Lead and Calcium Produce Rod Photoreceptor Cell Apoptosis by Opening the Mitochondrial Permeability Transition Pore

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Calcium overload is suggested to play a fundamental role in the process of rod apoptosis in chemical-induced and inherited retinal degenerations. However, this hypothesis has not been tested directly. We developed an in vitro model utilizing isolated rat retinas to determine the mechanisms underlying Ca\(^{2+}\) and/or Pb\(^{2+}\) induced retinal degeneration. Confocal microscopy, histological, and biochemical studies established that the elevated [Ca\(^{2+}\)] and/or [Pb\(^{2+}\)] were localized to photoreceptors and produced rod-selective apoptosis. Ca\(^{2+}\) and/or Pb\(^{2+}\) induced mitochondrial depolarization, swelling, and cytochrome \(c\) release. Subsequently caspase-9 and caspase-3 were sequentially activated. Caspase-7 and caspase-8 were not activated. The effects of Ca\(^{2+}\) and Pb\(^{2+}\) were additive and blocked completely by the mitochondrial permeability transition pore (PTP) inhibitor cyclosporin A, whereas the calcineurin inhibitor FK506 had no effect. The caspase inhibitors carboxenzoxy-Leu-Glu-His-Asp(CH\(_2\)F) and carboxenzoxy-Asp-Glu-Val-Asp(CH\(_2\)F) but not carboxenzoxy-Leu-Thr-Asp(CH\(_2\)F), differentially blocked post-mitochondrial events. The levels of reduced and oxidized glutathione and pyridine nucleotides in rods were unchanged. Our results demonstrate that rod mitochondria are the target site for Ca\(^{2+}\) and Pb\(^{2+}\). Moreover, they suggest that Ca\(^{2+}\) and Pb\(^{2+}\) bind to the internal metal (Me\(^{2+}\)) binding site of the PTP and subsequently open the PTP, which initiates the cytochrome \(c\)-caspase cascade of apoptosis in rods.

Apoptosis is an active mode of cell death that is induced by a variety of physiological and pathological stimuli. Convergent evidence suggests that mitochondria and caspases play a central and fundamental role in the effector or executioner phase of apoptosis. Early during the effector phase, the mitochondrial permeability transition pore (PTP), a megachannel in the inner mitochondrial membrane, is opened by a variety of apoptotic inducers such as elevated matrix Ca\(^{2+}\), pro-oxidants, and thiol-reactive agents (1–3). This leads to mitochondrial depolarization (decrease in \(\Delta\Psi_{\text{mt}}\)) and subsequently to the release of cytochrome \(c\) and/or apoptosis-inducing factor from mitochondria to cytosol or nuclei (1, 4, 5). Caspases are activated, which cleave downstream death substrates and activate endonucleases that cleave genomic DNA into fragments resulting in the apoptotic nuclear morphology (6, 7). The opening of the mitochondrial PTP and the apoptotic process can be inhibited by a diverse group of agents such as Bel-2, Bel-x\(_l\), bongkrekic acid, and CsA (1).

Sustained increases in intracellular [Ca\(^{2+}\)] trigger apoptosis in a diverse array of in vivo and in vitro systems (1, 8). Results from several studies suggest that elevated rod photoreceptor [Ca\(^{2+}\)] plays a key role in the process of apoptotic rod cell death in humans and animals during inherited retinal degenerations, retinal diseases and injuries, and chemical exposure. These include patients with retinitis pigmentosa and cancer-associated retinopathy (9, 10), lead-exposed rats (11–13), retinal degeneration mice (13, 14), rats injected with anti-recoverin monoclonal antibodies (15), rats with hypoxic-ischemic injury (16), and rats with light induced damage (17).

Several neurotoxic heavy metals, transition metals, and organometals produce neuronal apoptosis in whole animals and cultured cells (11, 12, 18–25). For example, low to moderate level Pb\(^{2+}\) exposure produces apoptotic rod and bipolar cell death in developing and adult rats (11, 12) and apoptotic neuronal cell death in primary cultured cells (23, 24). Similarly, Cu\(^{2+}\) and methyl mercury produce apoptosis in the developing and mature olfactory epithelium and cerebellum, respectively (18, 22). Although the molecular mechanisms underlying the apoptosis induced by these different metals are unknown, there are two possible, though not mutually exclusive, triggering mechanisms: one is Ca\(^{2+}\) overload and the other is the generation of ROS. Both in vivo and in vitro lead exposure increase rod and retinal intracellular [Ca\(^{2+}\)] during cell death (26, 27). Hg\(^{2+}\), tributyltin, and methyl mercury also cause Ca\(^{2+}\) overload in cultured neuronal cells prior to cell death (19, 20, 28). Moreover, Hg\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), and methyl mercury can induce apoptosis in neurons and other tissues by increasing ROS (21, 29–31).

As noted above, most studies of neuronal apoptosis are conducted using cell cultures or cell-free systems. The goals of this study were to establish an in vitro retinal model of in vivo...
lead-induced rod-selective apoptosis and to elucidate the cellular and biochemical mechanisms underlying the rod-selective apoptosis. Our results show that exposure of isolated adult rat retinas to Ca\(^{2+}\) and/or Pb\(^{2+}\) resulted in rod-selective apoptosis and suggest that Ca\(^{2+}\) and Pb\(^{2+}\) bind to the internal Me\(^{2+}\) binding site of the mitochondrial PTP, thereby opening the PTP and initiating the cytochrome c-caspase cascade of apoptosis in rods.

**EXPERIMENTAL PROCEDURES**

**Materials**—Materials were purchased from the following suppliers: DEVD-fmk, LEHD-fmk, and IETD-fmk were from Enzyme Systems Products (Dublin, CA); YVAD-cmk was from Alexis Biochemicals (San Diego, CA); DEVD-pNA and anti-PARP antibody (clone C-20) were from BIOMOL Research Laboratories Inc. (Plymouth Meeting, PA); DMQD-pNA and LEHD-pNA were from California Peptide Research, Inc. (San Diego, CA). The antibody to rabbit Ig A was from Sigma.

**Determination and Localization of Retinal (Ca\(^{2+}\) and Pb\(^{2+}\))—To determine the whole retinal elemental calcium and lead concentrations (Ca\(^{2+}\) and Pb\(^{2+}\)), flame atomic absorption spectrometry (n = 7–8 retinas/condition) was used as described (27). Fluo-3 Ca\(^{2+}\) imaging and confocal laser scanning microscopy were used as described (41) to localize the distribution and to determine the relative concentrations of free Ca\(^{2+}\) and Pb\(^{2+}\) in retinas (n = 3 retinas/condition). Retinas (180–200 μm thick) were mounted photoreceptor side down and scanned every 2 μm. Ninety to 100 images were aligned and stacked using the three-dimensional NIH Image software (version 1.62).

**Detection of Mitochondrial Membrane Potential and Swelling**—The techniques for utilizing JC-1 were essentially as described (42). Briefly, retinas (n = 4–8/condition) were stained with JC-1 (10 μg/ml), whole mounted on slides, and examined with the fluorescence microscope. The red fluorescence (585 nm) from the J-aggregate (hyperpolarized mitochondria) and the green fluorescence (520 nm) from the JC-1 monomer (depolarized mitochondria) were viewed with a rhodamine optical filter set and a fluorescein optical filter set, respectively.

**Enzymatic Assays for Caspase Activity**—Caspase activity was measured using three different tetrapeptide-pNA substrates (DEVD-pNA, DMQD-pNA, and LEHD-pNA) as described (43). The incubated retinas were swelled for 10 min on ice in cell lysis buffer, homogenized with a Polytron PT1200, and centrifuged (10,000 × g, 20 min, 4 °C). The supernatants were used for Western blot analysis. To detect PARP, proteins were prepared as described (44).

**Detection of HMW DNA Fragmentation**—To detect cleavage of genomic DNA into HMW fragments, single cell retinal suspensions were made from each pair of retinas (n = 5–7 pairs of retinas/condition) by incubating retinas in 500 μl of activated papain (26 units/ml) at 37 °C for 5 min followed by trituration. Field inversion gel electrophoresis was carried out as described (38). After field inversion gel electro-
er’s instructions. Primary antibodies were used at a dilution of 1:1000 (anti-cytochrome c, anti-caspase-3, -7, -9, and anti-PKC 6) or 1:10,000 (anti-PARP) followed by incubation with anti-mouse Ig-horseradish peroxidase (for anti-cytochrome c and anti-PARP) or with anti-rabbit Ig-horseradish peroxidase (for anti-caspase-3, -7, -9, and anti-PKC 6) at a dilution of 1:5000. To confirm that there was no mitochondrial contamination in the cytosolic fractions, immunoblots used for cytochrome c detection were stripped, incubated with anti-cytochrome oxidase IV (1:500), and incubated with anti-mouse Ig-horseradish peroxidase (1: 5000). The intensities of the cleaved bands were quantified using NIH Image software (n = 3–4 experiments/condition/mouse).

**Differences from Controls**—using the appropriate analysis of variance (ANOVA) and Fisher’s protected least significant difference posthoc comparisons (StatView, Abacus Concepts, Inc., Berkeley, CA). Differences from controls were regarded as significant if p < 0.05.

**RESULTS**

Retinal DNA Is Cleaved into HMW Fragments—Cleavage of DNA into HMW fragments is considered one of the hallmarks of apoptosis (38). Retinas incubated for 15 min in control buffer produced a minimal amount of 600- and 300- and no 50-kbp DNA fragments (Fig. 1, lane 1). Retinas incubated with 0.1–2 mM Ca2+ or 10 mM to 100 μM Pb2+ exhibited a concentration-dependent increase in the amount of HMW fragments (data not shown). The concentrations of Ca2+ and Pb2+ that produced an apparent 50% increase in the amount of HMW fragments (300 > 600 >> 50 kbp) were 0.5 mM Ca2+ and 1 μM Pb2+ (Fig. 1, lanes 2 and 3). These concentrations were employed for all other experiments. The amount of HMW fragments from retinas incubated with Ca2+ and Pb2+ was greater than with either divalent cation alone, and the pattern of fragmentation was different (300 > 50 > 600 kbp; Fig. 1, lane 4) suggesting an additive effect of these divalent cations. Moreover, the Ca2+- and Pb2+-induced changes were not the result of nonspecific divalent cation effects as the pattern of HMW DNA fragmentation for retinas incubated in buffers containing Zn2+ (1–3 mM) or no Mg2+ was not different than controls shown in Fig. 1, lane 1 (data not shown). The production of HMW DNA fragments by Ca2+ and Pb2+ was inhibited completely by 1 mM DEVD-fmk (Fig. 1, lane 5), 1 μM CsA (Fig. 1, lane 6), or 10 μM CsA (data not shown). In contrast, it was not blocked by 100 μM YVAD-cmk or 100 μM PFK506 (data not shown), indicating that group I caspases and calcineurin, respectively, were not involved under these conditions.

**Retinal Apoptosis Induced by Ca2+ and/or Pb2+ Is Rod Photoreceptor-specific**—AO/EtBr staining enabled us to distinguish between viable, early apoptotic, late apoptotic, and necrotic cells (39). All early and late apoptotic nuclei were identified as rod photoreceptor cells as determined by their localization within the outer nuclear layer and nuclear diameter. Electron microscopy confirmed that the Ca2+ and/or Pb2+-induced retinal cell death was rod-specific and apoptotic. Fig. 2A shows a typical apoptotic nucleus with fragmented and margined chromatin, whereas the surrounding rod nuclei appear normal. Retinas incubated in control buffer had 25 apoptotic early and late rod cells/mm² of retina (Fig. 2B). The number of apoptotic rods in retinas incubated with Ca2+ or Pb2+ increased 3–4-fold, whereas retinas incubated with Ca2+ and Pb2+ showed an 8-fold increase in apoptotic rods indicating an additive effect of both divalent cations similar to that observed with the HMW fragmentation. The Ca2+- and Pb2+-induced rod apoptosis was inhibited completely by 1 mM DEVD-fmk or 10 μM CsA, but not by 100 nM FKS06 (Fig. 2B). Moreover, AO/EtBr staining revealed that DEVD-fmk and CsA were cytoprotective, as the rod nuclei from these treated retinas were uniformly green indicating that these cells were viable and did not die instead by necrosis. The number of necrotic cells did not vary with any incubation condition: 1.9 ± 0.8 necrotic cells/mm² of retina. Taken together, these data show that Ca2+ and Pb2+ produce rod-selective apoptosis and that caspases are required for both nuclear apoptosis and cell death.

**Increased Retinal Ca2+ and Pb2+ Are Localized to the Photoreceptors**—The [Ca] and [Pb] in control retinas were 20.36 ± 0.85 and 0.03 ± 0.03 μg/mg wet weight of retina, respectively. The [Ca] of retinas incubated with 0.5 mM free Ca2+ or 0.5 mM Ca2+ and 1 μM Pb2+ was increased 23 and 37%, respectively. The [Pb] of retinas incubated with Pb2+ or Ca2+ and Pb2+ was increased 5–6-fold. Fluo-3 Ca2+ imaging and confocal laser scanning microscopy localized the increased free Ca2+ and Pb2+. The apparent dissociation constants (Kd) of the Ca2+-fluor-3 complex and Pb2+-fluor-3 complex are 450 nM (49) and 6 μM (50), respectively. Emission spectrum data show that...
$\text{Ca}^2+$ and $\text{Pb}^{2+}$ Produce Rod Photoreceptor Cell Apoptosis

$\text{Pb}^{2+}$ produces a concentration-dependent decrease in the $\text{Ca}^{2+}$-induced fluo-3 fluorescence (50). Therefore, we reasoned that the intracellular distribution of $\text{Pb}^{2+}$ might be localized by its ability to quench the $\text{Ca}^{2+}$-fluore-3 fluorescence. In controls, the fluo-3 signal was observed mainly in the photoreceptors (Fig. 3A). In retinas incubated with $\text{Ca}^{2+}$, the fluo-3 fluorescence was localized almost exclusively in the photoreceptors and was markedly increased as evidenced by saturation of the high affinity dye in several rods (Fig. 3B). In similar preparations, the free $[\text{Ca}^{2+}]$ in rods ranged from 5 to 10 $\mu$M (32). In retinas incubated with $\text{Ca}^{2+}$ and $\text{Pb}^{2+}$ (Fig. 3C) or $\text{Ca}^{2+}$ followed by $\text{Pb}^{2+}$ (data not shown), the $\text{Ca}^{2+}$-induced fluo-3 fluorescence was quenched to a level slightly below that observed in controls (Fig. 3A). These results indicate that $\text{Pb}^{2+}$ readily entered the retina, was localized to the photoreceptors, and did not block the entry of $\text{Ca}^{2+}$. The addition of 1 nM DEVD-fmk to the retinal incubation buffers did not affect the $\text{Ca}^{2+}$-induced fluo-3 fluorescence (data not shown) indicating that caspase-3 did not influence divalent cation entry into the photoreceptors.

Overall, the fluo-3 fluorescence was lower in the inner than outer retina and there were no treatment-related changes in the inner retinal $[\text{Ca}^{2+}]$. Retinas incubated with $\text{Pb}^{2+}$ alone had slightly lower levels of fluo-3 fluorescence in the photoreceptors than in controls (data not shown).

Mitochondrial $\Delta \psi_m$ Is Decreased in Retinas Incubated in $\text{Ca}^{2+}$ and/or $\text{Pb}^{2+}$—Accumulating evidence suggests that mitochondria play an important role in regulating apoptosis (1). The rod $\Delta \psi_m$ was monitored using JC-1 staining and fluorescence microscopy. As illustrated by a representative figure, control rod mitochondria throughout the retina displayed an intense fluorescence from J-aggregates (Fig. 4A) and very weak fluorescence from JC-1 monomers (Fig. 4F). Overall, the mitochondria from retinas incubated with $\text{Ca}^{2+}$ and/or $\text{Pb}^{2+}$ were depolarized as indicated by the decreased J-aggregate fluorescence and increased JC-1 monomer fluorescence. Representative images of rods from retinas incubated with $\text{Ca}^{2+}$ (Fig. 4, B and G) or $\text{Pb}^{2+}$ (Fig. 4, C and H) reveal markedly decreased J-aggregate formation (Fig. 4, B and C) and increased JC-1 monomers (Fig. 4, G and H). Closer examination of these figures reveals that a subpopulation of rod mitochondria was depolarized. Moreover, in retinas incubated in $\text{Ca}^{2+}$ and $\text{Pb}^{2+}$ the rod mitochondria were depolarized even further as shown by the large decrease in J-aggregate (Fig. 4D) and increase in JC-1 monomer (Fig. 4F). An electron micrograph of a transverse section through normal rod inner segments reveals that the circular pattern of mitochondria in a rod (Fig. 4K) is similar to the ring-shaped J-aggregate fluorescence observed in control retinas (Fig. 4A).

To determine the contribution of the mitochondrial PTP to the mitochondrial depolarization, retinas were incubated with 10 $\mu$M CsA plus $\text{Ca}^{2+}$ and $\text{Pb}^{2+}$. A representative pair of figures reveals that CsA markedly increased the rod $\Delta \psi_m$ as evidenced by an increase in J-aggregate (Fig. 4, E versus D) and a decrease in JC-1 monomer (Fig. 4, J versus I) indicating that the $\text{Ca}^{2+}$- and $\text{Pb}^{2+}$-induced opening of PTP significantly contributed to the rod mitochondrial depolarization. Similar data were obtained with CsA in the presence of $\text{Ca}^{2+}$ or $\text{Pb}^{2+}$ alone (data not shown). Interestingly, many rod mitochondria were still depolarized in the presence of CsA (compare Fig. 4, J to F). This latter result is consistent with our findings that $\text{Ca}^{2+}$ and $\text{Pb}^{2+}$...
produce a concentration-dependent transient stimulation of state 4 (ADP-independent) respiration in isolated retinal mitochondria that is inhibited by ruthenium red but not by CsA (Ref. 27 and data not shown). Alternatively, it is possible that Ca\(^{2+}\) and Pb\(^{2+}\) produce an initial mitochondrial depolarization that contributes to opening the mitochondrial PTP. This is consistent with the findings of Bernardi (3) on Ca\(^{2+}\)-induced opening of the PTP in liver mitochondrial populations. The temporal and spatial resolution of our techniques does not allow us to differentiate between these two alternatives. FK506 (100 nM) had no effect on the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced depolarization of rod mitochondria (data not shown), indicating that calcineurin is not involved in the opening of the PTP. To determine the contribution of the caspase activation to the mitochondrial depolarization, retinas were incubated with 1–10 nM DEVD-fmk plus Ca\(^{2+}\) and Pb\(^{2+}\). DEVD-fmk had no effect on the Ca\(^{2+}\)- and/or Pb\(^{2+}\)-induced mitochondrial depolarization (data not shown). Finally, verapamil (100 μM) had no effect on the pattern of JC-1 fluorescence in mitochondria (data not shown), indicating that the effects of CsA were on the PTP and not on the multidrug resistance pump (51).

Rod Redox Status Is Not Altered in Retinas Incubated in Ca\(^{2+}\) and/or Pb\(^{2+}\)—Decreases in GSH and NADH levels and the production of ROS occurs in many forms of apoptosis, are triggered by Ca\(^{2+}\) overload, and can initiate rod photoreceptor degeneration (1, 3, 45, 52). In addition, the probability of mitochondrial PTP opening is increased by the oxidation of glutathione and pyridine nucleotides (3). Therefore the concentrations of rod GSH, GSSG, NADH, and NAD\(^+\) and the peroxidation status of rod lipids were determined in retinas incubated with Ca\(^{2+}\) and/or Pb\(^{2+}\). In rods isolated from retinas incubated in control buffer, the mean ± S.E. concentrations of GSH and GSSG were 31.8 ± 1.8 and 4.1 ± 0.4 nmol/mg protein, respectively, and for NADH and NAD\(^+\) were 0.61 ± 0.06 and 3.44 ± 0.29 nmol/mg protein, respectively. The high NAD\(^+\)/NADH ratio is similar to that found in rat, rabbit, and monkey retinas (53, 54) and most likely results from the very high rate of retinal aerobic glycolysis (55). The mean ± S.E. mol% of the major fatty acids in rods was 9.58 ± 0.46 for palmitic acid (16:0), 27.44 ± 0.29 for stearic acid (18:0), 1.90 ± 0.09 for oleic acid (18:1), 2.43 ± 0.09 for arachidonic acid (20:4), and 55.69 ± 0.72 for docosahexaenoic acid (22:6). The phospholipid yield was 155.7 ± 7.0 nmol of lipid phosphorus/two retinas, and the conjugated diene content was 0.114 ± 0.004 nmol/mole of lipid phosphorus. The values were not significantly different in non-incubated control rods indicating that incubation per se did not change rod redox or lipid peroxidation status. Moreover, the values for each of the above rod measurements were not significantly different in retinas incubated in Ca\(^{2+}\) and/or Pb\(^{2+}\) compared with controls (96 ± 5% of control). These results demonstrate that Ca\(^{2+}\) and/or Pb\(^{2+}\) did not produce oxidative stress.

The Ca\(^{2+}\)- and/or Pb\(^{2+}\)-induced Cytochrome c Release and Mitochondrial Swelling Are Inhibited by CsA but Not by DEVD-fmk—Following a diverse array of apoptotic stimuli, cytochrome c is released from mitochondria to the cytosol where it participates in activating the caspase cascade leading to cell death (1, 4). Because cytochrome c release is not obligatory for apoptosis (56, 57), its role in Ca\(^{2+}\) and/or Pb\(^{2+}\)-induced apoptosis was examined. Retinas incubated in control buffers had no detectable cytochrome c in the cytosolic fraction (Fig. 5, lane 1), whereas approximately equal amounts of cytochrome c were detected in the cytosolic fractions of retinas incubated in Ca\(^{2+}\) (Fig. 5, lane 2) or Pb\(^{2+}\) (Fig. 5, lane 3). Retinas incubated with both divalent cations released twice the amount of cytochrome c (Fig. 5, lane 4) compared with retinas incubated with either divalent cation. The maximum amount of cytochrome c released was 15–20% suggesting that Ca\(^{2+}\) and Pb\(^{2+}\) released cytochrome c from the large, not small, pool that is localized to the inter cristal compartment (51). CsA completely blocked the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced release of cytochrome c (Fig. 5, lane 6), whereas 100 nM FK506 (data not shown) or DEVD-fmk (Fig. 5, lane 5) did not. Interestingly, CsA did not completely block mitochondrial depolarization (Fig. 4) or the Ca\(^{2+}\)- or Pb\(^{2+}\)-induced transient increase in state 4 respiration (Ref. 27 and data not shown) indicating that mitochondrial depolarization per se does not result in cytochrome c release; a similar result was obtained with rat brain mitochondria (57). These results also demonstrate that calcineurin or activation of group II caspases are not required for cytochrome c release. A mitochondrial fraction from control retinas was loaded as a positive control (Fig. 5, lane 7). The absence of cytochrome oxidase subunit IV in the cytosolic fractions (Fig. 5, lanes 1–6) indicates no contamination of mitochondria.

The findings that CsA, but not FK506, blocked cytochrome c release and reduced rod mitochondrial depolarization strongly
suggest that PTP opening was involved in these processes. In addition, previous electron microscopy studies show that rod mitochondria from lead-exposed rats are swollen (11) suggesting that cytochrome c may be released as a result of mitochondrial swelling and rupture of the outer membrane. Therefore, light scattering studies were conducted using isolated retinal mitochondria exposed to 475 nM Ca2+ and/or 585 µM Pb2+, concentrations that produce a transient stimulation of mitochondrial State 3 respiration and a 50–75% decrease in mitochondrial State 3 respiration (27). Large amplitude mitochondrial swelling was induced by Ca2+ (Fig. 6A) or Pb2+ (Fig. 6B), and the effects of Ca2+ and/or Pb2+ were blocked completely by 1 mM EGTA or 0.5 µM CsA (94 ± 5% of control) and partially blocked by 0.1 µM ruthenium red (65 ± 5% of control) or 0.5 µM ruthenium red (84 ± 7% of control). Similar results with CsA were observed in a sucrose buffer, whereas N-ethylmaleimide did not block the effects of Ca2+ and/or Pb2+ (data not shown).

**Caspase-9 and Caspase-3 Are Sequentially Activated**—Based on the K values of DEVDD-CHO for all the caspases (34), our results suggest that caspase-3, -7, and/or -8 may participate in the Ca2+- and Pb2+-induced rod apoptosis and that these caspases act downstream from the mitochondria. In addition, the apical caspase-9 interacts with cytochrome c to activate the group II executioner caspases (1). To determine if caspase-3, -7, -8, and/or -9 were activated during incubation with Ca2+ and Pb2+ and to delineate their sequence of activation, proteolytic activities associated with these caspases were measured by enzymatic assays and Western blot analysis. First, DEVDD-pNA, which is selectively cleaved by caspase-3, -7, and -8 (35, 59), was used as a substrate. Control retinas exhibited minimal activity, whereas retinas incubated with Ca2+ or Pb2+ exhibited a 3–4-fold increase in DEVDDase activity (Fig. 7A), whereas retinas incubated with Ca2+ or Pb2+ exhibited a 3–4-fold increase in DEVDDase activity. DEVDDase activity was increased 7-fold in retinas incubated in both divalent cations again showing the additive effect of Ca2+ and Pb2+ (Fig. 7A). The Ca2+- and Pb2+-induced increase in DEVDDase activity was inhibited completely by 1 mM DEVDD-fmk or 10 µM CsA, but not by 100 nM FK506 (Fig. 7A). These results indicate that PTP opening is upstream of caspase activation and that calcineurin is not involved. Second, to identify the specific caspases and their sequence of activation more selective caspase substrates and inhibitors were utilized. Retinas incubated in control buffers exhibited minimal caspase activity, as shown by the minimal cleavage of DEVDD-pNA, DMQQD-pNA, and LEHDD-pNA (Fig. 7B). The cleavage of these three substrates was increased 5–7-fold in retinas incubated with both Ca2+ and Pb2+. DMQQD-CHO, a selective caspase-3 inhibitor (36), completely inhibited the Ca2+- and Pb2+-induced increase in DEVDDase and DMQDase activity, whereas the caspase-8 selective inhibitor IETD-fmk (34) did not (Fig. 7A). These results provide evidence that caspase-9 was activated following the opening of the mitochondrial PTP, caspase-3 was activated by caspase-9, and caspase-8 did not participate in this apoptotic cascade. The fact that caspase activity was inhibited by CsA and that high concentrations of EGTA (2.5 mM) and dithiothreitol (10 mM) were in the assay buffers eliminated the possibility that the Ca2+-/or Pb2+-induced increase in caspase activity was a direct effect of these divalent cations on the caspases. This is consistent with the

![Fig. 5](http://www.jbc.org/Downloaded from)

**Fig. 5.** The Ca2+- and/or Pb2+-induced cytochrome c release from mitochondria is blocked by CsA but not by DEVDD-fmk. Retinas were incubated as described in the legend to Fig. 1. Western blot analysis and quantification of cytosolic cytochrome c (cyt c) and cytochrome oxidase IV (cyt ox. IV) were conducted as described under “Experimental Procedures.” A representative blot shows that no mitochondrial cytochrome c was released from retinas incubated in control buffer (lane 1), whereas similar amounts of mitochondrial cytochrome c were released from retinas incubated with Ca2+ (lane 2) or Pb2+ (lane 3). Twice (2.0–2.2x) as much cytochrome c was released from retinas incubated with Ca2+ and Pb2+ (lane 4) compared with Ca2+ or Pb2+ alone. The Ca2+- and Pb2+-induced release of cytochrome c was not blocked by 1 mM DEVDD-fmk (lane 5) or 100 nM FK506 (data not shown) but was blocked completely by 10 µM CsA (lane 6). A mitochondrial fraction (mito) was loaded as a positive control for cytochrome c (lane 7). The absence of cytochrome oxidase IV in the cytosolic fractions (lanes 1–6) indicates no mitochondrial contamination. These blots are representative of three to four experiments for each treatment condition.

![Fig. 6](http://www.jbc.org/Downloaded from)

**Fig. 6.** The Ca2+- and/or Pb2+-induced mitochondrial swelling is inhibited by EGTA, CsA, or ruthenium red (RR). Mitochondrial swelling induced from isolated retinal mitochondria exposed to 475 nM Ca2+ and/or 585 µM Pb2+, concentrations that produce a transient stimulation of mitochondrial State 4 respiration and a 50–75% decrease in mitochondrial State 3 respiration (27). Large amplitude mitochondrial swelling was induced by Ca2+ or Pb2+. The Ca2+- and Pb2+-induced increase in mitochondrial swelling was inhibited completely by 1 mM EGTA or 0.5 µM CsA and partially blocked by 0.1 and 0.5 µM ruthenium red. In B, the swelling induced by 585 µM Pb2+ and 475 nM Ca2+ was additive compared with either divalent cation alone, and it was blocked completely by 1 mM EGTA or 0.5 µM CsA and partially blocked by 0.5 µM ruthenium red.

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2. L. He, A. T. Poblenz, C. J. Medrano, and D. A. Fox, unpublished data.
findings that caspase activity is not affected by concentrations of Ca\(^{2+}\) below 100 mM (43).

The processing of procaspase-9, -3, and -7 was examined using Western blot analysis. Following incubation in Ca\(^{2+}\) or Pb\(^{2+}\), the 46-kDa proform of caspase-9 was processed into a 35-kDa fragment (p35) (Fig. 8A, lanes 2 and 3), and the amount of p35 doubled in the presence of both cations (Fig. 8A, lane 4). A quantitatively similar effect was observed for procaspase-3 such that its 32-kDa proform was processed into p17 fragments in retinas incubated in Ca\(^{2+}\) and/or Pb\(^{2+}\) (Fig. 8B). The 35-kDa proform of caspase-7 was detected in all retinas. However, it was not processed into either its p19 or p32 fragments (7) by Ca\(^{2+}\) and/or Pb\(^{2+}\) (data not shown), indicating that this group II caspase was not involved in the Ca\(^{2+}\)- or Pb\(^{2+}\)-triggered rod apoptosis. The effect of Ca\(^{2+}\) and/or Pb\(^{2+}\) on the processing of the death substrates PKC\(\delta\) and PARP also was examined (Fig. 8C and D). PARP is cleaved by group II and III caspases, whereas PKC\(\delta\) is cleaved only by caspase-3 (60). Following incubation in buffers containing Ca\(^{2+}\) and/or Pb\(^{2+}\), intact PKC\(\delta\) (78 kDa) and PARP (116 kDa) were cleaved into their signature 42-(Fig. 8C) and 85-kDa (Fig. 8D) fragments, respectively. The amounts of p42 PKC\(\delta\) and p85 PARP were doubled in retinas incubated with Ca\(^{2+}\) and Pb\(^{2+}\) compared with either divalent cation alone.
and p35 fragments (Fig. 8, lane 2). CsA, however, blocked the formation of both the p17 and p35 fragments (Fig. 8, lane 2) and did not affect the formation of the p35 fragment (Fig. 8, lane 3). CsA, however, blocked the formation of both the p17 and p35 fragments (Fig. 8, E, and F, lane 3). These results clearly demonstrate the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced sequential activation of caspase-9 and caspase-3.

Disruption of Mitochondrial Membrane Integrity Precedes the Activation of Caspases—Retinas were incubated in Ca\(^{2+}\)- and Pb\(^{2+}\)-depleted buffers or with Ca\(^{2+}\) and Pb\(^{2+}\) for 5, 10, or 15 min. Detection and quantification of mitochondrial \(\Delta \Psi\), cytochrome c release, DEVDase activity, and apoptosis were conducted as described under "Experimental Procedures." Quantitative analysis showed that significant mitochondrial depolarization and cytochrome c release occurred during the initial 5 min of incubation and increased further with 10 and 15 min of incubation. DEVDase activity was slightly, but significantly, increased during the first 5–10 min of incubation and then increased markedly by 15 min. The first significant increase in the number of apoptotic rods occurred at 10 min and then it increased dramatically with 15 min of incubation. The arbitrary values represent the mean ± S.E. of three to seven experiments for each treatment condition.

To corroborate the finding that Ca\(^{2+}\) and Pb\(^{2+}\) induced sequential activation of caspase-9 and caspase-3 activity, the processing of these caspases in the presence of 1 nM DEVD-fmk or 10 \(\mu\)M CaA was examined using Western blot analysis. As noted above, incubation in Ca\(^{2+}\) and Pb\(^{2+}\) resulted in the formation of a caspase-9 p35 fragment (Fig. 8E, lane 1) and a caspase-3 p17 fragment (Fig. 8F, lane 1). DEVD-fmk blocked completely the formation of the p17 fragment (Fig. 8F, lane 2) but did not affect the formation of the p35 fragment (Fig. 8E, lane 2). CsA, however, blocked the formation of both the p17 and p35 fragments (Fig. 8, E, and F, lane 3). These results clearly demonstrate the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced sequential activation of caspase-9 and caspase-3.

**DISCUSSION**

The results from this study identified rod photoreceptors as selective targets of Ca\(^{2+}\) and Pb\(^{2+}\) and delineated the biochemical mechanisms and temporal sequence of events in the apoptotic signaling cascade. The rod-selective apoptosis produced by pathophysiologically relevant concentrations of Ca\(^{2+}\) and/or Pb\(^{2+}\) is similar to that observed in a wide variety of human and animal retinal degenerations where Ca\(^{2+}\) overload appears to have a fundamental role (9–15).

The Ca\(^{2+}\)- and Pb\(^{2+}\)-induced Apoptosis Is Rod-selective—Several independent biochemical and morphological measures were used to establish that the cell death produced by Ca\(^{2+}\) and/or Pb\(^{2+}\) was apoptotic, rod-selective, and that the effects of these divalent cations were additive. First, genomic DNA was cleaved into HMW fragments and this result in the occurrence of morphological rod apoptosis. DEVD, Ac-DEVD-fmk; DMQD, Ac-DMQD-fmk; LEHD, Ac-LEHD-fmk.

FIG. 10. Schematic diagram of the cell signaling pathway mediating the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced rod apoptosis. Ca\(^{2+}\) and/or Pb\(^{2+}\) enter the rod photoreceptor outer segment via the cGMP-gated channel and subsequently enter the rod mitochondria through the ruthenium red (RR)-sensitive Ca\(^{2+}\) uniporter and bind to the internal metal (Me\(^{2+}\)) site of the mitochondrial permeability transition pore (PTP). This opens the PTP, which leads to mitochondrial depolarization, swelling, and osmosis-induced rupture of outer mitochondrial membrane. Cytochrome c (cyt c) is released from the mitochondria to cytosol where it interacts with apoptotic protease activating factor-1 (Apaf-1), dATP/dADP, and procaspase-9, and where caspase-9 is presumably activated. Caspase-9 activates procaspase-3. Caspase-3 subsequently activates endonucleases that cleave DNA into HMW fragments and this results in the occurrence of morphological rod apoptosis.

The Ca\(^{2+}\)- and Pb\(^{2+}\)-induced Rod-selectivity—Following incubation in Ca\(^{2+}\) alone or Ca\(^{2+}\) and Pb\(^{2+}\) for 5, 10, or 15 min, the number of rod apoptotic cells was determined as described under "Experimental Procedures." Quantitative analysis showed that significant mitochondrial depolarization and cytochrome c release occurred during the initial 5 min of incubation and increased further with 10 and 15 min of incubation. DEVDase activity was slightly, but significantly, increased during the first 5–10 min of incubation and then increased markedly by 15 min. The first significant increase in the number of apoptotic rods occurred at 10 min and then it increased dramatically with 15 min of incubation. The arbitrary values represent the mean ± S.E. of three to seven experiments for each treatment condition.

The Ca\(^{2+}\)- and Pb\(^{2+}\)-induced apoptosis was rod-selective. The rod selectivity is thought to result from a sustained increase in rod [Pb] and/or [Ca]. It was found in retinas of lead-exposed rats and retinal degeneration mice undergoing rod apoptosis (13, 26, 27). The finding that free Ca\(^{2+}\) and free Pb\(^{2+}\) were selectively accumulated in the photoreceptors is consistent with our observations that the apoptotic cell death was rod-selective. The rod selectivity is thought to result from a sustained increase in rod [Pb] and/or [Ca].
[Ca] compared with cones because the Na\(^+\)/Ca\(^{2+}\) exchanger in the rods is 8–10 times slower than in cones (33). The absence of free Ca\(^{2+}\) and/or Pb\(^{2+}\) in bipolar cells and the corresponding absence of bipolar cell apoptosis in the in vitro retinal preparation, compared with rats exposed to lead during development or adulthood (11, 12), most likely results from the lack of inhibition of bipolar cell cGMP phosphodiesterase by IBMX (34) during the short in vitro incubation period. This is supported by findings that only the rod, but not bipolar, cell cGMP levels were elevated in isolated rat retinas incubated in IBMX (61). These results suggest that the intracellular levels of Ca\(^{2+}\) and Pb\(^{2+}\) produced during in vitro incubation are pathophysiologically relevant and that the preparation is useful for determining the biochemical mechanisms underlying rod-selective death induced by Ca\(^{2+}\) overload and/or Pb\(^{2+}\) neurotoxicity.

The Mitochondrial PTP Is the Target Site of Ca\(^{2+}\) and/or Pb\(^{2+}\) —Our results reveal that mitochondria are the initial target site in rods responsible for mediating the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced apoptotic rod cell death. Both Ca\(^{2+}\) and Pb\(^{2+}\) rapidly entered the rod mitochondria through the Ca\(^{2+}\) uniporter as evidenced by the ruthenium red blockade of divalent cation uptake and the transient stimulation of state 4 respiration (27). Ca\(^{2+}\) and Pb\(^{2+}\) had an additive effect on decreasing \(\Delta \Psi_{\text{m}}\), producing mitochondrial swelling and releasing cytochrome c into the cytosol. These were early and critical events in the rod cell apoptotic process. CsA, but not DEVD-fmk or FK506, completely blocked the Ca\(^{2+}\)- and/or Pb\(^{2+}\)-induced cytochrome c release and markedly reduced the mitochondrial depolarization produced by these divalent cations. The molecular mechanisms of action of Ca\(^{2+}\) and Pb\(^{2+}\) on the rod mitochondrial PTP are not known. There are two classes of PTP pore agonists/antagonists: one modulates the membrane and surface potential of the voltage sensor, and the other modulates cyclophilin-D binding (3). Although Ca\(^{2+}\) and Pb\(^{2+}\) have diverse effects on mitochondria (2, 3, 27, 62), our current results suggest a few unifying, though not mutually exclusive, mechanisms. First, Ca\(^{2+}\) binds directly to the divalent metal (Me\(^{2+}\)) binding site on the matrix side of the mitochondrial PTP and induces its opening. These effects are inhibited by CsA (3, 51) but not by FK506 (37). In addition to CsA, which mediates its effect through cyclophilin-D, several group IIA and VIIA divalent cations competitively inhibit the Ca\(^{2+}\)-induced PTP opening at this Me\(^{2+}\) site. However, the divalent cation-induced inhibition of PTP occurs only when the Me\(^{2+}\)/Ca\(^{2+}\) ratio exceeds 10 (63). The findings that the Pb\(^{2+}\)/Ca\(^{2+}\) ratios in our experiments were 0.001–0.002, that the effects of Ca\(^{2+}\) and Pb\(^{2+}\) on all measures were additive and inhibited by CsA, and that Pb\(^{2+}\) can function as a potent Ca\(^{2+}\) agonist (62, 64) suggest one strong candidate mechanism for PTP opening is the occupation of the internal Me\(^{2+}\) binding site by Ca\(^{2+}\) and Pb\(^{2+}\).

Second, it is possible that Ca\(^{2+}\) and Pb\(^{2+}\) opened the PTP by oxidizing GSH and/or NADH resulting in the oxidation of the sensitive vicinal di thiol present in the “S-site” and “P-site” of the PTP, respectively (3). Based on our findings of no Ca\(^{2+}\)- and/or Pb\(^{2+}\)-induced alterations in rod redox status or ROS production, we conclude that oxidative stress is not the mechanism underlying the proapoptotic action of these divalent cations. This is in contrast to the proapoptotic effects of Hg\(^{2+}\), Mn\(^{2+}\), Cu\(^{2+}\), and methyl mercury that produce ROS (21, 29–31). Finally, it is possible that Pb\(^{2+}\) functions independently of Ca\(^{2+}\) and opens the PTP by cross-linking the di thiol in the S-site like the trivalent arsenite (1–3). However, preliminary experiments in isolated mitochondria using N-ethylmaleimide, a well-known blocker at both the S-sites and P-sites (3), showed no protective effect against Pb\(^{2+}\)- or Ca\(^{2+}\)-induced mitochondrial swelling. Altogether, these results indicate that both Ca\(^{2+}\) and Pb\(^{2+}\) bind to the matrix Me\(^{2+}\) binding site of the mitochondrial PTP and open the PTP.

Results from several different experimental systems suggest that cytochrome c is released from mitochondria either by a PTP-dependent mechanism (1) or by one of two PTP-independent mechanisms (4, 65). Our JC-1 staining and cytochrome c release studies are consistent with the PTP-dependent mechanism. Therefore, light scattering (mitochondrial swelling) experiments were conducted in isolated retinal mitochondria incubated in the presence of respiratory substrates, Mg\(^{2+}\) and P,

Ca\(^{2+}\) and/or Pb\(^{2+}\)-induced rapid and large amplitude mitochondrial swelling that was blocked by EGTA, ruthenium red, and CsA, indicating that the swelling was because of the opening of the mitochondrial PTP. In contrast, recent data showed that isolated rat brain mitochondria only exhibited Ca\(^{2+}\)-induced swelling and permeability transition in the absence, but not presence, of Mg\(^{2+}\) and ATP (58). The reason for these tissue differences is unknown especially because 1–3 log units lower free [Ca\(^{2+}\)] or CaCl\(_2\)/mg of protein was used in the retinal mitochondrial preparation (27) compared with the brain mitochondrial preparation (58).

Caspase-9 Is the Initiator Caspase and Caspase-3 Is the Executioner Caspase—Results from our pharmacological experiments establish that caspase-9 is the initiator caspase, whereas caspase-3 is the executioner caspase in Ca\(^{2+}\)- and or Pb\(^{2+}\)-induced rod apoptosis. Specifically, the caspase-3 selective inhibitor DMQD-CHO inhibited DEVDase activity but did not inhibit LEHDase activity, whereas the caspase-9 selective inhibitor LEHD-fmk inhibited the activity of all three tetrapeptide-pNA substrates indicating that caspase-9 was activated upstream of caspase-3. Interestingly, incubation with 1 mM DEVD-fmk did not inhibit the Ca\(^{2+}\)- and Pb\(^{2+}\)-induced LEHDase activity but it totally inhibited DEVDase activity, HMW DNA fragmentation, and nuclear chromatin condensation. This shows that activation of caspase-9 alone was not enough to cause apoptosis, whereas activation of caspase-3 was required for the cleavage of death substrates, activation of endonucleases to cleave DNA into HMW fragments, and morphological apoptosis. In vitro experiments have shown that caspase-9 activates procaspase-3 (6, 7, 66) and that this produces a feedback amplification loop that further activates procaspase-9 (6). In retinas incubated with Ca\(^{2+}\) and Pb\(^{2+}\), CsA blocked completely the activation of procaspase-9 and procaspase-3, whereas neither DMQD-CHO nor DEVD-fmk decreased caspase-9 activity or cleavage, respectively. These results conclusively demonstrate the Ca\(^{2+}\) and Pb\(^{2+}\) induced sequential activation of caspase-9 and caspase-3. In addition, the presence of similar amounts of the caspase-9 p35 fragment in retinas incubated with Ca\(^{2+}\) and Pb\(^{2+}\) in the absence or presence of DEVD-fmk suggests that there was no or limited activation of caspase-9 by caspase-3. Finally, there was no evidence of Ca\(^{2+}\)- and Pb\(^{2+}\)-induced caspase-8 activity or processing of caspase-7. These findings are consistent with recent results showing that caspase-9 and caspase-3 are activated sequentially in chemical and cytochrome c-induced apoptosis (7, 66), whereas caspase-8 is activated in receptor-mediated apoptosis (7).

In summary, we have demonstrated that elevation of intracellular Ca\(^{2+}\) and/or Pb\(^{2+}\) in rat rod photoreceptors produces rod-selective apoptosis and that the effects of Ca\(^{2+}\) and Pb\(^{2+}\) are additive. Based on the results, we have proposed a scheme for the Ca\(^{2+}\) - and Pb\(^{2+}\)-induced apoptotic process in rods (Fig. 10). A similar apoptotic cell-signaling cascade may underlie

\(^3\)L. He, A. T. Poblenz, C. J. Medrano, and D. A. Fox, unpublished observations.
rod-selective or neuronal apoptosis observed in humans and animals with different retinal and neural degenerations resulting from Ca\(^{2+}\) overload and/or Pb\(^{2+}\) neurotoxicity. The mechanistic knowledge gained from the delineation of the apoptotic cascade in rod photoreceptors will be useful for the development of specific neuroprotective strategies.

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