Basic Science Review

Calcium in Ischemic Cell Death

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Background—This review article deals with the role of calcium in ischemic cell death. A calcium-related mechanism was proposed more than two decades ago to explain cell necrosis incurred in cardiac ischemia and muscular dystrophy. In fact, an excitotoxic hypothesis was advanced to explain the acetylcholine-related death of muscle end plates. A similar hypothesis was proposed to explain selective neuronal damage in the brain in ischemia, hypoglycemic coma, and status epilepticus.

Summary of Review—The original concepts encompass the hypothesis that cell damage in ischemia-reperfusion is due to enhanced activity of phospholipases and proteases, leading to release of free fatty acids and their breakdown products and to degradation of cytoskeletal proteins. It is equally clear that a coupling exists between influx of calcium into cells and their production of reactive oxygen species, such as \( \cdot \text{O}_2^\cdot \), \( \cdot \text{H}_2\text{O}_2 \), and \( \cdot \text{OH} \). Recent results have underscored the role of calcium in ischemic cell death. A coupling has been demonstrated among glutamate release, calcium influx, and enhanced production of reactive metabolites such as \( \cdot \text{O}_2^\cdot \), \( \cdot \text{OH} \), and nitric oxide. It has become equally clear that the combination of \( \cdot \text{O}_2^\cdot \) and nitric oxide can yield peroxynitrate, a metabolite with potentially devastating effects. The mitochondria have again come into the focus of interest. This is because certain conditions, notably mitochondrial calcium accumulation and oxidative stress, can trigger the assembly (opening) of a high-conductance pore in the inner mitochondrial membrane. The mitochondrial permeability transition (MPT) pore leads to a collapse of the electrochemical potential for \( \text{H}^+ \), thereby arresting ATP production and triggering production of reactive oxygen species. The occurrence of an MPT in vivo is suggested by the dramatic anti-ischemic effect of cyclosporin A, a virtually specific blocker of the MPT in vitro in transient forebrain ischemia. However, cyclosporin A has limited effect on the cell damage incurred as a result of 2 hours of focal cerebral ischemia, suggesting that factors other than MPT play a role. It is discussed whether this could reflect the operation of phospholipase \( \text{A}_2 \) activity and degradation of the lipid skeleton of the inner mitochondrial membrane.

Conclusions—Calcium is one of the triggers involved in ischemic cell death, whatever the mechanism. (Stroke. 1998;29:705-718.)

Key Words: calcium ■ cerebral ischemia ■ free radicals ■ mitochondria

The calcium hypothesis of ischemic cell death was originally launched to explain the relationship between excessive calcium influx and the cell damage that is incurred in myocardial ischemia as well as in muscle dystrophy. In fact, studies conducted at that time led to the formulation of an excitotoxic hypothesis, predicting that excessive release of acetylcholine at the motor end plate was what caused damage to skeletal muscle. The work performed at that time on ischemic muscle damage was almost visionary since it forestalled the pivotal role of release of transmitters in enhancing the influx of \( \text{Ca}^{2+} \) at postsynaptic sites and also predicted that a nonphysiological rise in \([\text{Ca}^{2+}]\) could exert its adverse effects by overactivating cellular proteases and lipases. A link to mitochondrial dysfunction was also suggested since free fatty acids, liberated as a result of PLA2 activity, were supposed to increase the permeability of mitochondrial membranes and to uncouple respiration and oxidative phosphorylation in isolated mitochondria.

In 1977, Nicholson et al showed that anoxia triggers rapid translocation of \( \text{Ca}^{2+} \) from extracellular to intracellular spaces of cerebellar tissues. This, as well as other findings, led to the hypothesis of calcium-mediated neuronal death in ischemia/hypoxia, hypoglycemia, and status epilepticus. As applied to brain tissues, the hypothesis of calcium-mediated cell death has some special features. The most important of these is that since the BBB has a very low permeability to \( \text{Ca}^{2+} \), the calcium translocated into cells is, at least in the short perspective, that contained in the cerebral extracellular fluids. It was tempting to speculate, therefore, that certain neurons in the brain are selectively vulnerable to ischemic, hypoglycemic, and epileptic insults because they possess a high density of calcium channels in their plasma membranes. At that time, many neurons were known to have an innate proclivity to fire synchronously in an epileptic fashion, the epileptiform activity being driven by calcium spikes, ie, by calcium influx through VSCC, localized to the dendritic fields of these neurons. Accordingly, it could

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Selected Abbreviations and Acronyms

AMPA = α-amino-3-hydroxy-5-methyl-4-isoazolepropionate
BBB = blood-brain barrier
CaA = cyclosporin A
EAA = excitatory amino acid
ER = endoplasmic reticulum
IP<sub>3</sub>, IP<sub>4</sub> = inositol 1,4,5-triphosphate, inositol 1,3,4,5-tetraakisphosphate
MCA = middle cerebral artery
MPT = mitochondrial permeability transition
NMDA = N-methyl-d-aspartate
NO = nitric oxide
NOS = nitric oxide synthase
PBN = α-phenyl-N-tert-butyl nitrone
PA<sub>2</sub> = phospholipase A<sub>2</sub>
ROS = reactive oxygen species
VSCC = voltage-sensitive calcium channels

be assumed that selectively vulnerable neurons are those that show such calcium-related, “epileptogenic” spikes, neurons that thus could be assumed to accumulate the brunt of the calcium load under adverse conditions.

An important step forward was taken when it could be demonstrated in cultured neurons<sup>14</sup> and brain slices<sup>15</sup> that glutamate and related EAs trigger neuronal cell death. Such excitotoxic cell injury, which typically affects dendrites and neuronal somata, was originally assumed to be osmolytic, reflecting influx into cells of Na<sup>+</sup> and Cl<sup>-</sup>, with osmotically obliged water.<sup>16</sup> However, it was soon demonstrated by Choi<sup>17</sup> that whereas the early cell swelling was usually reversible, cells exposed to glutamate showed a type of delayed cell death that was calcium mediated. 18,19 This means that the excitotoxic hypothesis is a variant of the calcium hypothesis of cell death, but with agonist-operated channels playing the major role of translocating calcium into cells.

With this as a background, we will probe into the role of calcium in ischemic cell death in the brain. The first paragraphs will be devoted to calcium fluxes between extracellular and intracellular fluids and between blood and brain tissues. On the basis of the results quoted, we will discuss the role of calcium in rapidly occurring or delayed cell death. The notion will be considered that a coupling exists among calcium influx, generation of free radicals, and mitochondrial dysfunction, with mitochondria also emerging as important generators of ROS. It seems justified to begin, though, with a brief summary of cell calcium metabolism in normal cells. For further discussion of the topics discussed, the reader is referred to recent articles from the laboratory.<sup>20–23</sup>

**Cell Calcium Homeostasis**

There is normally a very large electrochemical driving force tending to translocate calcium into cells. This force has two components: the difference in calcium concentration (extracellular fluids having a 10 000-fold higher concentration than intracellular ones) and the electrical potential across plasma membranes (the inside being 60 to 90 mV negative to the outside). The electrochemical potential is upheld because calcium fluxes associated with signal transduction are usually small and tightly regulated and because a rise in [Ca<sup>2+</sup>], triggers the extrusion of calcium by 3Na<sup>+</sup>–Ca<sup>2+</sup> exchange and by an ATP-driven Ca<sup>2+</sup>–2H<sup>+</sup> exchanger.<sup>24–25</sup> The pump-leak relationships thus upheld normally maintain [Ca<sup>2+</sup>], at values of 100 to 200 nmol/L, but the concentration rises transiently during cell activation.

Presynaptic and postsynaptic calcium channels (“conductances”) at an excitatory synapse in which signal transduction is mediated by glutamate encompass N, P, L, and T types of VSCCs. The first two may be mainly localized to presynaptic endings, while the L and T types abound at postsynaptic membranes (see, for example, References 26 through 29). Two ionotropic glutamate receptors exist, one being selectively activated by AMPA and the other by NMDA.

Under normal circumstances, signal transduction begins with release of glutamate from the presynaptic ending and with activation of AMPA and NMDA receptors. Since the AMPA receptor–gated channel is permeable to monovalent cations, Na<sup>+</sup> will enter the cell along its electrochemical gradient, depolarizing the postsynaptic membrane. The depolarization has two effects: it relieves the Mg<sup>2+</sup> block of the Ca<sup>2+</sup>-permeable channel gated by the NMDA receptor, and it opens VSCCs. Calcium thus enters the cell by multiple pathways. At least in some cells additional VSCCs exist, and it is debated at present whether changes in the subunit composition of the AMPA receptor can render the channel it gates permeable to calcium.<sup>30–32</sup> Additional channels may be opened under adverse conditions, eg, those comprising unspecific cation channels and those activated by ROS, notably H<sub>2</sub>O<sub>2</sub> (F. Mendez and R. Penner, personal communication, 1997). It is of interest that the latter inactivate very slowly, if at all. Under some circumstances, such as depolarization and intracellular Na<sup>+</sup> accumulation, the 3Na<sup>+</sup>–Ca<sup>2+</sup> exchanger can operate in the reversed mode, causing calcium to accumulate in the cell (see below).

Since physiological signals usually have a short duration, mechanisms must exist for terminating the glutamate (and calcium) transients. This occurs by several mechanisms. The major mechanism involves reuptake of glutamate through an electrogenic Na<sup>+</sup>/glutamate symporter, which derives its energy from the electrochemical gradient for Na<sup>+</sup>.<sup>33</sup> Although some glutamate may be taken up by presynaptic and postsynaptic neuronal elements, glial cells represent major sinks for glutamate. The glial cells convert glutamate to glutamine or lactate, which are then exported to neurons for resynthesis of glutamate (and energy production).<sup>34,35</sup> Other mechanisms encompass activation of Ca<sup>2+</sup>- or ATP-dependent K<sup>+</sup> channels or of γ-aminobutyric acid–activated Cl<sup>-</sup> channels.<sup>36,37</sup> Activation of such channels would tend to repolarize or hyperpolarize membranes. Yet another mechanism, of theoretical and practical interest, can be deduced from the known dependence of NMDA-activated ion currents on pH. Since Ca<sup>2+</sup> ion conductance decreases steeply when extracellular pH is reduced below 7.0,<sup>34–40</sup> one can envisage that strong excitatory stimuli (which reduce intracellular and extracellular pH) are subjected to feedback inhibition by this mechanism. However, since the pK<sub>a</sub> for the effect of pH on NMDA channel currents is approximately 6.7, one can also deduce that alkalosis (ie, an increase in pH, above normal) increases the tendency to calcium-mediated cell firing, perhaps acting as a trigger for epileptogenic discharges.<sup>41</sup>
Fig 1 summarizes cell calcium metabolism in a wider perspective, taking into account not only pathways of Ca\(^{2+}\) entry but also mechanisms for extrusion, binding, and sequestration. The upper part of the figure illustrates what has already been discussed, ie, the pump-leak relationship for Ca\(^{2+}\) across the plasma membrane. Undoubtedly, this relationship will set, or at least modulate, the value of [Ca\(^{2+}\)]\(_i\). Another determinant is the corresponding calcium traffic across the membranes of the ER, which is supposed to contain fluids having a free cytosolic calcium concentration close to that of the extracellular fluid. The calcium sequestered by the ER can be released by the operation of a sequence of events that starts with activation of surface receptors coupled to phospholipase C, continues with the formation of IP\(_3\), and its activation of IP\(_3\) receptors on ER membranes, and ends with the release of Ca\(^{2+}\) from the ER. This is undoubtedly an important source of calcium, which could contribute to a rise in [Ca\(^{2+}\)]\(_i\). Furthermore, since sequestration of Ca\(^{2+}\) into the ER requires ATP, the traffic of Ca\(^{2+}\) across the ER membranes represents another aspect of the pump-leak relationship for Ca\(^{2+}\).

A third determinant of intracellular calcium movements, and thereby of [Ca\(^{2+}\)]\(_i\), is the mitochondrion. It is now widely accepted that the balance between influx and efflux of Ca\(^{2+}\) across the inner membrane regulates mitochondrial dehydrogenases, which are rate-limiting for citric acid cycle metabolism. In summary, when cell activity leads to a substantial or sustained rise in [Ca\(^{2+}\)]\(_i\), the mitochondrion may accumulate large amounts of calcium. This is because the uniporter, carrying calcium into the mitochondrion along the electrochemical gradient, has a much higher total activity than the extrusion pathway, which encompasses Na\(^+\)-Ca\(^{2+}\) exchange. In other words, if the net influx of Ca\(^{2+}\) exceeds the capacity of the extrusion pathway through Na\(^+\)-Ca\(^{2+}\) exchange, intramitochondrial Ca\(^{2+}\) concentration ([Ca\(^{2+}\)]\(_m\)) increases, and Ca\(^{2+}\) will be sequestered within the mitochondria. Under special circumstances, which will be discussed below, a large conductance pore will be opened. This, which is called the MPT pore, allows Ca\(^{2+}\) to leave the mitochondria.

Given this background, we will discuss calcium fluxes during and after ischemia. Before we do that, though, we wish to bring up two issues of conceptual importance. These relate to the total tissue and total cell calcium content and to the type of ischemia encountered.

As remarked, intracellular and extracellular (“free”) calcium concentrations are normally approximately 0.1 and 1000 μmol/L, respectively. However, since the total cell calcium content (excluding the extracellular spaces) is approximately 1000 μmol/L (ie, 1 mmol/L · kg\(^{-1}\)), it follows that more than 99% of the total cell calcium content is bound to proteins or phospholipids, or sequestered into ER, to so-called calciosomes and mitochondria. Clearly, although the source of a rise in [Ca\(^{2+}\)]\(_i\) is often the extracellular calcium content, the cell contains enough (bound or sequestered) calcium to markedly increase [Ca\(^{2+}\)]\(_i\). This could occur if the bound calcium is displaced from its binding sites or if the calcium sequestered in ER, or in mitochondria is released to the cytosol. An uncontrolled release of calcium from the ER (or related intracellular stores) is now believed to predispose to Ca\(^{2+}\)-related cell damage. Furthermore, calcium metabolism of the mitochondria has become the focus of interest (see below).

As discussed elsewhere, it appears justified that a distinction is made between ischemia of the “cardiac arrest” type and that of the “stroke” type. The former, which is usually of short duration (5 to 15 minutes), can be studied as such, ie, in models of cardiac arrest (eg, Reference 52); however, most workers in the field prefer to use forebrain ischemia in gerbils and rats. This is because these models allow some remaining flow to brain stem centers during ischemia, obviating the use of intensive care measures during reperfusion. The second type of ischemia (“stroke”) is a focal one, which is usually of much longer duration, if not permanent. It is commonly induced by permanent or transient occlusion of one MCA.

The most important differences between the two types of ischemia relate to the duration of the ischemia (and its density). In global/forebrain ischemia of brief duration, the tissue damage is usually confined to the neuronal population, and cell death is characteristically delayed by hours or days. Cells outside the selectively vulnerable areas are usually not affected, ...
nor are glial cells or vascular endothelium. Furthermore, any inflammatory component is of moderate degree. In contrast, focal ischemia of long duration leads to pannecrosis, ie, to the death of all types of cells (infarction). This implies that vascular damage is a prominent feature of the lesions and that a strong inflammatory response is elicited.

### Forebrain or Global Ischemia of Brief to Intermediate Duration

**Calcium Fluxes and Changes in Extracellular and Intracellular Calcium Concentration**

Under this heading, we will discuss events occurring after brief periods of forebrain ischemia, under optional reflow conditions. However, we will also consider results obtained with sudden, complete ischemia since they give unequivocal information about rates of calcium influx (for reviews, see References 22, 37, and 62).

Figure 2 illustrates calcium influx into cells of neocortical tissue after complete ischemia, as this influx is reflected in 

\[ \text{Ca}^{2+} \] . In normoglycemic animals the time to ischemic depolarization is approximately 50 seconds, and calcium influx is rapid. The raised plasma (and tissue) glucose concentrations in hyperglycemic subjects lead to delayed depolarization and to a two-phase influx of \( \text{Ca}^{2+} \), the second one being very slow.

Clearly, hyperglycemia delays depolarization, probably by providing additional substrate for (anaerobic) ATP production, and it reduces the rate of calcium influx. The latter effect is obviously due to the exaggerated tissue acidosis since excessive hypercapnia, induced in normoglycemic animals, duplicates the effects of preischemic hypercapnia. Finally, since the NMDA antagonist MK-801 has effects similar to those of hyperglycemia and of hypercapnia, the results must largely reflect the blocking effect of acidosis on NMDA receptor-gated \( \text{Ca}^{2+} \) influx.

![Figure 2](image-url)

**Figure 2.** Recordings of extracellular calcium concentration \( \text{Ca}^{2+} \) in complete ischemia during control (normoglycemic and normocapnic) conditions, in conditions with accentuated tissue acidosis (hyperglycemia and hypercapnia), and in animals given dizocilpine maleate (MK-801). Data from Reference 166.

During ischemia, \( \text{Ca}^{2+} \), decreases to values less than 10% of control, ie, to approximately 0.1 mmol/L. In addition, the extracellular fluid space decreases to approximately 50% of control. This means that almost all extracellular calcium is translocated into cells. With an extracellular fluid space of 20% of tissue volume and a \( [\text{Ca}^{2+}] \), of approximately 1.3 mmol/L, the calcium load to which cells are exposed is approximately 250 \( \mu \text{mol} \cdot \text{kg}^{-1} \) of tissue. Since it is not likely that much calcium uptake occurs into glial cells, the calcium load of neurons could well be twice that figure, meaning that the total neuronal cell calcium content may increase to 150% of control or more. Clearly, if some neurons have very high calcium conductances, they could be exposed to an even higher load.

In the hippocampus, CA1 cells have a very high density of NMDA receptors. It is of interest, therefore, that Silver and Erecinska demonstrated that ischemia causes \( \text{Ca}^{2+} \), to increase from approximately 0.1 \( \mu \text{mol} \) to values of 30 to 60 \( \mu \text{mol} \). Clearly, these are nonphysiological increases in \( \text{Ca}^{2+} \).

Additional information on ischemic calcium transients has been collected in experiments in which \( \text{Ca}^{2+} \), and DC potential shifts were measured in transient ischemia of the forebrain type. This type of ischemia has the drawback of giving a less distinct “onset” of ischemia but the advantage of readily allowing reperfusion events to be studied. Figure 3 illustrates the \( [\text{Ca}^{2+}] \), transients accompanying ischemia of 15 minutes’ duration in hypoglycemic, normoglycemic, and hyperglycemic animals. The results illustrate that hyperglycemic animals show a delay before depolarization occurs (see above) compared with normoglycemic animals but also that they repolarize earlier than their normoglycemic controls (see also Fig 4 for 5- and 2.5-minute ischemia). This means that the duration of the ischemic calcium transient is shorter in hyperglycemic than in normoglycemic animals (for review, see Reference 67); however, the ischemic damage is exaggerated in hyperglycemic animals.

These experiments challenge the postulate that any damage incurred by hypoxic cells is proportional to the duration of the calcium transient and to the rise in \( [\text{Ca}^{2+}] \), ie, to the time integral under the \( \text{Ca}^{2+} \) curve. Although it is noteworthy that hyperglycemic animals have a worse outcome after a 10-
minute period of ischemia, whether one uses a neurological or histopathological end point, the difference in duration of calcium uptake and DC potential shift between normoglycemic and hyperglycemic animals is relatively trivial. However, it becomes more obvious if the nominal ischemic period is only 5 minutes (Fig 4, data from Reference 69). With this period of ischemia, the duration of the Ca\(^{2+}\)/DC potential transient in hyperglycemic subjects is only 50% of that observed in normoglycemic animals. Despite that, the former incurred at least as much neuronal damage and, in contrast to normoglycemic animals. Despite that, the former incurred at least as much neuronal damage and, in contrast to normoglycemic animals, they showed a tendency to develop postischemic seizures. Clearly, although it is likely that Ca\(^{2+}\) constitutes a major mediator of ischemic cell death, it must act in concert with other mediators, such as exaggerated intracellular acidosis. Predictably, hyperthermia is an additional aggravating factor.

The relationship between calcium influx and neuronal injury is even more complicated than indicated in Fig 3. Thus, recent results demonstrate that hyperglycemic rats subjected to only 2.5 minutes of ischemia show 15% to 20% damage to the CA1 sector of the hippocampus even though the cells never depolarize during ischemia (Fig 4) and even though the [Ca\(^{2+}\)], REMAINS unchanged or increases somewhat. Very probably, depolarization does not occur because the increased glucose content provides additional ATP from anaerobic glycolysis. Clearly, any rise in the [Ca\(^{2+}\)] must have been small, and other factors must have contributed to the delayed neuronal death. Such factors could encompass acidosis caused by the anaerobic glycolysis and/or the redox change that accompanies ischemia/reperfusion.

**Calcium and Delayed Neuronal Death**

In this type of ischemia, the neuronal injury is truly delayed since cells repolarize and resume physiological and metabolic functions before they suffer secondary damage and die. Signs of ongoing adverse processes are present, however. These encompass a sustained depression of metabolic rate and of overall protein synthesis. Other signs of metabolic perturbation are the expression of mRNAs for immediate early genes and for neurotrophins and the synthesis of "stress" or "heat shock" proteins (for extensive discussions of these issues, see recent volumes edited by Moskowitz and Caplan and by Siesjö and Wieloch).

The question arises of how the perturbation of cell calcium metabolism during and immediately after a transient period of ischemia influences the cascade of events that leads to delayed cell injury. There are many possibilities since Ca\(^{2+}\) activates phospholipases, endonucleases, and proteases, since it affects protein phosphorylation by altering the activity of protein kinases and phosphatases, and since it activates enzymes that give rise to the production of ROS and NO.

Three schemes have been elaborated that purport to explain delayed neuronal death. One puts the emphasis on a sustained perturbation of the signal transduction pathway, ie, the sequence of events that starts with the activation of receptors for EAAs and neurotrophins, continues with the activation or deactivation of protein kinases and phosphatases, and ends with an altered activity of major response elements, mainly those regulating gene expression and protein. One feels intuitively that nuclear events, particularly if they involve fragmentation of DNA, could be devastating for cell survival. Furthermore, a sustained suppression of protein synthesis could be equally harmful since it bereaves the cells of molecules required for survival, such as antioxidative enzymes and trophic factors.

The second hypothesis is one in which cell death is assumed to be due to a sustained perturbation of cell calcium metabolism, leading to a slow, gradual rise in [Ca\(^{2+}\)], and to eventual mitochondrial calcium overload. We will describe the origin of that hypothesis, attempt to integrate it with data suggesting a failure of the signal transduction pathway, and discuss data that give a novel perspective on postischemic mitochondrial dysfunction.

In the beginning of the 1980s, it was known that ischemia is accompanied by translocation of Ca\(^{2+}\) from extracellular to intracellular fluids and also that reperfusion restored...
resulting in a gradual rise in \[\text{Ca}^{2+}\]. The results showed that the total tissue calcium content, nor was there information on calcium metabolism in the period of reperfusion preceding the delayed neuronal death. In 1984, Dienel reported results on transient forebrain ischemia of 20 minutes’ duration. His data revealed an increased incorporation of \([\text{Ca}^{2+}]\) into the subiculum of the CA1 sector and into the lateral caudoputamen after 24 hours of reperfusion; however, this increased incorporation seemed to occur without an increase in the total cell calcium content. On the basis of these results, our laboratory explored the time course of changes in the total tissue calcium concentration of the dorsal hippocampus, correlating it with light microscopic signs of cell death. The results showed that the total tissue calcium content during reperfusion did not increase until late (between 24 and 48 hours) and that an increase in \([\text{Ca}^{2+}]\) seemed to precede morphological signs of cell death. The results, which are illustrated in Fig 5, were subsequently confirmed by measurements with proton-intensified x-ray emission (PIXE), allowing analyses of the different layers of the CA1 and CA3 sectors. Based on these results and on the theory proposed by Alkon and Rasmussen, we advanced the hypothesis of delayed calcium-related cell death. This hypothesis predicts that the initial ischemic transient gives rise to a sustained perturbation of plasma membrane handling of \([\text{Ca}^{2+}]\), resulting in a gradual rise in \([\text{Ca}^{2+}]\). When the latter exceeds the “set point” for calcium sequestration in the mitochondria, these begin accumulating \([\text{Ca}^{2+}]\) until they are “overloaded” and become dysfunctional. It should be emphasized that the plasma membrane may not be the only type of membranes that are perturbed by the ischemic transient. Thus, evidence for an involvement of ER membranes in the delayed cell death was reported by Tsubokawa et al., who induced ischemia of 5 minutes in gerbils, allowed reperfusion for 2.5 to 3.5 days, and prepared hippocampal slices for patch clamping of CA1 cells. Tetanic stimulation of the input to these cells caused irreversible depolarization, as did injection of IP3 via the patch pipette. The depolarization could be prevented by antibodies to IP3 or to the kinase that converts IP3 to IP4. This suggests that release of calcium from the ER could contribute to the rise in \([\text{Ca}^{2+}]\), and that a perturbed signal transduction affecting the IP3-IP4 system could be part of the pathogenetic defect leading to dysregulation of calcium metabolism (see also Reference 45).

This hypothesis requires (1) that mitochondria accumulate \([\text{Ca}^{2+}]\) before cell death becomes manifest and (2) that \([\text{Ca}^{2+}]\) rises gradually during reperfusion. Both of these requirements seem to be fulfilled. Thus, Dux et al. showed that a second wave of \([\text{Ca}^{2+}]\) precipitates in the mitochondria of CA1 neurons after 24 hours of reperfusion following 5 minutes of transient ischemia in the gerbil. Furthermore, Zaidan and Sims demonstrated that although reperfusion in rats subjected to forebrain ischemia normalized \([\text{Ca}^{2+}]\), within 10 to 20 minutes, continued recirculation seemed to give rise to a secondary, gradual rise in \([\text{Ca}^{2+}]\).

The third hypothesis is that published by Abe et al. Like the second of the other two hypotheses discussed, it predicts that the ultimate cell damage is due to mitochondrial failure. However, the mechanisms proposed are different. The background is that the respiratory complexes, i.e., the enzymes that shuttle electrons along the respiratory chain and extrude \(H^+\), are encoded for by both mitochondrial and nuclear DNA. For mitochondria to gain genetic material for synthesis of all relevant proteins, the mitochondria must be transported to the nucleus by being propelled along cytoskeletal elements by transport proteins such as dynorphin and kinins. Abe et al. submit that this process is halted when the cytoskeleton is broken down by calcium-activated proteases and by calcium-dependent disassembly of microtubuli (see Reference 93). The long-term result of this would be reduced activities of respiratory enzymes, such as complex I or complex IV, with devastating effects on mitochondrial generation of ATP.

### Calcium Accumulation and Mitochondrial Dysfunction

The postulate that mitochondrial dysfunction contributes to delayed neuronal death has recently received support from results obtained with the immunosuppressant drug CsA. The background is as follows. It has been known for decades that massive calcium accumulation triggers mitochondrial damage. This was originally thought to reflect activation of mitochondrial PLA2 which, by breaking down the lipid backbone of the inner mitochondrial membrane, gives rise to a nonspecific increase in mitochondrial membrane permeability. However, a different mechanism giving rise to such an increase in permeability involves the formation (“assembly”) of a proteinaceous pore, the MPT pore, which has such a high conductance that it allows the passage of ions and molecules with a molecular mass less than 1500 D. According to the chemiosmotic theory of Mitchell, electron transport in the respiratory chain of mitochondria causes the extrusion of \(H^+\), creating a large electrochemical potential difference across the inner mitochondrial membrane. This potential \(\Delta(\Psi_H)\) consists of an \(H^+\) concentration gradient and an electrical potential difference \(\Delta(\Psi_e)\). The membrane is normally impermeable to \(H^+\) and other ions, and it only allows passage of ions (or substrates) for which specific transport systems exist. This means that \(H^+\) can normally only reenter
the mitochondria through the F1F0-ATPase. In that process, ATP is formed.

Mitochondrial ATP production thus depends on the regulated entry of H$^+$ across the inner mitochondrial membrane. However, the literature on mitochondrial function in vitro contains many reports demonstrating that exposure of mitochondria to calcium causes them to swell and to release intramitochondrial components into the medium.$^94,95$ It is now realized that this sequence of events reflects the assembly of an MPT pore in the inner mitochondrial membrane. This pore allows the release of Ca$^{2+}$ and Mg$^{2+}$ as well as of various low- and high-molecular-weight compounds. In this process the mitochondria show osmotic swelling. Furthermore, the assembly of the pore leads to the collapse of $\Delta \mu H^+$ and thereby to cessation of ATP production. As will be discussed below, an additional consequence is a burst of production of ROS.

The seminal work of Crompton and collaborators$^{98,101}$ identified the major factors triggering the assembly of an MPT pore in isolated mitochondria. These were a decrease in the ATP and increase in the P, concentration, oxidative stress, and calcium accumulation. The last two factors have emerged as major determinants in other experimental paradigms.$^{102,103}$ Furthermore, the coupling among a decrease in mitochondrial membrane potential, the assembly of an MPT pore, and enhanced mitochondrial production of ROS has been established in thymocytes that have been committed to die by an apoptotic mechanism, after exposure to dexamethasone.$^{104,105}$ In these and other rapidly proliferating cells, the first event in the sequence leading to (apoptotic) cell death is a decrease in $\Delta \Psi_m$. What then follows is a burst of free radical production, cell shrinkage, and cell death.

The conclusions that can be drawn from these experiments are that oxidative stress and mitochondrial calcium accumulation predispose to an MPT and to the consequences of such an event, eg, the depolarization-coupled production of ROS. This is where the action of CsA comes in. In all experimental paradigms studied in vitro, whether on cells of nonneuronal or neuronal origin, CsA proved to be an almost specific inhibitor of the MPT.$^7$ This is presumably because CsA, which combines with a series of cyclophilin proteins, blocks the MPT pore by competing with the effect of Ca$^{2+}$-cyclophilin for occupancy on the transition pore proteins.

Two years ago, our laboratory obtained results showing that CsA dramatically ameliorates the CA1 damage, provided that it can pass the BBB. The results were obtained in experiments in which growth factor–producing cells were injected into the CA1 sector of one hemisphere, CsA being given to suppress the immune response (Uchino et al$^{106}$). Analyses of the primary experiments and additional experiments revealed that the combination of systematically injected CsA and a unilateral needle lesion almost eliminated the CA1 damage after 7 or 10 minutes of forebrain ischemia (Fig 6). We interpreted the results to show that the needle lesion enhanced the BBB permeability of CsA and that CsA acted by preventing the assembly of an MPT pore in calcium-loaded mitochondria. At present, this is a tentative interpretation since CsA has effects other than that of blocking the MPT pore. These may be related to its immunosuppressant effects and its ability to combine with cyclophilin, a modulator of the phosphatase calcineurin. This enzyme affects several metabolic events, including NO production by NOS.$^{107}$ However, FK 506, which is a stronger immunosuppressant than CsA and which, like CsA, inhibits calcineurin, is less efficacious than CsA in forebrain ischemia.$^{108,109}$ Since FK 506 does not act as blocker of the MPT, the results suggest that CsA works by preventing a Ca$^{2+}$-triggered MPT during reperfusion.

### Calcium–Mediated Mechanisms of Delayed Neuronal Death: A Speculative Synthesis

The hypotheses discussed encompass events that are possibly linked. For example, a perturbation of signal transduction may alter the pump-leak relationship for calcium across plasma and intracellular membranes so that the result is a gradual rise in Ca$^{2+}$, and eventual mitochondrial calcium overload. Since the hypothesis of Abe et al$^{92}$ requires further support, we will discuss the hypothesis of delayed calcium-mediated cell death. In its simplest form, this hypothesis predicts that the initial ischemic transient gives rise to a sustained perturbation of membrane handling of calcium. This then sets the stage for a gradual, secondary rise in [Ca$^{2+}$], which eventually causes mitochondrial calcium overload. If this hypothesis is viewed against the background of our present knowledge of the MPT, affecting mitochondria that accumulate calcium, we can envisage a coupling among plasma membrane perturbation, gradual cell calcium accumulation, mitochondrial dysfunction, and delayed ischemic cell death.

Release of excitatory transmitters, depolarization, and an increase in [Ca$^{2+}$], trigger enhanced production of the traditional ROS (‘O$_2^-$, H$_2$O$_2$, and OH) as well as of NO.$^{110,111}$ In general, activation of PLA$_2$ leads to the production of ROS because arachidonic acid, generated by PLA$_2$ activity, is metabolized by cyclooxygenase (and lipoxygenase) to yield a variety of degradation products and, in that process, ‘O$_2^-$ is formed.$^{112}$ Another source of ROS is activation of the Ca$^{2+}$-calmodulin–dependent NOS pathway, which produces NO from arginine. As proposed by Beckman et al$^{113,114}$ NO can then react with ‘O$_2^-$ to yield peroxynitrite (ONOO$^-$), the latter decomposing with the production of ‘OH, a highly toxic free radical. The reaction sequences yield NO, peroxynitrate, and...
with reperfusion translocates protein kinase C to membranes, downregulates protein kinase A, alters the activity of Ca\textsuperscript{2+}-calmodulin–protein kinase II, reduces or arrests protein synthesis, and alters gene expression.\textsuperscript{80,124,125} Any of these events could modulate, over long periods, membrane handling of Ca\textsuperscript{2+}. We recognize that a rise in [Ca\textsuperscript{2+}], per se could trigger many of these effects. As discussed above, this does not necessarily imply that it is the plasma membrane function that is altered since a correspondingly altered pump-leak relationship for calcium could exist at the level of ER membranes. The important feature of the hypothesis is that ischemia and reperfusion perturb membrane handling of calcium in such a way that [Ca\textsuperscript{2+}] is gradually increased, triggering sequestration of calcium in mitochondria. Cell death could then be the direct result of a calcium–triggered MPT, since the latter induces a burst of free radical generation and leads to the collapse of Δ\(\mu\)H\textsuperscript{+}.

It should be emphasized that mitochondrial dysfunction and bioenergetic failure are delayed phenomena. Thus, in the early recirculation period a normal bioenergetic status is quickly achieved,\textsuperscript{126} and respiratory functions of isolated mitochondria are resumed.\textsuperscript{127–129} The mitochondria also have a normal capacity to respond to metabolic challenges. Thus, although the “resting” metabolic rate of brain tissues is reduced, challenges such as spreading depression (Reference 130 and T. Kristián, G. Gido, and B.K. Siesjö, unpublished data, 1996) and epileptic seizures\textsuperscript{131} trigger the expected responses, allowing maintenance of bioenergetic and ionic homeostasis. Clearly, it appears unlikely that the initial oxidative burst during recirculation causes direct damage to mitochondria. A more likely scenario is that slow calcium accumulation ultimately triggers an MPT, production of free radicals, and mitochondrial damage (see above) or that breakdown of the cytoskeleton with damage to the motor proteins arrests shuttling of mitochondria between the periphery and the nucleus, affecting proteins encoded for by nuclear DNA.\textsuperscript{122}

**Global or Focal Ischemia of Long Duration**

When global or forebrain ischemia is prolonged, the free interval between the insult and the final damage is shortened and, if the ischemia is of very long duration, recirculation may fail to restore mitochondrial function and cellular bioenergetic state. Experiments with extended periods of ischemia also reveal a rapidly developing, massive calcium accumulation in the tissue. We will consider in turn recovery of mitochondrial function and changes in calcium metabolism.

**Recovery of Mitochondrial Function**

Under adverse conditions, recirculation may fail to be accompanied by resumption of oxidative phosphorylation of isolated mitochondria or lead to partial recovery for a limited period only. Such conditions encompass ischemia of long duration and ischemia with superimposed hyperglycemia. For example, incomplete forebrain ischemia of 30 minutes’ duration in fed rats (which became hyperglycemic) was followed by additional deterioration of mitochondrial respiratory rates during recirculation.\textsuperscript{132,133} Furthermore, rapid maturation of mitochondrial damage has been found in hyperglycemic dogs subjected to anoxia-ischemia,\textsuperscript{129} in rats subjected to long ischemic peri-
ods,\textsuperscript{134,135} and in gerbils subjected to 30 minutes of forebrain ischemia.\textsuperscript{136}

The reason why mitochondrial respiratory functions are not resumed after long ischemic periods, particularly in hyperglycemic subjects, is not known. It has been generally held that mitochondria are either damaged by PLA\textsubscript{2}-mediated breakdown of the lipid backbone of the inner mitochondrial membrane or by oxidation of protein components mediating electron transport, H\textsuperscript{+} extrusion, or ATP production. However, no agreement has been reached on the targets. Thus, some studies implicate the pyruvate dehydrogenase complex, others one or more respiratory complexes (I through V), and still others the adenylate translocase.\textsuperscript{135–137} In fact, the pyruvate dehydrogenase complex has also been incriminated in ischemia of brief duration in starved animals.\textsuperscript{91,138} Since PLA\textsubscript{2} is a calcium-dependent enzyme and since calcium activates several enzymes producing ROS, the mitochondrial failure, whether acute or delayed, can be traced back to a perturbed calcium metabolism. However, the molecular defect remains unclarified.

In this context, recent results obtained in experiments on focal ischemia of 2 hours' duration are intriguing. In core and penumbral tissues, this period of MCA occlusion was accompanied by a decrease in tissue ATP content to approximately 10% and 25%, respectively, and by increases in lactate content to approximately 15 mmol/L \cdot kg\textsuperscript{-1}.\textsuperscript{139} After 1 hour of reperfusion, ATP increased to 50% to 70% of control. Since the adenine nucleotide pool (\(\Sigma-\text{ATP}+\text{ADP}+\text{AMP}\)) had decreased accordingly, this degree of recovery must reflect a virtually complete rephosphorylation of the ADP available to ATP. However, recirculation for 2 hours did not further increase ATP concentration or the size of the adenine nucleotide pool and, after 4 hours, signs of a secondary decrease in ATP concentration were apparent. Since tissue lactate concentrations showed little tendency to decrease at 1 and 2 hours and increased further at 4 hours, the results suggest partial recovery of cell energy metabolism and mitochondrial functions at 1 and 2 hours and secondary failure at 4 hours.

Subsequent results showed that the time course of changes in cellular bioenergetic state was paralleled by corresponding changes in ADP- and uncoupler-stimulated respiration of tissue homogenates in focal and penumbral tissues.\textsuperscript{140} These results suggest that the partial recovery of ATP concentrations and the failure of recovery of normal lactate concentrations reflect sustained mitochondrial dysfunction. It could further be shown that the free radical spin trap PBN and the immunosuppressant FK-506, both of which ameliorate tissue damage shown that the free radical spin trap PBN and the immunosuppressant FK-506, both of which ameliorate tissue damage due to transient ischemia,\textsuperscript{141–143} also prevent the secondary failure at 4 hours.

In view of the results obtained with brief to intermediate periods of ischemia, it is perhaps not surprising that mitochondria fail to resume normal respiratory functions after 2 hours of MCA occlusion. However, it is more surprising that the activity of respiratory complexes (I, II-III, IV) and of two associated enzymes (citrate synthase and glutamate dehydrogenase) did not decrease.\textsuperscript{146} These results suggest that failure of mitochondria to resume normal respiratory functions, in vivo or in vitro, may encompass mechanisms other than those discussed for global or forebrain ischemia. In vivo, and perhaps also when homogenates are used to study mitochondrial respiratory functions in vitro, increased FFA concentrations may uncouple mitochondria or inhibit their respiration.\textsuperscript{147–150} An additional possibility is that the mitochondria are partially calcium-loaded and prone to assemble a permeability transition pore.\textsuperscript{151} As remarked, this would promote mitochondrial production of free radicals and accelerate breakdown of mitochondrial phospholipids. Very likely, conditions during reperfusion favor the opening of an MPT pore, particularly if NO and peroxynitrate are formed.

**Calcium Metabolism**

After brief to intermediate periods of ischemia, massive calcium accumulation, reflecting net transfer of Ca\textsuperscript{2+} from blood to tissue, is observed many hours (or days) after the initial insult (see above). After long periods of ischemia, the "free" interval is shortened, and substantial amounts of calcium may accumulate during the first 2 to 3 hours of reperfusion.\textsuperscript{28,152} A similarly accelerated influx of calcium into the brain occurs in permanent or transient focal ischemia. In some published studies the calcium content was measured by atomic absorption spectrophotometry, giving quantitative values for calcium content and the rate of calcium flux from blood to tissue.\textsuperscript{11,153} In others, \textsuperscript{45}Ca autoradiography was used.\textsuperscript{154,155} This technique gives good spatial resolution, but the data cannot be used for quantitative estimates since the tissue activity must depend on both calcium concentration and the activity of respiratory complexes (I, II-III, IV) and of two associated enzymes (citrate synthase and glutamate dehydrogenase) did not decrease.\textsuperscript{146} These results suggest that failure of mitochondria to resume normal respiratory functions, in vivo or in vitro, may encompass mechanisms other than those discussed for global or forebrain ischemia. In vivo, and perhaps also when homogenates are used to study mitochondrial respiratory functions in vitro, increased FFA concentrations may uncouple mitochondria or inhibit their respiration.\textsuperscript{147–150} An additional possibility is that the mitochondria are partially calcium-loaded and prone to assemble a permeability transition pore.\textsuperscript{151} As remarked, this would promote mitochondrial production of free radicals and accelerate breakdown of mitochondrial phospholipids. Very likely, conditions during reperfusion favor the opening of an MPT pore, particularly if NO and peroxynitrate are formed.

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not normalized during recirculation? Provided that one can assume that calcium influx occurs along a transport gradient created by the low $Ca^{2+}$, where does the calcium accumulate? Does it accumulate in mitochondria? However, if mitochondria accumulate calcium, how can such accumulation be reconciled with the notion of an MPT, which should be accompanied by release of calcium to the cytosol? This leads to a general question: Where is the accumulated calcium localized? Furthermore, how is this localization/sequestration related to cell death? Does it cause cell death, or does it occur because dying cells accumulate calcium? To provide answers to some of these questions, we will examine in vitro experiments.

**Excitotoxicity, Calcium Influx, and Cell Death In Vitro**

There is now extensive literature on pathological calcium transients in cultured neurons. A major part of this work was inspired by the discovery that glutamate and related EAAs are neurotoxic and that the toxicity is related to $Ca^{2+}$ influx into cells.\(^{14,17}\) (for early reviews, see References 16 and 77). During the last 8 to 10 years, a large body of evidence has been accumulated that expands the central postulate, i.e., that damage is prone to develop when cells are exposed to sufficiently high concentrations of EAAs for a sufficiently long period of time and that the damage depends heavily on the $Ca^{2+}$ influx that occurs in response to the EAA exposure.\(^{157,158}\)

Exposure of cultured cells to EAAs in vitro has been claimed to be a useful paradigm for ischemic disease in vivo. Two facts suggest that the extrapolation from in vitro conditions is fraught with difficulties. First, while in vitro results demonstrate that 2 to 3 minutes of glutamate exposure leads to extensive neuronal necrosis in vitro,\(^{159}\) this duration of ischemia does not lead to cell damage in any region other than the CA1 sector of the hippocampus.\(^{160,169}\) In other words, cells are more vulnerable in vitro to anoxic/excitotoxic transients. Second, it has been demonstrated that acidosis in vitro protects cells against anoxic and excitotoxic insults.\(^ {160-162}\) However, it has been demonstrated beyond doubt that acidosis in vivo exaggerates ischemic brain damage (for reviews, see References 67 and 163).

It seems likely that these differences can be explained by the fact that, in vitro, cells are exposed to an unlimited source of calcium, allowing translocation of large amounts of $Ca^{2+}$ into cells.\(^ {164}\) This conclusion is supported by results obtained in vitro, demonstrating that the total calcium content during a 5- to 10-minute exposure to glutamate can increase severalfold.\(^ {165}\) Such a massive influx does not occur in vivo, since the amount acutely accumulated is restricted by the calcium content of the extracellular fluid (see above). By analogy, and recalling that acidosis reduces the rate of calcium influx through NMDA- and voltage-operated channels,\(^ {166}\) we can deduce that, in vitro, acidosis protects cells by limiting ischemia-induced calcium influx into cells. In vivo, acidosis can retard the rate of calcium influx in ischemia, but since the calcium source is restricted, factors other than the total calcium load determine the final outcome.\(^ {25}\)

Despite these reservations, it must be concluded that experiments on neurons in vitro represent a useful paradigm for studies of ischemia in vivo. The following results, obtained on cells in vitro, are particularly relevant.

1. A transient insult leads to a delayed increase in $Ca^{2+}$, after the initial increase. It has been documented in numerous publications that a glutamate transient leads to a marked rise in $Ca^{2+}$. A few studies have documented that if the excitotoxic insult is sufficiently severe or sufficiently prolonged, recovery of $Ca^{2+}$ occurs after the initial insult but is followed by a secondary rise in $Ca^{2+}$, obviously preceding deregulation of $Ca^{2+}$ homeostasis and cell death.\(^ {158,167,168}\) In fact, if the excitotoxic insult is sufficiently severe, $Ca^{2+}$ will not recover when the stimulus is removed.\(^ {158,166}\) We recognize that these results mimic those obtained in vivo after brief and sustained ischemic periods.

2. The cell damage incurred in EAA transients is not related to changes in $Ca^{2+}$, but to changes in total calcium influx. Results obtained from several laboratories\(^ {158,170,171}\) suggest that the (initial) rise in $Ca^{2+}$, is a poor indicator of the outcome. A variable that correlates better with the outcome of an excitotoxic insult is the total amount of calcium translocated into the cell.\(^ {165,172,173}\) (for review, see Reference 165). There are several possible implications of the findings, supporting this notion. One is that activation of NMDA receptors may be detrimental because the receptor-gated channel translocates more $Ca^{2+}$ into the cell than other agonist-operated channels or voltage-dependent channels. Another implication is that the vulnerability of neurons in vitro is exaggerated because much more calcium can be translocated into intracellular fluids in vitro because of the unlimited source of $Ca^{2+}$ available (see above).

3. The initial calcium load, at least in part, is sequestered by the mitochondria. Data obtained more than one decade ago suggested that the mitochondria served as a “sink” for calcium loads associated with intense physiological activity or pathological transients.\(^ {47}\) Subsequent work performed on neuronal cultures in vitro gave substance to this assumption and provided information on pathological calcium transients (Thayer and Miller\(^ {74}\) [1990], Werth and Thayer\(^ {175}\) [1994], White and Reynolds\(^ {176}\) [1995], Wang et al.\(^ {177}\) [1994], White and Reynolds\(^ {165}\) [1997]). The concept emerging from these studies is that when substantial amounts of $Ca^{2+}$ enter the cell, a large part is sequestered in the mitochondria. This process occurs by uptake of $Ca^{2+}$ through the electrogenic uniporter, while egress of $Ca^{2+}$ probably occurs by $Ca^{2+}$-2Na$^+$ exchange. The absolute amounts of $Ca^{2+}$ taken up are not known, however, i.e., we do not know how much of a given tissue calcium “overload” can be attributed to calcium sequestration.

4. Mitochondrial calcium accumulation is associated with enhanced production of ROS. As mentioned previously, ischemia with reperfusion is associated with enhanced production of ROS.\(^ {118-120}\) Evidence now exists that a substantial part of this production emanates from mitochondria.\(^ {176,179}\) This notion is supported by results obtained in vitro on primary neuronal cultures. The concept is based on the hypothesis that excitotoxic cell death is, at least in part, mediated by a coupling between glutamate-induced production of ROS and EAA-induced cell damage.\(^ {30}\) Subsequent results showed that exposure of cells to glutamate gave rise to production of ROS and that a substantial part of this production occurred in the mitochondrial fraction.\(^ {180,181}\)
(5) Cell death is preceded by mitochondrial membrane depolarization and is inhibited by CsA. Recently the EAA stimulation of surface receptors, increase in Ca$^{2+}$, sequestration of Ca$^{2+}$ by mitochondria, and mitochondrial generation of ROS have been further investigated. Thus, results have been published which show that the effect of EAA on cells encompasses a decrease in the mitochondrial ΔΨ; furthermore, CsA was found to not only reduce the incidence (and degree) of depolarization but also to reduce the incidence of cell death.$^{176,182}$ There are thus reasons to believe that EAs act by accelerating Ca$^{2+}$ influx into cells, with an ensuing rise in Ca$^{2+}$, that calcium is sequestered in mitochondria, and that this sequestration gives rise to the assembly of an MPT pore as well as to production of ROS by the mitochondria. Clearly, this sequence of events could explain the delayed cell death after brief periods of ischemia, the rapidly maturing cell death after long periods of ischemia, or cell death complicated by preischemic hyperglycemia.

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