Mitochondria and Neuronal Survival

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mechanisms by which isolated mitochondria generate ATP, sequester Ca\(^{2+}\), generate reactive oxygen species, and undergo Ca\(^{2+}\)-dependent permeabilization of their inner membrane are currently being applied to the function of mitochondria in situ within neurons under physiological and pathophysiological conditions. Here we review the functional bioenergetics of isolated mitochondria, with emphasis on the chemiosmotic proton circuit and the application (and occasional misapplication) of these principles to intact neurons. Mitochondria play an integral role in both necrotic and apoptotic neuronal cell death, and the bioenergetic principles underlying current studies are reviewed.

I. INTRODUCTION

The first great wave of mitochondrial research stretched from the 1940s, with the pioneering work of Lehninger, Chance, Slater, Ernster and others through the “chemiosmotic revolution” initiated by Peter Mitchell, to the early 1980s. During this period, the basic mitochondrial functions were identified and characterized with increasing precision, and the organelle emerged as a beautifully self-regulating machine for generating ATP (see Ref. 416 for review). In view of the central role played by mitochondria in cellular metabolism, it was assumed that the organelle was highly reliable and associated with few disease states. Indeed, almost the only mitochondrial dysfunction known was the extremely rare Luft’s disease (344) in which “loose coupling” was detected in mitochondria from a hypermetabolic patient. Mitochondrial research therefore became increasingly focused on the elucidation of the molecular machinery of the proton pumps. However, in recent years, there has been a resurgence of interest in the functional aspects of mitochondria with the realization that, far from being perfect machines, mitochondria are fragile structures operating near their physicochemical limits, whose dysfunction appears to underlie a host of degenerative disease states in the brain. Furthermore, it has recently been discovered that mitochondria have an additional role, participating directly in the signaling pathways that culminate in apoptosis.

A complex and disparate variety of factors can influence cell survival, but in this review we focus on bioenergetic aspects, and in particular how mitochondrial function controls cell survival. Initial sections review the bioenergetics of mitochondria both in isolation and within the healthy cell. This is followed by discussion of the application of these studies to pathophysiological and degenerative conditions. To keep this review within manageable bounds, the emphasis is on neurons and neuronal mitochondria, but general principles obtained with other cell types are also discussed. Related topics including mitochondrial mutation (105, 187, 199, 334, 505, 506, 510, 626) and mitochondria and aging (126, 187) have been recently reviewed and are not covered here.

II. MITOCHONDRIAL PROTON CIRCUIT

Mitochondria exert a multifactorial influence on cell function. In addition to ATP synthesis they can accumulate Ca\(^{2+}\) whenever the local cytoplasmic free Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_{c}\)) rises above a critical “set point” (11, 265, 410); under some conditions this can result in a nonspecific permeabilization of the inner membrane, the “mitochondrial permeability transition” (MPT) (for review, see Refs. 42, 634). In addition, changes in mitochondrial Ca\(^{2+}\) can regulate tricarboxylic acid cycle enzymes (367). The mitochondrial respiratory chain is also the major site for the generation of superoxide radicals (O\(_{2}^{-}\)) (for review, see Refs. 534, 575). Each of these parameters is influenced by, and can in turn influence, the mitochondrial membrane potential (Δ\(ψ_{m}\)). Through the ATP/ADP pool, mitochondria can influence glycolysis, the activity of Ca\(^{2+}\)- and Na\(^{+}\)-K\(^{+}\)-ATPases at the plasma membrane and consequently the activity of Na\(^{+}\)-coupled plasma membrane transporters.

Because the manipulation of a single mitochondrial parameter may affect many cellular functions, experiments must be designed with care. It follows that an understanding of the basic bioenergetics of isolated mitochondria is essential to be able to monitor and manipulate mitochondrial function in the much more complex environment of the intact cell.

A. Isolated Mitochondria

Electrons from NADH enter complex I of the respiratory chain (Fig. 1) at a redox potential of −300 mV and emerge to reduce a membrane-associated ubiquinol/ubiquinone (UQH\(_{2}/UQ\)) pool close to 0 mV. Electrons from flavin-linked dehydrogenases (such as succinate dehydrogenase) have an insufficiently negative redox potential to enter complex I and instead reduce the UQH\(_{2}/UQ\) pool via complex II. Ubiquinol transfers electrons to complex III, which in turn reduces cytochrome c (at a redox potential of approximately +250 mV). Cytochrome c then reduces the terminal acceptor complex IV (also called cytochrome-c oxidase) which then transfers four electrons to molecular oxygen. Complexes I, III, and IV function as proton pumps, acting in series with respect to the electron flux and in parallel with respect to the proton...
The fall in redox potential of the electrons passing through these complexes is used to generate a proton electrochemical potential gradient, \( \Delta \mu_H^- \), usually expressed in electrical potential units as the proton-motive force (\( \Delta p \)).

\[
\Delta p \text{ (mV)} = \Delta \psi_m - (2.3RT/F)\Delta pH.
\]

(At 37°C, \( \Delta p = \Delta \psi_m - 60\Delta pH \)) \((I)\)

where \( \Delta \psi_m \) is the mitochondrial membrane potential (a positive value corresponding to a negative matrix), \( \Delta pH \) is the pH gradient across the inner membrane (a positive value indicating an acidic matrix), and \( R, T \) and \( F \) refer to the gas constant, the absolute temperature, and the Faraday constant, respectively. Under most conditions, \( \Delta \psi_m \) is the dominant component of \( \Delta p \), accounting for 150–180 mV of the total proton-motive force of 200–220 mV \( (387, 408) \). A fourth component of the inner mitochondrial membrane, the energy-linked transhydrogenase \( (237) \), utilizes \( \Delta p \) to maintain a high level of reduction of the matrix NADP(H) pool by driving the following reaction to the right.

\[
\text{NADH} + \text{NADP}^+ \overset{\Delta p}{\rightarrow} \text{NAD}^+ + \text{NADPH} \quad (2)
\]

In a typical mitochondrion, the NAD pool is \( \sim 10\% \) reduced while the NADP pool is \( >90\% \) reduced \( (493) \).

The ATP synthase is the dominant pathway for the reentry of protons into the mitochondrial matrix. The proton-motive force forces this ATP-hydrolyzing proton pump to run in reverse and synthesize ATP. ATP is not a
“high-energy compound”; rather, the mitochondrion holds the ATP synthesis reaction (ADP + P_i = ATP) up to 10 orders of magnitude away from equilibrium (408, 482). The Gibbs free energy (ΔG) for a reaction in a cell is simply a measure of this displacement from equilibrium; at 37°C, this means that the ΔG for ATP synthesis in the cytoplasm is close to +60 kJ/mol.

An instructive analogy is to compare the proton circuit to an equivalent electrical circuit (Fig. 1), where Δp is the potential term, the effective proton conductance of the inner membrane corresponds to the conductance (reciprocal resistance) of a component, and the consequent proton current flowing round the circuit is governed by Ohm’s law (for review, see Ref. 416). Because the proton circuit is completed by the reentry of protons through the ATP synthase, which in turn is tightly coupled to ATP synthesis, it follows that the proton current responds automatically to ATP demand. Tight coupling between electron flux and proton pumping in each respiratory chain complex means that electron flux (and hence respiration) is in turn controlled by ATP demand, the phenomenon of respiratory control. The fixed H^+ /2e^- stoichiometry of the individual respiratory chain complexes means that the proton current can be calculated from the respiratory rate multiplied by the H^+ /2e^- (406). Because the potential term Δp can be calculated (see sect. iiiA), application of Ohm’s law allows the effective proton conductance of the membrane to be derived under a wide range of experimental conditions (406, 409).

Isolated mitochondria respire in state 4 (the state when there is no extramitochondrial ATP hydrolysis and hence no proton reentry via the ATP synthase) due to a constitutive proton leak across the inner membrane. This leak is highly potential dependent (“nonohmic”), being maximal in state 4 and dropping to almost zero in state 3 (the state when extramitochondrial ATP turnover and hence proton reentry via the ATP synthase is maximal) (409, 425, 480). The proton leak limits the respiratory control ratio (state 3 divided by state 4 respiration) to between 5 and 10 depending on the substrate (408, 409, 480). Substrates such as succinate that feed electrons into complex II generate a significantly higher Δp than complex I substrates and in consequence display a higher nonohmic proton leak (408). The nonohmic leak plays a major role in controlling basal metabolic rate (480) and in addition may limit the production of potentially dangerous reactive oxygen species (ROS) (534). The respiratory chain complexes are efficient transducers of redox potential into Δp; indeed, complexes I and III (but not IV) operate close to thermodynamic equilibrium. This means that changes in Δp affect the redox state of components within these complexes, and this in turn has important consequences for the cell’s susceptibility to oxidative stress (see sect. viD).

As with an electrical circuit, Δp drops slightly when the proton current is increased (409). The only communication between the respiratory chain and the ATP synthase is via the proton circuit, and it is the difference between the “static head” Δp, at which there would be no proton pumping, and the actual Δp that is responsible for controlling respiration. With isolated mitochondria, Δp in state 3 is 10–15% lower than in state 4 (387, 406). Although this difference is small, it can be further reduced in many cells under conditions of high metabolic activity by a Ca^2+-mediated activation of key dehydrogenases (for review, see Refs. 211, 367). This generates an enhanced reduction of NADH, increasing the static head Δp to compensate for the greater disequilibrium required to drive the more rapid ATP generation (479).

Protonophores can be used to titrate the proton conductance of the membrane, and in excess can virtually collapse Δp, leading to maximal, uncontrolled respiration (387, 409). The ATP synthase will reverse in the presence of a protonophore, hydrolyzing extramitochondrial ATP; as will be discussed in the cellular context, protonophores are a highly effective means of depleting extramitochondrial ATP.

Mitochondria possess an inherent monovalent cation/H^+ exchanger that prevents Na^+ or K^+ accumulation in the matrix in response to the high Δψ_m (386, 407). The ionophore nigericin (465) is a K^+ /H^+ -selective exchanger that in high K^+ media (equivalent to a physiological cytoplasm) can decrease ΔpH and cause a compensatory increase in Δψ_m. The K^+ uniport ionophore valinomycin collapses Δψ_m in high K^+ media and causes massive swelling.

B. Proton Circuit and the Intact Cell

Mitochondrial and cellular bioenergetics are intimately interlinked (Fig. 2). In neurons, the dominant mitochondrial substrate is pyruvate derived from glycolysis (230). Mitochondrial respiration is controlled by ATP turnover (“respiratory control”), which in neuronal cells is primarily a consequence of the ATP demand for plasma membrane ion pumping. The rate of glycolysis is controlled by mitochondrially generated ATP via the Pasteur effect (445); in neuronal preparations, inhibition of oxidative phosphorylation can lead to a 10-fold enhancement in the rate of glucose utilization (159, 160, 283). Importantly, a collapse in Δp will lead not only to a cessation of mitochondrial ATP synthesis, but to a rapid hydrolysis of cytoplasmic ATP as the ATP synthase reverses in an attempt to restore Δp. This can lead to profound depletion of ATP. Finally, to compound the complexity, the design of experiments to investigate mitochondrial function in intact cells must overcome the relative inaccessibility of the in situ mitochondria and the presence of other compartments and membrane systems.
The isolated nerve terminal (synaptosome) preparation retains an intact plasma membrane, cytoplasm, mitochondria, associated metabolic pathways, and all the machinery for the uptake, storage, and release of neurotransmitter (for reviews, see Refs. 160, 372, 411, 606). Although the synaptosomal preparation is enriched in presynaptic nerve terminals and not representative of an intact neuron, it represents one of the simplest preparations in which to model neuronal mitochondrial/cellular interactions. However, it must be kept in mind that synaptic and nonsynaptic mitochondria differ somewhat in their respiratory capacities; notably, synaptic mitochondria have a lower threshold before inhibition of complex I restricts state 3 respiration (115).

Respiration, and thus the proton current flowing across the inner membrane of the in situ mitochondria, is one of the few bioenergetic parameters that is not more difficult to determine in synaptosomes than in isolated mitochondria. The respiratory stimulation produced by protonophores indicates that the in situ mitochondria within rat cortical synaptosomes have 500% spare respiratory capacity (160, 517). The Na\(^+\)-K\(^+\)-ATPase is the major ATP-requiring process, thus its inhibition with ouabain substantially inhibits basal respiration (160, 272, 517), while addition of veratridine to prevent voltage-activated Na\(^+\) channels from inactivating results in a 300% increase in respiration driving the rapid cycling of Na\(^+\) and K\(^+\) across the plasma membrane (156, 160, 272, 517, 585, 588). The residual respiration in the presence of oligomycin (which inhibits the ATP synthase) can be used to quantify the proton leakage across the mitochondrial membrane and can detect classic “uncoupling” of the in situ mitochondria.

Accurate estimation of the \(\Delta G\) for the cytoplasmic adenine nucleotide pool is difficult. The \(\Delta G\) is a function of free \([ATP]/([ADP][P_i])\) in the cytoplasm rather than the ATP level per se. Whole cell ATP/ADP greater than \(\sim 5\) generally reflect a healthy synaptosomal preparation (73, 160), whereas ratios approaching 15:1 are characteristic of primary cultures of neurons and glia (526). However, it must be kept in mind that these values are a combination of the mitochondrial and cytoplasmic pools.

C. Use of Ionophores to Probe Mitochondrial Function in Intact Cells

The mitochondrial membrane potential \(\Delta \psi_m\) (or the proton-motive force \(\Delta p\)) is the central parameter controlling three fundamental cellular processes: ATP synthesis, mitochondrial Ca\(^{2+}\) sequestration, and the generation of ROS. Conversely, \(\Delta \psi_m\) is itself controlled by substrate availability, ATP demand, respiratory chain capacity, mitochondrial proton conductance, and mitochondrial Ca\(^{2+}\) sequestration. In this and subsequent sections we review experimental protocols that allow many of these parameters to be independently modulated.
Because ionophores induce ion permeabilities in lipid bilayers, they display virtually no membrane selectivity (465). Ionophores may induce relatively nonselective cation channels in membranes (e.g., gramicidin) or may be selective mobile carriers catalyzing the electrogenic uniport of a single ion [e.g., protonophores (H+) or valinomycin (K+ or Rb+) or the electroneutral exchange of two species [e.g., K+/-H+ (nigericin) or Ca2+/2H+ (ionomycin or A-23187)].

Protonophores have been widely employed in the cellular context to depolarize mitochondria (e.g., Refs. 118, 173, 292, 293, 392, 448, 449, 539, 549, 550, 559, 566, 598). However, protonophore addition to an intact cell has multiple consequences; the ionophore will acidify the cytoplasm by equilibrating protons across the plasma membrane (569, 597) and deplete synaptic vesicles of their contents, particularly amino acids, by collapsing the transvesicular Δp (80, 419, 599). At the mitochondrion, the protonophore will collapse Δψm (inhibiting mitochondrial Ca2+ accumulation and releasing any accumulated Ca2+), reverse the ATP synthase (leading to a rapid hydrolysis of cytoplasmic ATP, Ref. 73), inhibit the generation of O2−, and perhaps induce the MPT. Finally, the decreased cytoplasmic ATP may prevent Na+ and Ca2+ extrusion from the cell and lead to a subsequent failure of glycolysis if ATP levels are below those needed by hexokinase. For a cell to survive in the presence of protonophore, the capacity of glycolytic ATP generation must therefore exceed that required by the cell plus that consumed by the reversed ATP synthase; this only seems to be the case for cells with a limited population of mitochondria.

Great care must be taken in the use of other ionophores in the cellular context. The K+ uniport ionophore valinomycin cannot be used with intact cells unless it is the intention to swell and depolarize the in situ mitochondria (184, 489). Although nigericin (465) collapses intracellular ΔpH gradients between compartments containing an equal concentration of K+ and consequently allows mitochondria to hyperpolarize (604), the K+/Na+ selectivity for nigericin is only 25–45 (461), allowing high concentrations of the ionophore (>1 μM) to depolarize the plasma membrane, dissipate the Na+ and K+ gradients across the plasma membrane, and short-circuit the Na+-K+-ATPase (158, 160).

D. Respiratory Chain Inhibition in Intact Cells

A variety of inhibitors exist selective for each respiratory chain complex (Fig. 1). Because respiratory chain inhibitors target specific mitochondrial components, they are free of the inherent membrane nonspecificity characteristic of ionophores, although some complex IV inhibitors have additional, nonmitochondrial, sites of action. Inhibition of any one complex will block electron transfer through the entire respiratory chain. Complex III inhibition (by, for example, antimycin A or myxothiazol) or complex IV inhibition (by, for example, azide, cyanide, carbon monoxide, or NO+) will inhibit electron flow to the terminal oxidase. Even though two separate complexes (I and II) reduce complex III, inhibition of either will affect respiration in an intact cell, since the supply of succinate for complex II is dependent on preceding NAD+-linked dehydrogenases in the tricarboxylic acid cycle, whereas inhibition of succinate dehydrogenase or complex II will also block the cycle, limiting the generation of NADH.

Because redox components upstream of the site of inhibition will become reduced, whereas those downstream will be oxidized, the inhibitors can be used to control the redox state of specific regions of the respiratory chain. This is of particular interest in complex III, where myxothiazol inhibits upstream of the ubisemiquinone site, which is the major source of mitochondrial O2−, whereas antimycin A inhibits downstream, and, by increasing the occupancy of this site, increases O2− generation (576). The immediate bioenergetic consequences of respiratory chain inhibition are largely independent of the site of inhibition. Because respiratory chain-linked proton extrusion ceases, Δp decays, stopping ATP synthesis and allowing the ATP synthase to reverse. The resultant hydrolysis of cytoplasmic ATP occurs more slowly than in the presence of protonophore (see sect. μC) since the inner membrane retains its low permeability to protons. This slow hydrolysis of ATP is sufficient to maintain Δψm at levels only slightly lower than in the absence of inhibitor (517).

In synaptosomes, rotenone inhibition of complex I (517) or 3-nitropropionic acid (3-NPA) inhibition of complex II (157) leads to a fall in ATP and phosphocreatine levels. The ability of a cell to maintain function in the presence of respiratory chain inhibitors appears in the short term to be governed by the ability of glycolysis to supply sufficient ATP for cell metabolism as well as for this ATP synthase reversal. As any spare ATP-generating capacity of the cell is depleted, the susceptibility of cells to factors that increase ATP demand is enhanced proportionately.

E. Mitochondrial ATP Synthase Inhibition in Intact Cells

Oligomycin is a selective inhibitor of the Fo proton well of the mitochondrial ATP synthase (270), although high concentrations may also inhibit the plasma membrane Na+-K+-ATPase (169). Because both synthesis and hydrolysis of ATP by the mitochondrion are prevented, the organelle will not consume ATP regardless of subsequent bioenergetic manipulations. Oligomycin thus allows the key mitochondrial function of ATP synthesis to be
selectively inhibited, and the cell will survive as long as the glycolytic capacity of the cell is sufficient to maintain ATP in the absence of oxidative phosphorylation. Mitochondrial membrane potential is not only maintained in the presence of oligomycin but is increased by a few millivolts (46, 143, 489, 517) due to the inhibition of proton reentry via the ATP synthase. As a consequence, \( \Delta \psi_m \), dependent functions including mitochondrial Ca\(^{2+} \) transport and the generation of O\(_2^· \) will be maintained, or even increased as a consequence of the slight mitochondrial hyperpolarization. Most importantly, the further addition of a protonophore or respiratory chain inhibitor will not a priori cause a depletion of ATP, allowing \( \Delta \psi_m \) to be manipulated without the complications of bioenergetic failure (73, 74, 141, 188, 325).

The ability of cells to maintain bioenergetic homeostasis in the presence of oligomycin will depend on their glycolytic capacity. Cerebellar granule cells in culture have a high glycolytic capacity and can be maintained for several hours in the presence of the inhibitor (73, 74). However, cultured hippocampal neurons in some studies (63, 139, 508, 539) but not others (62) show signs of energetic limitation in the presence of the inhibitor.

### III. MITOCOCHONDRIAL MEMBRANE POTENTIAL, pH GRADIENT, AND PROTON-MOTIVE FORCE

#### A. Isolated Mitochondria

The membrane potential \( \Delta \psi_m \) is the mitochondrial parameter most frequently estimated in intact cells. We first review the methodologies that have been employed with isolated mitochondria, while bearing in mind that their extrapolation to intact cells may not always be valid.

The two components of \( \Delta \psi_m \) are determined separately. The \( \Delta \psi_m \) value is estimated from the gradient of a permeable cation across the inner membrane. Initially, K\(^+ \) or Rb\(^+ \) was employed in a low K\(^+ \) medium in the presence of valinomycin (387, 408). Subsequently, membrane-permeant phosphonium cations, triphenylmethylphosphonium (TPMP\(^+ \)), or tetrathylphosphonium (TPP\(^+ \)), were used, either as isotopes (543) or in combination with a selective phosphonium electrode (273).

To calculate \( \Delta \psi_m \) from the phosphonium ion accumulation, it is necessary to determine the matrix volume and probe binding. For this purpose, we have established a correction from diffusion potentials generated by valinomycin in the presence of defined extramitochondrial K\(^+ \) concentrations (609). The \( \Delta p \) is calculated from these experimentally determined values (Eq. 1).

Cationic membrane-permeant fluorescent probes have been much employed to monitor \( \Delta \psi_m \). Their use with isolated mitochondria is dependent on the quenching of their fluorescence due to stacking of the probe molecules at the high concentrations achieved within the polarized mitochondrial matrix (339). Under these conditions, the total fluorescence of a suspension of mitochondria will decrease if the organelles hyperpolarize and more probe is sequestered within the matrix. Because this technique depends largely on an empirical aggregation or stacking of the probe, results are highly dependent on dye concentration, and results are generally calibrated by reference to K\(^+ \) diffusion potentials obtained in the presence of valinomycin. Furthermore, great care must be taken to ensure that mitochondrial function is not compromised by the accumulated probe; for example, the cationic cyanine dye 3,3‘-dihexyloxycarbocyanine iodide [DiOC\(_6\)(3)] has been reported to be a particularly potent inhibitor of mitochondrial complex I (50% inhibition by <10 nM dye equilibrated with cells; Refs. 124, 488, 489).

In the presence of physiological concentrations of phosphate and Mg\(^2+ \), isolated mitochondria from liver, heart, brown fat, or brain (387, 408, 543) maintain a total \( \Delta p \) of some 220 mV in state 4, of which the mitochondrial potential \( \Delta \psi_m \) comprises 150–180 mV with a \( \Delta p \) of −0.5 to −1 pH unit contributing the remaining 30–60 mV (Eq. 1). In the presence of excess phosphate, \( \Delta p \) remains small and \( \Delta \psi_m \) generally shadows \( \Delta p \). This may justify the general failure to consider the \( \Delta p \) component in discussions of in situ mitochondrial energization, although as discussed in section 7.4 there are conditions associated with Ca\(^{2+} \) accumulation where this relationship falls down.

#### B. In Situ \( \Delta \psi_m \) and \( \Delta p \)

The determination of both components of \( \Delta p \) for mitochondria in situ is complex. In addition to the need to determine matrix volume and probe binding, the presence of the intervening plasma membrane, with its own membrane potential and pH gradient, has to be considered (128, 517). Hoek et al. (236) first quantified \( \Delta p \) for in situ hepatocyte mitochondria; \( \Delta \psi_m \) was determined from the Nernstian distribution of \(^{3}H\)TPMP\(^+ \) between the cytoplasm and matrix, after correcting for the plasma membrane potential (\( \Delta \psi_m \)), while \( \Delta p \) was measured by weak acid distribution, again controlling for the gradient across the plasma membrane. A value for \( \Delta p \) of >200 mV was consistent with values obtained with isolated mitochondria. Subsequently, the phosphonium cations have been used as isotopes or with a TPP\(^+ \)-selective electrode (273) to quantify the \( \Delta \psi_m \) component (typically some 150 mV).
for mitochondria within isolated nerve terminals (6, 517) and a variety of cells, including sea urchin sperm (504), basophilic leukemia cells (494), lymphocytes (59), thymocytes (60), hepatocytes (206, 425, 426), and perfused rat hearts (596).

The distribution of phosphonium cations such as TPMP$^+$ and TPP$^+$ is responsive to both $\Delta \psi_m$ and $\Delta \psi_p$

\[
\text{[Matrix]/[cytoplasm]} = 10^{\Delta \psi_m/60} (3)
\]

\[
\text{[Cytoplasm]/[medium]} = 10^{-\Delta \psi_m/60} (4)
\]

\[
\therefore \text{[Matrix]/[medium]} = 10^{(\Delta \psi_m - \Delta \psi_p)/60} (5)
\]

where the potentials have their normal sign conventions (resting $\Delta \psi_p$ is negative, $\Delta \psi_m$ is positive). The logarithmic nature of the above equations requires comment, particularly in the context of the fluorescent probes discussed below. A signal that is proportional to the concentration of the probe in the mitochondrion does not translate into a linear function of membrane potential. Thus, for example, a report of a 30% decrease in signal (e.g., Ref. 78) does not translate into a 30% decrease in $\Delta \psi_m$ (e.g., from 180 to 125 mV) but rather a much more modest decrease from 180 to 170 mV. Attempts to quantify $\Delta \psi_m$ from the extent of phosphonium cation binding after washing to remove free cation (82) are of course invalid.

The extension of the use of cationic fluorescent membrane potential indicators from isolated mitochondria to cells requires considerable care to avoid misleading artifacts. The probes are accumulated into intracellular mitochondria in the same way as the phosphonium cations: first across the plasma membrane in response to $\Delta \psi_p$ and then from cytoplasm to matrix in response to $\Delta \psi_m$. Although confocal microscopy can resolve the fluorescence of single intracellular mitochondria loaded with a membrane potential probe (340), direct determination of the Nernst gradient between matrix and cytoplasm by confocal microscopy is extremely difficult because of the small size of the mitochondria, the enormous dynamic range required to quantify the 300-fold gradient corresponding to a 150-mV potential, and the need to work at low concentrations of probe that do not produce quenching (or inhibition) within the matrix. Although it is virtually impossible to exclude extramitochondrial volume from the optical slice, Loew and co-workers (167) have devised compensating deconvolution algorithms for tetramethylrhodamine ester fluorescence from single in situ mitochondria. In addition, Ubl et al. (583) have evaluated the possibility of comparing the fluorescence intensity of mitochondria-poor regions (such as the nucleus in a thin optical section) with that of the mitochondria-rich cytoplasm and have concluded that with appropriate calibration (valinomycin-induced K$^+$ diffusion potentials in permeabilized cells) that an estimate of $\Delta \psi_m$ up to 140 mV may be obtained with an accuracy of $\pm$ 20 mV.

Plasma membrane and mitochondrial membrane potentials are determined from the free rather than bound concentrations of probe; thus the fixable cationic probe chloromethyl-tetramethyl-rosamine methyl ester (CMTMR; Mitotracker orange) (54, 593) poses serious limitations. The principle is that the cationic probe accumulates in the matrix and then reacts via its chloromethyl group with thiol groups, allowing the cells to be washed, fixed, and monitored. Unfortunately, it is not clear that the amount of chemically reacted CMT is directly proportional to the free matrix concentration and whether the thiol groups remain in excess or become saturated.

At single-cell resolution, two general protocols have been adopted. In the first, the putative change in $\Delta \psi_m$ is established before addition of the probe. Total cell fluorescence, i.e., the sum of nonmitochondrial and mitochondrial contributions, is monitored, a decrease relative to controls being ascribed to a mitochondrial depolarization. Unfortunately, cells are frequently washed in the absence of probe before or during measurement, thus disturbing the Nernst distribution across the plasma membrane. The fluorescence will then depend on the rate at which the membrane-permeant probe effluxes from the cell. In addition, most of these studies fail to take account of the extent of fluorescence quenching of the highly concentrated probe within the mitochondrial matrix (155).

Matrix quenching is deliberately exploited in the second protocol, which is suitable in principle for the continuous monitoring of $\Delta \psi_m$. If trans-plasma membrane equilibration of the probe is slow in relation to that across the mitochondrial inner membrane, a decrease in $\Delta \psi_m$ will be reported as an increased whole cell fluorescence as more probe moves from the relatively quenched environment of the matrix to the cytoplasm (339). Conversely, a mitochondrial hyperpolarization will cause a decreased whole cell fluorescence. Commonly employed dyes include rhodamine-123 (47, 73, 74, 141, 155, 257, 267, 286, 290, 463), DiOC$_6$(3) (84, 348, 349, 488, 489, 495, 623), and the tetramethylrhodamine methyl or ethyl esters [TMRM or TMRE (148, 340, 423, 508)]. The cationic 5,5’,6,6’-tetrachloro-1,1’,3,3’-tetraethylbenzimidazol-carbocyanine iodide (JC-1) (477) has been used in a different mode (107, 240, 476, 604). Above a critical concentration in the matrix, the probe forms red-emitting (590 nm) J-aggregates and below this concentration green-emitting (527 nm) monomers.

Redistribution of probe between the matrix and cytoplasm in response to a decrease in $\Delta \psi_m$ will of course increase the concentration in the latter compartment. However, because the probe is permeant across the plasma membrane (the pathway for its initial loading), it follows that the resultant increase in cytoplasmic concen-
tration should lead to a secondary efflux of probe from the cytoplasm to restore the plasma membrane Nernst equilibrium with a consequent decrease in signal. The actual shape of this biphasic fluorescent response to mitochondrial depolarization will depend on the relative permeability of the mitochondrial and plasma membranes to the probe and the geometry of the cell. The high surface-to-volume ratio possessed by small cells and cell processes such as neurites means that the secondary decrease in fluorescence follows rapidly upon the initial increase (415), whereas in large cell bodies the secondary efflux can be slower.

Because the cytoplasmic concentration of these probes at equilibrium is sensitive to $\Delta \psi_p$, the probes are also responsive to plasma membrane depolarization, as shown for the phosphonium cations (Eq. 2). This consideration is of particular relevance under conditions where $\Delta \psi_p$ changes, for example, KCl depolarization of neurons or N-methyl-d-aspartate (NMDA) receptor activation. The actual behavior of individual probes is largely empirical; thus the slow decrease in TMRM fluorescence seen when cultured neurons are challenged with glutamate (508) primarily reflects the decrease in $\Delta \psi_p$ accompanying receptor activation (415).

The fluorescent signal from some probes [e.g., rhodamine-123 (141) and JC-1 (495)] has been reported to be insensitive to changes in plasma membrane potential, although Davis et al. (119) observe a strong $\Delta \psi_p$ dependency of rhodamine-123 fluorescence in MCF-7 cells. Furthermore, changes induced by high KCl (141) or glutamate in the absence of Ca$^{2+}$ (604) fail to affect the red-to-green emission ratio of JC-1. This could either imply that redistribution across the plasma membrane of these probes is so slow that loss of probe from the cells is unimportant or that the signal results from predominantly bound rather than free probe, which would imply that the probes are not responding in the Nernstian mode required for interpretation of the fluorescence changes. A recent paper by Rottenberg and Wu (489) analyzed in great detail the use of DiOC$_{6}$ (6) for the determination of $\Delta \psi_m$ in lymphocytes by flow cytometry, a technique which has been widely employed to estimate changes in $\Delta \psi_m$ (84, 348, 349, 439, 623). Rottenberg and Wu (489) conclude that the concentrations at which the dye is normally employed (40–100 nM) result in a 90% inhibition of cell respiration (hence lowering $\Delta \psi_m$), quenching of the signal within the matrix, and a response selective for $\Delta \psi_p$, rather than $\Delta \psi_m$ (489).

It is important to remain cautious in the detailed interpretation of the fluorescence changes of $\Delta \psi_m$ monitoring probes and to seek some independent confirmation of the bioenergetic status of the in situ mitochondria. In particular, reliable application of a particular probe to the measurement of $\Delta \psi_m$ in one cell type should not be taken to mean that it can be used in the same way with other cells. Calibration with defined effectors of $\Delta \psi_m$ and $\Delta \psi_p$ should be performed with each new cell type.

Very recently, a promising technique for monitoring the pH of intracellular compartments has been described, based on the pH-sensitive fluorescence of targeted _Aequorea victoria_ green-fluorescent protein mutants (296, 336). Mitochondria in situ within HeLa cells displayed a $\Delta \mathrm{pH}$ of $-0.5$ units (336) consistent with values obtained with isolated mitochondria.

### IV. MITOCHONDRIAL CALCIUM TRANSPORT

#### A. Isolated Mitochondria

The inner mitochondrial membrane possesses a unipporter, which remains to be identified at a molecular level, able to transport Ca$^{2+}$, but not Mg$^{2+}$, into the matrix (for review, see Refs. 203, 211, 412). The high mitochondrial membrane potential dictates that thermodynamic equilibrium would be attained if the free matrix Ca$^{2+}$ rose to a value $10^5$–$10^6$ higher than in the extramitochondrial medium. This potentially lethal accumulation does not however occur, due to the presence in the inner membrane of a separate efflux pathway which exchanges Ca$^{2+}$ for protons (e.g., liver mitochondria) or for Na$^+$ (e.g., heart, brain, and brown fat mitochondria) (114). In the latter tissues, a mitochondrial Na$^+$/H$^+$ transporter is present, and the overall ion flux under conditions of constant mitochondrial Ca$^{2+}$ loading consists of sequential Ca$^{2+}$, Na$^+$, and H$^+$ cycling (113, 114), the last driven by the respiratory chain (Fig. 2).

In the presence of physiological concentrations of phosphate, an osmotically inactive Ca$^{2+}$-phosphate complex forms in the matrix at a critical Ca$^{2+}$ loading (318), and the activity of the efflux pathway is found to be invariant above this value (632). The complex is not a hydroxyapatite-like precipitate, since depolarization of the inner membrane leads to a rapid independent efflux of Ca$^{2+}$ via the reversed unipporter and phosphate on its transporter. The factors that prevent precipitation of calcium phosphate in the matrix are unclear, although phosphocitrurate has been proposed as an antinucleation agent preventing crystallization (564). When there is sufficient Ca$^{2+}$ and phosphate to form this complex, mitochondria behave as perfect buffers of extramitochondrial Ca$^{2+}$ ([Ca$^{2+}]_m$), accumulating the cation whenever its concentration rises above the set point at which uptake and efflux balance and releasing Ca$^{2+}$ below this value (40, 410). The set point can vary between 0.3 and 1 $\mu$M (58, 323, 410, 418), with the actual value depending on the kinetics of the two pathways. Thus the set point can be shifted to a higher [Ca$^{2+}]_m$ by activating the efflux pathway or inhibiting the uniporter. This can be observed with isolated brain mitochondria where increasing the Na$^+$...
concentration in the medium stimulates the efflux pathway (418). It is important that the set point is above the resting \( [Ca^{2+}]_c \) of typical cells, since otherwise there would be an inexorable increase in mitochondrial \( Ca^{2+} \). Ordinarily, this mechanism is adapted to cope with peak elevations in \( [Ca^{2+}]_c \) (for review, see Ref. 412).

In mitochondria operating below the set point, matrix \( [Ca^{2+}] ([Ca^{2+}]_m) \) is low and responsive to changes in \( [Ca^{2+}]_c \). Three key metabolic enzymes, pyruvate dehydrogenase, isocitrate dehydrogenase, and 2-oxoglutarate dehydrogenase, are activated by increases in \( [Ca^{2+}]_m \) and the increased flux of the citric acid cycle is reflected in an increased reduction state of NADH and a slight increase in \( \Delta p \) when \( [Ca^{2+}]_m \) responds to an increased \( [Ca^{2+}]_c \) (211, 367). This is a means of activating the respiratory chain in response to hormonal stimuli that lead to a physiological increase in \( [Ca^{2+}]_c \), allowing an increased "static head" \( \Delta p \) to offset the increased drop in potential as the proton current is increased in response to increased cellular ATP demand, or net \( Ca^{2+} \) accumulation itself (reviewed in Ref. 211). Thus an increase in \( [Ca^{2+}]_c \) in synaptosomes resulted in an increased activity of pyruvate dehydrogenase (212), while NAD(P)H autofluorescence (which largely reflects the mitochondrial pool) was enhanced by increase in \( [Ca^{2+}]_c \) in dissociated mouse sensory neurons (141).

Calcium accumulation by isolated mitochondria can lower \( \Delta \psi_m \) in one of three ways (Fig. 3). First, in the absence of phosphate, the capacity of isolated mitochondria to accumulate \( Ca^{2+} \) is restricted, since the protons extruded by the respiratory chain during \( Ca^{2+} \) accumulation are no longer neutralized by phosphate uptake (632). Under these conditions, a progressive alkalization of the matrix occurs, which can ultimately result in almost 2 pH units across the inner membrane and a \( \Delta \psi_m \) reduced to \(-100 \text{ mV} \) (410).

The second means by which \( Ca^{2+} \) can lower \( \Delta \psi_m \) occurs in the presence of excess phosphate (Fig. 3). Coaccumulation of phosphate with \( Ca^{2+} \) neutralizes \( \Delta p \) and forms the osmotically inactive but rapidly dissociable calcium phosphate complex. Under these conditions, there are transient depressions in both the \( \Delta \psi_m \) and \( \Delta p \) components of \( \Delta \psi_m \) during the uptake of the cation, due to the utilization of the proton gradient by the \( Ca^{2+} \) plus phosphate transport, and these can be sufficient to interrupt ATP synthesis or even to cause reversal of the ATP synthase (484). Acetate can also be used as a permeant anion to neutralize \( \Delta p \) (since acetate enters together with a proton), but this combination causes matrix swelling since calcium acetate is osmotically active.

The accumulation of \( Ca^{2+} \) in the presence of excess phosphate should be capable of continuing indefinitely, since phosphate neutralizes the alkalization of the matrix (Fig. 3), as long as there is space in the matrix for the osmotically inactive calcium-phosphate complex. In practice, mitochondria incubated in media approximating to the physiological cytoplasm (including adenine nucleotides and \( Mg^{2+} \)) can accumulate and retain in excess of 100 mM total \( Ca^{2+} \) (e.g., \( >200 \text{ nmol} Ca^{2+}/mg \) mitochondrial protein in a matrix volume of \(-1 \mu l/mg \) protein; Ref. 418) with no apparent deterioration in their capacity to maintain a high \( \Delta p \) or to generate ATP (418, 484).

The third mechanism by which \( Ca^{2+} \) accumulation may lower \( \Delta \psi_m \) (Fig. 3) is the condition of \( Ca^{2+} \) overload leading to mitochondrial swelling (as evidenced by a decreased 90° light scattering at 540 nm), loss of respiratory control, collapse of \( \Delta \psi_m \), and release of matrix \( Ca^{2+} \) caused by a permeabilization of the mitochondrial inner membrane to sucrose and other molecules up to \(-1.5 \text{ kDa} \) (11a). This MPT is most readily observed with isolated mitochondria incubated in the presence of phosphate and \( Ca^{2+} \), but in the absence of adenine nucleotides and \( Mg^{2+} \) (see Refs. 42, 44, 634 for reviews). It is unclear what relation free as opposed to total matrix \( Ca^{2+} \) has to mitochondrial dysfunction. Thus the deleterious effects of \( Ca^{2+} \) increase with phosphate concentration, whereas at the same time \( [Ca^{2+}]_m \) decreases due to the formation of the \( Ca^{2+}-\text{phosphate} \) complex (632).
That the MPT is mediated by a pore in the inner membrane, rather than by membrane damage during the isolation of mitochondria, was demonstrated by Hunter and Haworth and co-workers (246, 247). They showed, by using hyperosmotic solutions of polyethylene glycol of different molecular weights, that the MPT possessed a permeability cut off for solutes at \( \sim 1,500 \text{ Da} \) (247). Direct evidence for the existence of a pore was later obtained with electrophysiological measurements of a "mitochondrial megachannel" with properties qualitatively similar to the MPT (633). The maximum conductance of the megachannel is reported to be between 1 and 1.5 nS (633), with long-lasting closed states. The molecular identity of the pore remains elusive, but a number of components including the adenine nucleotide translocator, mitochondrial porin, and the peripheral benzodiazepine receptor are implicated in either its regulation or formation at membrane contact sites (208, 209).

The MPT is facilitated by factors that enhance oxidative stress or deplete the matrix adenine nucleotide pool such as pyrophosphate and atractylate (which locks the adenine nucleotide translocator in the C-conformation in which the binding site faces the cytoplasm). Conversely, the MPT is largely prevented by including Mg\(^{2+}\) and adenine nucleotides in the medium, by bongkrekate (which locks the adenine nucleotide in the matrix or M-conformation), and by cyclosporin derivatives that interact with a mitochondrial cyclophilin which associates with the adenine nucleotide translocator. Chelation of extramitochondrial Ca\(^{2+}\) with EGTA can also reverse the MPT (11a). However, a recent confocal study of immobilized mitochondria (248) has shown that individual mitochondria can undergo stochastic, cyclosporin-sensitive, large-amplitude fluctuations in \( \Delta \psi_m \) even in the absence of Ca\(^{2+}\).

The Ca\(^{2+}\) ionophores that have been commonly used in mitochondrial studies are ionomycin and A-23187, which function as electroneutral Ca\(^{2+}/2\text{H}^+\) exchangers. Their effect in mitochondria is to create an additional Ca\(^{2+}\) efflux pathway in the inner membrane, depleting the matrix of Ca\(^{2+}\) (456, 475). In contrast to the slow Ca\(^{2+}\) cycling that occurs between the uniporter and the relatively low activity endogenous efflux pathway, the rate of Ca\(^{2+}\) cycling in the presence of excess ionophore is only limited by the activity of the Ca\(^{2+}\) uniporter and is hence controlled by \([\text{Ca}^{2+}]_o\). In elevated \([\text{Ca}^{2+}]_o\) (\(>1-2 \mu\text{M}\)), this dissipative cycling can have a similar effect to a protonophore, collapsing \( \Delta \psi_m \) and inducing uncontrolled respiration (219).

**B. Ca\(^{2+}\) Transport by In Situ Mitochondria**

Calcium homeostasis, metabolism, and bioenergetics are intimately interconnected in the intact cell and must be considered as part of an integrated system (Fig. 2). Mitochondrial respiration drives both ATP synthesis and Ca\(^{2+}\) accumulation. Because these two processes compete for the proton circuit, they should be considered together in an analysis of the effects of cellular Ca\(^{2+}\) loading; thus, although Ca\(^{2+}\) accumulation by the mitochondria may affect ATP synthesis, alterations in ATP synthesis will in turn affect the activity of ion pumps responsible for removing Ca\(^{2+}\) from the cytoplasm.

The early studies with isolated mitochondria discussed above predicted that mitochondria would accumulate Ca\(^{2+}\) whenever \([\text{Ca}^{2+}]_o\) rose above the set point at which uptake and efflux balanced (410). A major goal of recent studies with intact neurons has been to determine how mitochondrial Ca\(^{2+}\) accumulation might affect cellular Ca\(^{2+}\) homeostasis, particularly in response to the Ca\(^{2+}\) loading via voltage-activated Ca\(^{2+}\) channels or NMDA receptor activation.

The cytoplasmic Ca\(^{2+}\) transients measured with fluorescent Ca\(^{2+}\) probes during depolarization of neurons with KCl (103, 141, 144), glutamate (102, 104, 473, 594), or trains of action potentials (109, 602) rise well above the predicted mitochondrial set point and should therefore be influenced by mitochondrial Ca\(^{2+}\) sequestration. Qualitative confirmation that the mitochondria have accumulated Ca\(^{2+}\) has been obtained by protonophore addition to a Ca\(^{2+}\)-loaded cell. This will release any accumulated mitochondrial Ca\(^{2+}\) to the cytoplasm, giving a transient elevation in \([\text{Ca}^{2+}]_c\) preceding any subsequent effects due to ATP depletion. When such experiments are performed with resting, polarized neurons, no cytoplasmic transients are observed, consistent with mitochondria being largely depleted of Ca\(^{2+}\) below their set point; however, protonophore addition to cells after Ca\(^{2+}\) loading produces the predicted spike in \([\text{Ca}^{2+}]_c\) (73, 565, 566, 598, 602, 603).

Although the Ca\(^{2+}/2\text{H}^+\) antiport ionophores ionomycin or A-23187 are frequently added to cells in an attempt to increase \([\text{Ca}^{2+}]_c\) (e.g., Refs. 89, 303, 312, 434, 447, 525), the incorporation of the ionophore into the inner mitochondrial membrane and resulting dissipative Ca\(^{2+}\) cycling can depolarize \( \Delta \psi_m \) with deleterious effects on ATP levels comparable to a protonophore (7).

The kinetics of mitochondrial Ca\(^{2+}\) transport suggested a model in which the organelles would act as temporary stores of Ca\(^{2+}\) during cytoplasmic Ca\(^{2+}\) peaks, blunting the cytoplasmic response and releasing the Ca\(^{2+}\) back to the cytoplasm when \([\text{Ca}^{2+}]_c\) recovered to below the set point (8, 412). In 1990, Thayer and Miller (565) obtained direct evidence for this by showing that brief KCl-mediated depolarization of dorsal root ganglion cells was followed by a recovery in \([\text{Ca}^{2+}]_c\) to a plateau of 200–600 nM (549, 565). This elevated plateau was interpreted to be a consequence of the slow release from the mitochondrion of Ca\(^{2+}\) accumulated at the peak \([\text{Ca}^{2+}]_c\).

A similar plateau was observed in these cells following...
trains of action potentials (602). Comparable plateaus have subsequently been reported for bullfrog sympathetic neurons (173) or chromaffin cells (29, 229).

The cationic fluorescent Ca\(^{2+}\) indicator rhod 2 can be localized within the mitochondrial matrix to monitor [Ca\(^{2+}\)]\(_{\text{m}}\) (268, 385, 447, 448, 570). Rhod 2 has been used to detect histamine-induced oscillations in [Ca\(^{2+}\)]\(_{\text{m}}\) (268) and mitochondrial Ca\(^{2+}\) accumulation in both glutamate-exposed striatal neurons (447, 448) and field-stimulated lizard nerve terminals (118). In the last example, a mitochondrial signal was only observed after a train of 15–20 pulses, consistent with the need to exceed the threshold imposed by the set point. A limitation, however, with rhod 2 is its relatively high affinity (dissociation constant \(K_d\) = 0.6 \(\mu\)M), which means that it is saturated by >5 \(\mu\)M free Ca\(^{2+}\).

The problem inherent in studies aimed at assessing the role of mitochondrial Ca\(^{2+}\) transport lies in the ability to perform appropriate control experiments in which mitochondrial Ca\(^{2+}\) uptake is selectively inhibited. There are currently no specific, cell-permeant inhibitors of the mitochondrial Ca\(^{2+}\) uniporter, although the ruthenium amine complex Ru-360 is a possible candidate (363). The hexavalent glycoprotein stain ruthenium red (343, 560) is effective with isolated mitochondria at low concentrations; however, there is little evidence that it can permeate across the plasma membrane of neurons. Experiments in which ruthenium red or La\(^{3+}\) have been shown to antagonize the neurotoxic effects of NMDA receptor activation (e.g., Refs. 131, 150, 257) may be primarily due to inhibition of plasma membrane voltage-activated Ca\(^{2+}\) channels or NMDA receptors (352, 560). Penetration of the stain into the somata of cultured neurons accompanying excitotoxicity has been reported (560), but it is not clear whether the appearance of ruthenium red in the cytoplasm preceded plasma membrane permeabilization. This criticism of course does not apply to experiments in which ruthenium red is loaded via the electrode in whole cell patch-clamp experiments (e.g., Refs. 221, 438, 565).

A possible inhibitor of the mitochondrial Ca\(^{2+}\) efflux pathway has been investigated by a number of groups (33, 241, 529, 604, 605). 7-Chloro-3,5-dihydro-5-phenyl-1H,4,1-benzothiazepine-2-one (CGP-37157) inhibits the mitochondrial Na\(^+\)/Ca\(^{2+}\) exchanger and would therefore be predicted to lower the mitochondrial set point and potentially to lead to massive, essentially irreversible, Ca\(^{2+}\) accumulation by the mitochondria. Addition of the inhibitor immediately subsequent to glutamate facilitates the restoration of basal [Ca\(^{2+}\)]\(_{\text{m}}\) in cultured forebrain neurons (605). Unfortunately, the inhibitor also inhibits voltage-gated Ca\(^{2+}\) channels, preventing its application to such studies (33). The lipophilic cation TPP\(^{+}\), discussed in section mB has been reported to inhibit the Na\(^{+}\)-dependent mitochondrial Ca\(^{2+}\) efflux pathway (278, 608), and the cation has been used in a recent study (559) in an attempt to establish the role of presynaptic mitochondrial Ca\(^{2+}\) transport in the posttetanic potentiation of synaptic transmission. However, the extracellular concentration employed, 10 \(\mu\)M, would give an estimated 100 \(\mu\)M within the mitochondrial matrix (see Eq. 5) which would result in extensive mitochondrial swelling and depolarization, with consequent compromised ATP synthesis and Ca\(^{2+}\) sequestration.

In the absence of selective, permeant mitochondrial Ca\(^{2+}\) uniport inhibitors, an indirect approach has to be taken to inhibit mitochondrial Ca\(^{2+}\) transport in intact neurons. That most frequently adopted has been to depolarize the mitochondria. However, addition of a protonophore can lead to profound ATP depletion as well as causing cytoplasmic acidification and releasing glutamate from synaptic vesicles. With this in mind, it has generally been reported that the addition of a protonophore leads to an increased cytoplasmic Ca\(^{2+}\) elevation in response to a Ca\(^{2+}\) load induced by elevated KCl (73, 549, 565) or glutamate activation of NMDA receptors (292, 566). However, in such experiments, it is difficult to distinguish between an increased [Ca\(^{2+}\)]\(_{\text{c}}\), as a consequence of failed mitochondrial Ca\(^{2+}\) sequestration and one due to inhibited Ca\(^{2+}\) extrusion after ATP depletion. This was already recognized in 1990, when Duchen et al. (144) reported enhanced cytoplasmic Ca\(^{2+}\) transients in dorsal root ganglion cells exposed to a range of metabolic inhibitors, including protonophores, cyanide, and glucose removal, but observed that these effects could be a consequence of impaired Ca\(^{2+}\) extrusion from the cells as well as inhibited mitochondrial sequestration. Similar results have been obtained with cortical neurons (472), hippocampal neurons (598), avian cochlear neurons (392), and cerebellar granule cells (73).

In cells with active glycolysis, the ATP synthase inhibitor oligomycin can be employed to reduce the number of parameters affected by mitochondrial depolarization. Comparison of the [Ca\(^{2+}\)]\(_{\text{c}}\) signal evoked, for example, by elevated KCl in cells in the presence of oligomycin with those incubated with oligomycin plus a respiratory chain inhibitor allows the effect of inhibited mitochondrial Ca\(^{2+}\) sequestration to be observed in the absence of ATP synthase reversal (73, 74, 85, 141, 517, 519). In designing such experiments, the reversibility of inhibitor binding needs to be taken into consideration. High-affinity inhibitors such as rotenone, antimycin A, and oligomycin (116) are effectively irreversible, whereas cyanide and ionophores including carbonyl cyanide p-trifluoromethoxyphenylhydrazone (FCCP) can be readily removed by washing.

In view of the evidence that mitochondria sequester much of the Ca\(^{2+}\) load imposed by KCl depolarization, it would be predicted that abolition of the mitochondrial pool would enhance a subsequent cytoplasmic Ca\(^{2+}\) transient. However, the results are surprising; granule cells maintaining a high ATP/ADP in the presence of rotenone/
oligomycin (73) and display reduced cytoplasmic Ca$_{2+}$ responses to KCl depolarization or NMDA receptor activation (73, 74, 85), whereas increased signals are obtained in cells treated with protonophore before Ca$_{2+}$ loading. Although a decreased bulk cytoplasmic Ca$_{2+}$ elevation when the cell’s main Ca$_{2+}$ sink is inactivated is counterintuitive, it is supported by the decreased total accumulation of 45Ca$_{2+}$ by neurons under these conditions (74).

V. REACTIVE OXYGEN SPECIES
AND MITOCHONDRIA

A. ROS Generation by Isolated Mitochondria

Oxidative stress is a common feature of many different forms of neurodegenerative disease (for review, see Ref. 38). To rationalize a large and complex field, it is helpful to first consider the mechanisms for the endogenous generation of ROS.

Although molecular oxygen is reduced to water in the terminal complex IV by a sequential four-electron transfer, a minor proportion can be reduced by a le$^-$ addition that occurs predominantly in complex III (56, 362, 544) but also in complex I (228, 577). The cyclic electron transfer pathway within complex III (the Q cycle; see Ref. 416 for review) involves a site close to the cytoplasmic face of the membrane where UQH$_2$ transfers a single electron to cytochrome c$_1$ via the Rieske iron-sulfur protein, leaving a highly reactive ubisemiquinone UQ$^-$.

Loss of the second electron and generation of UQ is therefore opposed by a high Δψ$_{im}$ such that a high Δψ$_{im}$ enhances the occupancy of the ubisemiquinone binding site, where a chance exists that this second electron can be transferred to molecular oxygen, generating the superoxide anion O$_2^-$ (576). The inhibitor antimycin A increases O$_2^-$ production by inhibiting reduction of the second b cytochrome, while myxathiazol has the opposite effect by inhibiting the initial generation of the semiquinone (307, 576). Recently, a Q cycle-related pathway of electron transfer has been proposed for complex I (146).

Very high membrane potentials (e.g., during succinate respiration in state 4) are required to generate significant amounts of O$_2^-$ (534) or H$_2$O$_2$ (57). Succinate-supported O$_2^-$ production is strongly inhibited in state 3 (57), although complex I substrates are unaffected by the decrease in Δψ$_{im}$ and may be the main contributors to O$_2^-$ production during state 3 respiration (228). The nonionic proton leak (408, 425) may function to limit Δψ$_{im}$ and hence potentially toxic O$_2^-$ generation (534). Proton-motive force can also be regulated by uncoupling proteins (UCP). In addition to the original UCP, UCP1 (220), other isoforms have recently been identified (168). The protein UCP2 is widely expressed and has been proposed to control ROS generation by limiting Δψ$_{im}$ (403).

Calcium loading of isolated mitochondria in the presence of phosphate increases the production of O$_2^-$ (147, 298), although the bioenergetic mechanism is not well understood, since the lowering of Δψ$_{im}$ during the uptake of Ca$_{2+}$ would be predicted to decrease O$_2^-$ generation. Because O$_2^-$ generation at complex III may require the abnormal access of protons to the outer UQ$^-$ binding site (427), it is possible that the formation of the calcium phosphate complex may alter the inner membrane structure to allow such access.

B. Mitochondria and the Cellular Generation of ROS

In this section we review some of the methodologies that have been employed to monitor the mitochondrial generation of ROS in cells. The application of these methods to investigations of excitotoxicity and apoptosis are discussed subsequently.

Techniques employed to detect O$_2^-$ and other reactive oxygen species include the formation of spin-trap adducts for subsequent electron paramagnetic resonance analysis (147, 314, 315) and the oxidation to fluorescent products of reduced, nonfluorescent probes (e.g., Refs. 45, 72, 145, 202, 463, 478, 545). The responsiveness of individual probes to the different ROS cannot be predicted on structural grounds and has to be determined empirically. Additionally, the extremely rapid interconversion of ROS within the cell can make it difficult to identify the originating species. Thus the oxidation of hydroethidine (HEt) to the fluorescent ethidium cation is selective for superoxide (45, 72, 519), whereas the oxidation of dihydorhodamine-123 to the fluorescent rhodamine-123 has been reported variously to be primarily responsive to hydrogen peroxide (222, 490) or peroxynitrite (297, 460). The superoxide dismutase (SOD) product H$_2$O$_2$ can be estimated by oxidation of 2,7’-dichlorofluorescin (DCF)-H$_2$ to the fluorescent oxidation product DCF (478, 490).

It must be emphasized that none of these assays is quantitative, since the dyes are competing with other enzymatic and nonenzymatic processes. The high activity of Mn-SOD within mitochondria minimizes the steady-state concentration of O$_2^-$ by generating H$_2$O$_2$ (111). In addition, the concurrent production of mitochondrial O$_2^-$ and cytoplasmic NO leads to the rapid formation of peroxynitrite, ONOO$^-$ (469). Therefore, in the case of HEt, the assay will compete for generated O$_2^-$ with Mn-SOD and NO, as well as with any protein or lipid targets. Thus the rate of production of these probes will reflect the steady-state concentration, rather than rate of production, of the ROS in the presence of these competing reactions.
A major complication with the use of many of these ROS probes is that the oxidized fluorescent product may not simply measure ROS. Thus ethidium cation (487) measures Δψm while dichlorofluorescein is also responsive to pH. Because the purpose of many experiments is to establish the interplay between ROS generation in cells and changes in these parameters, great care must be taken to avoid cross-talk. For example, the Δψm-responsive product of O2•−-mediated HET oxidation, ethidium cation, can undergo fluorescence dequenching on being released from the mitochondrial matrix to the cytoplasm after mitochondrial depolarization (72), which can be misinterpreted as a burst of superoxide production. This has led to some confusion; for example, in intact cells there have been reports that agents that lower or collapse Δψm increase the production of ROS (45, 145, 304, 622). A similar criticism can be leveled against the use of dihydorhodamine-123 (490), whose oxidation product, rhodamine-123, is also a membrane-permeant cation subject to fluorescence quenching within the matrix. To further complicate fluorescent investigations, dye-loaded mitochondria are photosensitized, such that their illumination can lead to ROS generation that can in itself artifactually induce the mitochondrial permeability transition (248, 627).

C. Oxidative Damage to Isolated Mitochondria

As well as being able to generate O2•− and H2O2, mitochondria are themselves susceptible to damage by these species. The redox state of intramitochondrial nicotinamide nucleotides is critical for the resistance of mitochondria to oxidative stress. NAD(P)H is of particular relevance, since the reduced nucleotide is responsible for maintaining the reduced matrix glutathione pool in the reduced form via glutathione transhydrogenase (237), the magnitude of Δp may be critical for mitochondrial survival; if it is too high, complex III will generate ROS, and if it is too low, ATP synthesis will be compromised and the NAD(P)H pool will become oxidized. Redox state also affects the opening of the MPT. Thus oxidative stress, induced for example by t-butylhydroperoxide (112), which oxidizes the mitochondrial NADPH pool and consequently the glutathione pool (422), is synergistic with Ca2+ and phosphate in inducing the MPT in isolated mitochondria (300).

VI. MITOCHONDRIA AND NEURONAL GLUTAMATE EXCITOTOXICITY

It has been recognized for 30 years (433) that glutamate is a powerful neurotoxin, capable of killing neurons in the central nervous system when its extracellular concentration is elevated (for reviews, see Refs. 96, 177, 354, 428, 430, 486). Mitochondria are implicated at multiple stages of glutamate excitotoxicity, including the initial bioenergetic collapse that triggers the ischemic release of the amino acid, the sequestration of Ca2+ entering via the NMDA receptor after reperfusion, the generation of ROS and, where relevant, the triggering of an apoptotic cascade. In addition to glutamate, a wide variety of mitochondrial inhibitors can induce neuronal cell death, although care is needed to distinguish between a direct effect of these agents on the target cells and an indirect excitotoxicity, particularly when cultured cells are maintained in a fixed volume of medium, where a primary bioenergetic inhibition can result in release of glutamate from cells by exocytosis and/or transporter reversal, leading to secondary activation of NMDA receptors.

A. Pathways of Ischemic Glutamate Release

The ability of glutamate to act both as a universal metabolite and a specific neurotransmitter is dependent on the compartmentation of the amino acid within the cytoplasm and synaptic vesicles by specific active transport mechanisms. A primary bioenergetic restriction induced by anoxia, ischemia, hypoglycemia, and metabolic restriction disturbs this compartmentation. During ischemia in vivo, the inhibition of mitochondrial respiration and depletion of the limited intracellular glucose causes a profound collapse in ATP levels; for example, within 90 s of ischemia, rat cerebrocortical [ATP]/[ADP] fell from 10 to 0.4 (281), extracellular K+ concentration increased to 55–60 mM, extracellular Na+ concentration declined to 80 mM, and Δψp depolarized to ~20 mV (reviewed in Ref. 551). In vivo dialysis indicates that the normal extracellular glutamate concentration, which in rat brain is in the range 2–5 μM (e.g., Refs. 41, 79, 276, 378), increases dramatically during transient cerebral ischemia (41, 189, 280). The stoichiometry of the plasma membrane glutamate transporters in neurons and glia is believed to be glutamate−cotransported with 2Na+ in exchange for one K+ and one OH− or HCO3− (55). Reversal of these transporters as a consequence of the collapse in ion gradients across the plasma membrane is sufficient to account for the massive nonvesicular efflux of glutamate (413), although there may be an additional contribution from glutamate exocytosis either before ATP levels have collapsed or subsequent to reperfusion (551).

The release of glutamate from neurons during ischemia in vivo can be mimicked in vitro during chemical anoxia (respiratory chain inhibition), hypoglycemia (inhibition of glycolysis), or ischemia (combined respiratory chain and glycolytic inhibition). With cultured neurons (458), glial cells (23), brain slices (77, 554), and synaptosomes (282, 419, 491, 500), the Ca2+-independent release of cytoplasmic glutamate predominates during chemical
anoxia, ischemia, and hypoglycemia. In neuronal culture, an initial exocytotic component can be detected after chemical ischemia or ouabain addition, consistent with a period of network firing before ATP decreases below the level required for exocytosis (458, 551, 595).

B. Role of the NMDA Receptor

Exposure of primary neuronal cultures to glutamate can reproduce many of the key features of in vivo excitotoxicity, including cell swelling dependent on Na\(^+\) entry into the cell (98, 132, 183, 359, 442, 457) and the final Ca\(^{2+}\)-dependent cell necrosis (reviewed in Refs. 97, 171). A period of exposure as short as 5 min can result in extensive cell death monitored 24 h later (16, 472, 473, 598). Death is primarily necrotic (96, 204), although a transition to an apoptotic mode of cell death with decreasing intensity of glutamate exposure has been reported (16, 563).

The key factor controlling the mode of cell death has been proposed to be the extent to which cytoplasmic ATP levels are lowered by the bioenergetic consequences of the glutamate exposure (16, 320, 421). Thus the bioenergetic status of mitochondria in situ may be a very important factor in deciding the fate of the cell in terms of survival, apoptosis, or necrosis. Each of the mutually interacting bioenergetic parameters may be implicated, including substrate availability, respiratory chain activity, Ca\(^{2+}\) accumulation, generation of ROS, mitochondrial swelling, release of cytochrome c, and cellular ATP generation. The challenge is to separate out the individual functions to determine the causal chain of events leading ultimately to cell death.

The NMDA-selective glutamate receptor is primarily responsible for initiating necrotic cell death, and NMDA receptor antagonists such as MK-801 are effective in many neuronal culture preparations in preventing the excitotoxicity resulting from subsequent glutamate addition. However, most preparations can display an MK-801-resistant component of glutamate excitotoxicity that can be enhanced by cyclothiazide to inhibit desensitization of D,L-\(\alpha\)-amino-3-hydroxy-5-methylisoxazolepropionic acid (AMPA)-selective glutamate receptors (88, 264, 366, 394). Kainate, which primarily induces a nondesensitizing activation of AMPA receptors, is excitotoxic in most preparations (61, 93, 166, 178, 205, 467, but see Refs. 101, 241, 582).

In cultured neurons, exposure to glutamate typically leads to three distinct phases of cytoplasmic Ca\(^{2+}\) elevation (Fig. 4). First there is a spike, which may be followed by a recovery until an elevated plateau is attained. This recovery may reflect the partial desensitization of NMDA receptors (317, 470, 481), a component due to fast-desensitizing voltage-activated Ca\(^{2+}\) channels (108), the delayed activation of Ca\(^{2+}\) extrusion pathways (294), or mitochondrial sequestration (598, 602). After the second, plateau, phase, an uncontrolled, essentially irreversible failure of cytoplasmic Ca\(^{2+}\) homeostasis, delayed Ca\(^{2+}\) deregulation (DCD) occurs (578). Delayed Ca\(^{2+}\) deregulation precedes plasma membrane lysis (since the fluorescent probe is still retained in the cytoplasm) but reliably predicts subsequent cell lysis. The second and third phases can proceed even after the removal of extracellular glutamate (16, 380, 472, 473, 578, 598).

C. Bioenergetic Consequences of Acute Glutamate Exposure

Glutamate exposure results in a massive accumulation of \(^{45}\)Ca\(^{2+}\) by cultured neurons (131, 149, 217). Much of this Ca\(^{2+}\) is accumulated within the first few minutes of glutamate exposure (85, 217, 498). Because the peak level of [Ca\(^{2+}\)], determined with Ca\(^{2+}\) indicators (particularly recent low-affinity indicators; Refs. 81, 249, 452, 471, 541) exceeds the mitochondrial set point, it would be predicted, just as for KCl depolarization of neurons, that the mitochondria would sequester much of the Ca\(^{2+}\) entering the cell. Consistent with such mitochondrial Ca\(^{2+}\) uptake is the ability of a protonophore to release Ca\(^{2+}\) from the mitochondria and produce a transient elevation in [Ca\(^{2+}\)]\(_{m}\) in cells exposed to glutamate, but not in control cells (74, 565, 598). More directly, the mitochondrially located Ca\(^{2+}\) indicator rhod 2 can detect a very rapid increase in [Ca\(^{2+}\)]\(_{m}\) after glutamate addition to striatal neurons (447, 448).
In a detailed series of papers, White and Reynolds (603–605) have analyzed the pathways of Ca$^{2+}$ removal from the cytoplasm after acute, nontoxic, glutamate exposure. Restoration of baseline [Ca$^{2+}$]$_{c}$, following as little as 15-s exposure to 3 μM glutamate was highly dependent on both extracellular Na$^{+}$ and Δψ$_{m}$, thus no recovery occurred during washout of glutamate by a Na$^{+}$-free medium containing protonophore.

Although NMDA receptor isoforms are more permeable to Ca$^{2+}$ than Na$^{+}$, the 100-fold greater concentration of the latter ion in the extracellular medium means that the inward flux of Na$^{+}$ is likely to exceed that for Ca$^{2+}$. Because the Na$^{+}$-K$^{+}$-ATPase has the greatest capacity of any ATP-hydrolyzing process within the neuron (182, 517), acute glutamate exposure will create a high bioenergetic demand on the neuron as the ATPase attempts to expel the cation.

Calcium entry through the NMDA receptor creates additional energy demands on the cell both at the plasma membrane and at the mitochondrion. Although the neuronal plasma membrane Ca$^{2+}$-ATPase has a substantially lower maximum velocity than the Na$^{+}$-K$^{+}$-ATPase (160), the increase in [Ca$^{2+}$]$_{c}$ will activate the pump. Second, the uptake of Ca$^{2+}$ by the mitochondria is driven by Δψ$_{m}$ and will compete in parallel with the mitochondrial ATP synthase for the protons of the proton circuit (Fig. 5). Calcium transport can dominate this competition (484), since in contrast to ATP synthesis, which has a thermodynamic threshold requirement for Δψ$_{m}$ (strictly Δp) only some 10% below resting values (414), Ca$^{2+}$ accumulation can proceed at a much lower Δψ$_{m}$ and Ca$^{2+}$ concentrations in excess of 1–2 μM lower Δψ$_{m}$ dramatically. The prediction for the glutamate-exposed neuron would therefore be that mitochondrial ATP synthesis may cease as the Ca$^{2+}$ floods from the cytoplasm to the matrix. The cell’s ATP demand during this peak response could be met by accelerated glycolysis and/or hydrolysis of phosphocreatine (370).

### D. Δψ$_{m}$ and Glutamate Excitotoxicity

As discussed in section III, the monitoring of in situ mitochondrial membrane potential is far from trivial, particularly during glutamate exposure when the plasma membrane will also be undergoing complex changes in potential. The key mechanistic question during the latent period culminating in DCD is whether the mitochondria maintain full bioenergetic competence, notably the ability to generate ATP, or whether they are partially or completely depolarized after the first minutes of glutamate exposure and after net Ca$^{2+}$ accumulation is largely complete. The general qualitative consensus seems to be that mitochondria remain partially depolarized for at least as long as glutamate is present (16, 141, 257, 286, 290, 462, 508, 604). However, particularly with JC-1, the pattern of response can be complex, with both hyperpolarizing and depolarizing responses being detected in adjacent cells (604). Even with the assumption that all the artifacts can be eliminated, these studies do not address the key question of the competence of the in situ mitochondria to generate ATP, since in addition to mitochondrial uncoupling or damage, a partial depolarization can reflect an increased rate of ATP synthesis and turnover, or could be masking a reciprocal increase in the Δp component of Δψ as occurs when isolated mitochondria accumulate Ca$^{2+}$ in the presence of limiting P$_{i}$.

The competence of in situ mitochondria to generate ATP during cellular glutamate exposure may be resolved by monitoring the immediate effect of ATP synthase inhibition on Δψ$_{m}$ (415). Isolated mitochondria that are generating ATP and are consequently in state 3 show a slight hyperpolarization when ATP synthesis stops through exhaustion of ADP or addition of oligomycin (state 4). Although this hyperpolarization may only amount to some 5 mV out of 150 mV, the logarithmic nature of the Nernst equilibrium means that this will be accompanied by about a 20% increase in matrix accumulation of a cationic Δψ$_{m}$ indicator (see Eq. 4). Conversely, mitochondria with inhibited respiration, or which are damaged or leaky to protons, will have a low Δψ$_{m}$ that is supported by ATP synthase reversal; in this condition, oligomycin will depolarize Δψ$_{m}$ (143, 415, 604).

By these criteria, the mitochondria in situ within cerebellar granule cells continue to remain bioenergetically competent and generate ATP throughout the elevated [Ca$^{2+}$]$_{c}$ plateau in the presence of glutamate concentrations that will ultimately lead to DCD (415). The retained bioenergetic integrity of the mitochondria during glutamate exposure is supported by the increased [Ca$^{2+}$]$_{c}$ seen on addition of a protonophore or respiratory chain inhibitor (85). Continued mitochondrial oxidative phosphorylation is also indicated by the ability of the nonglycolytic substrate pyruvate to support glucose-deprived cerebellar granule cells in the presence of glutamate (85, 110).

As long as NMDA receptors or voltage-activated Ca$^{2+}$ channels are not activated, cerebellar granule cells can maintain cytoplasmic Ca$^{2+}$ homeostasis in the presence of the respiratory chain inhibitors rotenone or antimycin A (74, 415). The Δψ$_{m}$ is maintained by hydrolysis of cytoplasmic ATP under these conditions (489, 517), and because the proton conductance of the inner mitochondrial membrane is low, cytoplasmic ATP demand to maintain mitochondrial polarization is minimal. This mitochondrial ATP consumption will increase dramatically when the cells are exposed to glutamate, as ATP synthase reversal attempts to maintain Δψ$_{m}$ during the uptake of Ca$^{2+}$ into the mitochondrial matrix. The granule cells are unable to generate sufficient ATP under these conditions, and an...
immediate cytoplasmic Ca\(^{2+}\) deregulation, visualized as a saturation of the fura 2 response, is seen within 2 min of glutamate addition to cells in the presence of rotenone, antimycin A, or a protonophore (74, 415), whereas a
similar deregulation is seen in cells exposed to glutamate in the absence of glucose or oxidizable substrate (85).

E. Mitochondrial Ca$^{2+}$ Sequestration and Glutamate Exposure

The total accumulation of $^{45}$Ca$^{2+}$ within cultured cerebellar granule cells exposed to glutamate can approach 20 nmol/µl of cell volume (equivalent to 20 mM) before the cells die (149). The mitochondrion is the only organelle capable of accumulating such amounts of Ca$^{2+}$, and it is of central importance to establish the role that this matrix Ca$^{2+}$ accumulation plays in excitotoxicity.

Two explanations have been advanced for the high toxicity resulting from NMDA receptor-mediated Ca$^{2+}$ uptake relative to that occurring through voltage-activated Ca$^{2+}$ channels in the presence of elevated KCl; either the absolute amount or rate of Ca$^{2+}$ entering through the NMDA receptor may exceed that through voltage-activated Ca$^{2+}$ channels (149, 217), or Ca$^{2+}$ entering through the NMDA receptor is focused onto a vulnerable excitotoxic locus within the cell (498, 578).

There is support for both possibilities; the extent of total $^{45}$Ca$^{2+}$ accumulated by cortical neurons (217, 342) or cerebellar granule cells (149) has been reported to correlate with the extent of subsequent cell death regardless of the pathway of entry. Additionally, the Ca$^{2+}$ elevation produced by glutamate may exceed that by KCl. Thus, although little difference is seen in the apparent elevation in [Ca$^{2+}$]c evoked with glutamate or KCl when determined with high-affinity Ca$^{2+}$ indicators such as fura 2 (139, 249, 377, 472, 604), the recent use of low-affinity indicators of free Ca$^{2+}$ capable of reporting far higher concentrations before saturating (81, 249, 452, 471, 541) as well as in vivo intracellular Ca$^{2+}$ microelectrodes (161) have indicated that glutamate-evoked [Ca$^{2+}$]c elevations may be considerably underestimated and may exceed 5 µM, considerably in excess of levels achieved with KCl depolarization.

The alternative view is that the extreme toxicity of glutamate-evoked Ca$^{2+}$ uptake may be due to the selective direction of the Ca$^{2+}$ onto an excitotoxic locus in the close vicinity of the intracellular face of the NMDA receptor (497, 498, 578). Thus Sattler et al. (498) have reported that an equivalent $^{45}$Ca$^{2+}$ uptake via NMDA receptors is more toxic than an equivalent extent of KCl-evoked $^{45}$Ca$^{2+}$ uptake (497). Fast-acting Ca$^{2+}$ chelators such as BAPTA, 1,2-bis(2-aminophenoxo)ethane-N,N,N',N'-tetraacetic acid (but not fura 2 or fluo 3, Ref. 5), are neuroprotective after loading into neurons in culture (579–581). Although this has been interpreted as a consequence of the dissipation by the chelator of local Ca$^{2+}$ gradients at excitotoxic loci, recent reports suggest that the protection may have a presynaptic action, by blocking glutamate exocytosis and thus preventing network firing in the culture (1).

An explanation for the selective toxicity of glutamate may be a close colocalization between mitochondria and NMDA receptors. Parallel determinations of [Ca$^{2+}$]c and [Ca$^{2+}$]m (with calcium green 1N and rhod 2, respectively) in striatal neurons indicate that although there is a detectable delay between the cytoplasmic and mitochondrial Ca$^{2+}$ elevations after activation of AMPA/kainate receptors or voltage activation Ca$^{2+}$ channels, NMDA receptor activation resulted in a virtually synchronous increase in both compartments, interpreted as a privileged access of NMDA receptor-mediated Ca$^{2+}$ entry to the mitochondrion (447).

Toxicity induced by the nondesensitizing activation of AMPA receptors by kainate, or by AMPA plus cyclothiazide, can occur as a consequence of Na$^{+}$ entry alone through Ca$^{2+}$-impermeable forms of the receptor, in which case death may result from osmotic rupture of the plasma membrane (291), or may be associated with Ca$^{2+}$ entry through Ca$^{2+}$-permeable AMPA/kainic acid receptors as occurs in a GABAergic subpopulation of cortical neurons (81).

F. Mitochondrial ROS During Glutamate Exposure

The generation of O$_2^-$· and derived ROS by cultured neurons is enhanced in response to activation of NMDA receptors (72, 314, 315, 364, 462, 478). Kainate also enhances ROS generation in some neurons (462, 466) but not others (315). There is extensive evidence that a major component of the neuronal degeneration subsequent to glutamate exposure is a consequence of the resulting oxidative damage (for reviews, see Refs. 38, 111). Glutamate excitotoxicity requires oxygen, thus hippocampal neurons exposed to glutamate under hypoxic conditions show no more cell death than due to hypoxia alone (136), whereas NMDA antagonists need only be present during reperfusion to protect against excitotoxic damage to cortical neurons after chemical ischemia (592).

The main ROS that have to be considered are O$_2^-$·, which is predominantly generated by the mitochondria; H$_2$O$_2$, produced from O$_2^-$· by the action of mitochondrial Mn-SOD and cytoplasmic CuZn-SOD, and peroxynitrite, ONOO$^-$, generated by the reaction of O$_2^-$· with nitric oxide (NO). Nitric oxide is in turn generated by cytoplasmic NO synthase (NOS) in response to elevated [Ca$^{2+}$]c (180).

The mitochondrially located Mn-SOD (SOD2) may have first access to mitochondrially generated O$_2^-$·; knock-out of the gene is embryonic lethal, whereas heterozygous SOD2$^{-/-}$ mice have an enhanced O$_2^-$· production and increased susceptibility to oxidative damage (396). In contrast, mice overexpressing human SOD-2 are protected against neuronal ischemic damage (287). The second enzymatic means of removal of O$_2^-$· is by glutathione
peroxidase (601); once again, overexpression is neuroprotective (601).

As discussed in section vB, it is virtually impossible to quantify total superoxide production in cells. As soon as the radical is produced, multiple pathways compete for its further metabolism. If attempts are made to assay \( O_2^- \) by spin trapping or by reaction with reduced precursors of fluorescent dyes, this will merely provide a further pathway for \( O_2^- \) removal that may not compete successfully with each of the endogenous pathways. Thus available assays may at best only assay the steady-state concentration of \( O_2^- \) rather than quantify its production. With this proviso, an enhanced neuronal production of \( O_2^- \) can be detected in neurons after NMDA receptor activation (45, 72, 478, 519). The increase is largely mitochondrial, since it is abolished by mitochondrial depolarization (478). Calcium accumulation by isolated mitochondria leads to an increase in \( O_2^- \) production (299, 300), and a plausible hypothesis is that it is the \( Ca^{2+} \) loading of the in situ mitochondria that is responsible for the increased \( O_2^- \) production after glutamate exposure. However, as discussed in section vA, there is currently no satisfactory molecular mechanism to explain the link between mitochondrial \( Ca^{2+} \) loading and \( O_2^- \) generation.

VII. MITOCHONDRIA AND GLUTAMATE-INDUCED DELAYED CALCIUM Deregulation

Individual neurons maintain a plateau \([Ca^{2+}]_c\) in the presence of glutamate for varying periods before DCD occurs. Cell survival fits a single exponential curve, consistent with a stochastic process (137). Delayed \( Ca^{2+} \) deregulation precedes and reliably predicts the necrotic death of the cultured neuron (578) and is thus an important and intensively investigated in vitro model. Although several steps preceding DCD remain to be clarified, there is evidence, which is now discussed, that DCD is the irreversible end point of a sequence involving \( Ca^{2+} \) loading of mitochondria, increased generation of ROS, and oxidative damage to the cell leading to a failure of \( Ca^{2+} \) extrusion. Deregulation of \([Ca^{2+}]_c\) in granule cells appears to be a result of failed efflux rather than enhanced influx, since the rise in \([Ca^{2+}]_c\) is not blocked by a cocktail of channel inhibitors (85), and the rate of \( Mn^{2+} \) quenching of fura 2 fluorescence, indicative of \( Ca^{2+} \) entry, does not increase as cells deregulate (288).

A. DCD and ATP Synthesis

A failure of \( Ca^{2+} \) extrusion could result from cytoplasmic ATP depletion; indeed, this is the cause of the immediate \( Ca^{2+} \) deregulation seen when cells are exposed to glutamate under conditions of energy depletion (74). A fundamental question is therefore whether DCD is itself a consequence of a collapse in cytoplasmic ATP levels. Although the ATP/ADP falls in populations of derestrregulating cells (16, 74), it has not so far been possible to determine the ratio in individual cells and to correlate this with the onset of DCD. Thus the population decline in ATP/ADP could reflect a population of surviving cells with high energy levels diluted by dying, ATP-depleted cells.

The requirement of glycolysis for ATP at two steps (hexokinase and phosphofructokinase) implies that a cell whose ATP levels are declining, even transiently, could suffer an irreversible collapse as glycolysis becomes limiting, exacerbating a further decrease in ATP. After respiratory inhibition by rotenone, synaptosomes show an initial enhancement of glycolysis, consistent with a Pasteur effect, followed by a progressive failure (284). This glycolytic failure has been subsequently analyzed in more detail (159) under conditions where the bioenergetic safety margin was eroded by increased energy demand (e.g., ionophore addition) and inhibited ATP generation (including rotenone addition). It was concluded that the ATP requirement for hexokinase was the limiting factor during glycolytic failure (159). Such a failure of glycolysis creates logistic difficulties for the cell, equivalent to that of a car with a flat battery, in restarting glycolysis and regenerating its ATP after termination of the bioenergetic insult. This is seen in granule cells exposed to glutamate in the presence of a respiratory chain inhibitor; inhibition of the NMDA receptor by MK-801 is insufficient to allow \( Ca^{2+} \) homoeostasis to be regained (85). However, if the continual drain on the ATP pool by the reversal of the mitochondrial ATP synthase is stopped by the addition of oligomycin, then the cells are able to restore a low cytoplasmic \( Ca^{2+} \) after the NMDA receptor is inhibited (85).

A more physiologically relevant mechanism to restart glycolysis in an ATP-depleted neuron is to utilize lactate and/or pyruvate, which are effective neuronal substrates, particularly in neonatal animals (133, 165, 262, 567, 589). Lactate readily permeates the plasma membrane and is converted to the mitochondrial substrate pyruvate by lactate dehydrogenase, a process which has no requirement for ATP. Thus lactate, predominantly supplied by glia (514), facilitates neuronal recovery after a bioenergetic insult (129, 150, 243, 261, 513, 515).

The timing and extent of glutamate-induced DCD in granule cells is unaffected by the presence of oligomycin (74). Thus a simple failure of mitochondrial oxidative phosphorylation can be eliminated as a cause of DCD. In other words, DCD occurs in cells whose mitochondria are polarized, transporting electrons and accumulating \( Ca^{2+} \) but not taking part in cellular ATP production. Evidence was presented in section vD that mitochondria within glutamate-exposed neurons continue to generate ATP during the latent period. If DCD were caused by a conti-
uously increasing ATP demand by the cell, it would therefore be predicted that the restriction of the maximal rate of cellular ATP synthesis imposed by oligomycin would greatly reduce the latency of DCD. Three further lines of evidence argue against bioenergetic failure as the cause, rather than an effect, of DCD. First, DCD is essentially independent of the pathway of ATP generation within the cell, occurring in glucose-maintained cells in the absence of oligomycin (glycolysis plus oxidative phosphorylation), in the presence of the inhibitor (glycolysis alone), and in glucose-depleted cells supported by lactate or pyruvate (tricarboxylic acid cycle-supported oxidative phosphorylation) (85). Second, cells supported by glucose and about to initiate DCD are not even temporarily rescued by the addition of lactate/pyruvate to provide additional substrate (85). Finally, in situ mitochondria remain competent to generate ATP until DCD is under way (415).

B. DCD and the MPT

Because mitochondria accumulate a considerable amount of Ca\(^{2+}\) during glutamate exposure, a reasonable hypothesis is that DCD may represent the final consequence of mitochondrial Ca\(^{2+}\) overload, namely, the MPT with the consequence that Ca\(^{2+}\) would flood out of the mitochondrial matrix and overwhelm the plasma membrane Ca\(^{2+}\) efflux pathways. However, although this may provide an attractive unifying hypothesis, particularly since oxidative stress facilitates the opening of the MPT, we would caution against an apparent consensus (see Ref. 304) that the MPT, which can be readily observed with isolated mitochondria, occurs in intact cells as a component of necrotic neuronal cell death. Although there is evidence in support of an activation of the MPT in myocardial reperfusion injury (12, 195, 196) and during oxidative and anoxic stress of hepatocytes (235, 255, 256, 279, 424, 440, 441, 527, 535, 571, 621), a role in glutamate-evoked neuronal excitotoxicity is currently much more speculative.

Much of the evidence brought forward in support of the MPT in this context relies on the use of the immunosuppressant cyclosporin A, which is an effective inhibitor of the MPT in isolated liver mitochondria (reviewed in Ref. 44) but may be less effective against brain mitochondria (303). Some measure of protection is afforded by cyclosporin A in vivo (328), but effects in vitro are variable with no effect (257), a slight delay in DCD (423, 508, 604), and more dramatic protection (17, 285) each being reported. Ready observation of the MPT with isolated mitochondria requires a distinctly nonphysiological combination of conditions, since it is inhibited by Mg\(^{2+}\) and adenine nucleotides and is difficult to see with NAD\(^{+}\)-linked substrates (43). The conditions within the cytoplasm of a glutamate exposed neuron would on each of these counts be predicted to be relatively resistant to the MPT.

The major limitation with the use of cyclosporin A is that it is a potent inhibitor of calcineurin, and it is likely that it is this, rather than the MPT, which underlies most of the observed effects in cultured neurons. Thus, although Ankarcrona et al. (17) found that cyclosporin A protected granule cells against both early necrosis and delayed apoptosis induced by glutamate, a similar protection was afforded by the more selective calcineurin inhibitor FK-506, which does not interact with the MPT pore. FK-506 is also neuroprotective in some in vivo models (75, 122, 251, 568). Calcineurin inhibition by cyclosporin A or FK-506 has multiple effects consistent with a hyperphosphorylation of the substrates for the phosphatase, including an increase in spontaneous action potential firing (590), prevention of neuronal NOS (nNOS) activation (122), and partial inactivation of the Na\(^{+}\)-K\(^{+}\)-ATPase (356), whereas chronic cyclosporin A induces neuronal apoptosis in cortical cultures (368, 369). Reported effects of cyclosporin A and FK-506 on NMDA receptors include inhibition (341), enhanced open time (329, 615), and blockade of desensitization (611). In granule cells, cyclosporin A, the more selective 4-N-methylvaline-cyclosporin (420), and bongkrekic acid (69) each only afforded an insignificant delay before the onset of delayed Ca\(^{2+}\) de-regulation (85).

“Rod” to “rounded” conformational changes in mitochondria and loss of \(\Delta \psi_m\) have been observed after addition of the Ca\(^{2+}\) ionophore 4-Br-A-23187 or glutamate to cultured hippocampal neurons (138). These changes have been ascribed to the MPT (138). However, although the ionophore induces swelling of isolated mitochondria (373), this may not in itself be due to the MPT (92). In addition, as discussed in section VI, studies with Ca\(^{2+}\)/2H\(^{+}\) exchange ionophores in intact cells are complicated by the direct effects on mitochondrial bioenergetics, including effective mitochondrial uncoupling due to Ca\(^{2+}\) cycling.

C. Mitochondrial Depolarization Protects Neurons Against DCD

Mitochondrial depolarization by rotenone (or antimycin A) plus oligomycin greatly prolongs the latency before DCD of glutamate-exposed cerebellar granule cells (74, 85). The equal effectiveness of the two respiratory chain inhibitors in the presence of oligomycin is surprising, since antimycin A, but not rotenone, increases the production of ROS by cultured neurons (72). This suggests that DCD cannot solely be a consequence of accelerated mitochondrial \(\text{O}_2^-\) generation. The observation that mitochondrial depolarization is neuroprotective has subsequently been confirmed with a variety of systems, includ-
ing hippocampal neurons in primary culture (139, 519) and cortical neurons (539).

Because prior granule cell mitochondrial depolarization with rotenone/oligomycin results in lower cytoplasmic Ca\(^{2+}\) responses to glutamate (74, 85) and a decreased accumulation of \(^{45}\)Ca\(^{2+}\) (74), a simple explanation for the increased survival of the cells might be that the decreased Ca\(^{2+}\) failed to activate cytoplasmic death processes. This can be eliminated since the enhanced survival is also seen in the presence of elevated external Ca\(^{2+}\), when [Ca\(^{2+}\)]\(_e\) responses to glutamate of cells with depolarized mitochondria are restored to control values (85). The increased [Ca\(^{2+}\)]\(_e\) and decreased matrix Ca\(^{2+}\) produced by cyanide (139) or protonophores (139) in cultured hippocampal neurons and cortical neurons (472, 539, 540) also demonstrate that an elevated [Ca\(^{2+}\)]\(_e\) may not be directly toxic. Indeed, an elevated [Ca\(^{2+}\)]\(_e\), by activating Ca\(^{2+}\)-dependent K\(^+\) channels, can hyperpolarize the plasma membrane and decrease excitability (140, 429). Furthermore, the concentration of glutamate required to produce a maximal elevation in [Ca\(^{2+}\)]\(_e\) is lower than that producing maximal excitotoxicity (598), consistent with a noncytoplasmic Ca\(^{2+}\) pool controlling cell death.

D. Superoxide, Oxidative Stress, and DCD

It is evident that oxidative stress will induce cytoplasmic Ca\(^{2+}\) deregulation and neuronal cell death (for reviews, see Refs. 36, 38, 111); the question that is addressed here is whether the enhanced mitochondrial production of ROS during glutamate exposure is directly responsible for DCD in cell culture models of glutamate excitotoxicity.

Imposed oxidative stress, for example by addition of menadione, which generates O\(_2\cdot\) by 1e\(^-\) redox cycling at the mitochondrial outer membrane (521), induces the MPT in isolated mitochondria (226, 499) and decreases the mitochondrial outer membrane (521), induces the direct toxic. Indeed, an elevated [Ca\(^{2+}\)]\(_c\), by activating Ca\(^{2+}\)-dependent K\(^+\) channels, can hyperpolarize the plasma membrane and decrease excitability (140, 429).

The cytoplasmic and mitochondrial glutathione pools represent the cell’s main redox buffer against oxidative stress. Total cellular glutathione levels in murine primary neuronal cultures were reported to be very much lower than in astrocytes (474), although mitochondrial glutathione in cultured cerebellar granule cells may be higher than in cerebellar astrocytes (245). Glutathione peroxidase works in parallel with Mn-SOD to trap intramitochondrial O\(_2\cdot\) (601), and glutamate excitotoxicity is accompanied by a depletion of reduced glutathione (GSH) (365) but can be delayed by increased GSH synthesis by the addition of precursors such as cystine (174). The reduced GSH pool is regenerated by glutathione reductase, which utilizes matrix NADPH. As discussed above, because the energy-linked transhydrogenase is in turn responsible for maintaining the high level of reduction of the NADP(H) pool in the mitochondrial matrix, driven by Δp (for review, see Ref. 237), there is a link between mitochondrial energization and GSH redox status. This is seen in hippocampal slices where NAD(P) reduction, which largely reflects mitochondrial pools (142), was monitored by surface fluorescence (453); a long-lasting postanoxic hyperoxidation of the nucleotide pool was seen that could be attenuated by exogenous reduced glutathione.

E. Role of NO\(^-\) in DCD

It is currently a matter of debate whether NO\(^-\) is protective or destructive in ischemic brain damage (reviewed in Ref. 250). Neuronal NOS knockout mice show decreased lesion sizes in response to stereotactic NMDA injection (25), whereas corresponding cortical cultures are more resistant to glutamate excitotoxicity (122). Nitric oxide synthase inhibitors have been reported to be neuroprotective against in vitro excitotoxicity (99, 120, 121, 260, 312), although this has been disputed (179, 231, 444). In contrast, nNOS expressing subpopulations of cortical neurons were resistant to glutamate excitotoxicity, and this protection was lost in the presence of a NOS inhibitor (252). In establishing the pathophysiological roles of NO\(^-\) and peroxynitrite (ONOO\(^-\)) with in vitro models, it is important to distinguish the effects of the endogenously generated species from those added exogenously, since the latter may not reproduce the spatial, temporal, and compartmental characteristics of the endogenous species. Thus the demonstration of an excitotoxic effect of NO\(^-\) donors or ONOO\(^-\) (e.g., Refs. 64, 319) does not in itself demonstrate that the glutamate-induced generation of these species is sufficient to cause such effects.

Although it is established that the generation of NO\(^-\) is increased during glutamate exposure of cultured neurons (315, 331, 600), the multiple potential effects of the radical on neuronal function make a molecular analysis extremely complex (for review, see Ref. 49). In addition to its signal transduction role in activating soluble guanylyl cyclase (435), NO\(^-\) competes reversibly with molecular oxygen at cytochrome oxidase (53, 65, 66) and provides an effective trap for O\(_2\cdot\) as a result of the nonenzymatic formation of ONOO\(^-\) (469). However, while decreasing the steady-state concentration of O\(_2\cdot\), peroxynitrite itself will nitrate tyrosine groups (587), inhibit Mn-SOD (350) and CuZn-SOD (258), and at very high concentrations irreversibly inhibit mitochondrial complex III (50, 335).
and plasma membrane glutamate transporters (572). The enhanced cell death associated with SOD-1 downregulation by antisense oligonucleotide required NOS, consistent with a toxic role of ONOO\(^{-}\) (573).

Many of the acute in vitro effects of exogenous NO\(^{\cdot}\) generators on cultured neurons (62–64) and synaptosomes (66) may be attributed to the inhibition of mitochondrial respiration. The extent of collapse in [ATP]/[ADP] in the presence of NO\(^{\cdot}\) is greater than that induced by protonophore, suggesting that the gas may additionally inhibit glycolysis (62). Inhibition is reversible, occurs at the level of cytochrome oxidase, and is competitive with oxygen, with half-inhibition by 270 nM NO\(^{\cdot}\) at \(\sim 145 \mu M O_2\) and by 60 nM NO\(^{\cdot}\) at \(\sim 30 \mu M O_2\) (66). Isolated cytochrome oxidase is inhibited by similar levels of NO\(^{\cdot}\); inhibition is transient as cytochrome oxidase can oxidize NO\(^{\cdot}\) to N\(_2\)O (629). These levels of NO\(^{\cdot}\) are within the measured physiological and pathological range for a number of tissues and conditions, suggesting that NO\(^{\cdot}\)-inhibition of cytochrome oxidase and the competition with oxygen may occur in vivo (66).

**F. Is DCD Caused by Oxidative Damage to the Plasma Membrane Ca\(^{2+}\)-ATPase?**

The plasma membrane Ca\(^{2+}\)-ATPase, which is the sole means of extruding Ca\(^{2+}\) from cells with compromised Na\(^{+}\) electrochemical gradients, is very sensitive to oxidative damage (238, 450). The alternative Ca\(^{2+}\)/3Na\(^{+}\) exchanger is thermodynamically incapable of Ca\(^{2+}\) extrusion under these conditions and indeed may reverse during the initial stages of glutamate exposure (239; but see Ref. 14). No increased rate of Ca\(^{2+}\) entry into granule cells can be detected during DCD (288). By elimination, the most likely cause of DCD is a failure of Ca\(^{2+}\) extrusion from the cell. Thus addition of an external Ca\(^{2+}\) chelator caused only a slow decrease in [Ca\(^{2+}\)]\(_{e}\) in hippocampal neurons exposed to 50 \(\mu M\) glutamate for 15 min, compared with a rapid decline in control cells (289). MK-801 fails to restore Ca\(^{2+}\) homeostasis to granule cells once DCD has begun (85), whereas a progressive loss of Ca\(^{2+}\) extrusion capacity is suggested by the ability of granule cells to extrude the Ca\(^{2+}\) released from mitochondria after 5-min glutamate exposure, but not after 35 min (86).

**G. At What Stage in DCD Do the Mitochondria Undergo Bioenergetic Failure?**

Although several mitochondrial enzymes, including aconitase (618) and pyruvate dehydrogenase (552), are sensitive to inhibition by ROS, the role that metabolic inhibition plays in DCD is unclear, since DCD can be induced independently of the source of ATP (85). Evidence that in situ mitochondria remain net generators of ATP throughout the plateau phase of glutamate exposure was presented above. In addition, although forebrain mitochondria rapidly prepared from rats subjected to 30 min of ischemia display a decreased respiratory control primarily ascribed to an inhibition of state 3 respiration using NAD-linked substrates (516), this was reversed in vitro by the addition of EGTA and may reflect extensive Ca\(^{2+}\) loading of the mitochondria, rather than an irreversible oxidative damage. Furthermore, most of the changes in function observed when mitochondria are prepared from ischemic brain are reversed when the organelles are isolated after reperfusion (for review, see Ref. 530).

Atlante et al. (22) incubated cerebellar granule cells with glutamate for 30 min and then isolated their mitochondria at intervals up to 5 h subsequently. They found a progressive decrease in both state 3 and state 4 respiration, although respiratory control (with succinate as substrate) was not lost until 5 h. Whole cell respiration became inhibited in parallel, although it is not possible to distinguish in these studies between a uniform, causal, failure of mitochondrial function and a population response that was the average of functional and damaged mitochondria in distinct subpopulations.

In conclusion, major mitochondrial depolarization, sufficient to reverse the ATP synthase, may be the final stage in the chain of events comprising DCD and the result of an uncontrolled increase in [Ca\(^{2+}\)]\(_{e}\) after failure of the Ca\(^{2+}\)-ATPase, rather than an early initiating signal.

**H. Synergy Between Bioenergetic Restriction and Excitotoxicity**

Although a fall in cellular ATP levels may be an effect rather than the direct cause of DCD (see above), there is abundant evidence, both in vitro and in vivo, that any restriction on the maximal capacity of the cell to generate ATP can exacerbate or even induce glutamate excitotoxicity. Furthermore, the extent to which ATP decreases may define the subsequent mode of cell death, with severe depletion being associated with necrosis and a more moderate decline leading to apoptotic cell death (16, 421). The “energy-linked excitotoxic hypothesis” (39, 110, 193, 224, 225, 346, 428) proposes that the synergism between excitotoxic damage and energy restriction is due to plasma membrane depolarization resulting from energetic limitation removing the voltage-dependent Mg\(^{2+}\) block of the NMDA receptor. Thus, when cerebellar granule cells are deprived of glucose for 40 min, the concentration of glutamate required to induce cell death is reduced 100-fold (346). However, additional bioenergetic factors are evidently involved, including mitochondrial control of oxidative stress and cytoplasmic ionic homeostasis. Furthermore, the depletion of ATP will directly release glutamate and activate NMDA receptors in the absence of exoge-
Nous glutamate, causing “indirect excitotoxicity,” so causal relationships are unclear (321).

Much of the impetus for these studies has come from observations that chemical restriction in the activity of respiratory chain complexes can induce pathological changes in specific brain regions remarkably similar to those observed in certain neurodegenerative diseases. Synergism between glutamate excitotoxicity and bioenergetic limitation has been proposed for Parkinson’s disease (PD), Huntington’s disease (HD), amyotrophic lateral sclerosis, traumatic central nervous system injury (26), and a range of degenerative ataxias (reviewed in Ref. 38). In many cases, specific respiratory chain complexes are implicated; however, it is unclear whether the distinctive pathology of each disorder is a consequence of the proposed site of energetic limitation or the location of affected neurons within the brain.

The two most intensively researched areas are the modeling of PD by complex I inhibition (for review, see Ref. 48) and HD by complex II inhibitors (for review, see Ref. 35). In addition, there is evidence for a link between a genetic restriction on complex IV activity and some forms of Alzheimer’s disease (520). Our discussion is restricted to in vitro models that focus on cellular bioenergetics.

The selective death of dopaminergic neurons of the substantia nigra after following exposure to 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) or its active metabolite 1-methyl-4-phenylpyridinium (MPP⁺), which results in complex I inhibition, provides a model for PD. Titration of synaptosomes prepared from the CA1 region of the rat hippocampus with rotenone revealed that the threshold beyond which complex I activity restricted respiratory chain activity was only 25% (115). Although MPP⁺ is a weak inhibitor of complex I in submitochondrial particles (398), its ability to accumulate across the plasma membrane and into the matrix in response to Δψp and Δφm, respectively, means that it becomes a potent respiratory chain inhibitor in intact cells. The transport of MPP⁺ is very similar to that of phosphonium cations such as TPMP⁺ (see sect. II B); thus accumulation across the mitochondrial membrane is facilitated by the coaddition of the cationic tetraphenylboron (117, 398). Very low concentrations of rotenone or MPP⁺ synergistically enhance the sensitivity of cultured mesencephalic dopaminergic neurons to glutamate (91, 358), whereas rotenone similarly sensitizes hippocampal neurons (508) toward glutamate.

The genetic defect in HD has been identified as an abnormally expanded CAG trinucleotide repeat in a protein of unknown function “huntingtin” (347). The main neuropathological features of the disease are seen within the neostriatum, and biopsies show the presence of abnormal mitochondria (324) with a decrease in respiratory chain activity that was most severe for complexes II and III, although complex IV was also decreased (200). In vivo studies extensively reviewed elsewhere (37, 67) have demonstrated that inhibition of complex II by the irreversible inhibitor 3-NPA or the reversible inhibitor malonate create lesions within the striatum with features in common with HD, although the mechanistic link remains to be elucidated. The damage induced by complex II inhibition in vivo is excitotoxic and can be protected against by MK-801 (227). Also, NMDA-induced death of cultured striatal (194), hippocampal (437), or mesencephalic dopaminergic neurons (625) is exacerbated by inhibition of complex II by 3-NPA or malonate generated intracellularly from methylmalonate (371).

VIII. MITOCHONDRIA AND APOPTOSIS

To keep the subsequent sections of this review within bounds, we have adopted a number of constraints: wherever possible we restrict discussion to neuronal apoptosis and, furthermore, focus on mitochondrial function. We shall also not review the extensive literature on growth factor modulation of apoptosis. However, because much of current research in this field has involved mitochondrial bioenergetics in nonneuronal cell lines, cell-free systems, or isolated mitochondria, we will discuss these findings where relevant. First we very briefly review the general features of programmed cell death.

A. Biochemistry of Apoptosis

The signaling pathway culminating in apoptosis or programmed cell death was first unveiled in the nematode Caenorhabditis elegans, where genetic analysis revealed three genes, ced-3, ced-4, and ced-9, which are important in the regulation of nematode cell death. Ced-3 and ced-4 encode effector components of the pathway, whereas the ced-9 product functions as a negative regulator, suppressing inappropriate death signals (154). Mammalian homologs to each of these genes were subsequently discovered and cloned (223, 635), and analysis revealed strong conservation in structure and function, suggesting that key components of apoptosis are evolutionarily conserved.

Caspases, a family of cysteine proteases homologous to CED-3, are implicated in neuronal apoptosis in vivo (91, 213, 338, 401, 616), in primary neuronal culture (19, 130, 134, 135, 151, 186, 214, 305, 360, 405, 512, 557, 561–563), and in neurally derived cell lines (216, 218, 459, 511, 537, 538, 584). The first caspase to be isolated, due to its sequence identity with CED-3, was interleukin-1β converting enzyme, ICE, or caspase 1 (388). Because caspase-1 was not found to be absolutely necessary for all forms of mammalian apoptosis (caspase-1 −/− thymocytes undergo apoptosis, Ref. 310), a search began for
other death effectors. To date, a family of at least 11 related cysteine proteases has been isolated (for review, see Ref. 100). The activation of caspases results in the cleavage of a variety of substrates at Asp-X residues, including α-fodrin, poly(ADP-ribose) polymerase (PARP), histone H1, and DNA fragmentation factor. Such proteolytic activity results in the dramatic and characteristic morphological changes observed during apoptosis (333).

Caspases are present in cells as minimally active pro-caspases, and this prevents their premature activation. However, as determined by the immunolocalization of cleaved, or active, caspase-3 (401), a low basal level of caspase activity is present in healthy postmitotic neurons, which may point to a physiological function for these proteases, perhaps in the modeling of cell architecture. This also implies that some form of regulation must exist to prevent inappropriate apoptosis; in this context, a family of inhibitors of apoptosis has been described that act directly on caspases (614).

It is currently uncertain whether each member of the caspase family mediates the cleavage of specific substrates, or whether there is some overlap of targets. However, some caspases appear to be more important than others for the induction of apoptosis. For instance, caspase-1 dominant negative mice appear normal (172), whereas caspase-3 knockout mice have severe developmental abnormalities and suffer premature lethality (311), and mutation of caspase-9 results in embryonic lethality (309). Caspase-3-related proteases cleave proteins of the cytoskeleton, nuclear matrix, transcription factors, and DNA repair enzymes (100). Because caspases are themselves activated after cleavage of the proenzymes at specific Asp-X residues, this suggests that an initial caspase activation can initiate a cascade of proteolytic activity through a hierarchy of caspases.

Bcl2 bears sequence similarity to the CED-9 protein (223) and is a member of a family of related proteins that includes both inhibitors (e.g., Bcl2, BclxL, and Mcl-1) and activators [e.g., Bax, Bak, Bad, Bik, Bid, Bim, and BclX(S)] of cell death. Bcl2 family members interact with each other and with nonfamily members via four domains of homology: the Bcl2 homology domains BH1 to BH4 (for review, see Ref. 374). Overexpression of the death agonists Bax or Bad in the absence of external death-inducing stimuli is sufficient to induce caspase activity and apoptosis (613). Overexpression of Bcl2, however, blocks apoptosis induced by a wide variety of stimuli (for reviews, see Refs. 4, 10, 13, 207, 374, 379).

Bcl2 seems primarily to play a role in physiological cell death, and mice completely lacking the Bcl2 gene show no gross anatomical abnormalities in the nervous system, although there is evidence of increased oxidative damage (232) and progressive peripheral neuronal degeneration during early postnatal development (376). Bcl2 was originally described as an inner mitochondrial membrane protein (233), but subsequent work showed that it is localized to outer mitochondrial membranes, endoplasmic reticulum, and nuclear membranes (10, 301, 391, 400, 404, but see Ref. 393). Mitochondrial Bcl2 may be enriched at contact sites between inner and outer membranes (127). Loss of the COOH-terminal hydrophobic signal that is responsible for its mitochondrial membrane localization reduces the protective effect of Bcl2 (234). Phosphorylation of Bcl2 (259) by a mitochondrially associated protein kinase C-α (492) may be required for its antiapoptotic function. The proapoptotic Bax translocates to the mitochondrion in response to an apoptotic signal (197, 242, 610) and interacts sufficiently closely with Bcl2 to allow fluorescence resonance energy transfer of the green fluorescence protein-tagged proteins (351). Apoptotic protease activating factor (Apaf-1), the mammalian homolog of CED-4, was the last of the three critical genes to be purified and cloned (635). Like CED-4, Apaf-1 can bind caspases and initiate a caspase protease cascade (327). One possibility is that this localization brings Bcl2 in proximity to its binding partners, i.e., Bcl2 may behave like CED-9, by binding to and preventing CED-4-like molecules from activating caspases (244). Proapoptotic relatives may free Apaf-1 from the death inhibitor.

With the isolation of this homolog came the surprising discovery that a component of the respiratory chain, cytochrome c, when released from the mitochondria interacts with Apaf-1 to initiate the caspase cascade (295, 327, 635). As recently reviewed by Zhivotovsky et al. (630), radiobiological studies dating back to the 1950s and 1960s indicated that mitochondria isolated from X-irradiated tissues could exhibit an inhibition of electron transport consistent with a controlled release of cytochrome c (502).

Consequently, a model has been proposed whereby Apaf-1 binds procaspase-9 in a reaction triggered by cytochrome c and dATP (327). This procaspase aggregation initiates the cleavage of procaspase-9 to the active protease, which then cleaves and activates caspase-3. The redox status of cytochrome c does not appear to be important for its activation of caspases (210).

A role of mitochondria in apoptosis was surprising in the light of an early finding that the morphological features of apoptosis can be observed in a fibroblast cell line depleted of mitochondrial DNA and that this can be inhibited by Bcl2 overexpression (263). However, although the mitochondria in these ρ0 cells lack a functional respiratory chain and F0F1-ATPase, they maintain an apparently normal ΔΨm (71), due to the electrogenericity of the adenine nucleotide translocator (454) in a process involving uptake of cytoplasmic ATP, hydrolysis by a functional but “uncoupled” F1-ATPase and export of the resulting ADP (71). Interestingly, maintenance of this membrane potential in ρ0 cells is essential for cell survival (71).
Subsequent research on the mitochondrial involvement in apoptosis has largely focused on the following questions: 1) How do Bcl2, Bax, and related proteins control the release of cytochrome c from mitochondria? In particular, is mitochondrial dysfunction (for example, the MPT and/or Δψm, collapse) accompanied by rupture of the outer membrane necessary to induce release? Therefore, do all proapoptotic signal transduction pathways require the release of cytochrome c from mitochondria? 2) Because cytochrome c can be released in response to a wide variety of apoptosis inducing factors, do the mitochondria themselves sense each type of insult?

B. Cytochrome c Release From Isolated Mitochondria

Cytochrome c release from intact mitochondria requires the transport of the protein across the outer membrane. Two theories prevail as to how this might be accomplished: 1) osmotically induced outer membrane rupture (for example, after activation of the MPT) modulated in some way by Bcl2 family proteins, or 2) direct formation of a specific outer membrane pore involving proteins of the Bcl2 family.

The deeply invaginated mitochondrial inner membrane allows the matrix volume to increase in response to a decrease in the concentrations of impermeant osmolytes, the entry of permeant species, or the induction of the MPT (277, 503, 619). Release of cytochrome c following the MPT is, however, not observed in the presence of the high-molecular-weight osmotic support PEG-1000 (619). In addition to cytochrome c, apoptosis in a cell-free system requires dATP (327, 332). Recently, dATP itself has been described as a specific factor inducing release of cytochrome c from isolated mitochondria (619).

In contrast to MPT-induced release, release induced by dATP was not prevented by PEG-1000, suggesting that osmotic swelling and outer membrane rupture were not involved. Brain mitochondria are relatively resistant to the MPT (303, 418), and a recent study (15) has shown that isolated brain mitochondria in the absence of ATP and Mg2+ can release up to 40% of their total cytochrome c upon exposure to very large Ca2+ loads (up to 3.2 μmol/mg protein) with only a transient depolarization and no induction of the permeability transition.

Mitochondria isolated from a Bcl2 overexpressing GT1–7 neural cell line and oxidizing malate/glutamate showed an enhanced capacity to accumulate Ca2+ before the induction of the MPT, although less effect was seen with succinate as substrate (397). Mitochondria from cells overexpressing Bcl2 (522) or BclxL (586) have also been reported to have unorthodox bioenergetic properties, such as resistance to protonophore uncoupling (522) or an altered ΔGATP to Δp thermodynamic relationship for the ATP synthase (586). However, it would seem prudent to confirm these findings by independent techniques before modifying the conventional understanding of the chemiosmotic proton circuit.

Although the release of cytochrome c from isolated mitochondria is facilitated upon in vitro addition of recombinant Bax or Bak (162, 269, 402), there is disagreement as to the role of the MPT. Jürgensmeier et al. (269) showed that submicromolar concentrations of recombinant Bak induce a release of cytochrome c from substrate-deprived isolated liver mitochondria that was prevented by the presence of recombinant BclxL and was not accompanied by mitochondrial swelling, arguing against the involvement of the MPT. Similarly, Eskes et al. (162) reported that the release of cytochrome c from isolated HeLa cell mitochondria after the addition of recombinant Bax (269) occurred by a mechanism that was Ca2+-independent, insensitive to cyclosporin A and bongkrekic acid, and facilitated by Mg2+ (162), conditions which should eliminate the MPT. In contrast, Narita et al. (402) incubated isolated rat liver mitochondria with recombinant human Bax or human Bak lacking the COOH-terminal hydrophobic domain. These authors found that addition of a high concentration (100 μg/ml) of Bax caused a spontaneous depolarization, within 20 min, of Ca2+-loaded mitochondria (assessed by rhodamine-123 de-quenching) and release of cytochrome c. Depolarization and cytochrome c release were sensitive to bongkrekic acid and cyclosporin A, prevented by BclxL, and accompanied by decreased light scattering and release of mitochondrial aspartate aminotransferase from the intermembrane space. Recombinant Bak lacking the BH3 domain, which mediates its interaction with Bcl2, was inactive.

Outer membrane rupture will release any inter-membrane protein in addition to cytochrome c. Single et al. (531) exposed Jurkat cells to a variety of apoptotic stimuli and monitored the mitochondrial release of cytochrome c and adenylate kinase by selectively permeabilizing the plasma membrane with digitonin. The authors found that cycloheximide, camptothecin, or actinomycin D, which each induce apoptosis in these cells, caused a parallel release of cytochrome c and adenylate kinase, as did staurosporine, which induces necrosis in these cells (531), consistent with a nonselective permeabilization or rupture of the outer mitochondrial membrane.

The crystal structure of BclxL indicates the presence of two central hydrophobic α-helices surrounded by five amphipathic helices (395). This is similar to the structure of the pore-forming bacterial diphtheria toxin. COOH-terminal truncated Bcl2 (507), BclxL (384), and Bak (18) can each form ion channels in synthetic lipid membranes. Chloride ion permeation across liposomal membranes with incorporated Bax or Bcl2 is optimal at pH 4–4.5 and has only 10% activity at pH 5.5, although Bax incorporated into planar bilayers...
is less pH dependent (509). The conductance properties of the channels are characteristic of rather nonselective cation (Bcl2 and BclxL) or anion (Bax) channels rather than the large channel that would be required to transport native holo-cytochrome c itself (509), although under some conditions large open pores ascribed to oligomerized subunits were detected.

The outer mitochondrial membrane possesses porin channels (also termed voltage-dependent anion channels) with pore diameters sufficient to allow the transport of adenine nucleotides but not cytochrome c (485). To establish the significance of the findings with Bcl2 and Bax, a number of questions need to be resolved: 1) Why can channels be formed in vitro by both pro- and antiapoptotic proteins? 2) How functional would the channels be at neutral pH? 3) Could channels be formed of sufficient size to allow transport of the holocytochrome c?

C. Release of Cytochrome c and Apoptosis-Inducing Factor by Mitochondria In Situ

Two techniques have been adopted to monitor cytochrome c release from mitochondria in situ in cells undergoing apoptosis: cellular subfractionation to separate membrane (plus mitochondria) from cytoplasmic fractions (76, 432, 439, 542, 584) and in situ immunofluorescence of the loss of punctate, mitochondrially located cytochrome c-like immunofluorescence (54, 130, 483). These techniques cannot determine what proportion of a cell’s pool of cytochrome c is required for the activation of Apaf-1. The consequences of cytochrome c release will likely depend on the cell type, size of cytochrome c pool, and energy requirements.

Kroemer et al. (304) have isolated a 57-kDa proapoptotic protein termed the apoptosis-inducing factor (AIF) that is released from mitochondria upon opening of the permeability transition and translocates to the nucleus, culminating in apoptosis (83, 123, 451, 546, 548, 622–624). Purified AIF was reported to be able to induce the whole pattern of nuclear alterations in the absence of any other factor (548). However, AIF fails to cleave PARP or nuclear lamin (548), which indicates that its nuclear substrates must be different from those of caspase-3. Apoptosis-inducing factor is a flavoprotein that induces chromatin condensation and large-scale fragmentation of DNA in isolated nuclei. In the presence of heat-inactivated cytosol, AIF additionally causes isolated mitochondria to swell and release cytochrome c, which would indicate positive-feedback amplification (546). Bcl2 is able to prevent the depolarization and release of the factor (548).

Although 10–20 μM cytochrome c injected into many cells is sufficient to induce a significant increase in apoptosis by 30 min (70, 631), in others an additional factor is required. Thus nerve growth factor (NGF)-maintained sympathetic neurons are insensitive to injected cytochrome c. However, if the cells are deprived of NGF for 15–20 h, they develop a protein synthesis-independent, Bax-independent “competence to die” upon injection of the cytochrome (130).

Some nonneuronal programmed cell death pathways have been reported to be independent of mitochondrial participation or release of active cytochrome c (2, 3, 8, 90, 558). For instance, apoptosis after CD95 (APO-1/Fas receptor ligation) is not always accompanied by signs of mitochondrial dysfunction. Ordinarily, after CD95 stimulation, caspase-3 activation is preceded by a defined sequence including recruitment of signaling proteins to form the death-inducing signaling complex, followed by caspase-8 and then caspase-3 activation (191, 198, 326, 345). However, after CD95 stimulation, mitochondrial release of cytochrome c is recruited under some circumstances (313, 501), perhaps to amplify the activation of caspase-3. Caspase-8 has been reported to disrupt mitochondrial function leading to the release of cytochrome c (361), and recently, a mechanism has been advanced whereby the proapoptotic protein Bid is cleaved by caspase-8 to liberate a fragment, tBid, which induces mitochondrial clustering followed by caspase-independent cytochrome c release (198, 326, 345).

In the C. elegans apoptotic pathway, the Apaf-1 homolog CED-4 does not require cytochrome c to activate the caspase CED-3 (95). Although it is not so extraordinary that the functions of Bcl2 and Apaf-1 are less simple than their counterparts in C. elegans, the recruitment of cytochrome c to this conserved pathway has, by all accounts, surprised everyone. Cytochrome c is a highly conserved protein, with a vital job in the electron transport chain. Bax expression in yeast can induce the in situ mitochondria to release cytochrome c (355); thus it is interesting that cytochrome c isolated from Saccharomyces cerevisiae is not able to initiate apoptosis in a Xenopus cell-free apoptotic model (295).

Because the redox properties of cytochrome c are redundant in this signaling (210, 295), the question arises as to why this protein has been selected to trigger apoptosis. An intriguing suggestion has been made (192) that the cytochrome c involvement is an evolutionary relic of the conflict between the original primitive mitochondria and their host eukaryotic cell, ensuring that cells that attempted to damage and reject mitochondria did not proliferate. Because ROS facilitate the opening of the MPT, Skulachev (533) has suggested that the permeability transition and consequent release of cytochrome c could provide a mechanism for the elimination of cells with oxidatively damaged mitochondria.
D. Role of the Permeability Transition in the Release of Cytochrome c

There are conflicting reports concerning the status of the mitochondrial membrane potential after the induction of apoptosis. Thus Kroemer et al. (304) have reported that mitochondrial depolarization induced by the MPT is the primary event in lymphocyte apoptosis (83, 84, 357, 622, 623), although the validity of techniques to monitor $\Delta \psi_m$ have recently been questioned (489). Kroemer et al. (304) have extended these findings to propose that mitochondrial depolarization and the MPT are not only early events in a wide range of apoptotic systems, but play the role of a general “central executioner” in the apoptotic cascade (190, 451, 547). However, apoptosis is an ATP-requiring process (16), yet the mitochondrial depolarization accompanying a general permeability transition would lead to reversal of the ATP synthase and an energy crisis comparable to that seen after protonophore addition. It is of course possible that only a small proportion of the mitochondria undergo the MPT, releasing sufficient cytochrome c to activate apoptosis, whereas the residual mitochondria maintain the cellular energetics.

Having said this, there is currently no single mechanism for the release of cytochrome c from mitochondria during apoptosis. In nonneuronal cells, such as hepatocytes, there is convincing evidence that an exogenously imposed oxidative stress, such as the addition of the pro-oxidant t-BuOOH, will induce the MPT in in situ mitochondria, resulting in NAD(P)H and glutathione oxidation, mitochondrial depolarization, and loss of calcein exclusion from the mitochondrial matrix (for review, see Ref. 322). On the other hand, although tributyltin induces apoptosis in Jurkat cells as a consequence of the MPT, releasing sufficient cytochrome c to activate apoptosis, whereas the residual mitochondria maintain the cellular energetics.

After transient chemical ischemia, the in situ mitochondria of the surviving GT1–7 cells (399) showed near-normal population respiratory parameters immediately after and 24 h after the insult, although immediately after the episode the control but not Bcl2-overexpressing cells showed a slight increase in state 4 respiration and decrease in protonophore uncoupled respiration. Thus any Bcl2-sensitive, death-inducing, mitochondrial dysfunction after chemical ischemia-induced apoptosis in this model is relatively subtle.

PC12 cells overexpressing Bcl2 display more negative (reduced) redox potentials for both their glutathione and NAD$^+$/NADH pools (153), a decreased radical-induced loss of electron probe resonance-detectable nitroxyl stearate spins labels (68) and are protected against high-oxygen-induced apoptosis by Bcl2 (308) and antioxidants (553). Additionally, PC12 cells overexpressing the mitochondrially located Mn-SOD are protected against apoptosis induced by $\beta$-amyloid peptides and exogenous oxidative stress (287).

A. Trophic Factor Withdrawal

PC12 cells differentiate into a neuronal phenotype in the presence of NGF and undergo apoptosis upon subsequent withdrawal of serum and NGF. Apoptosis is inhibited by mRNA and protein synthesis inhibitors added within 2 h of NGF removal (375, 511), by Bcl2 overexpression (34, 511, 523, 555), and by addition of cell-permeant caspase pseudosubstrates (218, 511).

Generation of ROS, monitored with DCF, increases after NGF deprivation, although the time course varies in different studies, peaking at 6 h (511) or showing a delayed increase (593). The increase in ROS appears to be of mitochondrial origin, since it can be inhibited by rotenone (511). Reactive oxygen species play a role in cell death, since the spin trap N-tert-butyl-\(\alpha\)-phenylnitrone enhances cell survival when added up to 4 h after NGF deprivation (511). Bcl2 overexpression, particularly together with BAG-1 (555), blocks caspase activation, ROS generation, and apoptosis after NGF withdrawal (511). The proposed sequence of events is thus new RNA and protein synthesis, followed by caspase activation (511, 538, 574) and subsequent ROS generation.
The $\Delta \psi_{\text{m}}$ in differentiated PC12 cells has been monitored after NGF deprivation. Using the nuclear background signal of cells as a reference, Wadia et al. (593) utilized the fixable cationic probe CMTMR (but see sect. wB) and reported progressive depolarization during the first 12 h of NGF withdrawal. The JC-1 emission spectra of individual mitochondria within a single PC12 cell were heterogeneous, and after NGF withdrawal, the proportion of green-emitting mitochondria increased, suggesting that individual mitochondria responded with a distinct decrease in $\Delta \psi_{\text{m}}$. A similar fluorescent heterogeneity with JC-1 is also seen with cultured cerebellar granule cells (16). It is not possible from this study to distinguish between a modest (10–20 mV) depolarization that could accompany increased ATP turnover or Ca$^{2+}$ accumulation and a dramatic depolarization associated with, for example, the MPT.

Because the ATP yield from glycolysis alone is some 15-fold lower than for glycolysis plus oxidative phosphorylation, a loss of mitochondrial ATP synthesis as a consequence of mitochondrial depolarization would be predicted to result in a dramatic enhancement in the rate of glycolysis, via the Pasteur effect, to maintain rates of ATP synthesis. However, glucose utilization by NGF-deprived PC12 cells was not enhanced relative to controls, whereas lactate actually decreased within 8 h (383), indicative of continued mitochondrial ATP synthesis.

Individual mitochondria within NGF-deprived PC12 cells show an increased fluorescence of the mitochondrially located Ca$^{2+}$ indicator rhod 2, which is maximal within 6 h and a progressive increase in ROS generation over 24 h (593). As discussed in section IVa, Ca$^{2+}$ accumulation by mitochondria can lower $\Delta \psi_{\text{m}}$ by a number of mechanisms less catastrophic than the MPT. That the mitochondria in NGF-deprived cells retain accumulated Ca$^{2+}$ (593) argues that they retain a substantial $\Delta \psi_{\text{m}}$.

Apoptosis of primary cultured cerebellar granule cells has been intensively investigated. Cells are normally maintained in 25 mM KCl in the presence of serum (176), since the combination of low (5 mM) KCl and serum deprivation leads to extensive apoptosis of rat granule cell death within 24 h (21, 175, 215, 405, 512, 524, 532, 557, 591, 617), although mouse granule cells are more resistant to low KCl (389, 390). Mouse cerebellar granule cells in culture from Bcl2-overexpressing mice show less caspase activation and are less susceptible than cells from control littermates to apoptosis in response to low KCl or serum deprivation (556, 557), whereas those from Bcl2-deficient mice are more susceptible (556). In addition, the enhanced survival of granule cells grown at high density in the absence of trophic factors has been correlated with a two- to threefold increase in the levels of Bcl2 and Bclx$_L$ per cell (431).

The commitment point for apoptosis after low KCl lies between 90 min and 4 h, and cells can be rescued before this point by restoring high KCl (175, 405). In common with most other forms of neuronal apoptosis (266), low K$^+$-induced apoptosis requires protein synthesis, and protection can be afforded by actinomycin D or cycloheximide added within 30–60 min of K$^+$ deprivation (19, 512, 561, 617). In the absence of these inhibitors, caspase activity is induced within 2 h (151, 360).

A burst of ROS production (monitored by dihydrorhodamine-123 or DCF oxidation) can be observed in granule cells after low KCl (512) or serum deprivation (20). Free radical trapping within 3 h of transfer to low KCl decreased apoptosis (512). In common with other forms of apoptosis, cell-permeant caspase-1 inhibitors prevent low KCl apoptosis in these cells (512).

There appears to be an early inhibition of metabolism in cerebellar granule cells exposed to low-KCl medium. Glucose uptake declines to <30% of basal within 2 h of serum deprivation in low KCl and before any detectable loss of viability (381). The absence of a detectable Pasteur effect is consistent with a maintained capacity of the mitochondria for ATP synthesis. Glucose-dependent cerebellar granule cell respiration is rapidly decreased after transfer of the cells to low KCl, and this inhibition is also apparent when the mitochondria are isolated and oxidize succinate (21). The impairment of cellular respiration is prevented by ROS scavengers (21), and ROS generation by cells undergoing apoptosis in low-KCl medium (512) can be inhibited by caspase-1 inhibitors and protein synthesis inhibitors.

B. Bioenergetic Limitation

Chronic KCN induces apoptosis in terminally differentiated but not undifferentiated PC12 cells and increases the generation of ROS detected by DCF (382). One report that challenges conventional concepts of mitochondrial bioenergetics is that the mitochondria within undifferentiated Bcl2-overexpressing PC12 cells maintained on nonglycolytic substrates fail to depolarize upon addition of KCN to inhibit their respiratory chains (523). In the absence of glycolysis, ATP levels collapsed within 30 min in both control and Bcl2 cells, yet $\Delta \psi_{\text{m}}$, monitored by TPP$^+$ accumulation or flow cytometry in the presence of rhodamine-123, was maintained for much longer in the Bcl2-expressing, but not control, PC12 cells. Because there is no obvious source of energy for proton extrusion from the mitochondria under these conditions, confirmation of these provocative experiments may be required.

As discussed in section wH, restriction of the bioenergetic capacity of mitochondria facilitates glutamate excitotoxicity. As in the case of glutamate excitotoxicity, moderate inhibition leads to apoptosis rather than necrosis. Thus transient oxygen/glucose deprivation (271) or low concentrations of MPP$^+$, rotenone, 3-NPA, exoge-
nous NO donors, and ONOO− each induces a largely apoptotic death in cerebellar granule cell cultures (52, 135, 319, 321) with associated caspase activation (315). It has recently been reported that respiratory chain inhibition induces indirect excitotoxicity in granule cell cultures by initiating a spontaneous presynaptic exocytosis of glutamate and activation of NMDA receptors, which can be prevented by inhibiting exocytosis with tetrodotoxin or botulinum neurotoxin C (319, 321). Interestingly, cyclosporin inhibits apoptosis of PC12 cells induced by MPP+(518) but potentiates apoptosis of SH-SY5Y neuroblastoma cells in the presence of MPP+(164).

Apoptosis induced by the K+-uniport ionophore valinomycin has been interpreted as a consequence of enhanced plasma membrane K+ conductance (620), although the insertion of the ionophore into the mitochondrial inner membrane causes mitochondrial swelling and depolarization (489, 517); therefore, cytochrome c release must also be considered.

C. Staurosporine-Induced Apoptosis

PC12 cells undergoing apoptosis in the presence of the nonselective protein kinase inhibitor staurosporine maintain an elevated 

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\text{[Ca}^{2+}\text{]}_\text{c}
\]

accompanied by ROS generation and subsequent mitochondrial depolarization (306). Each of these effects was prevented by caspase inhibition and was absent in Bcl2-overexpressing PC12 cells, indicating that the caspases act early in the apoptotic sequence. Not surprisingly, Bcl2 overexpression does not reduce the levels of ROS generated directly by exogenous oxidants (496). The loss of cytochrome c during staurosporine-induced apoptosis preceded the oxidation of the glutathione pool (76). As cytochrome c is progressively released from polarized mitochondria, there will come a stage when electron transfer from complex III to complex IV will become restricted (302). One effect will be an increased reduction of the ubiquinone binding site during \( \Delta \psi_m \) generation with a consequent enhanced \( \text{O}_2^\cdot \) generation (76).

Staurosporine induces apoptosis in cultured hippocampal neurons (305, 464), characterized by a rapid increase in caspase-1-like activity, peaking at 30 min, followed by a delayed rise in caspase-3-like activity, maximal at 8 h (305). Caspase-1 inhibitors are protective if present when staurosporine is added, but caspase-3 inhibition does not prevent cell death. Oxidative stress plays a major role in staurosporine-induced apoptosis, and adenovirus-mediated SOD-1 overexpression (464), the antioxidants manganese tetrakis(4- bezoyl acid)porphyrin, trolox, and \( \alpha \)-tocopherol (305) each protects against staurosporine-induced apoptosis. Superoxide generation, monitored by hydroethidine, peaked 8 h after staurosporine addition and was blocked by caspase-1-like protease inhibitors (305). Caspase-3-like activation was downstream of \( \text{O}_2^\cdot \), since it was blocked by \( \alpha \)-tocopherol. Cells can be protected by \( \alpha \)-tocopherol addition up to at least 6 h after staurosporine. Importantly, no decrease in \( \Delta \psi_m \) could be detected by single cell TMRE fluorescence for at least 8 h after staurosporine, although FCCP could elicit an immediate depolarization (305).

D. Ceramide-Induced Apoptosis

Ceramide, generated by sphingomyelin hydrolysis, has been proposed to be a second messenger in apoptosis induced by a variety of cytokines and physical stresses (reviewed in Ref. 446). Exogenous C2 ceramide induces apoptosis in PC12 cells (170, 216) associated with an increase in mitochondrial ROS generation (511, 512) and a large increase in caspase-1-like activity, peaking at 30 min, followed by a delayed rise in caspase-3-like activity, maximal at 8 h and causally associated with the subsequent cell death (170). Levels of ATP remained high for 12 h as did \( \Delta \psi_m \), assessed by rhodamine-123. Ceramides directly inhibit electron transport through complex III of isolated mitochondria (125, 201), and restriction of electron flow would have an effect similar to the addition of low concentrations of antimycin A (468) or the loss of cytochrome c in enhancing \( \text{O}_2^\cdot \) generation by complex III.

 generation of ROS by apoptosing PC12 cells (511) can be inhibited by caspase protease inhibitors and requires protein synthesis, implying that it is an event downstream of caspase activation and protein synthesis.

E. Glutamate-Induced Apoptosis

Although high glutamate concentrations result in predominantly necrotic cell death in cerebellar granule cell cultures, with decreasing time or concentration of exposure to glutamate, there is a shift toward apoptosis (16, 51, 134), resulting in cells displaying the morphological features of apoptosis, including nuclear condensation and internucleosomal DNA fragmentation. Glutamate-induced granule cell apoptosis is blocked by MK-801, implicating the NMDA receptor (16, 17, 134); however, in addition, kainate (93, 181, 528) and AMPA agonists in the presence of cyclothiazide to block desensitization (87, 88) can also induce apoptosis. In contrast, granule cell survival in low-KCl medium is enhanced by chronic NMDA receptor activation (30–32, 254), whereas brief blockade of NMDA receptors in vivo in the perinatal rat causes extensive apoptosis (253).

Glutamate-induced apoptosis is not prevented by inhibitors of RNA and protein synthesis (134), thereby contrasting with low KCl-induced apoptosis in these cells. However, caspase-3 activation and PARP cleavage both occurred within 24 h of glutamate addition (134). Cell-permeant caspase-3 inhibitors are neuroprotective.
against proapoptotic concentrations of glutamate but are ineffective against high glutamate concentrations leading to necrosis (19, 134).

The state of mitochondrial polarization during necrotic glutamate exposure was discussed at length in section VIII D, where evidence was advanced that high glutamate concentrations fail to depolarize mitochondria to the extent that they fail to generate ATP. If this is so, it would be reasonable to expect that in situ granule cell mitochondria remain bioenergetically competent during the more controlled exposure to glutamate that results in apoptosis. Ankarovina and co-workers (16, 17) employed the cationic probe JC-1 to monitor $\Delta \psi _{m}$ during and after a 30-min exposure to 3 mM glutamate. Somatic and neurite mitochondria showed distinct fluorescent responses; those in the somata showed faint green fluorescence before glutamate that was intensified after 30 min of glutamate exposure; in contrast, an initial red fluorescence of the neurite mitochondria was rapidly quenched with no detectable appearance of green fluorescence diagnostic of decreased JC-1 aggregation within the matrix (16). Complications due to plasma membrane depolarization accompanying glutamate addition would be largely avoided in these experiments that were performed in 25 mM KCl, but these experiments do not allow any quantification of the extent of mitochondrial depolarization. Importantly, the fluorescence of those cells that did not undergo immediate necrosis recovered to initial values on removal of glutamate, whereas the population “energy charge” (defined as $([\mathrm{ATP}] + 0.5[\mathrm{ADP}])/(\mathrm{[ATP]} + \mathrm{[ADP]} + \mathrm{[AMP]})$) was restored to initial values. Thus the bioenergetic status of the cells that survived the initial glutamate exposure but subsequently showed delayed apoptosis was not impaired.

Calcineurin inhibition has multiple consequences in the intact neuron; the protection afforded by cyclosporin A against glutamate in these cells was mimicked by FK-506 (17, 353) and thus cannot be used as evidence for the involvement of the MPT in cerebellar granule cells. We are aware of no direct determinations of cytochrome c release during apoptosis in these cells; however, the wasp venom peptide mastoparan, which induces apoptosis in granule cells (330), is known to induce the MPT and release cytochrome c from isolated mitochondria (152, 455).

In contrast to cerebellar granule cells, where there is general agreement that submaximal activation of glutamate receptors can induce apoptosis, the situation with cortical neurons is more controversial. Thus, although AMPA (316) or brief NMDA exposure (51, 563) has been reported to result in subsequent apoptosis, sensitive to caspase inhibitors (563), others (204, 536) have concluded that low concentrations of excitotoxin cause cortical neurons to die by necrosis even though transient internucleosomal fragmentation can be detected.

Apoptosis can be induced in cultured cortical neurons by staurosporine (186, 368, 620), serum deprivation (186, 620), $\beta$-amyloid peptides (163), $\mathrm{H}_{2}\mathrm{O}_{2}$ (607), methylmalonate, which generates the complex II inhibitor malonate intracellularly (371), or cyclosporin A (368, 369), but in contrast to glutamate-induced necrosis, which increases with the days in culture (94, 368), apoptosis induced by hypoxia (94), staurosporine (368), and cyclosporine A (368) is most readily observed in cells cultured for <10 days (368). Additionally, although oxygen/glucose deprivation normally induces a predominantly necrotic cell death (337), the presence of glutamate antagonists unmasks apoptosis (106, 186), which can be attenuated by caspase inhibitors (186).

An initial decrease in TMRE fluorescence after proapoptotic NMDA addition and interpreted as mitochondrial depolarization (563) was not prevented by caspase inhibition; however, the extent to which the fluorescence change can be ascribed to plasma membrane depolarization after NMDA receptor activation was not determined. The caspase inhibitors did, however, prevent the generation of ROS (563). Cortical neurons from Bax-deficient mice are resistant to apoptosis induced by serum deprivation or staurosporine addition, but there is disagreement as to whether Bax deficiency confers protection against glutamate toxicity in cortical cultures (612), or was ineffective (185).

X. CONCLUSIONS

The complex and frequently bewildering mutual interactions between mitochondrial and cellular bioenergetics discussed in this review allow some general principles to be derived. First, the mitochondrial membrane potential $\Delta \psi _{m}$ is at the center of the cell’s interactions, controlling ATP synthesis, mitochondrial $\mathrm{Ca}^{2+}$ accumulation, superoxide generation, and redox poise. Second, mitochondria are intimately involved with both necrotic and apoptotic cell death. In the former mode, modeled by exposing cultured neurons to pathological glutamate, mitochondrial $\mathrm{Ca}^{2+}$ loading and consequent generation of ROS appear to play a central role. The generation of ROS and the maintenance of a reduced environment are both favored by a high $\Delta \psi _{m}$, and the damaging effects of the one have to be balanced against the beneficial effects of the other. Sophisticated control mechanisms (the non-ohmic leak, uncoupling proteins) exist and may poise $\Delta \psi _{m}$ at the optimum balance between these opposing factors. The mitochondria themselves appear to retain bioenergetic competence and the capacity to generate ATP until a late stage, characterized in vitro by delayed cytoplasmic $\mathrm{Ca}^{2+}$ deregulation. Mitochondrial release of proapoptotic factors, such as cytochrome c or AIF, is integral to apoptosis induced by a wide variety of cellular
effectors of programmed cell death. Although the processes triggering the release are still controversial, it is likely that different effectors may invoke different release mechanisms so that the search for a unitary release mechanism, such as the MPT, may not be fruitful.

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