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Apoptosis in C3H-10T1/2 Cells: Roles of Intracellular pH, Protein Kinase C, and the Na^+/H^+ Antiporter

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Abstract Changes in intracellular ion concentrations have been correlated with the activation of an endogenous endonuclease and thus internucleosomal DNA cleavage during apoptosis in many cell types. We investigated whether intracellular pH could play a significant role in apoptotic initiation and progression in C3H-10T1/2 cells, a cell strain that does not exhibit double-stranded DNA cleavage during apoptosis. Protein kinase C and the Na^+/H^+ antiporter, known regulators of intracellular pH, also were assessed for their involvement in apoptosis of C3H-10T1/2 cells. When a H^+ ionophore was used to clamp intracellular pH to 6.0 or below, a significant level of apoptosis was induced in these cells within 6 h, whereas clamping at pH 6.75 did not induce significant amounts of apoptosis until 36 h after acidification. The acidified cells exhibited classic apoptotic morphology and chromatin condensation, similar to serum withdrawn cells, but failed to show internucleosomal DNA cleavage with electrophoresis of genomic DNA. Our results also suggest that the 12-O-tetradecanoylphorbol-13-acetate (TPA)-mediated inhibition of apoptosis in serum withdrawn C3H-10T1/2 cells functions through a sequential activation of protein kinase C and the Na^+/H^+ antiporter; thus, an alkalinization or an inhibition of acidification is involved in this apoptotic block. Serum withdrawal itself does not appear to act through a negative effect on either protein kinase C or the Na^+/H^+ antiporter. TPA was also capable of inhibiting the apoptosis induced by specific inhibitors of protein kinase C and the Na^+/H^+ antiporter, but the inhibition was successful only if the TPA was administered at least 20 min prior to the addition of the enzyme inhibitor. These results indicate that apoptosis in C3H-10T1/2 cells follows a pathway that involves intracellular acidification, but is independent of detectable endonuclease activity. J. Cell. Biochem. 67:231–240, 1997.

Key words: 5-(N,N-hexamethylene)-amiloride (HMA); 12-O-tetradecanoylphorbol-13-acetate (TPA); chelerythrine; protein kinase C (PKC); serum withdrawal; internucleosomal DNA cleavage

Apoptosis is a mechanism of cell death that plays an integral role in the development and maintenance of multicellular organisms. The hallmarks of this type of cell death are chromatin condensation (Afanas'ev et al., 1986), nuclear breakup, cellular fragmentation into apoptotic bodies (Wyllie et al., 1980; Arends and Wyllie, 1991), and phagocytosis of the apoptotic bodies by neighboring cells and macrophages (Arends and Wyllie, 1991). The cell membrane appears to remain intact both physically and biochemically, as indicated by the ability of cells undergoing apoptosis to exclude dyes such as trypan blue (Arends and Wyllie, 1991; Kerr and Harmon, 1991).

Although the morphological changes that occur when a cell undergoes apoptosis are well defined, the biochemical events that occur during this process are still poorly understood. One area that has attracted considerable attention involves changes in intracellular ion concentrations during apoptosis. Of particular interest are increases in ion concentrations that correlate with the internucleosomal DNA cleavage observed in a multitude of cell systems as they undergo apoptosis. The majority of reports suggest that rises in intracellular Ca^{2+} and Mg^{2+} concentrations activate the nuclease responsible for this DNA degradation (Umansky et al., 1988; Wyllie et al., 1992). Increases in intracellular H^+ concentration occur in several cell types as they undergo apoptosis, and this acidification correlates with internucleosomal DNA

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cleavage (Barry et al., 1993; Gottlieb et al., 1996; Ostad et al., 1996). Barry and Eastman (1993) proposed that intracellular acidification triggers activation of the acidic endonuclease DNase II, with the subsequent breakdown of the chromatin. Contrary reports have indicated that activation of a nuclease is not universal to all cell systems undergoing apoptosis and that this enzymatic activity is not necessary for apoptotic progression (Collins et al., 1992; Oberhammer et al., 1993; Schulze-Osthoff et al., 1994). In light of these conflicting reports, the question remains open as to the function of these observed changes in ion concentrations during apoptosis.

With the growing body of evidence suggesting that intracellular acidification is associated with apoptosis and that intracellular alkalinization may be sufficient to block the apoptotic process, it is important that the function of these pH changes be clearly determined. Changes in intracellular pH can be dependent on the activities of both protein kinase C (PKC) and the Na^+/H^+ antiporter. Rajotte et al. (1992) demonstrated that inhibition of either PKC or the Na^+/H^+ antiporter induced apoptosis in MO7-E (human megakaryoblastic leukemia) cells. In addition, stimulation of PKC with the tumor promoting phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA) results in a sustained intracellular alkalinization and suppression of apoptosis in these cells (Rajotte et al., 1992). Working with C3H-10T1/2 mouse embryonic fibroblast cells, Tomei et al. (1981) used TPA to inhibit apoptosis normally initiated by serum withdrawal. Of significance, C3H-10T1/2 cells undergoing apoptosis show characteristic changes in cellular morphology and chromatin condensation but do not exhibit the double-stranded internucleosomal DNA cleavage observed in many other cell types (Tomei and Shapiro, 1993).

The present study was designed to gain insight into any potential roles of intracellular pH changes, PKC, and the Na^+/H^+ antiporter in the apoptotic death of C3H-10T1/2 cells. Elucidation of these roles will be important in determining the full cascade of biochemical events that are critical for the induction of apoptosis in cells that do not appear to activate an endogenous endonuclease as they die. We used pharmacological agents to alter specifically the intracellular pH levels and to modulate the activities of PKC and the Na^+/H^+ antiporter. Our results demonstrate that C3H-10T1/2 cells exhibit apoptotic changes in cellular morphology and chromatin distribution when subjected to intracellular acidification directly or when treated with an inhibitor of either PKC or the Na^+/H^+ antiporter. The induction of apoptosis by intracellular acidification appears to be independent of endonuclease activation.

MATERIALS AND METHODS

All chemicals and reagents were purchased from AMRESCO (Solon, OH) or Sigma Chemical Company (St. Louis, MO) unless otherwise noted. All cell treatments were for 24 h unless indicated otherwise. For all drug administrations, control cells were treated with medium containing the respective solubilizing agent for that drug. In all cases, addition of the solubilizing agent alone did not significantly alter the level of apoptosis relative to the untreated control cells.

Cell Culture

Mouse C3H-10T1/2 embryonic fibroblast cells were cultured in basal medium Eagles (BME; Life Technologies, Gaithersburg, MD) supplemented with 10% fetal bovine serum (FBS; Hyclone Laboratories, Logan, UT). Cells were seeded at an initial density of 2×10^3 cells/cm^2 and were grown at 37°C in a 5% CO_2 atmosphere until they reached 75% confluency. Passage-16 cells were used in all experimental protocols.

Serum Withdrawal

Serum withdrawal was accomplished by changing the cell culture medium to BME without FBS.

Clamping Intracellular pH

Complete medium was buffer expanded by the addition of 16 mM final concentration each of MES (2-[N-Morpholino]ethanesulfonic acid; pKa 6.1), PIPES (piperazine-N,N′-bis[2-ethanesulfonic acid]; 1,4-piperazinediethanesulfonic acid; pKa 6.8), and HEPES (hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pKa 7.5). The buffer expanded medium (BEM) was equilibrated overnight to 37°C and 5% CO_2 prior to final pH adjustment. The pH of the medium was lowered or raised by the addition of HCl or NaOH, respectively. The proton ionophore car-

bonyl cyanide m-chlorophenylhydrazone (CCCP; CalBiochem, LaJolla, CA) was added to the medium at a final concentration of 20 µM to equilibrate the intracellular and extracellular pH of the treated cells. The medium of the cultures was replaced with the pH-adjusted BEM, and the cells were harvested at 6, 12, 24 and 36 h posttreatment. Control groups included cells treated with unaltered complete medium (pH 7.25) or with BEM containing CCCP (pH 7.25).

Inhibition of the Na^+/H^+ Antiporter

5-(N,N-hexamethylene)-amiloride (HMA) is an amiloride analogue reported to be more specific than the parent compound for inhibition of the Na^+/H^+ antiporter (Rajotte et al., 1992). Doses of HMA were used that induced a significant level of apoptosis in the cultured cells but also spared a sufficient number of cells to allow for later comparison of coadministration of different drugs. Complete medium was supplemented with 40 µM HMA from an 80 mM stock in DMSO dimethyl sulfoxide, and this medium was transferred to the cells.

Regulation of PKC Activity

TPA is a tumor-promoting phorbol ester known to activate PKC (Livne et al., 1991). TPA was added to medium at a final concentration of 0.1 µM from a 1.62 mM stock in DMSO. The TPA-containing medium was then transferred to the cells. The nontumor-promoting phorbol ester 4α-phorbol-12,13-didecanoate (4αPDD) was used at the same concentration as was TPA and served as a negative control in TPA experiments.

Chelerythrine is a PKC inhibitor that acts at the diacylglycerol binding site within the regulatory domain of PKC (Jarvis et al., 1994). This drug was chosen over more popular protein kinase inhibitors (H7 and staurosporine) because of its high specificity for PKC relative to other protein kinases (Jarvis et al., 1994; Chmura et al., 1996). Cells were treated with 4.0 µM chelerythrine dispensed from a 2.6-mM stock in distilled, deionized water.

Administration of Treatments in Combination

Inhibitors of PKC and the Na^+/H^+ antiporter were combined with serum withdrawal to assess the effect on the level of apoptosis induced. HMA was added to serum-free medium at a final concentration of 40 µM, and this medium was transferred to the cell culture for 24 h. In a separate experiment, chelerythrine was added to serum-free medium at a final concentration of 4.0 µM and subsequently transferred to the cells for 24 h.

Preliminary experiments involving coadministration of TPA and HMA indicated that the order of drug addition had a significant effect on the level of apoptosis that was induced; thus, time-dependent experiments were developed. The medium to be added to the cells was divided into aliquots. Using the same drug concentrations, the first experiment involved adding TPA to 80% of the medium and HMA to 20% of the medium. The cells were incubated for 20 min with the TPA-containing medium followed by addition of the HMA-containing medium. In a subsequent experiment, HMA was added to 80% of the medium and TPA was added to 20% of the medium. The HMA medium was added to the cells 20 min before adding the TPA-containing medium. Identical protocols were followed for experiments involving coadministration of TPA and chelerythrine.

Evaluation and Quantitation of Apoptosis

Cell counting. When C3H-10T1/2 cells undergo apoptosis, they detach from the culture dish. This response to apoptotic induction allows easy separation of the two cell populations because apoptotic cells are suspended in the medium and nonapoptotic cells remain attached to the culture vessel. Nonadherent cells were collected from the aspirated culture medium by centrifugation at 400*g* for 10 min. Cell pellets were resuspended in 100 µl each of appropriate culture medium. Adherent cells were harvested by trypsinization (Life Technologies), collected by centrifugation, and resuspended, as described earlier. Apoptotic cells were distinguished from necrotic cells by assessing their ability to exclude trypan blue. Apoptotic cells are impermeable to trypan blue, whereas necrotic cells readily take up the dye (Kerr and Harmon, 1991). Cells were counted by using either a Cell-Dyn 1600 Cell Counter or by manual counting on a hemacytometer. All cell counts for treatments that were compared directly were performed with the same counting method.

Fluorescent microscopy. To evaluate for the presence or absence of chromatin condensation, cells were stained with the fluorescent

nucleic acid stain acridine orange. Five microliters of the respective cell suspension were placed on a glass slide, and 5 µl of acridine orange [5 µg/ml in phosphate buffered saline (PBS; AMRESCO)] were placed on a coverslip. The coverslip was placed over the cell suspension so that the two solutions mixed. The cells were immediately viewed and photographed with an Olympus Bmax-50 fluorescent microscope equipped with a wideband green excitation cube.

Electrophoresis. Genomic DNA was isolated from cells with the Invitrogen (LaJolla, CA) Easy DNA kit according to the manufacturer's instructions. Ten-microgram samples were electrophoresed through 1.2% agarose gels in 0.5× TBE buffer (0.045 M Tris-borate; 1 mM EDTA, pH 8.0) at 90 V for 1.5 h. Ethidium bromide was included in the gels at a concentration of 0.5 µg/ml. DNA bands were visualized on an ultraviolet transilluminator, and the gels were photographed with Polaroid 667 film.

Data Analysis

Statistical analyses were performed by using Statview^{512+}™ for the Macintosh. Experiments were analyzed by analysis of variance. Statistical difference between treatments was established with the Fisher PLSD test and the Sheffé F-test, both at a significance level of 95%. The Q-test was used to determine whether an outlying datum was part of the normal data set or should be rejected.

RESULTS

Buffer-expanded acidified culture medium was used to assess the influence of intracellular pH changes on apoptosis in C3H-10T1/2 cells. The equilibration of intra- and extracellular pH was accomplished by including the proton ionophore CCCP. Preliminary experiments demonstrated that dropping the intracellular pH of C3H-10T1/2 cells to below pH 6.0 induced apoptosis in almost 100% of the cells within 6 h of treatment (Fig. 1A). Because decreases in pH of this magnitude are unlikely to be physiologically relevant, we assessed cells for apoptotic induction between pH 6.0 and 7.0. Increasing intracellular acidification correlated directly with increased levels of apoptotic induction (Fig. 1B). Notably, intracellular acidification to pH 6.75 did not induce a level of apoptosis that was significantly different from the controls until 36 h after the drop in pH was imposed. These results indicate that intracellular acidification alone, even at levels close to neutral, was sufficient to induce apoptosis in C3H-10T1/2 cells if the intracellular pH was clamped for a sufficient period of time.

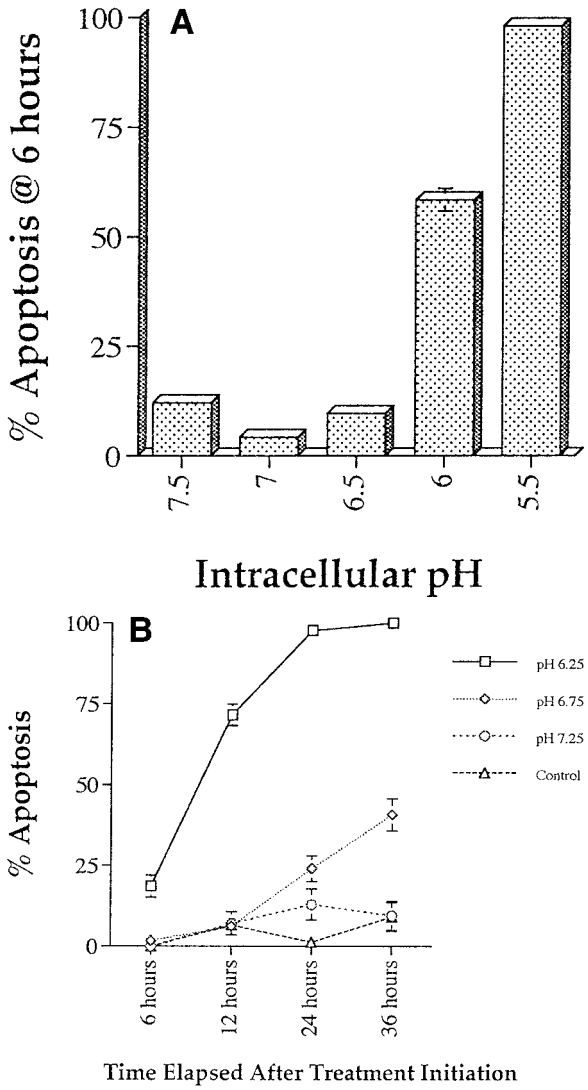

Fig. 1. Induction of apoptosis in C3H-10T1/2 cells by intracellular acidification. Cells grown to 75% confluency were clamped at the indicated intracellular pH by replacing the medium with BEM containing the proton ionophore CCCP. **A:** Clamping intracellular for 6 h. **B:** Intracellular pH was clamped over a 36-h period. The medium for the control group was replaced with fresh complete medium (pH 7.25) without any additional buffers or CCCP. At the indicated time points, cells were harvested and counted, as described in Materials and Methods. Values represent mean ± SE, n = 6.

Apoptosis was confirmed by trypan blue dye exclusion and acridine orange staining. For up to 24 h, nonadherent cells retained their ability to exclude trypan blue, which is consistent with their being apoptotic and not necrotic. With incubations extending beyond 24 h, an increasing number of cells clamped at pH 6.25 stained

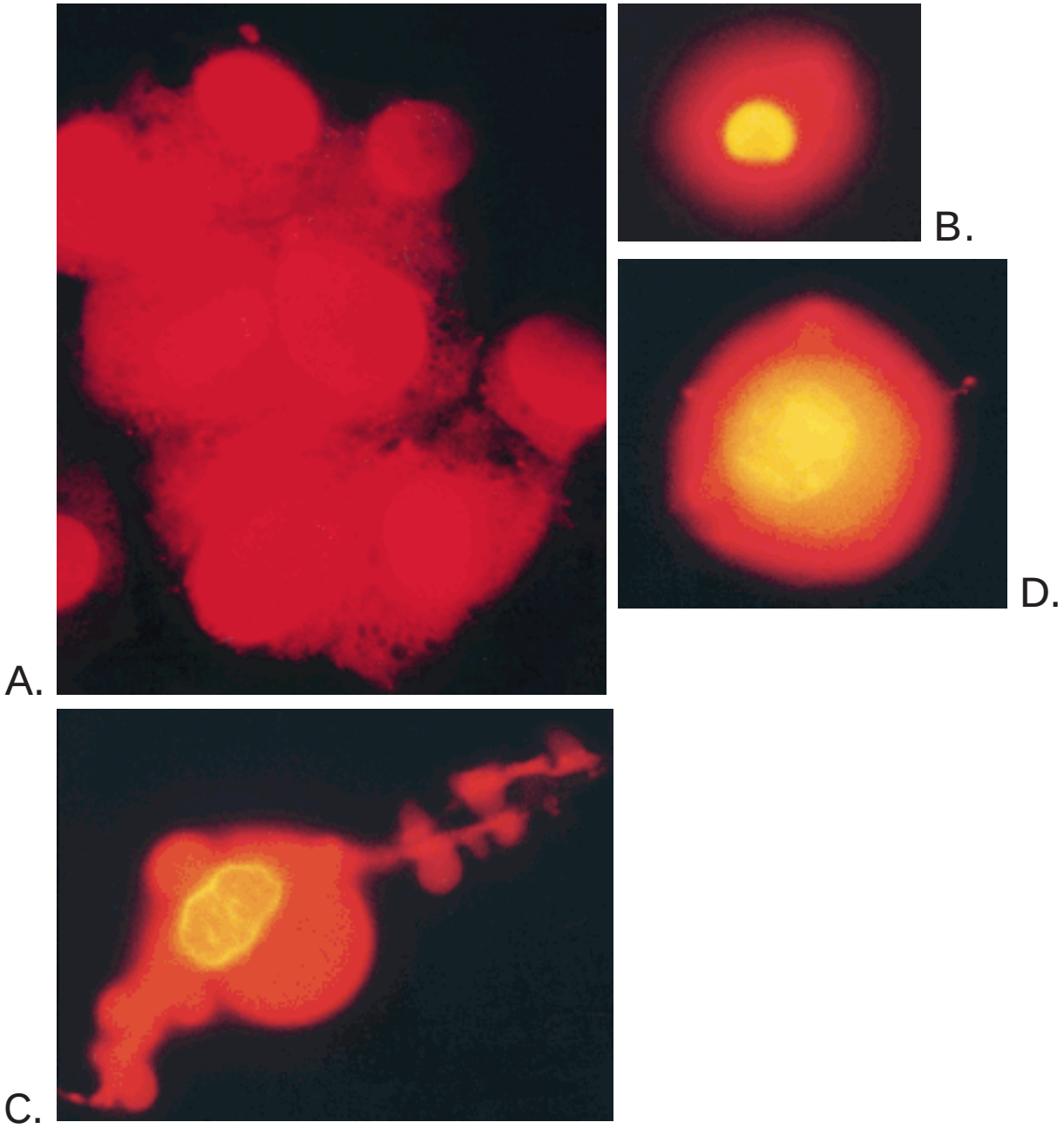

Fig. 2. Nonadherent C3H-10T1/2 cells exhibit apoptotic morphology. Cells were harvested, pelleted by centrifugation, resuspended in their respective culture medium, and stained with acridine orange. All nonadherent cells have multiple membrane blebs, a decreased cell size, and a shrunken nucleus containing condensed chromatin (bright-yellow fluorescent areas). **A:** Untreated adherent cell showing normal morphology. **B:** Nonadherent cell 24 h after serum withdrawal. **C:** Nonadherent cell 24 h after clamping at pH 6.75. **D:** Nonadherent cell 24 h after clamping at pH 6.25. Magnification, ×1,000.

blue with trypan blue, presumably because of secondary necrosis of cells that had apoptosed early in the treatment period (Kerr and Harmon, 1991).

The nucleic acid stain acridine orange was used to examine nuclear morphology and the chromatin distribution in the nucleus. Although the nucleus is the primary target for this stain, there was a sufficient level of cytosolic fluorescence to allow for an analysis of cellular morphology. Figure 2A depicts normal C3H-10T1/2 cells that exhibited evenly distributed chromatin, unfragmented nuclei, and intact plasma membranes. Also shown are representative nonadherent cells 24 h after serum withdrawal (Fig. 2B) and the imposition of an intracellular

pH of 6.75 (Fig. 2C) or 6.25 (Fig. 2D). The serum-withdrawn cells and the cells at both acidic pH levels showed classic signs of apoptosis including plasma membrane blebbing, cell shrinkage, and condensation of the chromatin.

Electrophoretic analysis of genomic DNA isolated from apoptotic serum-withdrawn cells, or acidified cells, revealed only a high-molecular-weight band. The classic "ladder" of DNA fragments, indicative of internucleosomal DNA cleavage, was not observed in any sample, independent of the stimulus that induced apoptosis (Fig. 3).

Having established that intracellular acidification alone was sufficient to induce apoptosis in C3H-10T1/2 cells, we next investigated cellular components that may be involved in controlling intracellular pH during C3H-10T1/2 cell apoptosis. First, we confirmed the results of Tomei et al. (1981) by showing that TPA can rescue C3H-10T1/2 cells from apoptosis initiated by serum withdrawal; the nontumor-promoting phorbol ester 4αPDD had no effect on the level of apoptosis observed (Fig. 4). Phorbol esters such as TPA stimulate the activity of PKC (Kikkawa et al., 1989). Therefore, we designed experiments to determine whether activation of this enzyme was involved in the inhibition of apoptosis in C3H-10T1/2 cells. We employed chelerythrine to specifically inhibit PKC activity. The cells treated with 4.0 μM chelerythrine showed a significant level of apoptosis as opposed to control cells (Fig. 5), whereas a 5.0-μM dose of this drug induced more than 95% of the cells to apoptose (unpublished observation). These results are consistent with reports from other investigators for the inhibition of PKC (McCabe and Orrenius, 1994). Also shown in Figure 5 are results obtained when different combinations of cell treatments were administered. When serum withdrawal was combined with chelerythrine administration, the level of apoptosis induced was additive of the levels induced by the individual stimuli alone. Combining the serum withdrawal plus chelerythrine treatment with TPA administration caused opposite results that were dependent on the time of TPA administration in the protocol. Addition of chelerythrine 20 min prior to TPA in serum-withdrawn cells resulted in no significant change in the level of apoptosis observed relative to the serum withdrawal plus chelerythrine treatment. In contrast, when TPA was added at the time of serum withdrawal but 20 min prior to chelerythrine administration, apoptosis was almost completely inhibited. These data suggest that apoptosis initiated by serum withdrawal or chelerythrine administration is blocked by TPA stimulating PKC.

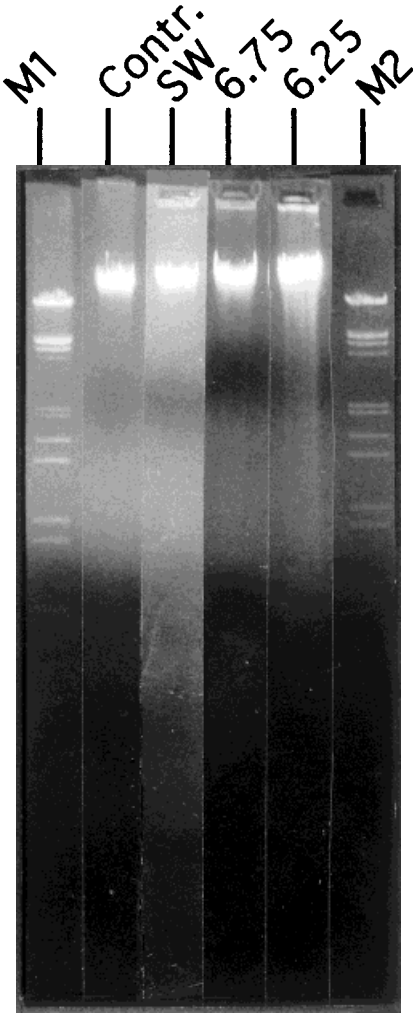

Fig. 3. Electrophoretic analysis of DNA from apoptotic C3H-10T1/2 cells. Genomic DNA was isolated from C3H-10T1/2 cells, as described in Materials and Methods, and 10 μg per sample were electrophoresed through a 1.2% agarose gel in 0.5× TBE. **M1,M2:** λ EcoRI–Hind III–digested marker DNA. **Contr.:** DNA from untreated control cells. **SW:** DNA from serum-withdrawn cells. **6.75:** DNA from cells at intracellular pH 6.75. **6.25:** DNA from cells at intracellular pH 6.25. Control DNA was extracted from adherent nonapoptotic cells grown in unaltered complete medium. All other DNA was collected from apoptotic (nonadherent) cells 24 h after treatment initiation. Similar results were obtained using DNA extracted from cells at 6, 12, and 36 h post-treatment.

An important substrate for PKC in the plasma membrane is the Na^+/H^+ antiporter (Ober and Pardee, 1987; Livne et al., 1991; Rajotte et al., 1992), which regulates intracellular pH. Block-

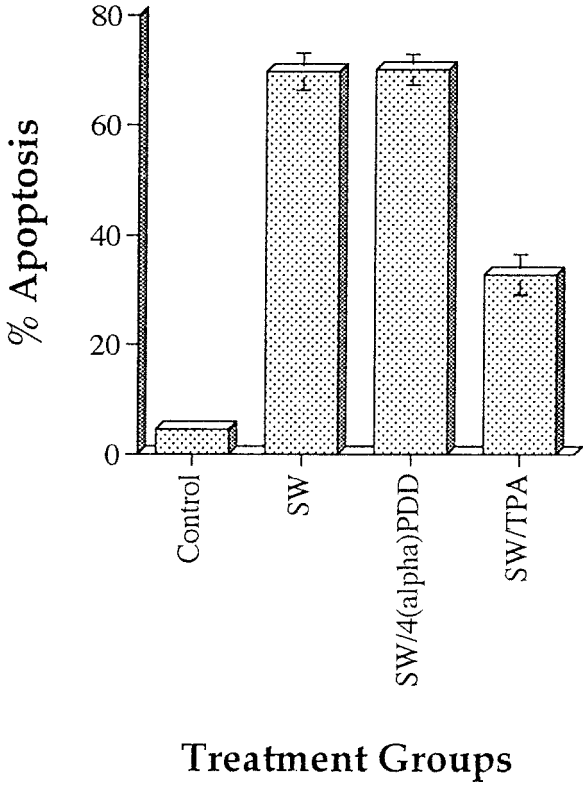

Fig. 4. Effect of phorbol ester administration on serum-withdrawal-induced apoptosis. The 75% confluent cultures were changed to complete medium (control), serum-free medium (SW), serum-free medium containing 4α-phorbol-12,13-didecanoate (SW/4αPDD) or, serum-free medium containing 12-O-tetradecanoylphorbol-13-acetate (SW/TPA). Cultures were incubated for 24 h at 37°C, 5% CO_2, at which time cells were harvested and counted. Values represent mean ± SE, n = 5.

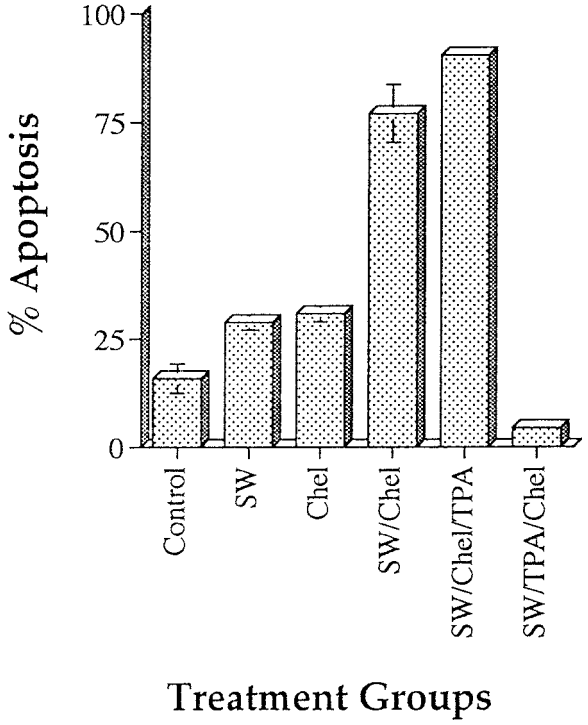

Fig. 5. Influence of PKC-modulating agents on C3H-10T1/2 cell apoptosis. The 75% confluent cultures were changed to complete medium (control), serum-free medium (SW), complete medium containing chelerythrine (Chel), serum-free medium containing chelerythrine (SW/Chel), serum-free medium containing chelerythrine with the addition of TPA after 20 min (SW/Chel/TPA) or, serum-free medium containing TPA with the addition of chelerythrine after 20 min (SW/TPA/Chel). All cultures were incubated for 24 h at 37°C, 5% CO_2 after the medium change. Values represent mean ± SE, n = 6.

ing this antiporter with amiloride or amiloride analogues causes cells to retain H^+ ions, resulting in intracellular acidification (Hendey and Mamrack, 1991). Inhibition of the Na^+/H^+ antiporter with 40 μM HMA caused a significant induction of apoptosis in the C3H-10T1/2 cells (Fig. 6). Dose–response experiments indicated that increasing HMA concentration to 50 μM induced greater than 90% apoptosis in these cells (unpublished observation). Furthermore, apoptosis resulting from inhibition of the Na^+/H^+ antiporter with 40 μM HMA was additive to that induced by serum withdrawal alone, indicating that serum withdrawal did not cause a significant inhibition of this antiporter.

We then investigated whether PKC and the Na^+/H^+ antiporter were acting in conjunction during TPA-mediated inhibition of apoptosis. Importantly, coadministration of TPA and HMA elicited quite different responses from the cells, depending on the order of the drug addition. When HMA was added first, with the subsequent addition of TPA, a level of apoptosis was induced that was not significantly different from that induced by HMA alone. However, when TPA was administered before the addition of HMA, the apoptotic induction was significantly lower (Fig. 7). These results strongly suggest that the Na^+/H^+ antiporter is a target substrate for PKC during TPA-mediated apoptotic inhibition. These same results also indicate that prior activation of PKC and, presumably, phosphorylation of the Na^+/H^+ antiporter at the PKC target site, blocks the ability of HMA to act as an effective inhibitor of this transporter.

DISCUSSION

A number of reports have documented decreases in intracellular pH when cells are exposed to various apoptosis-inducing stimuli (Barry et al., 1993; Eastman and Li, 1994; Pérez-Sala et al., 1995). In these reports, the

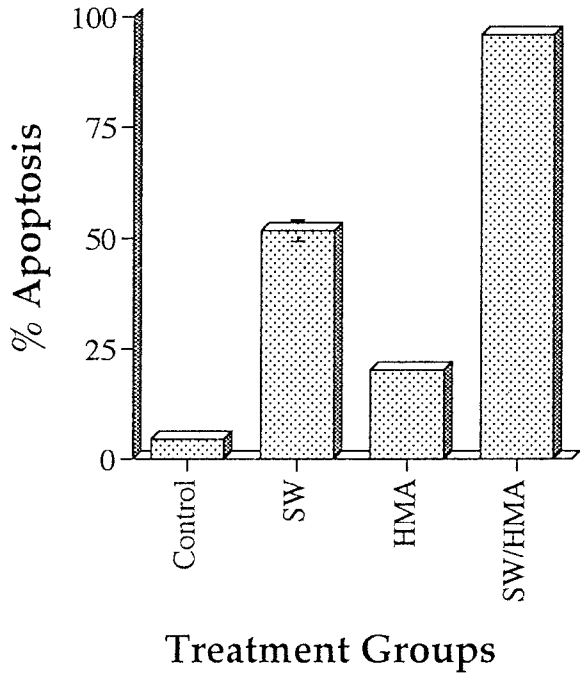

Fig. 6. Effect of Na^+/H^+ antiporter inhibition on C3H-10T1/2 cell apoptosis. The 75% confluent cultures were changed to complete medium (control), serum-free medium (SW), complete medium containing HMA (HMA) or, serum-free medium containing HMA (SW/HMA). All cultures were incubated for 24 h at 37°C, 5% CO_2 after the medium change. Values represent mean ± SE, n = 5.

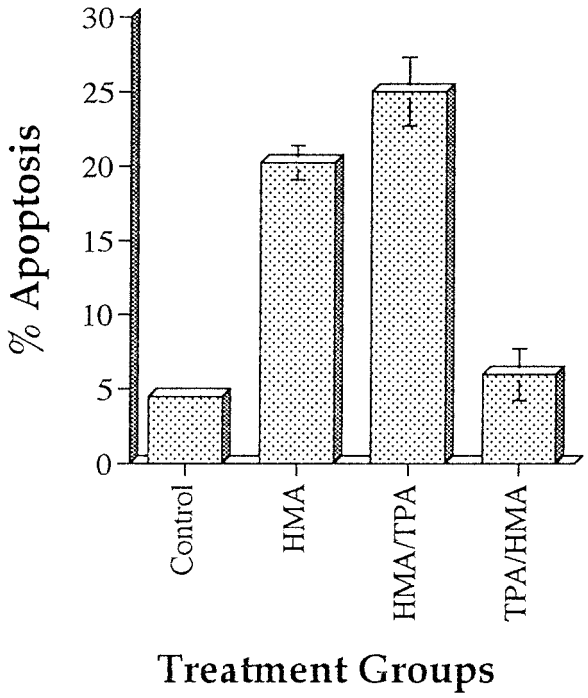

Fig. 7. Apoptotic effect of PKC stimulation and Na^+/H^+ antiporter inhibition in combination. The 75% confluent cultures were changed to complete medium (control), complete medium containing HMA (HMA), complete medium containing HMA with the addition of TPA after 20 min (HMA/TPA) or, complete medium containing TPA with the addition of HMA after 20 min (TPA/HMA). All cultures were incubated for 24 h at 37°C, 5% CO_2 after the medium change, at which time the cells were harvested and counted. Values represent mean ± SE, n = 5.

investigators have correlated the intracellular acidification with activation of an acidic endonuclease and, ultimately, DNA fragmentation. In C3H-10T1/2 cells, Tomei and Shapiro (1993) demonstrated that double-stranded breaks are not produced when cells undergo apoptosis. In our hands, whereas the level of apoptosis steadily increased with decreasing intracellular pH at all time points analyzed, no double-stranded breaks were detected in C3H-10T1/2 cell DNA. This result indicates that an acidic endonuclease is not activated during acidification-induced apoptosis in this cell line. Alternatively, a DNA repair process may be coordinately activated with the putative nuclease, rendering a transient DNA fragmentation undetectable. In either case, our results are consistent with reports that nuclease activation and the associated DNA fragmentation are not required for apoptosis in all cell types (Oberhammer et al., 1993) and that some cells may not even require the presence of a nucleus to undergo apoptosis (Schulze-Osthoff et al., 1994). Intracellular pH changes have a broad range of effects on a cell including altering the activity of many pH-sensitive proteins and regulating the cell cycle (Madshus, 1988). Several regulatory proteins are known to play significant roles in both the cell cycle and apoptosis (Steller, 1995). It is conceivable that several cellular components involved in apoptosis are coordinately affected by intracellular acidification and ultimately act to trigger the death of the cell.

The results of the present study agree with the work by Tomei et al. (1981), which show that C3H-10T1/2 cells undergo apoptosis when transferred to a serum-free environment and that TPA can prevent serum withdrawn C3H-10T1/2 cells from dying. Although the mechanism of serum withdrawal-induced apoptosis is still poorly defined, our results indicate that PKC and Na^+/H^+ antiporter are not blocked by removal of serum from the culture medium. If serum withdrawal was blocking the activity of either of these proteins, then addition of the respective inhibitors would have little or no effect on the level of apoptosis induced, which is

clearly not the case. Administration of chelerythrine (an inhibitor of PKC) or HMA (an inhibitor of Na^+/H^+ antiporter) to C3H-10T1/2 cells at the time of serum withdrawal induces a level of apoptosis that is additive to that stimulated by serum withdrawal and the respective inhibitor when each is administered alone. One scenario that cannot be ruled out is that serum withdrawal has an impact on isoforms of PKC or the Na^+/H^+ antiporter that are not responsive to the inhibitors used in the present study (Dekker and Parker, 1994; Noël and Pouysségur, 1995). Although this is possible, it seems unlikely because our preliminary results showed that slight increases in the concentrations of each inhibitor cause dramatic increases in the levels of apoptosis induced.

Although apoptosis induced by serum withdrawal does not appear to involve PKC or Na^+/H^+ antiporter, the inhibition of apoptosis by TPA in these cells under serum-free conditions does appear to act through the sequential activation of these two proteins. Because phosphorylation of the Na^+/H^+ antiporter by PKC stimulates the extrusion of H^+ ions, causing an intracellular alkalinization (Ober and Pardee, 1987; Livne et al., 1991; Rajotte et al., 1992); our data suggest that an alkalinization subsequent to TPA administration is responsible for the observed apoptotic block.

Using MO7-E cells, Rajotte et al. (1992) measured the change in intracellular pH when cells deprived of growth factor were preincubated with an inhibitor of the Na^+/H^+ antiporter or treated with PKC inhibitors prior to the addition of interleukin-3, granulocyte macrophage colony-stimulating factor, or TPA. They concluded that TPA could not block apoptosis in cells pretreated with inhibitors of PKC or the Na^+/H^+ antiporter. Although our data agree with their primary conclusions for pretreatment with the inhibitors, we observed a quite different effect when the order of the drug administrations was reversed. When cells were pretreated with TPA, chelerythrine and HMA were not effective at inducing apoptosis in C3H-10T1/2 cells. Pretreatment with TPA may cause a sustained activation of the Na^+/H^+ antiporter, effectively clamping the intracellular pH in the neutral to alkaline range. Thus, phosphorylation by PKC may "lock" the antiporter in the "open" conformation, with the conformational change being propagated to the extracellular domain of the protein, which in turn could be responsible for disabling the binding site for HMA. The phosphorylation and conformational change of the exchanger would take time to occur; hence, when 20 min were allowed to elapse between addition of TPA and HMA, the conformational change could occur, intracellular alkalinization would take place, and apoptosis would be inhibited. Conversely, when HMA is added first, the antiporter may be "locked" in the "closed" conformation, and PKC cannot phosphorylate the antiporter or the phosphorylation cannot effect a conformational change to stimulate ion exchange. Either way, the Na^+/H^+ antiporter would be closed; the cells would accumulate H^+ ions and acidify and eventually undergo apoptosis. Conformational changes are known to affect activity of the Na^+/H^+ antiporter (Madshus, 1988).

In summary, our results show that intracellular acidification is sufficient to induce apoptosis in C3H-10T1/2 cells in the absence of detectable nuclease activation. We also report that, although apoptosis induced by serum withdrawal does not appear to result from inactivation of either PKC or the plasma membrane Na^+/H^+ antiporter, the TPA-mediated inhibition of serum-withdrawal-induced cell death does appear to function through the sequential activation of these proteins. Thus, it is likely that an intracellular alkalinization or at least an inhibition of acidification is sufficient to block apoptosis initiated by serum withdrawal. In addition, our evidence suggests that the induction of apoptosis by inhibition of the Na^+/H^+ antiporter requires the antiporter to be present in the unphosphorylated state. Based on these results, it is very likely that apoptosis-inducing therapeutic strategies, targeted at inhibition of the Na^+/H^+ antiporter, will be successful at killing only those cells that do not have a phosphorylated Na^+/H^+ antiporter due to a previously activated PKC.

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REFERENCES

Afanas'ev V, Korol B, Mantsygin YA, Nelipovich PA, Umansky SR (1986): Flow cytometry and biochemical analysis of DNA degradation characteristic of two types of cell death. FEBS Lett 194:347–350.

Arends MJ, Wyllie AH (1991): Apoptosis: Mechanisms and roles in pathology. Int Rev Exp Pathol 32:223–254.

Barry MA, Eastman A (1993): Identification of deoxyribonuclease II as an endonuclease involved in apoptosis. Arch Biochem Biophys. 300:440–450.

Barry MA, Reynolds JE, Eastman A (1993): Etoposide-induced apoptosis in human HL-60 cells is associated with intracellular acidification. Cancer Res 53:2349–2357.

Chmura SJ, Nodzenski E, Weichselbaum RR, Quintas J (1996): Protein kinase C inhibition induces apoptosis and ceramide production through activation of a neutral sphingomyelinase. Cancer Res 56:2711–2714.

Collins RJ, Harmon BV, Gobé GC, Kerr JFR (1992): Internucleosomal DNA cleavage should not be the sole criterion for the identifying apoptosis. J Radiat Biol 61:451–453.

Dekker LV, Parker PJ (1994): Protein kinase C—A question of specificity. TIBS 19:73–77.

Eastman A, Li J (1995): Apoptosis in an interleukin-2-dependent cytotoxic T lymphocyte cell line is associated with intracellular acidification: Role of the Na^+/H^+ antiport. J Biol Chem 270:3203–3211.

Gottlieb RA, Nordberg J, Skowronski E, Babior BM (1996): Apoptosis induced in Jurkat cells by several agents is preceded by intracellular acidification. Proc Natl Acad Sci USA 93:654–658.

Hendey B, Mamrack MD (1991): WS-1 human fibroblasts contain distinct calcium and protein kinase C–mediated pathways for activation of Na^+/H^+ exchange: Contrasting effects of thrombin and PMA. J Cell Physiol 146:290–297.

Jarvis WD, Turner AJ, Povirk LF, Traylor RS, Grant S (1994): Induction of apoptotic DNA fragmentation and cell death in HL-60 human promyelocytic leukemic cells by pharmacological inhibitors of protein kinase C. Cancer Res 54:1707–1714.

Kerr JFR, Harmon BV (1991): Definition and incidence of apoptosis: An historical perspective. In Tomei LD, Cope FO (eds): "Apoptosis: The Molecular Basis of Cell Death." Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, pp 5–29.

Kikkawa U, Kishimot A, Nishizuka Y (1989): The protein kinase C family: Heterogeneity and its implications. Annu Rev Biochem 58:31–44.

Livne AA, Sardet C, Pouysségur J (1991): The Na^+/H^+ exchanger is phosphorylated in human platelets in response to activating agents. FEBS Lett 284:219–222.

Madshus IH (1988): Regulation of intracellular pH in eukaryotic cells. Biochem J 250:1–8.

McCabe MJ Jr, Orrenius S (1994): Protein kinase C: A key enzyme determining cell fate and apoptosis? In Kuo JF (ed): "Protein Kinase C." New York: Oxford University Press, pp 290–304.

Noël J, Pouysségur J (1995): Hormonal regulation, pharmacology, and membrane sorting of vertebrate Na^+/H^+ exchanger isoforms. Am J Physiol 268 (Cell Physiol 37): C283–C296.

Ober SS, Pardee AB (1987): Both protein kinase C and calcium mediate activation of the Na^+/H^+ antiporter in chinese hamster embryo fibroblasts. J Cell Physiol 132: 311–317.

Oberhammer J, Fritsch G, Schmied M, Pavelka M, Printz D, Purchio T. Lassmann H, Schulte-Hermann R (1993): Condensation of the chromatin at the membrane of an apoptotic nucleus is not associated with activation of an endonuclease. J Cell Sci 104:317–326.

Ostad M, Shu WP, Kong L, Liu BC (1996): Ha-ras oncogene expression abrogates a pH dependent endonuclease activity of apoptosis in normal rat kidney cells. Cancer Lett 98:175–182.

Pérez-Sala D, Collado-Escobar D, Mollinedo F (1995): Intracellular alkalinization suppresses lovstatin-induced apoptosis in HL-60 cells through the inactivation of a pH-dependent endonuclease. J Biol Chem 270:6235–6242.

Rajotte D, Haddad P, Haman A, Crago EJ, Hoang T (1992): Role of protein kinase C and the Na^+/H^+ antiporter in suppression of apoptosis by granulocyte macrophage colony-stimulating factor and interleukin-3. J Biol Chem 267:9980–9987.

Schulze-Osthoff K, Walczak H, Droege W, Krammer PH (1994): Cell nucleus and DNA fragmentation are not required for apoptosis. J Cell Biol 127:15–20.

Steller H (1995): Mechanisms and genes of cell suicide. Science 267:1445–1449.

Tomei LD, Shapiro JP (1993): Apoptosis in C3H-10T1/2 mouse embryonic cells: Evidence for internucleosomal DNA modification in the absence of double-strand cleavage. Proc Natl Acad Sci USA 90:853–857.

Tomei LD, Cheney JC, Wenner CE (1981): The effect of phorbol esters on the proliferation of C3H-10T1/2 mouse fibroblasts: Consideration of both stimulatory and inhibitory effects. J Cell Physiol 107:385–389.

Umansky SR, Beletsky IP, Korol BA, Lichtenstein AV, Nelipovich PA (1988): Molecular mechanisms of DNA degradation in dying rodent thymocytes. Mol Cell Biol 7:221–228.

Wyllie AH, Kerr JFR, Currie AR (1980): Cell death: The significance of apoptosis. Int Rev Cytol 68:251–306.

Wyllie AH, Arends MJ, Morris RG, Walker SW, Evan G (1992): The apoptosis endonuclease and its regulation. Semin Immunol 4:389–397.