Calcium and Calpain as Key Mediators of Apoptosis-like Death Induced by Vitamin D Compounds in Breast Cancer Cells

The active form of vitamin D$_3$ (1,25(OH)$_2$D$_3$) induces an increase in the intracellular free calcium ([Ca$^{2+}$]) and caspase-independent cell death in human breast cancer cells. Here we show that the treatment of MCF-7 breast cancer cells with 1,25(OH)$_2$D$_3$ or its chemotherapeutic analog, EB 1089, releases Ca$^{2+}$ from the endoplasmic reticulum. The increase in [Ca$^{2+}$], was associated with the activation of a calcium-dependent cysteine protease, ?-calpain. Interestingly, ectopic expression of a calcium-binding protein, calbindin-D$_{28k}$, in MCF-7 cells not only attenuated the elevation in [Ca$^{2+}$], and calpain activation, but also reduced death triggered by vitamin D compounds. Similarly, the inhibition of calpain activity by structurally unrelated chemical inhibitors increased the survival of the cells and reduces the amount of annexin V-positive cells. Despite the complete absence of effector caspase activation, transmission electron microscopy of MCF-7 cells treated with 1,25(OH)$_2$D$_3$ or EB 1089 revealed apoptosis-like morphology characterized by the condensed cytoplasm, nuclei, and chromatin. Overall, these results suggest that calpain may take over the role of the major execution protease in apoptosis-like death induced by vitamin D compounds. Thus, these compounds may prove useful in the treatment of tumors resistant to therapeutic agents dependent on the classical caspase cascade.

The ability of the active form of vitamin D$_3$ (1,25(OH)$_2$D$_3$)$^1$ to induce growth arrest, differentiation, and cell death in malignant cells of various origins has formed the basis for the development of vitamin D analogs as possible anticancer agents (1–4). A desired profile of such analogs consists of a high potency for induction of antiproliferative effects, differentiation, and apoptosis combined with reduced effects on systemic calcium homeostasis compared with the parental compound. The vitamin D analog EB 1089 displays such a profile and is currently in phase III clinical trials for the treatment of cancer (5–7).

Very little is known about the signaling pathways mediating vitamin D-induced cell death. A single family of proteases, the caspas, has until recently been considered the pivotal executioner of all programmed cell death (8). Therefore it is interesting to note that breast cancer cells treated with vitamin D compounds die in the complete absence of effector caspase activation (4). Despite the lack of the caspase activation, dying cells present several characteristics of apoptosis, i.e. rounding, shrinkage, and detachment of cells as well as DNA strand breaks and DNA fragmentation (4, 9, 10). Furthermore, anti-apoptotic proteins Bcl-2 and Bcl-X$_L$, can rescue breast cancer cells from death induced by the active form of vitamin D or its analogs (4). This apoptosis-like death program appears also independent of cytochrome c release and p53 tumor suppressor protein (4, 11). Instead, the elevation in the intracellular free calcium ([Ca$^{2+}$]) brought about by vitamin D compounds correlates with the induction of apoptosis in breast cancer cells (9, 12, 13).

Data from studies employing various pharmaceutical modulators of calcium homeostasis have suggested that the elevation in [Ca$^{2+}$] is a sufficient signal to induce apoptosis in several model systems, even though it may also have the opposite effect in other systems (14). Further supporting the idea that the elevation in [Ca$^{2+}$] may mediate apoptosis, studies based on modulated expression of calcium-binding proteins, calbindin-D$_{28k}$ or glucose-regulated proteins GRP78 and GRP94, have shown that Ca$^{2+}$ buffering can confer protection against various apoptotic stimuli (15–19). The calcium-dependent neutral cysteine proteases, calpains, are frequently activated in apoptosis models involving elevated [Ca$^{2+}$] (20–22).

Two forms of calpains, ?-calpain and m-calpain or type I and type II calpain, respectively, are ubiquitously expressed in human cells (23–25). The active forms of the enzymes consist of a variable large subunit (80 kDa) and a common small subunit (30 kDa). To become active, calpains require an elevation in [Ca$^{2+}$], and the autoproteolytic cleavage of the enzymes further enhances their activity. Whereas m-calpain requires Ca$^{2+}$ at a millimolar range, micromolar concentrations are enough for the activation of ?-calpain (in vitro; lower in cells). So far no difference in the substrate specificity of the two isozymes has been found. Growing evidence suggests that calpains may play a central role in the execution of apoptosis either upstream or downstream of the classical caspase pathway.
downstream of caspases in, e.g. glucocorticoid-treated and irradiated thyocytes, neuronal cells exposed to various stresses, or MCF-7 breast cancer cells treated with β-lapachone (20, 22, 26–29). Interestingly, caspases and calpains share several substrates (e.g. endogenous calpain inhibitor calpastatin, fodrins, focal adhesion kinase, calmodulin-dependent kinases, actin, vimentin, and keratins), suggesting that these enzymes may execute the cell in a partially indistinguishable manner (23). Calpains can also cleave other potentially interesting apoptosis-related proteins including caspase-12, Bax, Bcl-XL, GRP94, c-Fos, and p53 (19, 20, 30).

This study was undertaken to enlighten the poorly understood signaling pathway mediating vitamin D-induced caspase-independent death of MCF-7 breast cancer cells. The focus was centered on finding a responsible protease for this form of cell death and characterization with respect to the ultrastructural morphology of the dying cells.

**EXPERIMENTAL PROCEDURES**

**Compounds and Plasmids**—1,25(OH)2D3 and EB 1089 were generous gifts from Christina Maar Hansen and Lise Binderup (LEO Pharmaceutical Products, Ballerup, Denmark). Both compounds were stored at −20 °C and diluted in isopropyl alcohol. Control cells were always treated with the isopropyl alcohol vehicle (1% v/v). TNF was a generous gift from Anthony Cerami (Kenneth Warren Laboratories, Tarrytown, NY). Recombinant human TNF-related apoptosis-inducing ligand (Alexis Corp., San Diego) was used at 1 μg/ml in anti-FLAG M2 monoclonal antibody (F316S, Sigma). N-Acetyl-Leu-Leu-Ne-CO-CHO (calpain inhibitor I) dissolved in ethanol to 10 mM was used at 10 μM final concentration. N-Acetyl-Leu-Leu-Met-CO-CHO (calpain inhibitor II) dissolved in dimethyl sulfoxide to 20 mM was used at 20 μM final concentration. (2S,3S)-trans-Epoxy-succinyl-l-leucylamido-3-methylbutane ethyl ester (EST or E64d) dissolved in ethanol to 100 μM was used at 100 μM final concentration. PD 150606 dissolved in dimethyl sulfoxide to 50 mM was used at 50 μM. All were purchased from Calbiochem-Novabiochem Co. (Z-Van-Ala-i-Asp-fluoromethylketone (ZVAD-fmk) was dissolved in ethanol to 10 mM and used at 1 μM. The compound was purchased from Bachem (Bubendorf, Switzerland). Thapsigargin, Pluronic F-127 and fura-2/AM were from Molecular Probes (Eugene, OR). The pEBST-calbindin-D28k plasmid was created by subcloning a 1-kb EcoRI fragment of pGEM-calbindin (kindly provided by Dr. Diane Dowd, St. Louis University, St. Louis, MO) containing the sequence encoding for the complete chicken calbindin-D28k (31) into the pEBST plasmid.

**Cell Lines**—The MCF-7S8 subclone (34) of MCF-7 breast carcinoma cells with a sensitivity to vitamin D-induced apoptosis comparable with that of parental cells was used throughout the study (4, 10). The MCF-7S8 and MCF-7S8 calbindin28 cells are pools of MCF-7S8 cells transfected with the pEBS7-calbindin-D28k vector or with empty pEBS7 vector, respectively. MCF-S8 cells were transfected by electroporation (960 microfarads, 330 V) and selected for hygromycin (0.15 mg/ml) resistance. The cells were grown in RPMI 1640 with Glutamax (Invitrogen), 10% fetal calf serum, and 1% antibiotic–antimycotic (Glostrup, Denmark). The compound was dissolved in ethanol to 10 mM and used at 100 nM final concentration. Image and data analyses were performed as described previously (35, 36).

**RESULTS**

**Vitamin D-induced Apoptosis Is Mediated by Calpain**

Mobilization of Ca2+ from the ER stores was accomplished by using thapsigargin, which specifically inhibits ER Ca2+-ATPase and results in the rapid Ca2+ release from the ER. To measure Ca2+ release from the ER stores, 1 μM thapsigargin was added to the cell preparations after recording the basal [Ca2+]i for 1–3 min, and the peak values of the [Ca2+]i increase were measured. The fluorescence of the Ca2+ signal was followed at the end of preincubation experiments (35). Ca2+ was saturated at this concentration by adding 5 μM ionomycin in the presence of 5 mM Ca2+ and virtually zero Ca2+ by the further addition of 10 mM EGTA; 5 mM MnCl2 was subsequently added to obtain background fluorescence. The concentration of vehicle, dimethyl sulfoxide, never exceeded 0.1%; the vehicle did not affect [Ca2+]i at this concentration. Image and data analyses were performed as described previously (35, 36).

**Western Blot Analysis**—Immunoblot analysis of protein samples separated by SDS-PAGE was performed essentially as described before (11). Cell samples were separated by 8% (weight of fodiin, μ-calpain, calpastatin, and 70-kDa heat shock cognate protein, Hsc70) or 12% SDS-PAGE (calbindin-D28k). After electrophoresis the proteins were transferred onto a Hybond ECL membrane (Amersham Biosciences) in a trans-blot system (ECL Blotter, Amersham Biosciences, Arlington Heights, IL) for 3 h at 20 mA in 0.1% SDS, 10% glycine, 20% methanol) using a semidyblotter (Millipore, Amicon) and detected with the respective antibodies and enhanced chemiluminescence reagents (Amersham Biosciences). The primary antibodies used were mouse monoclonal antibodies against calbindin-D28k (1:100) (Sigma) (the estimated size of the detected protein was, however, double the expected size), fodrin (1:1,000) (Chemicon International Inc., Temecula, CA, USA), μ-calpain (1:1,000) (monoclonal antibody 3082, Chemicon), calpastatin (1:500) (monoclonal antibody 3084, Chemicon), and Hsc70 (1:8,000) (Borís A. Margulis, St. Petersburg, Russia). The appropriate peroxidase-conjugated secondary antibodies were from DAKO A/S (Glostrup, Denmark).

**Annexin V Staining and Analysis by FACS**—Cells (5 × 106 cells) suspended in phosphate-buffered saline and detached cells from medium, as well as cells detached by trypsinization, were collected, centrifuged, and washed twice in binding buffer (10 mM Heps pH 7.4, 150 mM NaCl, 5 mM KCl, 1 mM MgCl2, 1.8 mM CaCl2) before incubation in binding buffer supplemented with 1:100 annexin V (Roche Molecular Biochemicals) and 1 μg/ml propidium iodide for 20 min at room temperature protected from light. After staining, the cells were handled ice-cold, washed twice in phosphate-buffered saline with calcium and magnesium before analyzing using a Becton Dickinson flow cytometer. The amount of cells presented in the plots was 20,000.

**Transmission EM**—The cells were grown in Petri dishes and fixed for 30 min in 50% Karnovsky solution at 25 °C. Cells were collected by scraping with a rubber policeman, transferred to a centrifugation tube, and centrifuged at 1,500 × g for 5 min. After removal of the supernatant the pellet was washed once and subsequently stored in 70% Karnovsky solution at 4 °C until embedding. After two 10-min washings in 0.1 M cacodylate buffer and centrifugation as above, the pellet was embedded in agar, cut into smaller cubes, and fixed in osmium and embedded in Epon according to standard procedures. Ultrathin sections were collected on Formvar-coated copper grids, stained according to standard procedures, and examined in a Jeol 1210 electron microscope.

**RESULTS**

**MCF-7 Cells Expressing Calbindin-D28k Are Protected against Apoptosis Induced by 1,25(OH)2D3**—After treatment of MCF-7 cells with 1,25(OH)2D3, the appearance of apoptotic cells becomes evident first after 3–5 days (4). During this relatively long latent period, the cells are growth-arrested, and unknown cell changes may occur. Previous studies have shown that the treatment of breast cancer cells with vitamin D compounds results in the elevation of [Ca2+]i (9, 12, 13). To test whether this calcium signal plays a role in vitamin D-induced apoptosis, we introduced a cDNA encoding a calcium-binding protein, calbindin-D28k, into MCF-7 cells. Immunoblot analysis employing anti-calbindin-D28k antibodies did not reveal a detectable level of the protein in MCF-7 cells left untreated or treated with 100 nM 1,25(OH)2D3 (not shown). Analysis of the
MCF-7 cell pool transfected with pEBS7-calbindin-D_{28k} and selected for hygromycin resistance (MCF-calb28) revealed a single band detectable with the anti-calbindin-D_{28k} antibody (Fig. 1A). Based on the migration in the SDS-PAGE, the size of the detected protein was double the expected size (56 kDa). Because a single anti-calbindin-D_{28k}-reacting protein of the same size was also observed in MCF-7 cells transiently transfected with pEBS7-calbindin as well as in MG-63 osteosarcoma cells known to express calbindin-D_{28k} (16), but not in vector-transfected MCF-7 cells (MCF-cont), the detected protein was likely to be calbindin-D_{28k}. As analyzed by the MTT cell density assay, 1,25(OH)_{2}D_{3} treatment for up to 4 days resulted in similar growth inhibition in MCF-calb28 and MCF-cont cells (Fig. 1B). Interestingly, after day 4 of the treatment, the MCF-calb28 cells were, however, clearly more resistant to 1,25(OH)_{2}D_{3}, suggesting that the antiproliferative effect of the compound was unaffected by the presence of calbindin-D_{28k}, but the onset of apoptosis occurring after day 4 was inhibited. These data correlated with the microscopic examination showing clearly reduced appearance of cells with apoptotic morphology in MCF-calb28 cells compared with MCF-cont cells (not shown). Although MCF-calb28 cells were almost completely rescued from apoptosis induced by 10 nm 1,25(OH)_{2}D_{3}, only partial inhibition was observed when a 10× higher concentration of 1,25(OH)_{2}D_{3} was used. Contrary to this form of cell death, MCF-7 cell apoptosis induced by 1–10 ng/ml TNF or 10–100 ng/ml TNF-related apoptosis-inducing ligand was not affected by the ectopic expression of calbindin-D_{28k} (Fig. 1C and data not shown).

Calbindin-D_{28k} confers resistance to MCF-7 cell apoptosis induced by vitamin D compounds. A, Western blot analysis for calbindin-D_{28k} expression in the MCF-7 cells stably transfected with calbindin-D_{28k} (MCF-calb28) or empty vector (MCF-cont). MCF-7 cells transiently transfected with calbindin-D_{28k} (MCF-7 transient calb28) and the osteosarcoma cell line MG-63. B, time course of the relative cell density of MCF-calb28 cells (dashed lines) and MCF-cont cells (solid lines) treated with the indicated doses of 1,25(OH)_{2}D_{3} and compared with vehicle-treated cells. At day 6 there was a significant difference between densities of MCF-cont and MCF-calb28 cells treated with 100 nm (paired t test; p < 0.01) and 10 nm 1,25(OH)_{2}D_{3} (paired t test; p < 0.001). The cell density was analyzed by the MTT assay. The data are accumulated from four individual experiments with triplicate determinations. C, MCF-calb28 (gray bars) or MCF-cont (white bars) cells were treated for 48 h with 10 ng/ml TNF or 100 ng/ml TNF-related apoptosis-inducing ligand (TRAIL) before analysis of cell density by the MTT assay. The experiment was conducted twice with triplicate determinations. Error bars indicate S.D.

FIG. 1. Calbindin-D_{28k} confers resistance to MCF-7 cell apoptosis induced by vitamin D compounds. A, Western blot analysis for calbindin-D_{28k} expression in the MCF-7 cells stably transfected with calbindin-D_{28k} (MCF-calb28) or empty vector (MCF-cont). MCF-7 cells transiently transfected with calbindin-D_{28k} (MCF-7 transient calb28) and the osteosarcoma cell line MG-63. B, time course of the relative cell density of MCF-calb28 cells (dashed lines) and MCF-cont cells (solid lines) treated with the indicated doses of 1,25(OH)_{2}D_{3} and compared with vehicle-treated cells. At day 6 there was a significant difference between densities of MCF-cont and MCF-calb28 cells treated with 100 nm (paired t test; p < 0.01) and 10 nm 1,25(OH)_{2}D_{3} (paired t test; p < 0.001). The cell density was analyzed by the MTT assay. The data are accumulated from four individual experiments with triplicate determinations. C, MCF-calb28 (gray bars) or MCF-cont (white bars) cells were treated for 48 h with 10 ng/ml TNF or 100 ng/ml TNF-related apoptosis-inducing ligand (TRAIL) before analysis of cell density by the MTT assay. The experiment was conducted twice with triplicate determinations. Error bars indicate S.D.
Vitamin D-induced Apoptosis Is Mediated by Calpain

FIG. 2. Calbindin-D_{28k} inhibits the elevation in [Ca^{2+}]_{i} in MCF-7 cells treated with 1,25(OH)_{2}D_{3} or EB 1089. The kinetics of [Ca^{2+}]_{i} in MCF-cont and MCF-calb28 cells during treatment with vehicle (A), 100 nM 1,25(OH)_{2}D_{3} (B), or 100 nM EB 1089 (C) are shown. In both cell lines the treatment with vitamin D compound evoked a significant (t test, \( p < 0.05 \)) increase in [Ca^{2+}]_{i}, compared with vehicle-treated cells. Expression of calbindin-D_{28k} in the MCF-7 cells significantly (t test, \( p < 0.05 \), indicated with an asterisk) diminished the increased [Ca^{2+}]_{i} evoked by 1,25(OH)_{2}D_{3} or EB 1089. D, peak values of thapsigargin-evoked [Ca^{2+}]_{i} increase in MCF-cont (white bars) and MCF-calb28 (gray bars) cells treated for 1 day with vehicle, 1,25(OH)_{2}D_{3}, or EB 1089. In the MCF-calb28 cells the increase in [Ca^{2+}]_{i} was significantly diminished compared with MCF-cont cells. Asterisks indicate a significant (t test, \( p < 0.05 \)) difference from vector-transfected cells having the same treatment (*) or a significant difference from vehicle treatment in same cell line (**). E, relative values of thapsigargin-evoked [Ca^{2+}]_{i}, increase related to basal level of [Ca^{2+}]_{i}, in MCF-cont cells (A) expressed as a percent in E. In all samples thapsigargin resulted in Ca^{2+} release from the ER. Moreover, MCF-cont cells treated with 1,25(OH)_{2}D_{3} or EB 1089 displayed a significantly (t test, \( p < 0.05 \)) lower increase in [Ca^{2+}]_{i}, compared with vehicle-treated cells.

points (data not shown). This suggests that the buffering capacity of calbindin-D_{28k} in the MCF-calb28 cells is exceeded during the sustained treatment of the cells with vitamin D compounds. Overall, these data indicate that 1,25(OH)_{2}D_{3} or EB 1089 results in increased [Ca^{2+}]_{i}, by inducing a sustained release of Ca^{2+} from the ER. Furthermore, the forced expression of calbindin-D_{28k} increases the Ca^{2+} buffering capacity of the cells, thus partially counteracting the Ca^{2+}-mediated signaling induced by 1,25(OH)_{2}D_{3} and EB 1089.

Vitamin D Compounds Activate Calpain—To investigate whether calcium-dependent proteases, calpains, are activated during treatment of MCF-7 cells with vitamin D compounds, we analyzed protein samples from cells treated with 1,25(OH)_{2}D_{3} or EB 1089 for characteristic signs of calpain activity by immunoblot analysis. Although caspases and calpains share a number of substrates, including fodrin and calpastatin, their cleavage during treatment with 1,25(OH)_{2}D_{3} or EB 1089 in MCF-7 cells would indicate calpain activity because effector caspases are not activated in this model (4). As shown in Fig. 3, fodrin processing becomes evident in cells treated with vitamin D compounds for 4 days and increases at days 5 and 6. These data strongly suggest the presence of active calpain, and therefore we subsequently analyzed whether the \( \mu \)-calpain or the intracellular inhibitor of calpain, calpastatin, was cleaved (23). MCF-7 cells express a high level of the large subunit of \( \mu \)-calpain. In contrast to vehicle-treated control cells, cells treated with 1,25(OH)_{2}D_{3} or EB 1089 also contained a cleaved form of \( \mu \)-calpain, suggesting that the enzyme is, indeed, activated by vitamin D compounds (Fig. 3). Moreover, the level of the calpain substrate, calpastatin, was clearly reduced after 6 days treatment with 1,25(OH)_{2}D_{3} or EB 1089 (Fig. 3). Overall we conclude that the treatment of MCF-7 cells with 1,25(OH)_{2}D_{3} or EB 1089 leads to the activation of \( \mu \)-calpain occurring in parallel with the onset of apoptosis.

Calpain Inhibitors Partially Inhibit Apoptosis Induced by Vitamin D Compounds—The apoptosis-like cell death induced by vitamin D compounds is associated with DNA fragmentation (10). To test whether the activation of calpain is necessary for this nuclear sign of apoptosis, the effect of protease inhibitors on the degree of vitamin D-induced DNA fragmentation was analyzed by an enzyme-linked immunosorbent assay measuring histone-associated cytoplasmic DNA. As expected, the pancaspase inhibitor zVAD-fmk at a concentration specific for caspases (1 \( \mu \)M) could not inhibit the DNA fragmentation induced by a 6-day treatment with 100 nM 1,25(OH)_{2}D_{3} (Fig. 4A). A 10 \times higher concentration of zVAD-fmk was also without effect on the survival of 1,25(OH)_{2}D_{3}-treated MCF-7 cells (data not shown). On the contrary, three structurally different calpain inhibitors, calpain inhibitor II, EST, and PD 150606, all proved partially inhibitory (Fig. 4A). Also, the reduction in cell density in cultures treated with 100 nM 1,25(OH)_{2}D_{3} for 6 days was partially attenuated in the presence of calpain inhibitors (Fig. 4B). It should be noted that the treatment of the MCF-7 cells with vitamin D compounds reduces cell density in a 6-day assay by two mechanisms, i.e. first by the inhibition of proliferation and then by the induction of cell death (4). Therefore, only a partial protection against cell reduction can be expected even if the latter was completely inhibited; e.g. enforced expression of Bcl-2 that confers complete protection against apoptosis.
induced by vitamin D compounds results only in an ~70% increase in cell density (Ref. 4 and Fig. 4).

Studying annexin V staining of the cells clearly show that the treatment of MCF-7 cells with 100 nM 1,25(OH)2D3 or EB 1089 for 6 days results in increased phosphatidylserine exposure at the surface compared with the control cells (Fig. 4C, top panels). In these dot blots from the FACS analysis of annexin V-stained cells the percentages of cell counts in the two lower quadrants (cells not stained with propidium iodide, i.e. with intact cell membrane) are indicated with numbers. Vehicle-
noblot analysis of cleaved fodrin in proteins samples from MCF-7 cells treated with vitamin D compounds (A and B) and MCF-calb28 or MCF-cont cells (C) is shown. MCF-7 cells were treated for 6 days with vehicle (I), 100 nm 1,25(OH)2D3 (V), or 100 nm EB 1089 (E), alone or in combination with calpain inhibitor II (A) or PD 150606 (B) for the last 3 days. MCF-calb28 or MCF-cont cells were treated with the indicated doses of 1,25(OH)2D3 for 4 days (C). Detection of cleaved fodrin reveals a band of 150 kDa visible in some samples and attenuated in the presence of inhibitors. Lower panels, detection of HSC 70 (internal protein control). The experiments were repeated twice with similar results.

treated cells (left dot blot) displayed 4.6% cells in the quadrant, indicative of annexin V-positive propidium iodide-negative cells, whereas cells treated with 1,25(OH)2D3 (middle dot blot) or EB 1089 (right dot blot) displayed 9.6% or 13.6%, respectively. At later stages in this death process after the cells have been annexin V-positive only the membrane integrity is lost, and the cells become propidium iodide-positive too. Presenting the annexin V staining of the cells as a histogram with control and the cells become propidium iodide-positive too. Presenting the annexin V staining of the cells as a histogram with control.

Vitamin D-induced Apoptosis Is Mediated by Calpain

In addition to typical apoptotic changes, the EM of the dying MCF-7 cells after treatment with vitamin D compounds. Contrary to control cells (left), cells treated with either EB 1089 (right) or 1,25(OH)2D3 (middle) showed apoptosis-like morphology characterized by condensed fragmented chromatin marginalized along the nuclear envelope, condensed cytoplasm and condensed nuclei (Fig. 6). Furthermore, the dying cells had several light vesicles caused by dilated ER, an effect that could be expected during ER stress occurring after depletion of the calcium store. Such light vesicles were never present in control cells (Fig. 6).

DISCUSSION

In the present study we have identified a novel death pathway in breast carcinoma cells treated with 1,25(OH)2D3 or its chemotherapeutic analog EB 1089. This pathway involves Ca2+ release from the ER and μ-calpain activation. Despite the lack of the activation of known caspases, transmission EM images of the MCF-7 cells treated with vitamin D compounds demonstrated an apoptosis-like morphology characterized by condensed cytoplasm, nuclei, and chromatin. Compared with classical caspase-dependent apoptosis models displaying geometrical condensation of chromatin (38), chromatin of the cells treated with vitamin D compounds condensed to lumpier and less compact structures. Similar “incomplete” chromatin condensation has been observed in several other caspase-independent apoptosis models, implying that the activation of caspases may be required for the complete chromatin condensation (39–44). It should be noted, however, that also TNF-treated tumor cells (including MCF-7 cells) dying in a caspase-dependent manner show incomplete chromatin condensation (42, 45).

In addition to typical apoptotic changes, the EM of the dying cells revealed dilated ER, indicative of ER stress. The ER may participate in the initiation of apoptosis by at least two different mechanisms, i.e. Ca2+ signaling and unfolded protein re-
spontaneous (14, 46). Thus, it is interesting to note that the treatment of the MCF-7 cells with 1,25(OH)2D3 or EB 1089 for 3–5 days caused an elevation in [Ca2+]i. This was associated with a sustained reduction in the ER Ca2+ stores, suggesting that vitamin D compounds, indeed, induced a release of Ca2+ from the ER. The late occurrence of the calcium signal correlates well with previous literature, suggesting that vitamin D-induced changes in gene expression are needed for the Ca2+ release to occur in breast cancer cells (12, 13, 47). Furthermore, the kinetics of Ca2+ release correlated well with the onset of apoptosis suggesting that Ca2+ may play a direct role in the apoptosis induction. In support of this idea, the partial stabilization of [Ca2+]i by the enforced expression of calbindin-D28k partially protected cells against apoptosis induced by vitamin D compounds. Interestingly, modulation of ER Ca2+ fluxes has also been suggested to be one of the modes by which Bcl-2 inhibits apoptosis (49, 50). In line with this, Bcl-2 protects the MCF-7 cells and other cancer cells against apoptosis induced by vitamin D compounds (4, 51).

Although calbindin-D28k in some models has been shown to confer protection against apoptosis by directly inhibiting caspases, such a mode of action is, however, unlikely in our model system because the death is independent of known caspases (4, 16, 17, 52). Furthermore, MCF-7 cells expressing calbindin-D28k were as sensitive as the control cells to death receptor-induced caspase-dependent apoptosis. Instead of inhibiting caspases, the calbindin-D28k-mediated stabilization of [Ca2+]i inhibited the activation of a calcium-dependent neutral cytostine protease, calpain. The activation of calpain upon a 4–6–day treatment with vitamin D compounds was demonstrated by the cleavage of fodrin and calpastatin, which are specific markers for calpain activation in the absence of parallel effector caspase activation (23). Furthermore, cleavage of the large subunit of μ-calpain paralleled the cleavage of the substrates. Because no difference in the substrate specificity of the calpain isozymes has been found (23–25), we conclude that vitamin D compounds at least activated μ-calpain and possibly also other calpains.

The role of calpain in the apoptosis pathway was assessed by employing calpain inhibitors acting by binding either to the active site (calpain inhibitor II and EST) or to the Ca2+ binding site (PD 150606) of the enzyme (43). Remarkably, both types of inhibitors protected MCF-7 cells against DNA fragmentation and cell density reduction induced by vitamin D compounds. Furthermore, PD 150606 also inhibited increased phosphodiesterase exposure at the cell membrane after treatment with 1,25(OH)2D3 as detected by FACS analysis of annexin V-stained cells. It should be noted that calpain inhibitors conferred only a partial protection against apoptosis induced by vitamin D compounds. The lack of complete protection could be caused by subtoxic effects or too low a concentration of the inhibitors (all inhibitors displayed some toxicity during the long term application or at higher doses; not shown). The latter possibility is supported by the fact that the inhibitors reduced the activation of calpains only partially. Alternatively, other effector molecules in addition to calpains may be involved in this apoptosis pathway.

Except for the lack of geometrical condensation of chromatin, our data suggest that calpains can mimic caspases as the major execution proteases in apoptosis-like cell death induced by vitamin D compounds. Previously, calpains have been demonstrated to mediate differentiation and necrosis and to participate in caspase-dependent apoptosis pathways (19–23, 28, 29, 43, 53–55). In most apoptosis models calpains act upstream of caspases (43). Why calpain does not activate caspases in vitamin D-treated MCF-7 cells is as yet unclear. It is, however, unlikely to be the result of any dominant caspase inhibitor induced by vitamin D compounds because pretreatment of MCF-7 cells with 1,25(OH)2D3 does not inhibit but, in fact, enhances TNF-induced caspase activation (11, 56). Furthermore, it is not because of the lack of caspase-3 expression in MCF-7 cells. Vitamin D compounds also fail to induce any measurable effector caspase activity in MCF-7 cells expressing ectopic caspase-3 or in T47D cells expressing high levels of endogenous caspase-3 (4). Treatment of cancer with vitamin D compounds seems to be an attractive suggestion for the future, in particular for the treatment of cancers originating from cells in which calpains are readily activated, but indeed also for combination therapy with agents inducing different apoptotic pathways. The fact that vitamin D compounds induce a form of apoptosis different from the caspase-dependent pathway induced by the majority of anticancer drugs may prove very useful. Defects in caspase-dependent apoptosis signaling pathways are common in cancer cells (57). The problem of resistance may, of course, also hamper vitamin D-based therapies. Long term treatment with vitamin D compounds may in some cancer types induce resistance against themselves via the induced expression of calbindin-D28k (58). No induction of calbindin-D28k was, however, detected in MCF-7 cells treated with vitamin D compounds, indicating that this induction is not a universal phenomenon (not shown). Furthermore, putative resistance of renal cancer, osteoblastic tumors, as well as other malignancies with high constitutive expression levels of calbindin-D28k should be kept in mind. The use of combinations of therapeutic agents that trigger cell death by independent pathways may circumvent resistance problems. Promising in vitro data showing synergistic apoptosis-inducing effects of combination treatments employing vitamin D analogs with agents triggering other death pathways strongly support this idea (11, 48, 56).

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