{2+} uptake on propagated $[\text{Ca}^{2+}]_c$ waves

A shows the effect of mitochondrial energization on the properties of $[\text{Ca}^{2+}]_c$ waves in *Xenopus* oocytes. $[\text{Ca}^{2+}]_c$ waves in a *Xenopus* oocyte triggered by flash photolytic release of a caged, non-hydrolysable analogue of IP_3 were initially chaotic and small in amplitude. Following microinjection of the mitochondrial substrates pyruvate and malate (at the time indicated by the arrows), mitochondrial potential increased (measured separately) and the waves became increasingly co-ordinated, larger in amplitude, and the rate of propagation increased. A(i) shows examples of the waves before (90 s) and after (at 240 and 480 s) injection of the substrates. A(ii) shows a line image to illustrate the characteristics of the waves with time, and a plot of amplitude with time for a single point in the cell is shown in (iii) (modified from Jouaville *et al.* 1995, with permission). B, mitochondrial Ca^{2+} uptake has opposite effects on the propagation of $[\text{Ca}^{2+}]_c$ waves in rat cortical astrocytes in culture. B(i) shows a plot of intensity with time at a series of equidistant points through a single cell. The images in B(ii) illustrate the spread of the $[\text{Ca}^{2+}]_c$ signal through the cell with time. To obtain the images shown in (iii) and (iv), which illustrate the propagating wave front, the image series was differentiated (a process of sequential subtractions that reveals a signal only in pixels in which the signal changed). A line was then selected along the axis of the cell and the colour-coded intensity profile along that line was plotted as a function of time, revealing the wave front as a diagonal band as it progressed across the cell with time. The rate of propagation of the wave clearly increased in cells in which mitochondrial Ca^{2+} accumulation was abolished by dissipating $\Delta\Psi_m$ (iv), using antimycin A1 combined with oligomycin, to limit mitochondrial ATP consumption (M. R. Duchen, R. Rea & E. Boitier, unpublished observations).

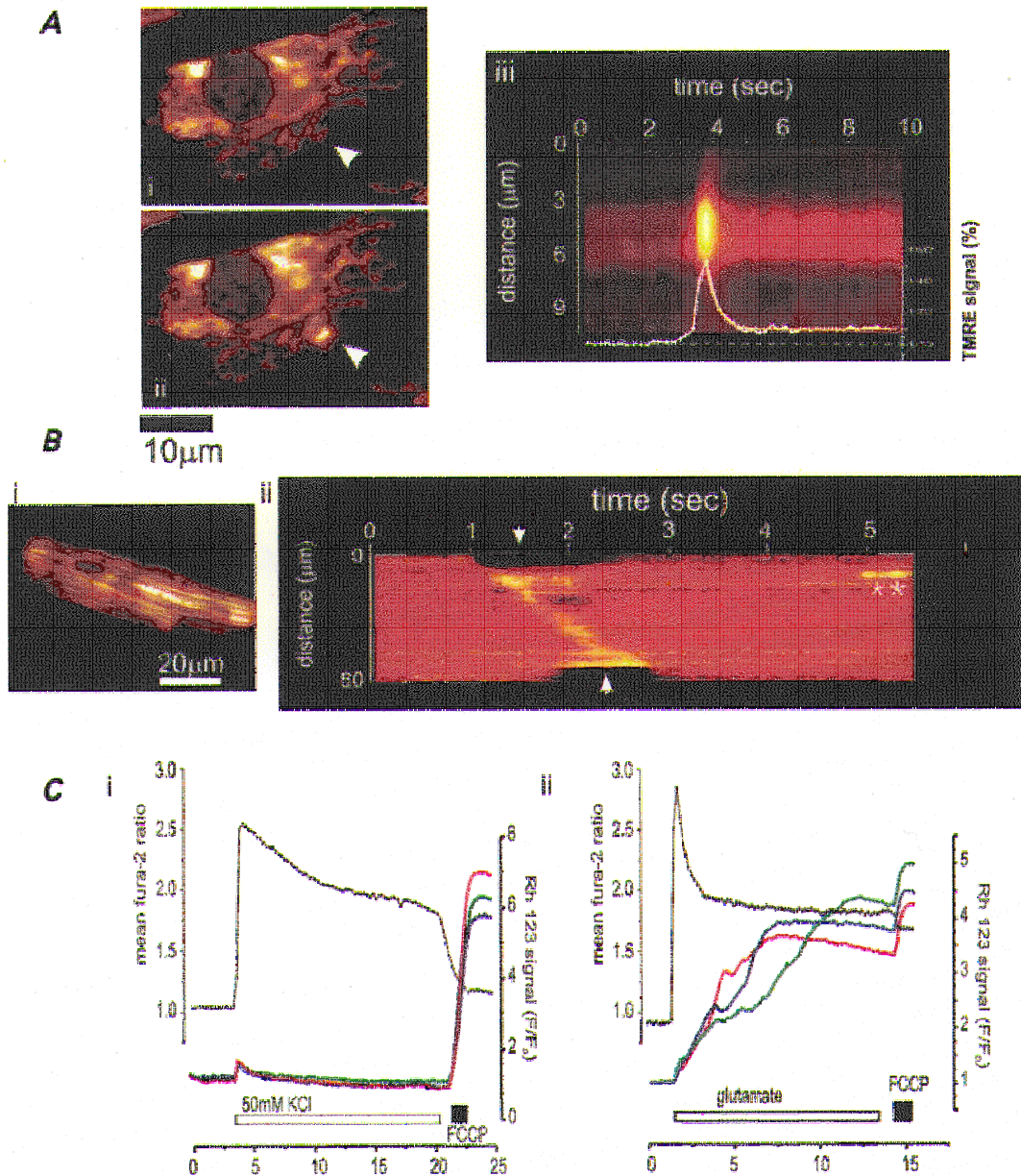


Figure 5. Impact of mitochondrial Ca^{2+} uptake on mitochondrial potential

A, microdomains of high $[\text{Ca}^{2+}]_i$ due to release from SR or ER cause brief transient depolarizations of mitochondria. These events were seen in single cells loaded with the potentiometric probe, tetramethyl rhodamine ethyl ester (TMRE), involving either clusters of mitochondria or even a single mitochondrion, as shown in (ii) in a neonatal cardiomyocyte in culture. The time course of the event is illustrated in (iii) in which the intensity profile along a line selected through the mitochondrion is shown with time. The mean intensity plotted with time is superimposed. These events were abolished by ryanodine or intracellular calcium chelation. *B*, a similar process operates during co-ordinated $[\text{Ca}^{2+}]_i$ signalling, illustrated during a $[\text{Ca}^{2+}]_i$ wave in a cardiomyocyte (also loaded with TMRE). A $[\text{Ca}^{2+}]_i$ wave in these cells causes a contractile wave which was associated here with a wave of mitochondrial depolarization, seen as an increase in fluorescence signal. The line image (ii) illustrates the evolution of the signal with time along a line selected along the axis of the cell (shown in (i), white line). The contraction started first at the upper margin (the top left in (i) and propagated along the length of the cell. The contraction can be seen at the edges of the line image (arrows) as the line extended beyond the edges of the cell (modified from Duchen *et al.* 1998, with permission). Two spontaneous transient events of similar amplitude as the wave were seen later (*). *C* shows simultaneous measurements of $[\text{Ca}^{2+}]_i$ and $\Delta\Psi_m$ made in rat hippocampal neurons in culture using fura-2 (●) and rhodamine 123 (continuous lines). (i), depolarization of the plasma membrane with 50 mM KCl for 10 min raised $[\text{Ca}^{2+}]_i$ but caused only a small transient mitochondrial depolarization. (ii), depolarization for 10 min with glutamate under conditions that caused 75% cell death 24 h later, also raised $[\text{Ca}^{2+}]_i$ but

amplitude of cytosolic $[Ca^{2+}]_c$ signals were modulated by CsA, which inhibits the MPT. It would be nice to be completely confident that the CsA was doing nothing else in the cell, as CsA is also a potent inhibitor of the Ca^{2+} -dependent phosphatase, calcineurin, but the possibility that mitochondria may play an active role in $[Ca^{2+}]_c$ signalling, at least in some cells, remains highly intriguing.

At the very least, it should be clear from the arguments presented that mitochondria play a significant, although varied, role in shaping cellular $[Ca^{2+}]_i$ signals. $[Ca^{2+}]_i$ signalling is so central to all normal physiological functions in all cell types that any disturbances in the subtleties of $[Ca^{2+}]_i$ signalling that result from mitochondrial dysfunction will inevitably have a substantial impact on cell function, and more broadly, on the integrative function of more complex organ systems.

Consequences of mitochondrial Ca^{2+} uptake for mitochondrial function: from cell signalling to cell death

The most immediate consequence of mitochondrial Ca^{2+} uptake is a mitochondrial depolarization. This is most probably simply the consequence of the flow of a Ca^{2+} current, as the uniporter is electrogenic. It is seen as a response to the addition of Ca^{2+} to preparations of isolated mitochondria, and is seen in intact cells as a response to physiological $[Ca^{2+}]_c$ signals. Transient mitochondrial depolarizations have been described in excitable cells in response to depolarization in sensory neurons (Duchen, 1992), hippocampal neurons in slices (Bindokas *et al.* 1998) or in culture (Keelan *et al.* 1998 and see Fig. 5C) and in response to a propagating $[Ca^{2+}]_c$ wave in cardiomyocytes (Fig. 5B; Duchen *et al.* 1998), and have also been seen in inexcitable cells in response to mobilization of ER Ca^{2+} (Peuchen *et al.* 1996; Boitier, Rae & M. R. Duchen, submitted). Recently we have also described focal mitochondrial depolarizations in relation to focal SR/ER Ca^{2+} release (Duchen *et al.* 1998; Fig. 5A), a functional consequence of the close apposition of ER Ca^{2+} release sites and mitochondria.

The depolarization of $\Delta\Psi_m$ in response to a rise in $[Ca^{2+}]_c$ lies at the borderlines between mitochondrial physiology and mitochondrial pathology. It has been clear for many years that mitochondrial Ca^{2+} accumulation during a state of oxidative stress may precipitate opening of the megachannel or mitochondrial permeability transition pore MPT (see above, and Lemasters *et al.* 1998). While flickering subconductance states may play roles in $[Ca^{2+}]_c$ signalling (Ichas *et al.* 1997), the MPT generally represents a catastrophe for the cell and will lead inexorably to cell death, either through ATP consumption, acute energy failure and necrosis or through the initiation of apoptosis.

The latter may be driven by the release of apoptosis-inducing factor (AIF; Kroemer *et al.* 1998) or of cytochrome *c* (see Liu *et al.* 1996) or both, leading to caspase (cysteine protease) activation and a suicidal (apoptotic) cascade. It seems increasingly likely that a key step in several routes to programmed cell death involves the release of mitochondrial cytochrome *c* from the intermembrane space and activation of caspases, initiating a positive feedback cycle in which caspase activation releases more cytochrome *c* or AIF, which in turn triggers the permeability transition (i.e. pore opening), and drives a relentless progression to cell death. There is some controversy at present about the exact sequence – perhaps even by the time this essay has appeared on paper the answer will be known, so fast is this field evolving (for recent reviews, see Kroemer *et al.* 1998; Green & Reed, 1998). It is also perhaps worth remarking on the paradox embedded in this story: apoptosis is an ATP-dependent process and yet opening of the megachannel is likely to lead rapidly to ATP depletion, much as seen in response to an uncoupler (see above). How then is the MPT compatible with a role in apoptosis? Perhaps initiation of apoptosis requires only flickering transient openings of the megachannel sufficient to release some cytochrome *c*. Perhaps the full opening of the megachannel would lead rather to ATP depletion, energetic collapse and necrotic cell death. Perhaps apoptosis will only result from MPT if mitochondrial ATP consumption is somehow limited. Clearly, the details of this process remain to be clarified. It is also important to emphasise that the interpretation of experimental evidence in favour of a role of the MPT in apoptotic cell death relies heavily on the use of pharmacological agents, such as cyclosporin A, which itself is not specific, and to measurements of $\Delta\Psi_m$ which are fraught with inaccuracies and misinterpretations, and so this remains an area in which it is necessary to tread carefully.

Most of the models of apoptotic cell death involve the application of toxic agents to or the withdrawal of growth factors from cell lines in culture. Perhaps the most actively studied model that bears an obvious relationship to cell death in a common human pathology involves glutamate toxicity. During periods of anoxia and ischaemia in the CNS, during the evolution of a stroke or following neonatal asphyxia, for example, glutamate accumulates in the extracellular space. The toxicity of glutamate under these conditions has been recognized and attributed to a cellular Ca^{2+} overload for many years (see Choi, 1994). However, recent evidence suggests that a key process that links the rise in $[Ca^{2+}]_c$ to toxicity is mitochondrial Ca^{2+} accumulation and resultant mitochondrial depolarization. The loss of mitochondrial potential in this model is clearly linked to cell death, and may result from MPT, but the evidence for a role

caused a progressive and very nearly complete dissipation of $\Delta\Psi_m$ (note that the response could barely be increased further by application of FCCP; J. Keelan, O. Vergun & M. R. Duchen, unpublished observations, but see Keelan *et al.* 1998).

of the MPT in this model is still weak, and other processes – notably inhibition of the respiratory chain – could produce similar consequences (Fig. 5C; Schinder *et al.* 1996; Nieminen *et al.* 1996; Khodorov *et al.* 1996; Castilho *et al.* 1998; Keelan *et al.* 1998). A similar process may take place in the heart following reperfusion after an ischaemic episode – again conditions in which mitochondrial Ca^{2+} loading will be combined with oxidative stress, leading to MPT and cell death (Duchen *et al.* 1993a; Griffiths & Halestrap, 1995).

Why should the cell place such fundamental decisions about life and death in the hands of the mitochondrial interlopers? Perhaps the key lies in the heart of the evolutionary history of the organelles. Although mitochondria have become obligate symbionts, dependent on the cellular machinery for production of many proteins, they still retain many features of their bacterial origins. The pore that underlies the ‘permeability transition’ consists of a protein complex that includes at least VDAC – the voltage-dependent anion channel that has a strong homology to bacterial porins, situated in the outer mitochondrial membrane – and the adenine nucleotide translocase (ANT), which sits in the inner mitochondrial membrane but especially at contact sites between inner and outer membranes. Cyclophilin D and the benzodiazepine receptor are also attached to this complex and appear to regulate pore opening (Crompton *et al.* 1998). Apparently in close association with, or even a constituent of the complex, is the proapoptotic protein bax (Marzo *et al.* 1998), a channel-forming protein reminiscent of the bacterial pore-forming colicins, which are used by bacteria for self defence. It seems likely that the anti-apoptotic proteins bcl-2 and bcl-x_L also act as mitochondrial pores or regulators (Vander Heiden *et al.* 1997). Is it not remarkable that if you wish to express bax in bacteria you must also express bcl-2 or the bacteria die? Why cytochrome c should have become a major trigger to cell death is harder (for me) to understand but perhaps its presence in the cytosol informs the cell that its mitochondria are damaged, that energetic function will become compromised and the mitochondria may become a source of free radicals and oxidative injury (see also Skulachev, 1996). As oxidative stress may promote DNA damage and possible neoplastic transformation, it is perhaps safer for the cell to commit suicide than to put the host organism at risk.

CODA

Mitochondria have become recognized as fascinating structures that have a major impact on the physiology and pathophysiology of the organism that they inhabit at many levels. Their relative inaccessibility and the difficulties involved in trying to study them in their native habitat – the cytosol – has delayed much of this understanding and they still have many secrets to yield. What is perhaps most fascinating is the combination of a curiously wild and independent streak, a residue of their independent bacterial history, with an extraordinary integration with cellular specializations. However, why do mitochondrial structures

and shape vary between one cell type and another? To what extent are mitochondrial functions different in different cell types? How do cells regulate mitochondrial numbers, morphology, function? And to what extent are changes in mitochondrial function at the heart of many diseases?

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Corresponding author

M. R. Duchen: Department of Physiology, University College London, Gower Street, London WC1E 6BT, UK.

Email: m.duchen@ucl.ac.uk