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CALCIUM-MEDIATED MECHANISMS IN CHEMICALLY INDUCED CELL DEATH

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INTRODUCTION

Biochemical mechanisms involved in toxic cell injury have attracted increased interest during recent years and have been the subject of several reviews (1–6). In this chapter we discuss one particular aspect of chemically induced cell death, i.e. the role of the calcium ion in the early development of damage. We summarize current evidence for an association between a perturbation of intracellular Ca^{2+} homeostasis and cytotoxicity and discuss various Ca^{2+}-mediated mechanisms that may contribute to the development of cell damage.

Ca^{2+} OVERLOAD AND CYTOTOXICITY

The role of Ca^{2+} as an intracellular regulator of many physiological processes is well established. During the past several years, however, it has also become increasingly clear that Ca^{2+} can play a determinant role in a variety of pathological conditions. Disruption of the mechanisms that regulate intra-

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cellular Ca^{2+} homeostasis is often an early event in the development of irreversible cell injury (7,8).

There is convincing evidence that sustained increases in intracellular Ca^{2+} can activate cytotoxic mechanisms in various cells and tissues. For example, intracellular Ca^{2+} accumulation appears to mediate the toxicity of chemicals such as cyanide and chlordecone in the brain, as well as the neurotoxic effects of several metals, including lead, mercury, and organotin compounds (9). In addition, intracellular Ca^{2+} accumulation resulting from abusive stimulation of neuronal ionotropic glutamate receptors is most likely involved in the toxicity of excitatory amino acids and in the degeneration of neurons following ischemia (10–12). More recently, it has also been shown that astrocytes possess glutamate receptors and that the administration of the glutamate receptor agonist, quisqualate, can stimulate intracellular Ca^{2+} accumulation in these cells (13). Since cell killing induced by other neurotoxins such as MPTP (1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine) can be prevented by the administration of excitatory amino acid antagonists (14), it is conceivable that stimulation of these receptors and intracellular Ca^{2+} accumulation may be a general mechanism in neuronal cell killing. Notably, Ca^{2+} channel blockers can also prevent the neurotoxicity of the HIV-1 coat protein, gp120 (15), and a calmodulin antagonist, flunarizine, appears to protect neuronal cells from death induced by nerve growth factor (NGF) deprivation (16).

Myocardial injury occurring upon post-ischemic reperfusion has been linked to the generation of oxygen radicals as well as to intracellular Ca^{2+} overload (17, 18). Previous reports have indicated that Ca^{2+} overload occurs in myocytes following anoxia and reoxygenation (18). More recently, it has been shown that oxygen radical-induced myocyte injury is mediated by intracellular Ca^{2+} entry through voltage-operated Ca^{2+} channels (17), suggesting a link between the oxygen radical and Ca^{2+} mechanisms of cell death during ischemia-reperfusion of the heart. Moreover, an increased intracellular Ca^{2+} level has been reported to be the determinant factor in the development of cardiotoxicity following exposure to the environmental contaminant 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) (19).

Evidence for the involvement of Ca^{2+} overload in acute and chronic cell injury in the kidney has also been reported (20, 21), and a study in individual LLC-PK1 cells exposed to nephrotoxic haloalkenyl cysteine S-conjugates has recently demonstrated that intracellular Ca^{2+} increases concomitantly with the appearance of plasma membrane blebs (20, 21). Oxidative damage to kidney membranes during cold ischemia has also been associated with a sustained increase in intracellular Ca^{2+} (22).

Intracellular Ca^{2+} accumulation also seems to contribute to cell killing caused by several hepatotoxic agents, such as acetaminophen (23–25), diquat, CCl_4 (25), quinones (26), cyanide (27), and maitotoxin, a polyhydroxy

polyether compound that stimulates Ca^{2+} influx in cells (28). There is also increasing evidence that Ca^{2+} plays an important role in cell death in the immune system. The killing of immature thymocytes by glucocorticoids (29, 30), the cytolytic activity of cytotoxic T lymphocytes (31), natural killer cells (32), and complement (33) all appear to be Ca^{2+}-dependent. Furthermore, the immunotoxicity of certain environmental contaminants may also be mediated by Ca^{2+}-dependent processes. This seems to be the case for thymocyte killing caused by TCDD (34) and by tributyltin (35). It has also been reported that macrophage death is triggered by disturbances in intracellular Ca^{2+} homeostasis and is linked to the onset of programmed cell death in these cells (36). Finally, recent studies have suggested that viruses (37) and viral components (15) can promote cell killing by increasing intracellular Ca^{2+} levels.

Normally, intracellular Ca^{2+} homeostasis is controlled by the concerted operation of plasma membrane Ca^{2+} translocases and intracellular compartmentalization systems (38). Disturbances of this operation during cell injury can result in enhanced Ca^{2+} influx, release of Ca^{2+} from intracellular stores, and/or inhibition of Ca^{2+} extrusion through the plasma membrane. This can lead to an uncontrolled, sustained rise in intracellular Ca^{2+} concentration (39). Such sustained increases in intracellular Ca^{2+} will obliterate the transient Ca^{2+} responses normally caused by hormone stimulation, compromise mitochondrial function and cytoskeletal organization, and ultimately activate irreversible catabolic processes (Figure 1). The following sections address the mechanisms involved in the regulation of intracellular Ca^{2+} homeostasis and summarize existing evidence for the involvement of Ca^{2+}-dependent processes in cell killing.

INTRACELLULAR Ca^{2+} HOMEOSTASIS AND THE ROLE OF Ca^{2+} IN CELL SIGNALING

Recent studies using selective indicators have shown that the Ca^{2+} concentration in the cytosol of unstimulated cells is maintained between 0.05 and 0.2 μM (38). Extracellular Ca^{2+} levels are approximately four orders of magnitude higher (1.3 mM). This produces a large, inwardly directed, electrochemical driving force that is primarily balanced by active Ca^{2+} extrusion through the plasma membrane and by the coordinated activity of Ca^{2+}-sequestering systems located in the mitochondrial, endoplasmic reticular, and nuclear membranes (Figure 2). In excitable tissues, different types of voltage-operated Ca^{2+} channels have been identified and characterized as well as receptor-operated channels that are involved in Ca^{2+} entry during hormone stimulation. It is still unclear whether voltage-operated Ca^{2+} channels exist in

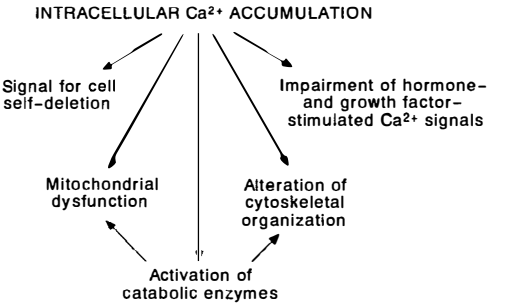

Figure 1 Ca^{2+}-dependent mechanisms of cell killing.

nonexcitable cells, whereas, receptor-operated channels mediate Ca^{2+} influx in both excitable and nonexcitable tissues (40).

Although isolated mitochondria can accumulate large amounts of Ca^{2+}, the affinity of the uniport carrier for Ca^{2+} uptake is low, and the mitochondria appear to play a minor role in buffering cytosolic Ca^{2+} under normal conditions. Electron probe X-ray microanalysis of rapidly frozen liver sections has shown that mitochondria contain little Ca^{2+} in situ (about 1 nmol Ca^{2+} per mg protein), whereas the endoplasmic reticulum represents the major intracellular Ca^{2+} store (41). However, it should be noted that several physiological constituents (e.g. polyamines) can potentially increase the affinity of the uniport carrier for Ca^{2+}, at least to the level that is reached in the cytosol during agonist stimulation (about 400 nM) (42). Thus, the role of mitochondria in the modulation of the agonist-stimulated Ca^{2+} transients deserves further consideration.

Recent studies have shown that liver nuclei possess an ATP-stimulated Ca^{2+} uptake system responsible for intranuclear Ca^{2+} accumulation (43). The more recent finding that the nucleus has a high Ca^{2+}-buffering capacity and that Ca^{2+} can be released from a nuclear compartment in response to intracellular messengers (44), suggests the possibility that the nucleus may have self-regulating mechanisms to control its Ca^{2+} level and to modulate intranuclear Ca^{2+} responses to hormones and growth factors.

The mechanisms whereby Ca^{2+}-mobilizing hormones elicit Ca^{2+} transients have been extensively studied in recent years (45). The signal transduction pathway leading to an elevation of cytosolic Ca^{2+} can be summarized as follows: upon binding of the agonist to its plasma membrane receptor, a specific phospholipase C is activated via stimulation of a G protein, resulting in the hydrolysis of phosphatidylinositol 4,5-bisphosphate and the generation of two second messengers, inositol 1,4,5-trisphosphate ($Ins(1,4,5)P_3$) and diacylglycerol (Figure 3). Diacylglycerol is a potent activator of protein

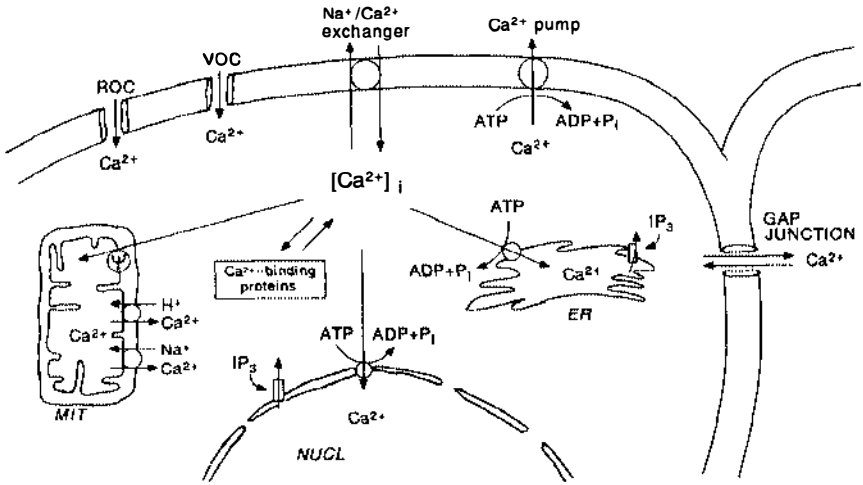

Figure 2 Schematic representation of the mechanisms regulating intracellular Ca^{2+} compartmentalization. ROC: receptor-operated Ca^{2+} channel; VOC: voltage-operated Ca^{2+} channel; MIT: mitochondrion; NUCL: nucleus; ER: endoplasmic reticulum; IP_3: inositol (1,4,5)-trisphosphate.

kinase C (46), whereas Ins(1,4,5)P_3 binds to specific receptors and stimulates Ca^{2+} release from nonmitochondrial intracellular stores (45). The exact intracellular localization of the Ins(1,4,5)P_3-sensitive Ca^{2+} pool is not known. Subcellular fractionation and immunocytochemical studies to determine the localization of the Ins(1,4,5)P_3 receptor have suggested that at least part of this pool is located within the endoplasmic reticulum (47, 48). In addition, the existence of a distinct Ins(1,4,5)P_3-sensitive Ca^{2+} store located in the hepatocyte nucleus has recently been suggested (44); however, the possible contribution of this pool to the agonist-induced elevation of cytosolic Ca^{2+} has yet to be elucidated.

In addition to mobilizing Ca^{2+} from intracellular stores, hormones can stimulate Ca^{2+} influx from the extracellular compartment through specific receptor-operated Ca^{2+} channels (49). With the recent development of techniques to study Ca^{2+} changes in single cells, it has become possible to study the spatial and temporal distribution of transients within the cell. This has led to the observation that at low, close-to-threshold concentrations of Ca^{2+}-mobilizing hormones, many cells respond to these agents with relatively rapid, oscillating spikes (50). It has been suggested that such oscillatory patterns may carry a frequency-encoded message (51). Furthermore, slow propagating Ca^{2+} waves have been detected after agonist stimulation in some

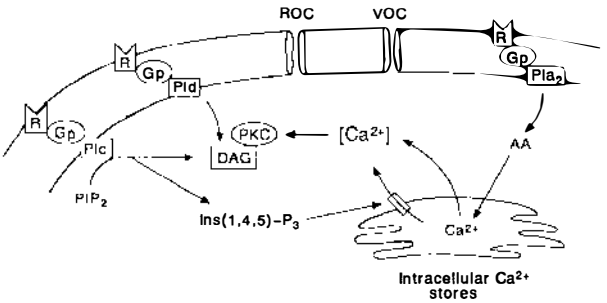

Figure 3 Ca^{2+}-coupled signaling systems. ROC: Receptor-operated Ca^{2+} channel; VOC: voltage-operated Ca^{2+} channel; R: receptor; Gp: G protein; Plc: phospholipase C; Pld: phospholipase D; Pla_2: phospholipase A_2; DAG: 1,2-diacylglycerol; PIP_2: phosphatidylinositol 4,5-bisphosphate; Ins(1,4,5)-P_3: inositol 1,4,5-trisphosphate. The mechanisms for phosphatidylinositol 4,5-bisphosphate breakdown and the generation of Ca^{2+} transients by this signaling system are described in the text. (For a comprehensive review on the other two pathways described in this figure (activation of Pla_2 and Pld) see (76).) Influx of Ca^{2+} during cell signaling can occur through both ROC and VOC (40) and the control of Ca^{2+} entry during hormone stimulation is quite complex and still subject to debate. For simplicity, the regulation of Ca^{2+} entry during agonist stimulation has been omitted.

cell types, suggesting that Ca^{2+} fluctuations are not uniform in time and spatial distribution (52). However, the possible implications of these phenomena to cell regulation have yet to be identified.

ALTERATIONS IN CELL SIGNALING AND CYTOTOXICITY

Alterations in cell signaling may play a determinant role in a variety of pathological and toxicological processes. Xenobiotics can interfere with signal transduction at different levels with a resulting loss of normal Ca^{2+} responses to hormones and growth factors (53–55). Chemical, bacterial, and viral toxins can interact with receptors, G proteins and other enzymes involved in cell signaling or can directly affect intracellular Ca^{2+} homeostasis by interfering with Ca^{2+} pumps or Ca^{2+} channels. It has long been recognized that bacterial toxins can inhibit the generation of intracellular messengers linked to the phosphoinositide pathway and thus result in a loss of hormone and growth factor stimulation. Toxic chemicals can also inhibit the generation of inositol polyphosphates (53), whereas oxidative stress may result in either stimulation or inhibition of protein kinase C (54). Although the implications of these effects for cell survival have yet to be established, it is becoming increasingly apparent that the inhibition of hormonal responses may

result not only in the loss of a trophic stimulus, but also the activation of a program for cell self-deletion in some instances (56).

Other environmental toxins that may interfere with cell signaling include various metal ions. Several metals have been shown to interfere with intracellular Ca^{2+} transport systems and with Ca^{2+} channels (58) and to compete with Ca^{2+} for Ca^{2+}-binding proteins, including calmodulin (59). Voltage-operated Ca^{2+} channels also admit Ba^{2+} and Sr^{2+}. The influx of Ca^{2+} through these channels is inhibited by Cd^{2+}, Co^{2+}, Ni^{2+}, Mn^{2+}, and Mg^{2+} (60). In addition, mercuric mercury can travel across sodium or calcium channels, competing with these ions, causing cell depolarization and the modification of neurotransmitter release (61). Several divalent metals, including Cd^{2+} and Hg^{2+}, can also interact with protein thiol groups and inhibit Ca^{2+} transport systems within the cell (62, 63) and thus inhibit Ca^{2+} efflux and release of Ca^{2+} from intracellular stores. Metal-induced disruption of intracellular Ca^{2+} homeostasis has been implicated in the onset of aluminum encephalopathy (64) and lead poisoning (65). Moreover, experiments using synaptosomal preparations have indicated that the effects of certain metals within a given class on intracellular Ca^{2+} levels can be directly correlated with their neurotoxic effects in vivo. For example, the neurotoxic organotin derivative, trimethyltin, is much more potent than the closely related nonneurotoxic mono- and dimethyltin in causing increases in intracellular Ca^{2+} (66). At extremely low concentrations certain metals can directly affect protein kinase C activity and inositol polyphosphate generation (67).

Abnormal activation of pathways involved in Ca^{2+} signaling is another mechanism whereby toxic agents can cause cell death. For example, both the HIV envelope glycoprotein, gp 120 (15), and excitatory amino acids may cause intracellular Ca^{2+} overload and cytotoxicity by abusive stimulation of cell surface receptors (10). An intriguing example of cytotoxic mechanisms activated via stimulation of Ca^{2+} signals has recently been provided by Snyder and coworkers (68), who found that intracellular Ca^{2+} accumulation produced by abusive stimulation of the glutamatergic pathway in neurons activates the calmodulin-dependent nitric oxide (NO) synthase, which catalyzes the conversion of arginine to NO and citrulline. The release of NO into the extracellular space is followed by the killing of neighboring neurons, while NO-producing cells appear to be unaffected.

ACTIVATION OF Ca^{2+}-DEPENDENT DEGRADATIVE ENZYMES

Ca^{2+} is an activator of several enzymes involved in the catabolism of proteins, phospholipids, and nucleic acids. Thus, a sustained increase in intracellular Ca^{2+} concentration above the physiological level can potentially

result in an uncontrolled breakdown of macromolecules of vital importance for the maintenance of cell structure and function.

Ca^{2+}-Activated Proteases

Several studies (69–73) have noted the possible involvement of Ca^{2+}-activated neutral proteases in the cytotoxic effects of certain agents in various cell types. Ca^{2+}-activated proteases (calpains) are essentially nonlysosomal, have a neutral pH optimum, and are primarily located in the soluble cytosolic compartment (74). Upon Ca^{2+} activation, they associate with the membrane in combination with a specific inhibitory protein (calpastatin) (74). The involvement of this proteolytic system in cytotoxic reactions is supported by two lines of evidence. First, the detection of an increase in TCA-soluble radioactivity in [^{14}C]-leucine-labeled cells exposed to toxic agents suggests the activation of an endogenous degradative process (69). Second, the demonstration that selective calpain inhibitors, such as leupeptin, or chelation of extracellular or intracellular Ca^{2+} prevent, or reduce, both the extent of proteolysis and cell killing (69–73) indicates that a Ca^{2+}-dependent proteolytic system plays a critical role in the overall toxic process.

Little is known about preferential substrates of Ca^{2+}-activated proteases. However, the observation that leupeptin, under certain conditions, can prevent the appearance of plasma membrane protrusions caused by toxic agents (75) suggests that cytoskeletal proteins or cytoskeletal anchoring-integral proteins are degraded by the Ca^{2+}-dependent proteases (see also the section on cytoskeletal alterations).

Ca^{2+}-Activated Phospholipases

Several phospholipases depend on Ca^{2+} for activity and play important physiological roles, e.g. phospholipase C, which catalyze the hydrolysis of phosphatidylinositol 4,5-bisphosphate to generate second messengers (see above; Figure 3; 45). Another group of phospholipases, collectively designated phospholipase A_2, regulate the release of arachidonic acid from phospholipids for ecosanoid biosynthesis (76; Figure 3) and have recently also been implicated in the detoxication of phospholipid hydroperoxides because of their ability to release fatty acids from peroxidized membranes (77). Polyunsaturated fatty acids, on the other hand, can mobilize Ca^{2+} from intracellular stores. Activation of phospholipases coupled to receptor–G protein stimulation has also been proposed to function as an intracellular signal (76; Figure 3). Phospholipase A_2 is a Ca^{2+}- and calmodulin-dependent family of enzymes and thus is susceptible to activation after increases in the cytosolic Ca^{2+} concentration. When this Ca^{2+} increase is sustained, the activation of phospholipase A_2 can result in extensive membrane breakdown and in the generation of toxic metabolites. Ca^{2+}-dependent activation of phospholipase

A_2 has been detected during ischemic (78) and anoxic (79) injury and has been associated with cytotoxicity caused by carbon tetrachloride (80) and quinone imines (71) in hepatocytes, by the toxin pyrularia thionin in 3T3 fibroblasts (81) and by Ca^{2+} ionophore in alveolar epithelial cells (82). However, the lack of specific inhibitors of phospholipase A_2 activity has limited extensive investigations to determine the role played by this degradative pathway in chemical toxicity.

Ca^{2+}-Activated Endonuclease(s)

Programmed cell death (apoptosis) is characterized by morphological changes such as plasma and nuclear membrane blebbing, organelle relocation and compaction, and chromatin condensation (83). The most characteristic biochemical feature of this process is the endonuclease-mediated cleavage of DNA at internucleosomal linker regions in fragments of multiples of approximately 200 base pairs (84). Endonuclease activity is stimulated by Ca^{2+} and Mg^{2+} and has been implicated in the killing of target cells by cytotoxic T lymphocytes (31) and natural killer cells (32) and of thymocytes exposed to glucocorticoids (30) or to an antibody to the CD3/T cell-receptor complex (85). In thymocytes treated with glucocorticoids, endonuclease activation and DNA fragmentation are preceded by a sustained increase in intracellular Ca^{2+} concentration and are blocked by pretreatment of the cells with intracellular Ca^{2+} chelators or calmodulin antagonists (30). This observation has led to the proposal that Ca^{2+} overload caused by toxic agents could promote cell killing by endonuclease activation. The Ca^{2+} ionophore A 23187 stimulates apoptosis in thymocytes (86), and Ca^{2+}-dependent endonuclease activation has been detected in thymocytes treated with the immunotoxic agents TCDD (34) and tributyltin (35).

Although endonuclease activation has been extensively studied in thymocytes, this process is also important in other tissues. For example, a constitutive Ca^{2+}-Mg^{2+}-dependent endonuclease is present in liver nuclei and is activated by submicromolar Ca^{2+} concentrations upon stimulation of nuclear Ca^{2+} uptake with ATP (87). In addition, recent studies employing video-image analysis have shown that human mammary adenocarcinoma (BT-20) cells exposed to tumor necrosis factor alpha (TNF-α) undergo programmed cell death following a selective increase in intranuclear Ca^{2+} (88), suggesting that a compartmentalized increase in Ca^{2+} may be sufficient to stimulate DNA fragmentation. However, although studies using different experimental models of programmed cell death have reported similar selective nuclear Ca^{2+} increases (89), other observations suggest that the signaling involved in the activation of programmed cell death is more complex and that additional mechanisms are involved. Thus, in some experimental systems, apoptosis can occur in the absence of any detectable increase in intracellular Ca^{2+}, whereas

in others, it can be prevented even in the presence of high intracellular Ca^{2+} levels. Other studies have indicated that stimulation of protein kinase C activity by phorbol esters or growth factors can prevent DNA fragmentation in the presence of high Ca^{2+} levels (90). More recently, it has been shown that agents known to modify chromatin structure (i.e. polyamines, namely spermine), can prevent DNA fragmentation and apoptosis in thymocytes exposed to glucocorticoids, Ca^{2+} ionophore, or organotin compounds (91). The ability of the polyamines to modify the chromatin structure is responsible for the inhibition of DNA fragmentation. Conversely, agents that cause chromatin unfolding seem to stimulate DNA fragmentation, and polyamine-depleted cells appear more susceptible to the onset of programmed cell death. This suggests that the endonuclease-mediated internucleosomal cleavage occurs only when the linker regions are made accessible through a decondensation or local reduction in histone-DNA interaction. Thus, the arrangement of the chromatin structure may play a determinant role in the activation of the endonuclease in programmed cell death. Since Ca^{2+}, other ions, and various growth stimuli are known to influence chromatin conformation, it is conceivable that multiple signaling systems are necessary to initiate apoptosis in otherwise resistant cells.

Ca^{2+} overload may also stimulate other enzymatic processes that result in DNA damage. Elevated Ca^{2+} levels can lock topoisomerase II in a form that cleaves, but does not religate, DNA. Topoisomerase II-mediated DNA fragmentation has been implicated in the cytotoxic action of some anticancer drugs (92). DNA single strand breaks in cells exposed to oxidative stress can also be generated through a Ca^{2+}-dependent process (26), although the specific mechanism involved is still unclear. Thus, further work is required to identify not only other situations in which endonuclease activation can mediate Ca^{2+}-dependent cell killing, but also additional Ca^{2+}-mediated processes that may generate DNA damage.

MITOCHONDRIAL DAMAGE

Work from several laboratories has indicated that mitochondrial damage may be a common event in the development of cell injury caused by various toxic agents (93–96). Mitochondrial damage is initially manifested by a decrease in the mitochondrial membrane potential followed by ATP depletion. Protons are continuously pumped from the matrix into the intermembrane space in mitochondria of living cells (97). Since the inner mitochondrial membrane is relatively impermeable to anions, a considerable portion of the energy resulting from the proton concentration gradient is stored as membrane electric potential (98). The proton gradient and the transmembrane potential represent the electrochemical forces employed for ATP synthesis as well as for other

metabolic activities, including the maintenance of Ca^{2+} homeostasis within mitochondria. As described above, Ca^{2+} can be actively transported into mitochondria via an electrophoretic uniporter. The driving force for the continuous Ca^{2+} pumping is provided by the transmembrane potential. However, studies performed in isolated mitochondria have demonstrated that during Ca^{2+} uptake the membrane potential decreases and the extent of the decrease is proportional to the amount of Ca^{2+} taken up by the mitochondria (99). Thus, under conditions that cause massive amounts of Ca^{2+} to accumulate in the mitochondria, it appears that their membrane potential would collapse. This proposal has recently been confirmed by studies in which isolated rat hepatocytes were incubated with sodium orthovanadate to inhibit the endoplasmic reticular and the plasma membrane Ca^{2+} translocases. Under these conditions, cytosolic Ca^{2+} increases and large amounts of Ca^{2+} are accumulated in the mitochondria (100). Concomitantly, the membrane potential decreases to an extent proportional to the amount of Ca^{2+} accumulated and ATP depletion invariably follows. Other studies, in which cells were exposed to the Ca^{2+} ionophore, ionomycin, or were permeabilized with digitonin in the presence of Ca^{2+}, have recently demonstrated that intracellular Ca^{2+} overload causes the collapse of mitochondrial membrane potential (101, 102).

The existence of different Ca^{2+} uptake and release pathways in mitochondria provides a basis for Ca^{2+} cycling (99). This process continuously uses energy supplied by the membrane potential. Several investigations, using mitochondria isolated from different sources, have demonstrated that the oxidation of intramitochondrial NAD(P)H can activate the release route and accelerate Ca^{2+} cycling across the mitochondrial membrane (103–108). This condition is associated with a decrease in the mitochondrial membrane potential that parallels the rate of Ca^{2+} cycling. Moreover, chelation of extramitochondrial Ca^{2+} with EGTA or inclusion of ruthenium red in the incubation medium to abolish the reuptake of the released Ca^{2+} prevents the collapse of the membrane potential (109, 110). The mechanisms of Ca^{2+}-dependent mitochondrial damage have been extensively investigated by Pfeiffer and coworkers (111–114). They have proposed that phospholipase A_2 activation may induce permeability changes in mitochondria exposed to Ca^{2+} and oxidizing agents. According to this view, Ca^{2+} uptake, or Ca^{2+} cycling, results in phospholipase A_2 activation and the accumulation of deacylated phospholipids, whose reacylation is inhibited by the concomitant depletion of intramitochondrial GSH and the increase in glutathione disulfide. Evidence that deacylated phospholipids can accumulate, and that the process is inhibited by dibucaine, supports the involvement of phospholipase A_2. As a consequence of the overall process, a proteinaceous pore opens, leading to the release of ions and other small molecules as well as proteins into the ex-

tramitochondrial environment (114). This process has also been shown to occur in heart mitochondria exposed to Ca^{2+}, inorganic phosphate, and oxidizing agents and has been claimed to be a mechanism for mitochondrial dysfunction in the ischemic, reperfused heart (115).

A more detailed analysis of mitochondrial damage induced by the combined effects of Ca^{2+} and active oxygen species has been performed by Malis & Bonventre (116). In the presence of Ca^{2+}, oxygen radicals cause a marked increase in mitochondrial membrane permeability and a major functional breakdown in the electron transport chain. Furthermore, there is a 50% reduction in ATPase activity, a decrement in ADP translocation and uncoupling of respiration, reflecting the inability of the mitochondria to synthesize ATP. It is important to stress the role of Ca^{2+} in triggering these alterations, as the omission of this ion from the incubation medium minimized the damaging effects of the oxygen free radicals.

An interesting aspect of the relationship between mitochondrial membrane potential and intracellular free Ca^{2+} is that Ca^{2+}-independent, direct mitochondrial toxins can subsequently cause a toxic increase of cytosolic Ca^{2+} concentration. Due to the energy requirements of the different Ca^{2+} transport systems that control Ca^{2+} homeostasis, mitochondrial dysfunction and subsequent ATP depletion invariably result in the disruption of intracellular Ca^{2+} homeostasis. Treatment of cultured rat hepatocytes with the uncoupler protonophore carbonylcyanide-3-chlorophenylhydrazone (CCCP) causes an early, modest and transient increase in cytosolic Ca^{2+} concentration that is restricted to the peripheral regions of the cell and is likely the result of Ca^{2+} release from mitochondrial stores (101). However, at early time points (0–5 min), the intracellular concentration of ATP and the ATP/ADP ratio are sufficient to allow the plasma membrane and the endoplasmic reticular Ca^{2+}-ATPases to function in lowering the cytosolic Ca^{2+} concentration. At later time points, when the intracellular ATP concentration has fallen to very low levels and the ATP-dependent Ca^{2+} pumps cannot transport Ca^{2+} efficiently, the cytosolic Ca^{2+} concentration increases to high levels and potentially triggers a series of Ca^{2+}-dependent events that ultimately lead to cell death.

A different type of mitochondrial damage induced by Ca^{2+} has been described by Pascoe & Reed (4, 117). They report that removal of Ca^{2+} from the incubation medium of freshly isolated rat hepatocytes results in the collapse of the mitochondrial membrane potential and the appearance of signs of oxidative cell injury that were abolished by inhibitors of mitochondrial Ca^{2+} cycling, such as ruthenium red and lanthanum. These findings have led to the hypothesis that mitochondrial Ca^{2+} cycling alone can lead to oxidative stress and cell injury. However, the mechanism by which Ca^{2+}-free medium stimulates mitochondrial Ca^{2+} cycling and the relationship between the latter and the onset of oxidative stress remain to be elucidated.

CYTOSKELETAL ALTERATIONS

An early event in cell injury caused by toxic conditions is the appearance of multiple surface protrusions, often referred to as "blebs" (7, 118). The pathophysiology of bleb formation has not been fully elucidated, but it is generally accepted that a disruption of cytoskeletal organization and interaction with the plasma membrane plays a critical role. Hence, ultrastructural studies show a marked reorganization of several cytoskeletal elements accompanying the appearance of plasma membrane blebs (21, 120), and well known cytoskeletal toxins, such as cytochalasins and phalloidin, cause bleb formation in different cell types (121).

It also appears that Ca^{2+} plays an important role in bleb formation resulting from toxic cell injury (7). This assumption is supported by several lines of evidence. A perturbation of intracellular Ca^{2+} homeostasis with a sustained increase in cytosolic free Ca^{2+} concentration is associated with bleb formation caused by various toxic compounds, including Ca^{2+} ionophores (122). Furthermore, the omission of Ca^{2+} from the incubation medium or pretreatment of target cells with intracellular Ca^{2+} chelators prevent or delay the appearance of blebbing following chemical injury (27,120,123). Finally, many investigators have clearly demonstrated the importance of Ca^{2+}-dependent events in the control of cytoskeletal organization and function (124). In particular, a key role for Ca^{2+} has been detected in the reaction sequence that controls the appearance of plasma membrane protrusions under physiological conditions. For example, pseudopod formation following platelet activation is triggered by an elevation of cytosolic Ca^{2+} concentration and by Ca^{2+}-dependent proteolysis of an actin-binding protein (125). Furthermore, neurite outgrowth can be induced in mouse NB2a/d1 neuroblastoma cells by the calcium ionophore A 23187 and this process involves alterations in actin and microtubule dynamics (126).

The three main classes of cytoskeletal fibers—microfilaments, microtubules and intermediate filaments—are differentiated by their size, protein composition, and activity and function (127). The disruption of the normal organization of these three classes of cytoskeletal fibers by increased intracellular Ca^{2+} concentration is attributable to the activation of at least three different mechanisms.

Modification of the Association Between Actin and Actin-binding Proteins

Microfilaments are primarily composed of actin and several actin-binding proteins. The latter modulate the state of actin polymerization to form fibers, the self-association of fibers to form bundles, and the association of these bundles with the plasma membrane (128). Many actin-binding proteins re-

quire Ca^{2+} to interact with other cytoskeletal constituents. Typical examples include caldesmon (which binds to actin and prevents myosin binding), gelsolin (which severs actin microfilaments), and villin (which severs actin microfilaments into short fragments) (128). Moreover, Ca^{2+} regulates the function of three other actin-binding proteins that are directly involved in the association of microfilaments with the plasma membrane. Among these proteins, alpha-actinin is involved in the normal organization of actin filaments into regular, parallel arrays. However, in the presence of micromolar Ca^{2+} concentrations (e.g. after exposure of cells to Ca^{2+} ionophore), alpha-actinin dissociates from the actin filaments (129). The other two actin-binding proteins, vinculin and ABP (actin-binding protein, in platelets), are substrates for Ca^{2+}-dependent proteases (see below). Another example of Ca^{2+}- mediated modification of actin-binding proteins is found in the work by Harris & Morrow on fodrin (130). Fodrin is an ubiquitous cytoskeletal protein that is able to link integral membrane proteins to cortical actin filaments. Thus, it is involved in the organization of receptor domains and in the control of vesicular traffic at the plasma membrane. When the cytosolic Ca^{2+} level increases, fodrin can either bind calmodulin, or it can be cleaved by a Ca^{2+}-dependent protease. Both processes result in the loss of the ability of fodrin to bind actin and in the dissociation of microfilaments from membrane integral proteins. There is convincing evidence for the involvement of Ca^{2+}-dependent processes in the alterations of actin microfilaments and actin-binding proteins induced by chemical toxins. For example, the incubation of human platelets with the prooxidant, menadione, which promotes a marked increase in cytosolic free Ca^{2+} concentration, results in a net decrease of polymerized actin and in the dissociation of alpha-actinin from the cytoskeleton (131). These changes are largely inhibited by conditions that prevent an increase in cytosolic free Ca^{2+} and by pretreatment of the platelets with intracellular Ca^{2+} chelators. Furthermore, immunocytochemical investigations using anti-alpha-actinin antibodies and NBD-phallacidin to stain actin reveal a dissociation of alpha-actinin from the actin filaments and suggest this phenomenon as one of the factors responsible for bleb formation (132).

Microtubule structure and distribution are also controlled by Ca^{2+}. Microtubule polymerization obtained in vitro by warming tubulin solutions at 37°C in the presence of GTP is abolished when micromolar Ca^{2+} concentrations are present in the incubation medium (133). Moreover, the activity of microtubule-associated proteins (MAPs), which control the turnover and distribution of microtubules, can be modulated by Ca^{2+} and calmodulin (134). Studies performed by Shelanski and coworkers (135) have shown that microinjection of Ca^{2+}/calmodulin complexes in 3T3 fibroblasts results in a total depolymerization of microtubules that is spatially limited to the site of injection.

Although microtubule depolymerization during toxic cell injury has been reported (136) the role of Ca^{2+} in this process has not yet been identified.

Activation of Ca^{2+}-Dependent Proteases

Ca^{2+}-dependent proteases catalyze the proteolysis of several cytoskeletal proteins including spectrin, fodrin, caldesmon, adducin, tubulin, MAP-2, tau factor, vimentin, and cytokeratin (137–139). Two of the cytoskeletal proteins that are directly involved in the anchoring of microfilaments to the inner surface of the plasma membrane, vinculin and actin-binding protein, have been reported as preferential substrates for Ca^{2+}-dependent proteases (139). An increase in cytosolic Ca^{2+} concentration to micromolar levels (high enough to activate the protease) results in the proteolysis of these two polypeptides. This occurs physiologically during platelet activation (125) and toxicologically during oxidative stress caused by the metabolism of menadione in CG5 cells (120) and in platelets (131). Investigations performed by Steenbergen and coworkers (140), using anti-vinculin antibodies in the canine heart during ischemia and reperfusion, reveal a progressive loss of vinculin staining along the lateral margin of myocytes. The loss in vinculin staining is associated with the appearance of subsarcolemmal blebs and breaks in the plasma membrane. Since a marked intracellular Ca^{2+} overload is concomitantly occurring during the ischemia-reperfusion injury, the loss of vinculin and bleb formation have been attributed to the activation of Ca^{2+}-dependent proteases. The assumption that proteolysis of cytoskeletal proteins is one of the pathophysiological factors in bleb formation during chemical injury is also supported by the fact that Ca^{2+}-dependent proteolysis of actin-binding proteins can occur under these conditions (131) and that protease inhibitors can protect from blebbing (75).

Activation of Protein Kinases and Alterations in Protein Phosphorylation

The Ca^{2+} ion has been implicated in the regulation of mitosis in plant and animal cells (141). Transient increases in intracellular free Ca^{2+} precede the breakdown of the intermediate filament-rich nuclear envelope that is prevented when the Ca^{2+} increase is buffered by Ca^{2+} chelators (142, 143). The activation of the multifunctional Ca^{2+}/calmodulin-dependent protein kinase (144) appears to be necessary for nuclear envelope breakdown. Since several cytoskeletal elements are phosphorylated by the Ca^{2+}/calmodulin-dependent kinase or by other Ca^{2+}-dependent kinases, it is conceivable that Ca^{2+}-stimulated phosphorylation is associated with the rearrangement of the cytoskeleton during mitosis. Other important regulatory reactions that control cytoskeletal organization involve the continuous dephosphorylation of the constitutent proteins. It has recently been reported that microcystin-LR, a

cyclic peptide toxin isolated from certain species of cyanobacteria, (blue-green algae), can induce blebbing and a rapid reorganization of microfilaments in isolated rat hepatocytes (145). These effects are apparently independent of any appreciable variation in intracellular free Ca^{2+} concentration, ATP level, or oxidation or alkylation of macromolecules. Subsequent studies revealed that microcystin-LR is a potent inhibitor of type 1 and type 2A protein phosphatases (146), suggesting that an altered balance of protein kinase/phosphatase activity can induce abnormal phosphorylation of various cellular proteins (including cytoskeletal proteins), ultimately leading to cell injury and death. Calyculin-A, another inhibitor of cellular phosphatases, causes enhanced phosphorylation of vimentin and 20-kd myosin light chain in 3T3 fibroblasts and promotes their detachment from the substratum (147), providing further support for this proposal.

Other Mechanisms of Cytoskeletal Damage and Bleb Formation during Chemical Injury

The activation of Ca^{2+}-dependent functions is clearly not the only mechanism responsible for chemically induced cytoskeletal damage, which also involves Ca^{2+}-independent mechanisms. Thus, ATP depletion can cause depolymerization of actin (148), breakdown of the actomyosin network (149), a change in the lipid order of the plasma membrane (150), and membrane blebbing. Furthermore, oxidative stress induced by incubation of hepatocytes with diamide or menadione causes the oxidation of thiol groups in cytoskeletal proteins and a disruption of Ca^{2+} homeostasis. (151). This results in the formation of gross actin aggregates and in the relocalization of microfilaments, generating weak sites in the periphery of the cell for potential bleb formation.

CONCLUSIONS

It is evident that the calcium ion plays an important role in cell killing and recent research has revealed some of the biochemical mechanisms whereby intracellular Ca^{2+} overload can activate lethal processes. In addition, the consequences of impaired Ca^{2+} signaling are becoming apparent and may be reflected in the development of chronic toxicity. During acute toxicity, different mechanisms are likely to be recruited, contributing with the Ca^{2+} increase to accelerate cell death. On the other hand, most cases of toxic injury in vivo develop over a period of time. The hypothesis that altered cell signaling plays a role in toxic injury and that programmed cell death may often be the mechanism involved appears very convincing.

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