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Matrix Regulation of Skeletal Cell Apoptosis III: Mechanism of Ion Pair-Induced Apoptosis

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Abstract Our previous work has demonstrated that while the Ca^{2+} and Pi ions acting in concert function as a potent osteoblast apoptogen, the underlying mechanisms by which it activates cell death is not known. We hypothesize that the ion pair causes release of Ca^{2+} from intracellular stores ($[Ca^{2+}]i$); the increase in intracellular calcium prompts the mitochondria to uptake more calcium. This accumulation of calcium eventually results in the loss of mitochondrial membrane potential (MMP) and, subsequently, apoptosis. To test this hypothesis, we evaluated apoptosome formation in MC3T3-E1 osteoblast-like cells treated with the ion pair. Western blot analysis indicated migration of cytochrome-*c* and Smac/DIABLO from mitochondria to the cytoplasm. Inhibition of either the electron transfer chain (with antimycin a and rotenone), or the activation of a MMP transition (with bongkrekic acid) inhibited apoptosis in a dose-dependent manner. Pre-treating osteoblasts with ruthenium red, a Ca^{2+} uniporter inhibitor of both mitochondria and the endoplasmic reticulum (ER), also completely abolished Ca^{2+}·Pi-induced apoptosis. Moreover, we showed that an increase in $[Ca^{2+}]i$ preceded the increase in MMP over the first 45 min of treatment; a mitochondrial membrane permeability transition was evident at 75 min. To determine the role of ER, Ca^{2+} stores in the generation of the apoptotic signal by the ion pair, cells were treated with several inhibitors. Apoptosis was inhibited when cells were treated with dantrolene, an inhibitor of ER ryanodine receptors, and 2-aminodiphenylborate, an IP3 Ca^{2+} channel inhibitor, but not cyclopiazonic acid, an ER Ca^{2}-ATPase inhibitor. Together, these data demonstrate that Ca^{2+} Pi-induced osteoblast apoptosis is characterized by the generation of an apoptosome and that Ca^{2+} release from ER stores may promote ion pair-dependent cell death. J. Cell. Biochem. 100: 703–715, 2007.

Key words: osteoblast; calcium; phosphate; apoptosis; endoplasmic reticulum; mitochondria

Removal of damaged or non-functional cells from a tissue can be regarded as a physiological response to environmental cues. In both cartilage and bone, the induction of apoptosis maintains cell number during tissue remodeling, turnover, and growth. Initiation and completion of apoptosis is dependent on recognition and decoding of incoming death signals and the encoding and activation of executioner enzyme systems [Hengartner, 2000]. These proteins dismantle the organized structure of the cell by hydrolyzing membrane pumps, depolymerizing cytoskeletal elements, and fragmenting critical macromolecules [Coleman and Olson, 2002; Breckenridge and Xue, 2004]. During this process, changes in membrane structure generate signals that result in scavenger cells removing skeletal cell debris [Fadok et al., 1992; Koopman et al., 1994].

The mechanism by which skeletal cells recognize and transduce death signals has received cursory study. In vivo, bone cell apoptosis increases with estrogen withdrawal [Tomkinson et al., 1997] and glucocorticoid treatment. In culture, osteoblasts are sensitive to a number of apoptotic stimuli that include undue mechanical strain [Weyts et al., 2003], radiation [Szymczyk et al., 2004], the presence of cancer cells [Mastro et al., 2004], and at resorption sites, extracellular matrix fragments [Perlot et al., 2002]. We have explored the possibility that at these resorption sites, pH gradients solubilize the mineral phase of bone and generate a local elevation in both Ca^{2+} and Pi.

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We have shown that a small rise in the environmental Ca^{2+} concentration promotes Pi-induced apoptosis [Meleti et al., 2000; Adams et al., 2001]. This observation raises a number of fundamental question concerning the mechanism of apoptosis, in particular, the role of Ca^{2+} in the death response.

The objectives of the current investigation are twofold. First, is to assess the role of Ca^{2+} in the apoptotic process. More specifically, we explore the mechanism by which the Ca^{2+} and Pi ion pair causes a loss of mitochondrial function and activation of bone cell apoptosis. We test the hypothesis that release of Ca^{2+} from the endoplasmic reticulum triggers osteoblast death. Second, we examine the notion that activation of the apoptotic pathway requires the formation of an apoptosome. The apoptosome enhances the recruitment of executioner caspases, enzymes that are required for the activation of bone cell death.

METHODS

Design of the Study

The goal of this investigation was to explore the mechanism by which the Ca^{2+}·Pi ion pair induces osteoblast-like cell death. The first series of experiments were designed to test the hypothesis that the intrinsic apoptotic pathway is activated by the ion pair. For this study, we first determined whether ion pair treatment would cause mitochondrial cytochrome-*c* to redistribute to the cytosol and serve as a molecular framework for apoptosome formation. Next, we determined whether Smac/DIABLO accompanies the re-distribution of cytochrome-*c*. To verify apoptosome formation, and the involvement of mitochondrial proteins in this process, we treated cells with ruthenium red, an inhibitor of the mitochondrial membrane permeability transition (MMPT), and evaluated Ca^{2+}·Pi-induced apoptosis. Furthermore, we evaluated the effect of ion pair on mitochondrial complex I and III and the adenine nucleotide translocase using a series of site-specific inhibitors. As our previous work had suggested that an elevation of intracellular Ca^{2+} is correlated with the loss of mitochondrial membrane potential (MMP), we examined the relationship between these two factors during the initial activation of apoptosis by the Ca^{2+} Pi ion pair using confocal microscopy. Since much of the cell Ca^{2+} is stored in the endoplasmic reticulum, we then tested the hypothesis that apoptosis was due to release of the cation from this organelle. For this study, osteoblast-like cells were treated with carbachol, an activator of intracellular Ca^{2+} release, and cell death was determined by the MTT assay. To further probe the role of Ca^{2+} in the death process, we used inhibitors of the Ca^{2+} ATPase and ryanodine and inositol 1,4,5-trisphosphate (IP3) receptors. In addition, cells were treated with an inhibitor of IP-3 formation. The impact of each of these blockers on the ion pair death response was then determined. Finally, to determine if ion pair killing activated the apoptotic form of death, the treated cells were incubated with a fluorescent substrate, and caspase-3 activity was evaluated.

Cell Culture

We used MC3T3-E1 (subclone 4), a cell line that differentiates into mature osteoblast-like cell for all of the experiments described herein [McCauley et al., 1995]. Cells were grown to confluence in Dulbecco's Minimum Essential Medium (DMEM)(Gibco, Grand Island, NY) supplemented with 10% fetal calf serum, 5 mM L-glutamine, and antibiotics in 5% CO2–95% air. The medium was changed every 48 h. After 7 days, the cells were released from the tissue culture plastic with 2.5% trypsin (Gibco) in Hank's Balanced Salt Solution and sub-cultured in 24-well plates at a density of 4×10^3 cells/well in the presence of agents mentioned below.

Induction of Ion Pair-Dependent Apoptosis

Osteoblast viability was assessed as a function of the medium Ca^{2+}·Pi concentration (3 mM Pi and 1.9 mM Ca^{2+} or 3 mM Pi and 2.9 mM Ca^{2+}) in the presence of rotenone (1–10 μM) antimycin A (1–15 μM), Ruthenium Red (1–15 μM), 2-aminodiphenylborate (50–200 μM), cyclopiazonic acid (1–30 μM), or dantrolene (1–30 μM). These inhibitors were contained in DMEM supplemented with the raised concentration of Pi. After 1–2 h, the Ca^{2+} concentration was raised to 1.9 or 2.9 mM. For carbachol (100–500 μM), cells were treated with the inhibitor in DMEM in the presence of 3–7-mm Pi. In all cases, cell death was determined by the MTT assay. All inhibitors were purchased from Sigma Chemicals (St. Louis, MO).

Detection of Cell Death

This assay is based on the ability of mitochondria in live cells to oxidize thiazolyl blue (MTT), a tetrazolium salt (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-terazolium bromide), to an insoluble blue formazan product. Cells were treated with inhibitory agents, followed by the ion pair, washed, and then incubated with the MTT solution at 37°C for 2.5 h. The reagent was removed and 200 μl of 0.04 M HCl in isopropanol was added to each well. The optical density was read at 590 nm in an ELISA plate reader [McGahon et al., 1995]. Since the generation of the blue product is proportional to the cellular dehydrogenase activity, the absorbance change provides a direct measurement of the number of viable cells.

Evaluation of Cytochrome-*c* and Smac/DIABLO by Western Blot

Mitochondrial and cytoplasmic fractions were separated using the following protocol. Briefly, apoptosis was induced in osteoblasts using the Ca^{2+} and Pi ion pair. Untreated osteoblasts were used as controls. Cells were collected by centrifugation at 600*g* for 5 min at 4°C, washed with PBS, and recentrifuged. The supernatant was removed and the cells resuspended in a cytosol extraction buffer (Cytochrome-*c* Assay Kit, BioVision, Inc., Mountain View, CA) on ice for 10 min. The cells were homogenized in an ice-cold dounce tissue grinder. The homogenate was transferred to a new tube and centrifuged at 700*g* for 10 min at 4°C. The supernatant was collected in a new tube and centrifuged at 10,000*g* for 30 min at 4°C. The supernatant was collected at the cytosolic fraction, while the pellet was resuspended in a mitochondrial extraction buffer mix containing DTT and protease inhibitors (BioVision, Inc), incubated on ice for 10 min, vortexed for 10 s and was saved as the mitochondrial fraction. The fractions were then run probed by Western blot analysis for using antibodies for cytochrome-*c* and Smac/DIABLO.

Analysis of Intracellular Events Using Confocal Microscopy

The treated adherent cells were treated with Mitotracker Red (0.5 μM), a voltage sensitive mitochondrial dye, or Calcium Green-AM (5 μM) (Molecular Probes), a cell-permeable green fluorescent dye that, upon binding with calcium ions, exhibits an increase in fluorescence emission intensity. In some studies, to assess whether there was induction of apoptosis, cells were incubated with PhiPhiLux-G1D2 (10 μM; OncoImmunin, Inc., Gaithersburg, MD), a fluorescent caspases-3 substrate, for 1 h at 37°C. Cells were washed to remove excess dye and the change in fluorescence was monitored using an Olympus Fluoview inverted confocal microscope (Olympus, Melville, NY). A long-working distance lens fitted with a specialized cap was used to permit evaluation of the cells through the plastic dish. The plane of maximum fluorescence was determined and the photomultiplier tube voltage set at that point for the control wells. To permit quantification, the photomultiplier tube voltage was established for each fluorophore. Images were recorded from three fields for each time point. Individual cellular fluorescence was quantified using the ImagePro software.

Quantitative Analysis

Data from MTT assays were normalized to control well values and expressed as a percentage of the control. The values were analyzed using a one-way variance test (ANOVA), examining the effect of treatment of each well on cell viability. All experiments were repeated three to five times. Significance was assessed when $P < 0.05$. Fluorescence data was analyzed using a Kruskal–Wallis Analysis of Variance on Ranks Test with Dunn's method of multiple comparisons.

RESULTS

Ca^{2+}·Pi Ion Pair Treatment Causes Redistribution of Cytochrome-*c* and Smac/DIABLO

To test the hypothesis that cell death was mediated through the intrinsic pathway, we analyzed, by Western blot, the movement of both cytochrome-*c* and Smac/DIABLO from the mitochondrion into the cytoplasm (Fig. 1). In untreated (control) osteoblast-like cells, Smac/DIABLO is present predominantly in the mitochondrial fraction (Fig. 1A). When treated with the Ca^{2+}·Pi ion pair, Smac/DIABLO is localized exclusively in the cytoplasm. Similarly, in untreated osteoblasts, cytochrome-*c* is present in the mitochondrial fraction (Fig. 1B). Induction of the death response by the ion pair results in the re-location of the protein to the cytoplasmic fraction; Western blot

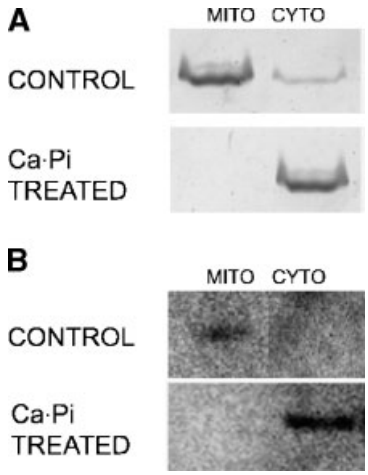

Fig. 1. Western blot analysis of cytochrome-*c* and Smac/DIABLO in mitochondrial and cytoplasmic fractions of cells challenged with the ion pair. In untreated (control) osteoblast-like cells, Smac/DIABLO is present predominantly in the mitochondrial (mito) fraction (**A**). When treated with the Ca^{2+}·Pi Pi ion pair, Smac/DIABLO is seen exclusively in the cytoplasm (cyto). Similarly, in untreated (control) osteoblasts, cytochrome-*c* (cyto-*c*) is present exclusively in the mitochondrial fraction (**B**). When treated with the ion pair, cytochrome-*c* is localized to the cytoplasmic fraction.

analysis indicates that cytochrome-*c* is absent from the mitochondrial fraction. These results lend strength to the concept that ion pair treatment produces an MMPT and formation of an apoptosome.

To further evaluate the importance of mitochondrial function in the death process, we treated cells with inhibitors of complex I (rotenone) and III (antimycin A). We reasoned that in the absence of a functioning respiratory chain, no outward transport of cytochrome-*c* should occur [Chauvin et al., 2001; Dairaku et al., 2004]. Figure 2A shows that in the absence of rotenone, about 70% of cells are killed by the ion pair. As might be expected, rotenone itself killed osteoblasts, however, in the presence of rotenone, ion pair-mediated cell death is proportionally reduced. Thus, at a killing ion pair concentration (3 mM Pi and 2.9 mM Ca^{2+}), the proportion of viable cells has approached the level of those killed by rotenone alone.

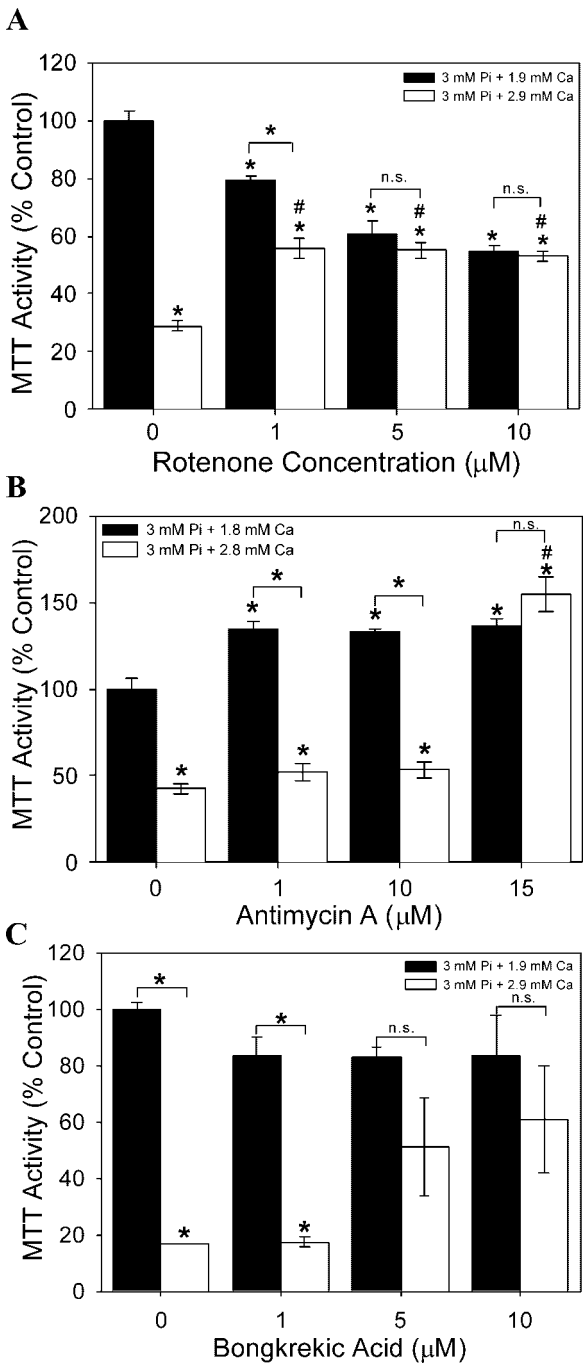

Fig. 2. Effect of inhibitors of mitochondrial function on sensitivity of osteoblast-like cells to Ca^{2+}·Pi ion pair-induced apoptosis. MC3T3-E1 cells were pre-treated with inhibitors of mitochondrial complex I (rotenone; **A**); mitochondrial complex III (antimycin A; **B**); and an inhibitors of the adenine nucleotide translocator (bongkrekic acid; **C**). The cells were then challenged with 3 mM Pi and 1.9 mM Ca^{2+} and 3 mM Pi and 2.9 mM Ca^{2+}. Cell death was measured using the MTT assay. Values shown are mean ± SEM. A: Increasing concentration of rotenone markedly affects osteoblast viability. In the presence of 1 μM rotenone, there is a significant increase in live cells; at 5 and 10 μM, there is no significant difference between ion pair-treated and non-treated cells. B: At low concentrations (1 and 10 μM), antimycin does not protect osteoblasts from the ion pair challenge. At a concentration of 15 μM, antimycin completely inhibits cell death. **C**: As the bongkrekate concentration is raised, there is progressive protection against ion pair-induced cell death.

In contrast to rotenone, when challenged with a low ionic pair concentration, antimycin A causes an increase in MTT signal, probably due to an overall increase in dehydrogenase activity. Thus, the MTT activity is significantly higher for cells treated with 1 to 15 μM antimycin A than with the ionic pair alone. Nevertheless, when osteoblast-like cells are pre-treated with 15 μM antimycin A (Fig. 2B), there is complete inhibition of apoptogen-induced cell death. To assess whether a transition pore is created by ion pair treatment, an inhibitor of the adenine nucleotide translocator was used. Pre-treatment of osteoblast-like cells with 5–10 μM bongkrekic acid (Fig. 2C), maintained the viability of the osteoblast-like treated with 3 mM Pi and 1.9 mM Ca^{2+}. Moreover, at the highest ion pair concentration (3 mM Pi and 2.9 mM Ca^{2+}), there appeared to be protection from the apoptogen, as no significant difference was seen between the two concentrations of ion pair for either 5 or 10 μM bongkrekic acid. Results of these studies and those with rotenone and antimycin reinforce the observation that the ion pair disrupted mitochondrial function and the consequential release of cytochrome-*c* mediates cell death.

Since the proton motive force across the mitochondrial membrane serves to drive Ca^{2+} into the mitochondria and because we had previous identified increases in intracellular Ca^{2+} as correlated with loss of the MMP, we tested the hypothesis that the uptake of that cation initiated the MMPT. To inhibit Ca^{2+} influx, we treated ion pair treated cells with ruthenium red, a non-competitive inhibitor of the mitochondrial Ca^{2+} uniporter [Kruman and Mattson, 1999; Gunter et al., 2000b]. Figure 3 shows that while ruthenium red caused a small increase in the MTT signal, at both 10 and 15 μM, it inhibited ion pair-induced apoptosis.

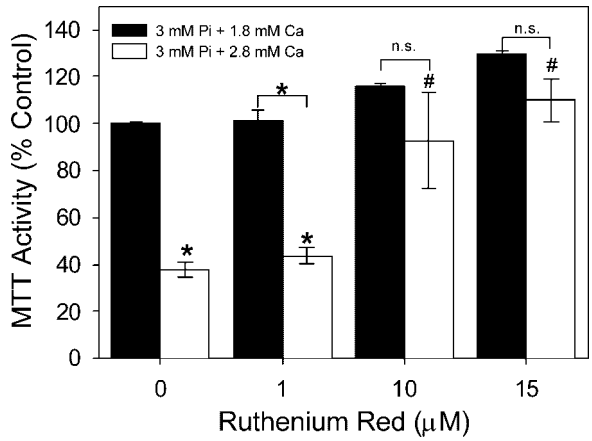

Fig. 3. Effect of ruthenium red on ion pair-mediated osteoblast death. Osteoblast like cells were pre-treated with 1–15 μM ruthenium red, a non-competitive inhibitor of the mitochondrial Ca^{2+} uniporter, in the presence of 3 mM Pi and 1.9 mM Ca^{2+} and 3 mM Pi and 2.9 mM Ca^{2+}. Cell death was measured using the MTT assay. As the inhibitor concentration is raised, there is progressive protection against ion pair-induced cell death. Values shown are mean ± SEM.

Changes in Mitochondrial Membrane Potential Are Coincident With an Increase in Intracellular Ca^{2+}

To test the hypothesis that loss of the MMP was closely linked to levels of intracellular calcium, osteoblast-like cells were treated with the Ca^{2+}·Pi ion pair for 0–90 min. Following staining with MitoTracker Red or Calcium Green-AM, fluorescence was quantified. The changes in each fluorochrome every 15 min are shown in Figure 4A–G. While both Mitrotracker Red and Calcium Green-AM fluorescence are initially low (Fig. 4A), they progressively increase and are maximal at 30 and 45 min, respectively (Fig. 4B–D). At 60 min (Fig. 4E), Mitotracker Red fluorescence returns to pre-treatment levels, while Calcium Green-AM fluorescence remains elevated. At both 75 and 90 min, Mitotracker Red fluorescence decreases precipitously, while the Calcium Green-AM fluorescence returns to basal levels (Fig. 4F,G). Quantification of these images is shown in Figure 4H. A significant increase in Calcium Green-AM signal is seen at 15, 30, and 45 min following ion pair treatment. No significant changes from basal levels are seen at the three later time points. While there is an increasing trend in the MitoTracker Red signal at both 15 and 30 min, these differences are not statistically significant. However, there is a significant increase in MitoTracker Red fluorescence at 45 min. This is followed by a significant decrease in fluorescence at both 75 and 90 min. Of interest is the observation that, with the exception of the 45 min time point, there is a significant correlation between the intracellular MitoTracker Red and Calcium Green-AM fluorescence at all treatment times (Fig. 4I,J present representative data for the 30 and 45 min time point, respectively). These results suggest a causal relationship between intracellular Ca^{2+} accumulation and the MMP.

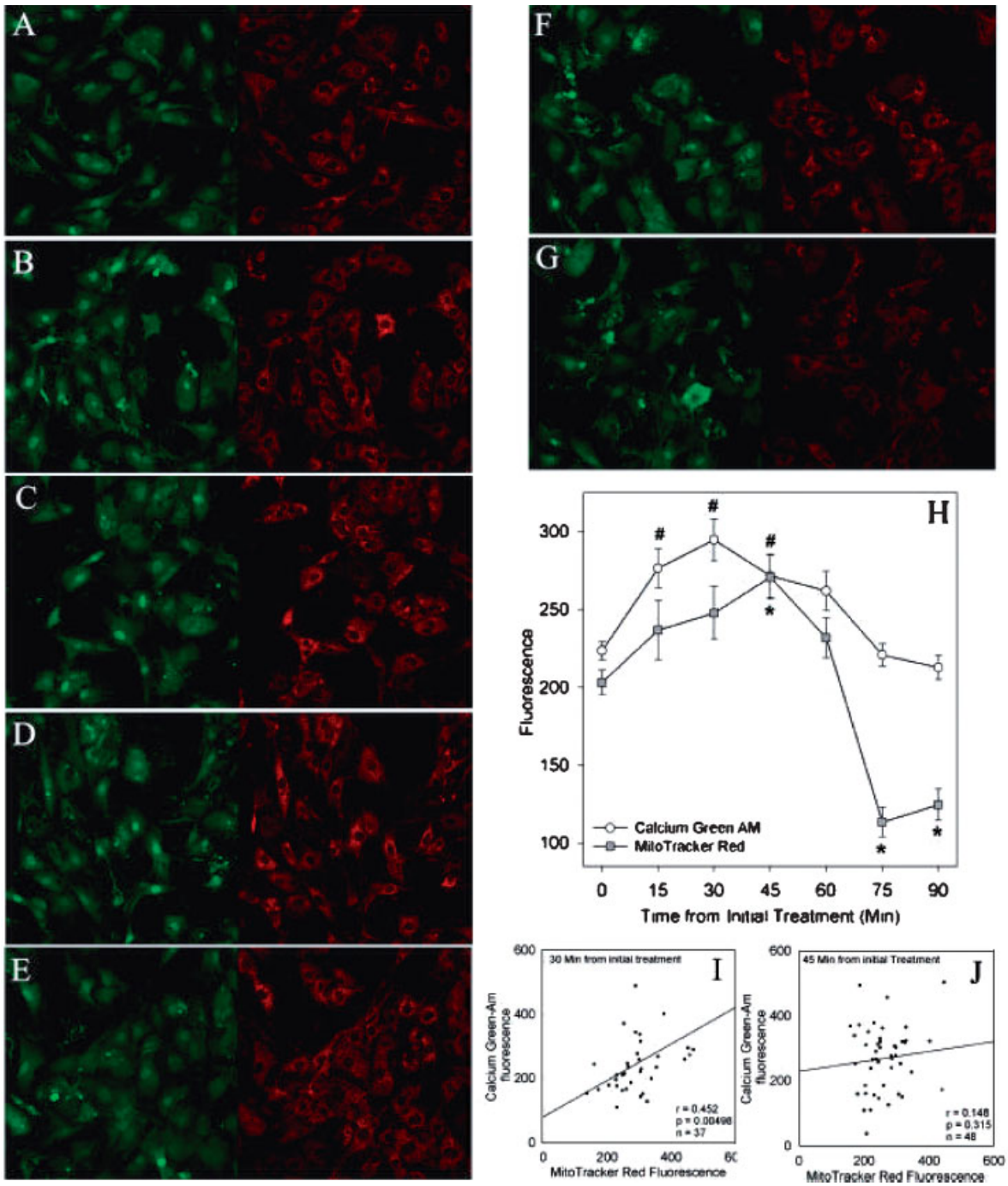

Fig. 4. Changes in MMP and intracellular Ca^{2+} levels in osteoblasts treated with Ca^{2+} Pi ion pair Changes in the MMP and ionized Ca^{2+} levels of MC3T3-E1 cells treated with 3 mM Pi and 2.9 mM Ca^{2+} were monitored using Mitotracker Red (500 nM)(red images) or Calcium Green-AM (5 mM) (green images) and laser scanning confocal microscopy. Cells were analyzed at 0 (**A**), 15 (**B**), 30 (**C**), 45 (**D**), 60 (**E**), 75 (**F**), and 90 min (**G**). Initial photomultiplier tube voltages were set for controls and used for subsequent images. For each time period, three images were collected. Time-dependent changes in individual cellular fluorescence yield for both probes was determined by analysis with ImagePro Software and plotted as a function of time (**H**). Note the increase in Calcium Green-AM at 15, 30, and 45 min ($^{\#}P < 0.05$ in comparison to 0 time point controls). There was also a significant increase in MitoTracker Red fluorescence at 45 min and the significant decrease in fluorescence at 75 and 90 min ($^{*}P < 0.05$ in comparison to 0 time point controls). For each time point, individual cellular MitoTracker Red and Calcium Green-AM fluorescences were subjected to Pearson's correlation analysis and plotted against each other (representative data for the 30 min (**I**) and 45 min (**J**) time points are presented). The 30-min time point is representative of the other time points as each showed a statistically significant correlation. Note, however, that at 45 min, there is no correlation between the MMP and the concentration of intracellular calcium. Mag = 400×.

Release of Intracellular Ca^{2+} Enhances Pi-Dependent Osteoblast Apoptosis

To test the hypothesis that the ion pair-induced apoptosis is mediated by an increase in intracellular Ca^{2+}, cells were treated with carbachol in the presence of increasing concentrations of Pi (extracellular Ca^{2+} concentration maintained constant at 1.9 mM). Figure 5 shows that when osteoblast-like cells are treated with carbachol alone, there is an increase in cell death. Thus, at 500 μM Carbachol, there is a significant proportion of cell killing. However, when the Pi concentration is raised to 5 mM, an elevation in the medium carbachol concentration exacerbates cell death. Since carbachol releases Ca^{2+} from intracellular stores, it was concluded that the rise in Ca^{2+} level promotes Pi-dependent osteoblast apoptosis.

Apoptosis Is Modulated Through the Release of Ca^{2+} From the Endoplasmic Reticulum

Three different types of ER Ca^{2+} channel inhibitors were used to block apoptosis: cyclopiazonic acid (CPA), a potent inhibitor of the endoplasmic reticulum Ca^{2+} ATPase; dantrolene, an inhibitor of the ryanodine receptor Ca^{2+} channel; and 2-aminodiphenylborate, an IP3 Ca^{2+} channel inhibitor. While no concentration of CPA exerts a significant effect on ion pair-induced apoptosis (Fig. 6A), higher doses of CPA (10–30 μM) cause osteoblast cell death. In contrast, dantrolene has a significant effect on the induction of apoptosis by the ion pair (Fig. 6B). Thus, 10 μM dantrolene provides 80% protection from ion pair-induced apoptosis, while 30 μM completely inhibits killing. Similarly, pre-treatment with 2-aminodiphenylborate causes a significant decrease in the number of cells killed by the ion pair (Fig. 6C). These results clearly indicate that Ca^{2+} release from both the ryanodine and IP3 channels is a critical step in the activation of cell death by the Ca^{2+}·Pi ion pair.

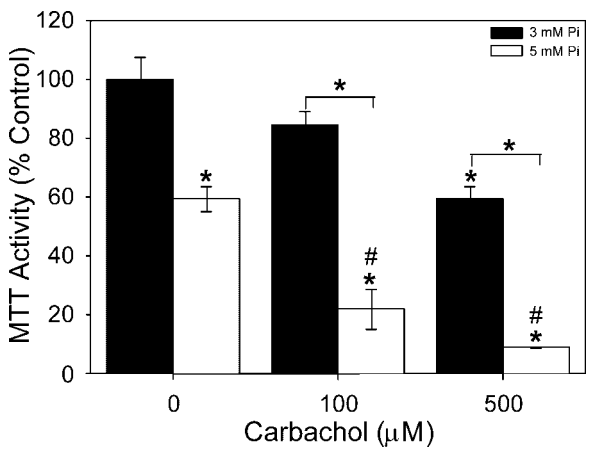

Fig. 5. Effect of carbachol on Pi-mediated cell death. MC3T3-E1 osteoblast-like cells were pre-treated with 100 and 500 μM carbachol in the presence of 3 and 5 mM Pi. After 24 h, cell death was measured using the MTT assay. Carbachol itself causes a significant increase in cell death. However, when challenged with 5 mM Pi, in the presence of carbachol, there is a considerable increase in the proportion of dead cells. Values shown are mean ± SEM.

Evidence That Ion Pair-Mediated Cell Death Is Through the Induction of Apoptosis

While use of the MTT assay provides a quantitative measure of cell death, it does not provide information on the mechanism of killing. To ascertain if the agents used above are modulating the apoptosis, ion pair-treated cells were incubated with the fluorescent caspase-3 substrate, Phiphilux G1D2. Immediately following treatment with the phiphilux, osteoblast-like cells present low levels of fluorescence (Fig. 7A). In the presence of the Ca^{2+}·Pi ion pair, there is an increase in substrate fluorescence (compare Fig. 7A,B). Phiphilux fluorescence in the presence of antimycin A (Fig. 7C), ruthenium red (Fig. 7D), 2-ADB (Fig. 7E), or dantrolene (Fig. 7F) is similar to that of the control (Fig. 7A). These results confirm that the ion pair kills through apoptosis and that the agents listed above do not activate the apoptotic response.

PI Hydrolysis Is Activated During Ion Pair-Induced Apoptosis

Finally, we examined the importance of the calcium receptor in the process of ion pair-induced apoptosis. We pre-treated cells with neomycin, an agent that can inhibit IP3 binding to the endoplasmic receptor. Figure 8 shows that at concentrations above 200 μM, neomycin caused a significant decrease in ion pair-induced apoptosis.

DISCUSSION

In earlier publications, we showed that chondrocytes, primary osteoblasts, and an osteoblast cell line was sensitive to small changes in the extracellular concentration of Ca^{2+} and Pi and that in a 2–24 h period, there was chemical and histochemical evidence of cell

death [Mansfield et al., 2001, 2003; Adams et al., 2001; Meleti et al., 2000]. In those studies, we related the activity of the ion pair to both the functional state of the tissue and the maturational status of the dying cells. How these ions mediated apoptosis was unclear, although it was noted that there were changes in mitochondrial function, possibly leading to development of an MMPT. The major objective of this investigation was to explore this observation in more detail and determine the mechanism by which the ion pair can induce osteoblast apoptosis. Initially, we focused on mitochondrial function and apoptosome formation. We showed that release of Ca^{2+} from intracellular stores preceded events mediated by the mitochondrion. The transient increase in intracellular Ca^{2+} was followed by a change in membrane potential that was associated with release of Smac/DIABLO and cytochrome-*c* from the mitochondrion. Since Ca^{2+} is stored in the endoplasmic reticulum and can interact with the mitochondrion, we evaluated the role of this organelle in controlling ion flux and osteoblast apoptosis. Studies with inhibitors revealed that protection of mitochondria from Ca^{2+} prevented cell death and that both the ryanodine receptor and IP3 channels were implicated in ion pair-induced apoptosis. From a functional viewpoint, the study highlighted an as yet unexplored role for the osteoblast endoplasmic reticulum in mediating Ca^{2+} flux, and thereby regulating the life history of the bone-forming cell.

There are two possible ways in which the ion pair might modulate bone cell death. The ion pair could trigger the extrinsic pathway, which involves the activation of death receptors on the cell membrane; or the intrinsic pathway, which involves mitochondrial dysfunction and the subsequent release of apoptotic factors into the cytosol. Since we had prior evidence that the ion pair generated an MMPT [Adams et al., 2001], we focused the study on the intrinsic pathway. Our previous publication demonstrated that the observed loss of MMP occurred in parallel with an increase in intracellular Ca^{2+} [Adams et al.,

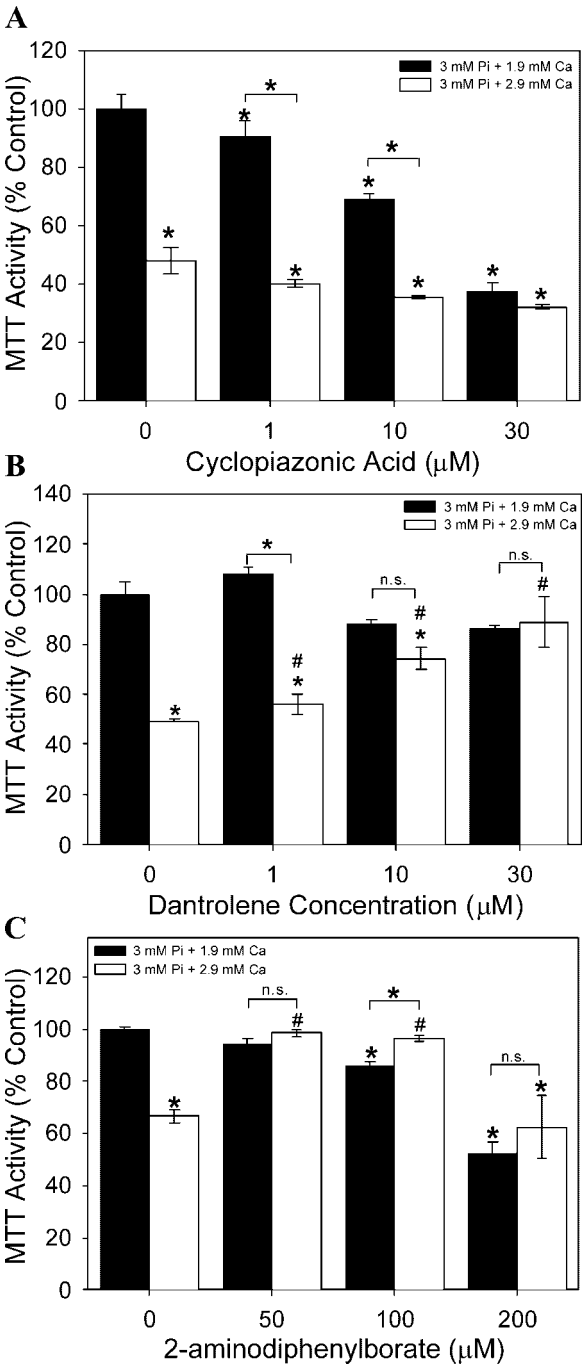

Fig. 6. Effect of Cyclopiazonic acid, dantrolene, and 2-aminoethoxyphenyl borate on Ca^{2+}·Pi ion pair-induced osteoblast death. MC3T3-E1 cells were pre-treated with (**A**) cyclopiazonic acid (1-30 μM), (**B**) dantrolene (1–30 μM), and (**C**) 2-aminoethoxyphenyl borate (50–200 μM.) The cells were then challenged with 3 mM Pi and 1.9 mM Ca^{2+} and 3 mM Pi and 2.9 mM Ca^{2+}. Cell death was measured using the MTT assay. At a low ion pair concentration, cyclopiazonic acid caused an enhancement of cell death on its own; at the higher ion pair concentration, cyclopiazonic acid show no inhibition of ion pair-mediated cell death (A). Dantrolene, on the other hand, displayed a significant effect on the induction of apoptosis. Thus, at a concentration of 10 μM, there was 80% protection from ion pair-induced apoptosis, while at 30 μM, there was complete inhibition of cell death. Similarly, pre-treatment with 2-aminodiphenylborate, an IP3 Ca^{2+} channels inhibitor caused a significant decrease in the number of cells killed by the ion pair. Values shown are mean ± SEM.

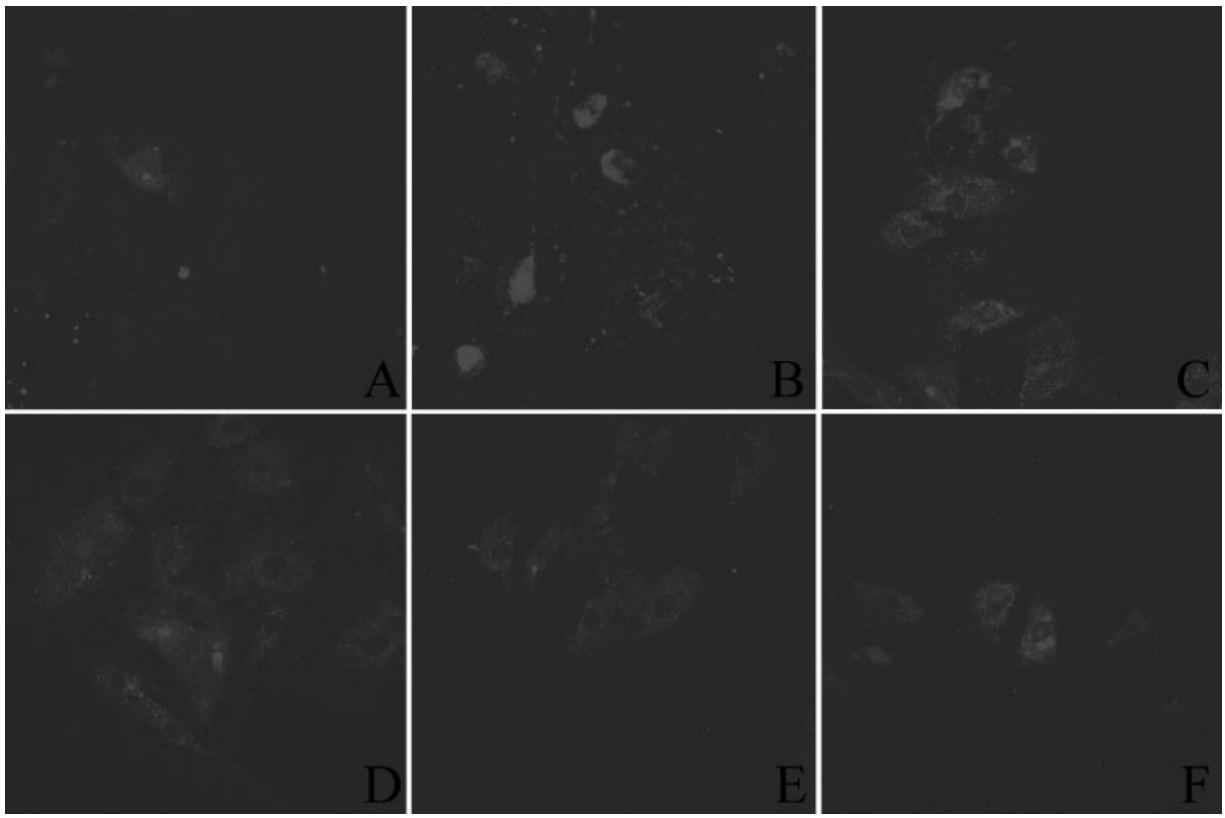

Fig. 7. Evaluation of cell killing by Ca^{2+}·Pi ion pair (**A**, **B**) in the presence of antimycin A (**C**), ruthenium red (**D**), 2-aminoethoxyphenyl borate (**E**), and dantrolene (**F**). To confirm that osteoblast-like cells underwent apoptosis, the cells were treated with the fluorescent caspase-3 substrate, Phiphilux G1D2. When initially treated with the fluorescent substrate in the presence of a non-killing concentration of the ion pair (3 mM Pi + 1.9 mM Ca^{2+}), osteoblast-like cells present low levels of fluorescence (A). In the presence of the ion pair (3 mM Pi + 2.9 mM Ca^{2+}) (B), there is an increase in substrate fluorescence as caspase-3 is activated (compare A with B). In the presence of antimycin A (C), ruthenium red (D), 2-ADB (E), or dantrolene (F), the low level of fluorescence is similar to that of the control (A). Mag = 600×.

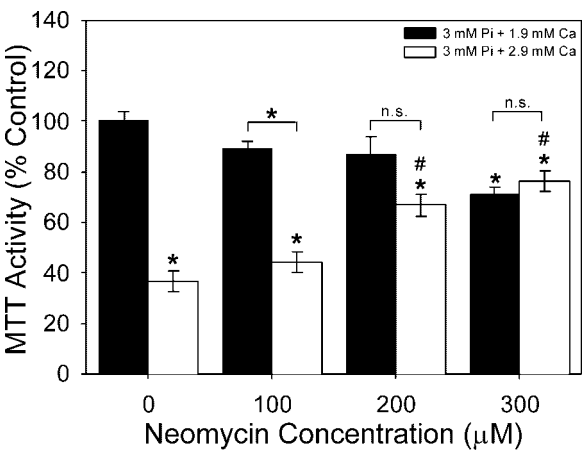

Fig. 8. Effect of Neomycin on Ca^{2+}·Pi ion pair-induced osteoblast death. Osteoblast-like cells were treated with neomycin, an agent that can inhibit inositol 1,4,5-triphosphate (IP3) binding to the ER receptor. The cells were then challenged with 3 mM Pi and 1.9 mM Ca^{2+} and 3 mM Pi and 2.9 mM Ca^{2+}. Cell death was measured using the MTT assay. At concentrations above 200 μM, neomycin caused a significant decrease in ion pair-induced apoptosis. Values shown are mean ± SEM.

2001]. To explore this relationship further, we examined the correlation between intracellular Ca^{2+} and MMP using more discrete time points. Surprisingly, the impact of ion pair treatment was to cause a sustained Ca^{2+} transient beginning at the 15 min time point and maintained through 45 min. This spike in Ca^{2+} concentration could have been due to the entry of Ca^{2+} across the plasma membrane or release of ions from the endoplasmic reticulum. During the remaining 45 min, the intracellular Ca^{2+}concentration returned to baseline. The increase in Ca^{2+} was mirrored by an increase in the MMP. This was supported by analysis of the individual cellular signals. At 0, 15, and 30 min, there was a significant positive correlation between the two signals. However, at 45 min, this correlation is lost. Thus, there is an increase in the mean MMP fluorescence accompanied by a decrease in the intracellular Ca^{2+} signal. This decoupling of the intracellular Ca^{2+} signal from the MMP suggests that the mitochondria are

buffering the increase in intracellular Ca^{2+}. As an MMPT is seen between the 60 and 75-min time points, it is possible that this is the point at which apoptosis is activated. It is likely that the accumulated Ca^{2+} would subsequently hinder oxidative phosphorylation and lower the energy charge.

The cumulative Ca^{2+}-directed events discussed above would be expected to lead to an MMPT, with the subsequent loss of cytochrome-*c* and the formation of an apoptosome. Indeed, when treated with the ion pair, cytochrome-*c* partitions from the mitochondrial fraction to the cytoplasmic fraction. Likewise, when treated with the Ca^{2+}·Pi ion pair, Smac/DIABLO was localized exclusively to the cytoplasm. These results support the concept that ion pair treatment and the release of Ca^{2+} produces an MMPT and formation of an apoptosome. Although we did not explore the mechanism by which mitochondria lose cytochrome-*c*, recent work suggests that it is dependent on the activity of the pro-apoptotic protein Bid binding to the mitochondrial membrane and releasing cytochrome-*c* [Rostovtseva et al., 2004]. Ongoing studies are directed at evaluating the role of Bid and other Bcl2 family members in regulating events at the level of the mitochondrion.

To explore the role of the mitochondrion in ion pair-mediated cell death, we challenged osteoblasts with high and low concentrations of apoptogens in the presence of inhibitors of mitochondrial function. We blocked mitochondrial energy generation using inhibitors of the electrochemical gradient. Thus, we blocked complex I with rotenone and complex III with antimycin A. We reasoned that in the absence of a functioning respiratory chain, Ca^{2+} would be excluded from the mitochondria and, as a result, there would be no outward transport of cytochrome-*c*. Consequently, we noted that antimycin A provided considerable protection against ion pair-induced cell death, as did rotenone. Finally, we reasoned that if the mitochondria were required for induction of the death process, then agents that inhibited generation of an MMPT would protect the cell from apoptosis. Treatment of the cells with bongkrekate, a glycoside that locks the substrate binding site of the translocase in a matrix-directed state [Klingenberg, 1989], provided protection against the apoptotic activity of the ion pair. In a parallel study, to inhibit influx of Ca^{2+} into the mitochondria, cells were treated with ruthenium red; this agent is a non-competitive inhibitor of the mitochondrial Ca^{2+} uniporter [Gunter et al., 2000a]. Again, we noted that this agent profoundly protected the cells from ion pair-induced cell death.

As we have demonstrated the close correlation between intracellular Ca and MMP during the activation of ion pair-induced apoptosis, we examined the premise that the release of endoplasmic reticulum Ca^{2+} can modulate the apoptotic effect of the ion pair. Earlier work indicated that mitochondria of skeletal tissues avidly take up Ca^{2+} [Shapiro and Lee, 1975] and that they are in very close physical proximity to the endoplasmic reticulum [Cherradi et al., 1997]. Accordingly, mitochondria can shape the Ca^{2+} signal, and depending on the context, can potentiate or inhibit Ca^{2+} oscillations. That intracellular Ca^{2+} release was implicated in ion pair-induced apoptosis was reinforced by treating osteoblasts with carbachol. Significantly, when challenged with Pi, the carbachol-treated cells evidenced a marked increase in cell death. As carbachol causes release of Ca^{2+} from IP3-sensitive intracellular stores, and because the endoplasmic reticulum is the primary IP3 target, we suggest that the Ca^{2+} spikes that are characteristic of ion pair-activated apoptosis originate from this organelle.

Although little attention has been directed at the importance of the endoplasmic reticulum in controlling osteoblast apoptosis, this topic has received considerable attention in a number of other cell types, notably neuronal cells, macrophages, and cardiomyocytes [Kong and Rabkin, 1999; Chen and Lin-Shiau, 2000; Gotoh et al., 2002; Hamada et al., 2004; Wootz et al., 2004]. To evaluate the importance of this Ca^{2+} pool in osteoblasts, we used three different types of channel inhibitors to block apoptosis: cyclopiazonic acid, dantrolene, and 2-aminoethoxyphenyl borate. When pre-treated with cyclopiazonic acid, a potent inhibitor of the endoplasmic reticulum Ca^{2+} ATPase [Goeger et al., 1988], at a low ion pair concentration, there was a dose-dependent increase in cell death. A similar result was seen with thapsigargin (data not shown). Dantrolene, an inhibitor of the ryanodine receptor Ca^{2+} channel [Ward et al., 1986; Simpson et al., 1995] and 2-aminoethoxyphenylborate, an inhibitor of Ca^{2+} release via IP3 channels [Lee et al., 2002] provided significant protection from ion pair-induced apoptosis. To

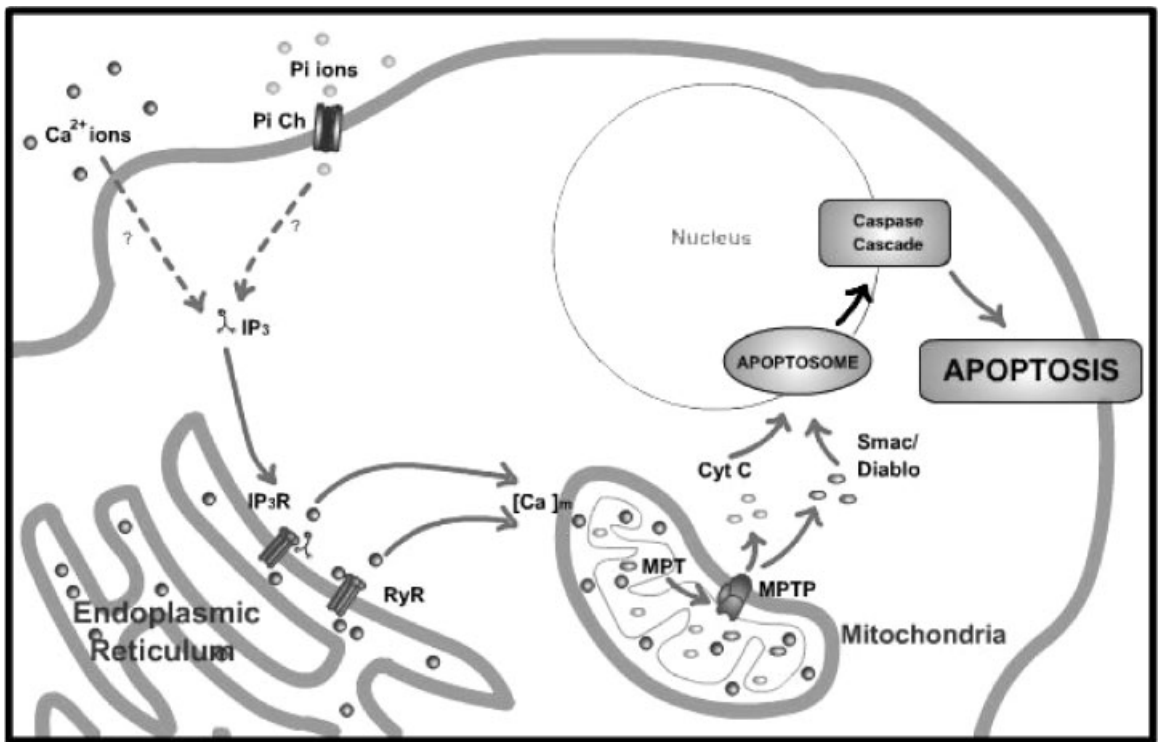

Fig. 9. A suggested pathway for the activation of cell death by the Ca^{2+} and Pi ion pair. Influx of Ca2+ and Pi ions into the cytoplasm results in the release of Ca^{2+} ions from the endoplasmic reticulum. This increase in Ca^{2+} causes the mitochondria to uptake Ca^{2+} and eventually activates a mitochondrial potential transition (MPT), causing the opening of the mitochondrial pore (MPTP) and the release of cytochrome-*c* and Smac/ DIABLO, activation of the apoptosome and apoptosis.

confirm that these channels were activated, cells were treated with neomycin, an agent that inhibits IP3 formation [Orsulakova et al., 1976; Schacht, 1976; Marche et al., 1983; Prentki et al., 1986]. We noted a dose-dependent increase in protection against ion pair-mediated apoptosis confirming that the inositide mediates the death response. Together, these results clearly indicate that Ca^{2+} stored in the endoplasmic reticulum is released by treatment of cells with the Ca^{2+}·Pi ion pair. Thus, a very early step in ion pair-mediated apoptosis is regulated at the level of the endoplasmic reticulum. Whether the ion pair contributes to the endoplasmic reticulum, Ca^{2+} response is at this time unknown. However, since osteoblasts accumulate considerable quantities of Ca^{2+} [Imai et al., 1992], these cells would be particularly sensitive to modest fluxes in extracellular Pi levels. This observation may help to explain why osteoblast apoptosis is most marked at sites of rapid bone formation or resorption [Bronckers et al., 1996; Noble et al., 1997; Jilka et al., 1998, 1999; Weinstein et al., 1998].

In summary, findings from this study indicate that Ca^{2+} pools, in the endoplasmic reticulum and the mitochondrion, mediate osteoblast death (Fig. 9). Treatment with the ion pair leads may result in uptake of Ca^{2+} into the endoplasmic reticulum, possibly as a stress response. When the capacity of this organelle is exceeded, release of Ca^{2+} and its uptake by the mitochondrion would lead to loss of organelle function and ATP depletion. Opening of the membrane pore and loss of cytochrome-*c* and APAF results in formation of an apoptosome, activation of downstream executioner enzymes and eventually programmed cell death.

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