Killing of Cancer Cells by the Photoactivatable Protein Kinase C Inhibitor, Calphostin C, Involves Induction of Endoplasmic Reticulum Stress

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Abstract
Calphostin C (cal-C) is a photoactivatable inhibitor that binds to the regulatory domain of protein kinase C (PKC) and to other proteins that contain diacylglycerol/phorbol ester binding sites. Cal-C is cytotoxic against many types of cancer cells, yet the basis for this activity remains poorly understood. Here, we show that one of the earliest effects of cal-C is an impairment of glycoprotein export from the endoplasmic reticulum (ER), accompanied by formation of ER-derived vacuoles. Vacuolization of the ER is correlated with induction of an ER stress response that includes activation of c-Jun N-terminal kinase and protein kinase R-like ER kinase, as well as increased expression of CCAAT/enhancer binding protein homologous transcription factor (CHOP; GADD153). These effects of cal-C are not mimicked by staurosporine, an inhibitor of PKC catalytic activity, indicating that ER stress is due to interaction of cal-C with targets other than PKC. In conjunction with the induction of ER stress, breast carcinoma cells undergo caspase-dependent cell death with early activation of caspases 9 and 7 and cleavage of poly(ADP-ribose)polymerase. Reduction of CHOP expression by short hairpin RNA decreases the sensitivity of the cells to cal-C, suggesting that induction of apoptosis by cal-C is related, at least in part, to ER stress triggered by disruption of ER morphology and transport function. Antineoplastic drugs that work by inducing ER stress have shown promise in preclinical and clinical trials. Thus, the present findings raise the possibility that cal-C may be useful for photodynamic therapy based on induction of ER stress in some forms of cancer.

Introduction
Protein kinase C (PKC) isoforms have been implicated as regulators of signaling pathways that promote proliferation, survival, metastasis, and drug resistance in cancer cells [1,2]. Elevated levels of PKC expression or activity have been noted in human malignancies such as gliomas [3], breast tumors [4], and metastatic gastric carcinoma [5]. Many investigations have explored the antineoplastic activity of PKC inhibitors, with particular emphasis on the indocarbazoles (e.g., staurosporine (STS)), which target the ATP binding site of PKC, and the macrocyclic lactones (e.g., bryostatin 1), which antagonize PKC activation and stimulate ubiquitin-mediated degradation [1,6]. Despite encouraging preclinical results, the efficacy of these compounds in clinical trials has been disappointing, possibly because of the number of functionally distinct PKC isoforms and the lack of kinase specificity [7].

The fungal perylenequinone, calphostin C (cal-C), was initially described as a potent inhibitor of PKC that operates through a novel mechanism, binding to a Ca2+-induced hydrophobic site on the PKC regulatory domain and preventing activation by diacylglycerol (DAG) and phorbol esters [8,9]. The inhibitory activity of cal-C is strictly dependent on photoexcitation, which causes irreversible site-specific oxidative modification of PKC [10,11]. This has raised the prospect...
that cal-C might be a useful agent for photodynamic cancer therapy [12]. Thus far, the evaluation of cal-C has been limited to preclinical studies. The results have established that this inhibitor can induce apoptosis in a broad spectrum of human cancer cell lines, including glioma [13–15], cervical carcinoma [16], lymphoblastic leukemia [17], prostate cancer [18], and nasopharyngeal carcinoma [12]. However, important questions about the molecular mechanisms underlying the cytotoxicity of cal-C remain to be answered. In particular, accumulating evidence suggests that the compound could operate through mechanisms that are independent of PKC. For example, cal-C can directly inhibit phospholipase D [19], and at concentrations that trigger apoptosis, it can cause a general increase in reactive oxygen species (ROS) [16], oxidation and degradation of lamin B1 [16], disassembly of Golgi membranes [20,21], inhibition of endocytosis [21], and mobilization of calcium from intracellular stores [17]. Further complicating matters, a study of the effects of cal-C in wild type and drug-resistant MCF-7 breast cancer cells indicated that the inhibitor can kill the cells by a novel mechanism involving the accumulation of cytoplasmic vacuoles of unknown origin [22]. In the present study, we have clarified the latter mechanism of calphostin-induced cell death by showing that the cytoplasmic vacuoles arise from the endoplasmic reticulum (ER) and that cell death is preceded by disruption of ER to Golgi trafficking and induction of a robust ER stress response. These findings suggest that cal-C may merit further evaluation as a potential photodynamic agent that could be used to inhibit tumor progression by stimulating ER stress.

Materials and Methods

Cell Culture and Transfection

MCF-7 breast carcinoma cells were a gift from A. Parisenti, northeastern Ontario Regional Cancer Centre. U251 glioblastoma cells were from the DCT Tumor Repository (National Cancer Institute, Bethesda, MD). 501 MEL cells were from the Cell Culture Core Facility at the Yale Skin Disease Research Center. All other cell lines were from the American Type Culture Collection (Rockville, MD). Cells were cultured at 37°C in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum with a 5% CO2 atmosphere. The pDsRed2-ER mammalian expression vector was purchased (Diagnostic Instruments, Inc, Sterling Heights, MI).

Generation of CCAAT/Enhancer Binding Protein Homologous Transcription Factor Knockdown MCF-7 Cells

CCAAT/Enhancer Binding Protein Homologous Transcription Factor (CHOP; GADD153) short hairpin RNA (shRNA) and control (scrambled) shRNA lentiviral particles were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). MCF-7 cells were infected with lentiviral particles as per the manufacturer’s protocol. The transduced cells were selected in medium containing 1 μg/ml puromycin for a week. Cells remaining after the selection were pooled and maintained continuously in medium containing puromycin for all subsequent experiments.

Drug Treatments and Photoactivation of Cal-C

Calphostin C (Tocris, Ellissive, MO) was dissolved in dimethylsulfoxide (DMSO). Cells were preincubated in medium containing cal-C in the dark at 37°C for 30 minutes, followed by exposure to a 30-W fluorescent light at 3 inches from the source for another 30 minutes to activate cal-C. Time points indicated in the figures start after the light was switched off. Control dishes were incubated with an equivalent volume of DMSO under the same conditions. During cotreatments, cal-C and z-VAD-fmk (Bachem Bioscience, Inc, King of Prussia, PA) or z-LEVD-fmk (BioVision, Inc, Mountain View, CA) were added simultaneously to the medium. The cultures were then preincubated at 37°C for 1 hour, followed by photoactivation of cal-C, as described previously. In some experiments, cells were treated with 500 nM STS (Cayman Chemical Company, Ann Arbor, MI) or 2.5 μM brefeldin A (Sigma, St Louis, MO) as noted in the figure legends.

Cell Viability

Cell viability was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT)–based cell growth determination kit from Sigma following the manufacturer’s protocol. The MTT assays were performed in quadruplicate wells of a 96-well plate 24 hours after photoactivation of cal-C. The absorbance at 570 nm was measured with a SpectraMax Plus plate reader (Molecular Devices, Sunnyvale, CA). Statistical significance of differences between wells treated with a specific drug concentration compared with the control wells was assessed by Student’s t test. Half-maximal inhibitory concentration (IC50) values for cal-C in different cell lines were determined by performing MTT assays on cells exposed to drug concentrations of 0, 1, 5, 10, 25, 50,100, and 150 nM for 24 hours. IC50 values were calculated from linear regression analysis of sigmoidal dose-response curves plotted with GraphPad Prism software (La Jolla, CA).

For colony-forming assays, cells were harvested 24 hours after treatment with 50 nM cal-C or DMSO. Attached cells were harvested by trypsinization, pooled with the detached cells, counted, and replated in 100-mm-diameter dishes. MCF-7 and PANC-1 cells were seeded at 1600 cells per dish, and U251 cells were plated at 1400 cells per dish. The medium was replaced every 3 to 4 days. Colonies were stained and counted after 2 weeks for U251 cells and 3 weeks for MCF-7 and PANC-1 cells, as described [23].

Western Blot Analysis

The following antibodies used for Western blot analysis were purchased: c-Jun N-terminal kinase (JNK), phospho-JNK, caspase 7, and caspase 9 (Cell Signaling Technology, Danvers, MA); protein kinase R-like ER kinase (PERK), phospho-PERK and CHOP (Santa Cruz Biotechnology); poly(ADP-ribose)polymerase (PARP; BD Pharmingen, San Jose, CA); caspase 4 (MBL International, Woburn, MA); and α-tubulin (Sigma). The BX6 antibody against the β-amyloid precursor protein (β-APP) was a gift from Elan Pharmaceuticals (South San Francisco, CA). Cells were lysed directly in SDS sample buffer [24], and protein concentrations were determined using the Bio-Rad colorimetric assay (Bio-Rad Laboratories, Hercules, CA). Aliquots of lysate containing equal amounts of protein were subjected to SDS-
PGE and immunoblot analysis using the procedures for chemiluminescence detection described previously [25]. Chemiluminescence signals were quantified using an imaging system (FluorChem HD2; Alpha Innotech, San Leandro, CA).

Annexin V Assay

Attached cells were harvested by trypsinization and pooled with any detached cells in the medium. The cells were then stained with the Guava Nexin reagent (Guava Technologies, Hayward, CA) containing premixed annexin V–PE and 7-amino-actinomycin D following the manufacturer’s protocol. Annexin V–positive cells were quantified by flow cytometry using a Guava Personal Cytometer.

Metabolic Labeling and Immunoprecipitation of β-APP

MCF-7 cells were washed twice with methionine-free DMEM and incubated with either 50 nM cal-C or DMSO in methionine-free DMEM, first for 30 minutes in the dark at 37°C and then exposed to a 30-W fluorescent light source for 30 minutes. For pulse labeling, 100 μCi/ml [35S] methionine/cysteine mix (Easy Tag EXPRESS; NEN/Perkin Elmer, Waltham, MA) was added to the cultures, and cells were incubated at 37°C for 30 minutes. The “pulse” samples were harvested immediately. Parallel cultures (the “chase” samples) were washed with PBS and incubated in DMEM containing 10% fetal bovine serum, 200 μM methionine, and 200 μM cysteine, with or without cal-C. The cultures were exposed to light for 30 minutes after addition of the chase medium containing fresh cal-C and then incubated at 37°C for 1.5 hours (2 hours of total chase period). Cells were harvested and lysed in PBS containing 1.0% Triton X-100 and protease inhibitor cocktail (Roche), and radiolabeled β-APP was immunoprecipitated as described previously [26], using the anti-BX6 polyclonal antibody [27]. Immunocomplexes were collected by incubation for 1 hour at 4°C with protein A-Sepharose (Amersham Biosciences, Uppsala, Sweden) coupled to goat anti–rabbit immunoglobulin G (MP Biomedicals, Inc, Solon, OH). Immunoprecipitated proteins were subjected to SDS-PAGE and fluorography as described [26]. The relative amounts of mature and immature β-APP were determined by densitometry using a Kodak 440CF image station (Eastman Kodak Co, Rochester, NY).

Fluorescence Microscopy

MCF-7 cells were grown on glass coverslips for 24 hours and treated with 50 nM cal-C or DMSO as described previously. Two hours after cal-C treatment, cells were washed with Hank’s balanced salt solution, fixed in ice-cold methanol for 10 minutes, and blocked with 10% goat serum in PBS. For visualization of the Golgi apparatus, cells were incubated for 1 hour with the anti-GM130 antibody (BD Transduction Laboratories, San Jose, CA) diluted in PBS + 10% goat serum, followed by incubation with goat anti–mouse immunoglobulin G conjugated with Alexa Fluor 568 (Molecular Probes/Invitrogen, Eugene, OR). Images were obtained using an Olympus IX70 fluorescence microscope equipped with a digital camera. Merger of phase-contrast and fluorescent images was accomplished with the SPOT imaging software.

For visualization of acidic lysosomal or late endosomal compartments, cells were incubated with LysoTracker Red DND-99 (Invitrogen Corp, Carlsbad, CA) as described previously [23], and live cell imaging was performed as described previously for DsRed.

Statistical Analysis

All data are expressed as mean ± SD. Results were analyzed with Student’s t test for unpaired samples using the GraphPad Prism software. P ≤ .05 was considered as statistically significant.

Results

Cytoplasmic Vacuolization Precedes Loss of Viability in Tumor Cell Lines Treated with Activated Cal-C

Exposure of MCF-7 breast carcinoma cells to photoactivated cal-C in the concentration range of 15 to 100 nM caused a significant reduction in cell viability within 24 hours as determined by the MTT cytotoxicity assay (Figure 1A). The effects of cal-C on cell viability were confirmed by colony-forming assays (Figure 1B). Loss of viability was accompanied by increased cleavage of the caspase substrate, PARP, which was detectable as early as 2 hours after termination of the light activation of cal-C (Figure 1C). The 85-kDa PARP fragment was identical in size to that generated in cells treated with STS, a known PKC inhibitor and a potent inducer of apoptosis (Figure 1D). As reported previously [22], death of the MCF-7 cells treated with cal-C was preceded by extensive cytoplasmic vacuolization (Figure 2A). We were able to detect the accumulation of phase-lucent vacuoles within 1 to 3 hours after activation of cal-C. If the photoactivation of cal-C was omitted, vacuoles did not form, and there was no increase in cell death (Figure 2B). Shortly after the onset of vacuolization, some of the cells began to detach from the surface of the culture dish, so that by 24 hours, more than half of the cells in the culture were floating. Examination of PARP cleavage in the detached versus attached cell populations (Figure 1E) indicated that most of the floating cells were undergoing cell death. Unlike the cells treated with cal-C, MCF-7 cells treated with STS rounded and detached from the dish but did not undergo vacuolization (Figure 2B).

To determine whether early cytoplasmic vacuolization is a general characteristic of cal-C treatment in other types of cancer cells, we exposed U251 glioblastoma and PANC-1 pancreatic carcinoma cells to similar concentrations of the compound. In both cases, vacuoles accumulated within 3 hours (Figure 2A), with substantial loss of cell viability (Figure 1, A and B) and increased cleavage of PARP (Figure 1, D and E) occurring by 24 hours. A broader survey of established cancer cell lines indicated that cal-C generally induced substantial cytotoxicity within 24 hours, with IC50 values ranging from 15 to 37 nM (Table 1). Most of the adherent cell lines exhibited extensive vacuolization, but a few did not (e.g., SW480, SKOV3).

Cytoplasmic Vacuoles Are Derived from the ER

The close temporal correlation between the onset of cytoplasmic vacuolization (Figure 2A) and PARP cleavage (Figure 1C) prompted us to conduct further studies with MCF-7 cells to establish the origin of the vacuoles induced by cal-C. Phase-lucent vacuoles can arise from various subcellular compartments. For example, inhibition of the class III phosphatidylinositol-3-kinase, Vps34, causes swelling and vacuolization of late endosomes [28,29], whereas accretion of misfolded proteins in cells treated with inhibitors of Hsp90 and the ubiquitin-proteasome pathway causes distension of the ER [30]. Most recently, we described a novel cell death pathway in which cells are disrupted by the accumulation of large fluid-filled vacuoles derived from macropinosomes [23].

To test the hypothesis that the vacuoles induced by cal-C are derived from the ER, we transfected MCF-7 cells with a vector encoding a red
fluorescent protein (DsRed) that contains specific ER targeting and retention sequences. In nontreated MCF-7 cells, the marker was localized diffusely in the perinuclear region, a pattern typical for the ER (Figure 3A). However, within 3 hours after light activation of cal-C, the DsRed-ER was concentrated in discrete globular structures that matched the lucent vacuoles observed by phase-contrast microscopy (Figure 3A). In contrast to these results, the phase-lucent vacuoles induced by cal-C did not incorporate the late endosome/lysosome tracer, LysoTracker Red (Figure 3B). Moreover, these structures did not internalize detectable quantities of fluid-phase tracers such as Lucifer yellow or Dextran–Alexa Fluor 488 (not shown). Essentially identical results were obtained when the studies were performed with U251 and PANc-1 cells. These findings support the conclusion that the cytoplasmic vacuoles induced by cal-C are formed by dilation of ER cisternae.

**Induction of Vacuoles by Cal-C Is Accompanied by ER Stress**

In light of the known association between accumulation of unfolded proteins and swelling of the ER, we undertook a series of studies with MCF-7 cells to determine whether cal-C induces a prototypical ER stress response concurrent with vacuolization. One of the earliest signposts of ER stress is the phosphorylation of the stress-activated kinase, JNK. JNK activation seems to be mediated through a signaling cascade triggered by inositol-requiring enzyme 1 (IRE1), an ER resident kinase/endoribonuclease that senses accumulation of misfolded proteins [31,32]. As shown in Figure 4A, treatment of MCF-7 cells with cal-C caused a spike in JNK phosphorylation that was maximal at 15 minutes to 1 hour. The level of JNK phosphorylation tapered off slightly after 1 hour, consistent with a report that cells respond to unmitigated ER stress by first activating and then attenuating IRE1 and JNK [32].

A second major branch of the ER stress response involves the activation of PERK, a transmembrane protein kinase. PERK is phosphorylated in response to ER stress, resulting in the activation of its cytoplasmic kinase domain, which targets eukaryotic initiation factor 2α and attenuates translation [33]. Activation of PERK is sustained with prolonged ER stress [32]. Figure 4B shows that within 15 minutes after activation of cal-C, phosphorylation of PERK was increased to approximately twice the level seen under baseline culture conditions and remained elevated for the duration of the 4-hour experiment.

The preceding studies were extended by examining the expression of CHOP (GADD153), a bZIP transcription factor that is upregulated during the ER stress response [34] and is implicated in apoptosis...
caused by ER stress [35]. As shown in Figure 5A, treatment of MCF-7 cells with cal-C caused a substantial increase in CHOP commencing around 2 hours, coinciding with the timing of cytoplasmic vacuolization and the first detectable increase in PARP cleavage (Figure 1C). In contrast to the robust increase in CHOP triggered by cal-C, STS, which targets the catalytic domain of PKC, did not increase the expression of CHOP even after 18 hours (Figure 5A). To determine whether induction of CHOP was required for subsequent death of the cells treated with cal-C, we used a lentiviral vector to introduce shRNA directed against CHOP into MCF-7 cells. As shown by the Western blots in Figure 5B, the induction of CHOP was reduced by approximately 70% when these cells were treated with cal-C for 24 hours. Parallel cytotoxicity assays indicated that suppression of CHOP expression was able to partially protect the cal-C–treated cells from the loss of cell viability (Figure 5C).

Death of Calphostin-Treated Cells Is Caspase-Dependent
The close temporal correlation between ER vacuolization, induction of ER-stress markers, and onset of PARP cleavage in MCF-7 cells treated with cal-C suggests that, contrary to a previous report [22], cell death is mediated by a caspase-dependent apoptotic pathway. To directly test the importance of caspase activation in the death of cal-C–treated MCF-7 cells, we added the broad-spectrum caspase inhibitor, z-VAD-fmk together with 50 nM activated cal-C for 24 hours. As shown in Figure 6 (A and B), z-VAD effectively inhibited the cleavage of PARP and significantly increased cell survival. Similar results were obtained with U251 and PANC-1 cells and with a separate MCF-7 cell line obtained directly from the American Type Culture Collection (not shown).

Independent evidence that the cal-C–treated MCF-7 cells were undergoing apoptosis was obtained by staining them with the apoptosis marker, annexin V. Cal-C caused an increase in the percentage of annexin V–positive MCF-7 cells, similar to what was observed in cultures treated with STS (Figure 6C).

Cal-C has been reported to induce rapid activation of mitochondrial death pathways and cleavage of procaspase 3 in several cell lines [36,37]. Separate studies have implicated the mitochondrial apoptosome and caspase 3 as important mediators of ER stress–induced apoptosis [38,39]. Consistent with these reports, we observed substantial activation of the mitochondrial initiator caspase, caspase 9, within 1 hour after photoactivation of cal-C (Figure 7A). This coincided with the initial appearance of vacuoles (Figure 2A) and the early spike in JNK and PERK phosphorylation (Figure 4, A and B). By comparison, STS, which targets the catalytic site of PKC but does not cause ER vacuolization or ER stress, did not begin to cause procaspase 9 cleavage until 2 hours (Figure 7A).

| Table 1. IC50 Determined for Cal-C in Various Cell Lines. |
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| Cell Line | Vacuolization | IC50 (nM) |
| MCF-7 (breast carcinoma) | + | 16.7 |
| ZR-75 (breast carcinoma) | + | 31.9 |
| U251 (glioblastoma) | + | 16.4 |
| T98G (glioblastoma) | + | 26.6 |
| 501 MEL (melanoma) | + | 12.9 |
| U2OS (osteosarcoma) | + | 18.4 |
| PANC-1 (pancreatic carcinoma) | + | 24.9 |
| SW480 (colon carcinoma) | – | 14.7 |
| SKOV3 (ovarian carcinoma) | – | 37.1 |
| CaOV3 (ovarian carcinoma) | – | 29.8 |

Each cell line was treated with graded concentrations of cal-C for 24 hours, and the IC50 was calculated from the results of the MTT assay. Separate cultures of each cell line were treated with cal-C at concentrations of 30 to 50 nM and observed by phase-contrast microscopy after 3 hours to determine whether cytoplasmic vacuolization occurred.

Figure 2. Calphostin induces early accumulation of cytoplasmic vacuoles in multiple tumor cell lines. (A) MCF-7 and PANC-1 cells were treated with 50 nM cal-C. U251 cells were treated with 30 nM cal-C. Parallel control dishes were incubated with vehicle (DMSO). (B) MCF-7 cells were incubated with 50 nM cal-C, 500 nM STS, or DMSO (control) for 3 hours, with or without a 30-minute light activation step. Phase-contrast images (original magnification, ×400) of live cells were obtained after 3 hours.

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Because MCF-7 cells lack the executioner caspase, caspase 3, we examined the activation of caspase 7. The latter is widely viewed as an alternative executioner caspase that can act downstream from caspase 9 [40,41], but it has also been implicated as an initiator caspase in the ER [42]. As shown in Figure 7B, the time course of procaspase 7 cleavage after treatment with cal-C closely matched that observed for procaspase 9 (Figure 7A).

Finally, we examined the effects of cal-C on caspase 4. Some studies have pointed to a role for caspase 4 in ER stress–induced cell death [43,44], whereas others have argued that caspase 4 is not required [45]. We observed an increase in procaspase 4 cleavage by 24 hours after addition of cal-C, although the increase was not as great as that seen with brefeldin A, a commonly used inducer of the ER stress response (Figure 7C). However, we did not find a consistent increase in cleavage of procaspase 4 above baseline levels during the critical early period (1–4 hours) when ER vacuolization and ER stress markers were induced (Figure 7C). In agreement with these observations, the inhibitor, z-LEVD-fmk, used at a concentration reported to specifically

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**Figure 3.** Vacuoles induced by cal-C are derived from the ER. (A) MCF-7 cells were transfected with the pDsRed2-ER vector as described in the Materials and Methods section. Twenty-four hours after transfection, the cells were treated with 50 nM cal-C or DMSO (control). Live-cell phase-contrast images and fluorescent images showing the localization of the DsRed-ER protein (original magnification, ×400) were obtained 3 hours after activation of cal-C. (B) Untransfected MCF-7 cells were treated with cal-C as in panel A, and cells were incubated with LysoTracker Red for 1 hour before phase-contrast microscopy and fluorescence imaging.

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**Figure 4.** Cal-C treatment induces an ER stress response concurrent with ER vacuolization. (A) MCF-7 cells were harvested at the indicated times after treatment with 50 nM cal-C, and equal amounts of protein were subjected to immunoblot analysis for phospho-JNK (Thr183/Tyr185) or total JNK. Tubulin served as a loading control. The ratio of phospho to total proteins was determined by quantifying the immunoblot signals with the FluorChem HD2 imaging system (Alpha Innotech). The upper panel depicts a representative immunoblot of phospho-JNK and total JNK. The bar graph shows the mean (± SD) of the ratio of phospho-JNK to total JNK determined from three cultures. Asterisks denote values that were significantly increased relative to the 0-hour time point (*P* ≤ .01). (B) Immunoblot showing the time course of PERK (Thr981) phosphorylation after treatment with cal-C. The ratios of phospho-PERK to total PERK (mean ± SD) determined from three cultures are shown in the bar graph. Asterisks denote values that were significantly increased relative to the 0-hour time point (*P* ≤ .02).
block caspase 4 [46,47], failed to protect MCF-7 cells from cal-C–induced cell death (Figure 7D).

**Protein Trafficking between the ER and Golgi Compartment Is Blocked by Cal-C**

The observation that ER vacuolization and stress were caused by cal-C, but not STS, suggested that the induction of ER stress could be due to PKC-independent effects of cal-C. In this regard, our attention was drawn to an early study showing that cal-C, used at concentrations similar to those used in the present study, can block export of proteins from the ER in a PKC-independent manner [48]. At significantly higher concentrations (>500 nM), cal-C can also cause disassembly of the Golgi apparatus [20,48]. To determine whether the swelling of the ER observed within 1 to 2 hours after adding cal-C might reflect an underlying inhibition of protein export from the ER, we took advantage of the fact that MCF-7 cells, like most other cultured cell lines, express measurable amounts of the Alzheimer β-amyloid precursor protein (β-APP). The latter has been used as an endogenous tracer to detect perturbations in vesicular trafficking of proteins from the ER to the Golgi compartment [26,49].

β-APP is a type 1 transmembrane glycoprotein that exhibits an increase in its apparent molecular mass on SDS gels after it is exported from the ER and enters the Golgi apparatus. The mobility shift is due to O-glycosylation of the exodomain [27,50]. The modified protein is...
then further processed by endoproteases (α- or β-secretases) that cleave off the glycosylated exodomain, which is secreted as soluble APP. Subsequent proteolytic events catalyzed by γ-secretases can result in the formation of amyloid β peptides from the residual C-terminal membrane–anchored remnant of the protein [51]. To determine whether cal-C causes an early block in export of β-APP from the ER, we performed a pulse-chase study depicted in Figure 8. After a 30-minute pulse with 35S-methionine, the radiolabeled β-APP immunoprecipitated from control cells migrated predominantly as the immature (approximately 115 kDa) ER resident form, with a lesser amount of β-APP migrating as the mature Golgi form (approximately 135 kDa; Figure 8A, left panel). A similar distribution was observed for β-APP when cal-C was included during the 30-minute pulse, although there was a decrease in overall 35S-methionine incorporation into β-APP, possibly because of a decline in the rate of protein synthesis. The key observation is that when the cells were chased in the absence of 35S-methionine for an additional 2 hours to allow time for the immature β-APP to be exported to the Golgi apparatus, a significant impairment of β-APP maturation was noted in cells treated with cal-C compared with the controls (Figure 8A, right panel). In the control cells, the movement of the labeled β-APP into the Golgi compartment was manifested by a marked decrease in intensity of the radiolabeled immature band, so that the ratio of mature to immature β-APP increased significantly (Figure 8B). In contrast, the immature ER form of β-APP remained the predominant species in the cells exposed to cal-C (Figure 8A, right panel), and the ratio of the mature Golgi to the immature ER form at the end of the chase was essentially unchanged compared with the ratio at the end of the 30-minute pulse (Figure 8B). These findings are similar to what happens to processing of newly synthesized β-APP when cells express a dominant-negative form of the Rab1b GTPase, which regulates vesicular trafficking between the ER and Golgi compartments [26].

Western blot analyses of the steady state levels of β-APP in control-versus cal-C–treated cells were consistent with the results of the pulse-chase experiment (Figure 8C). During the first 30 minutes, the total amounts of mature and immature forms of β-APP in cells treated with cal-C were modestly reduced compared with the controls, but both forms were readily detected. However, by 2 hours after activation of cal-C, there was a notable loss of the mature form of β-APP. This is exactly what one would expect if the preexisting pool of mature β-APP continues to be proteolytically processed and secreted, whereas the block in ER export of immature β-APP prevents replenishment of the mature β-APP pool.

Taken together, the results of the preceding studies indicate that cal-C causes a rapid arrest of ER → Golgi protein trafficking in MCF-7 cells. To determine whether this could be related to the effects of cal-C on integrity of the Golgi stack, we performed immunofluorescence localization of the Golgi marker, GM130. This revealed a similar perinuclear Golgi staining pattern in both control- and cal-C–treated cells (Figure 8D). Some cells exhibited a discrete juxtanuclear cluster of GM130, whereas others showed a more dispersed staining pattern. However, the distended ER-derived vacuoles in the cal-C–treated cells were not stained with anti-GM130, suggesting that the Golgi stack did not collapse into the ER. This implies that the observed block in ER → Golgi trafficking of β-APP is most likely related to functional impairment of the vesicular transport mechanism, rather than complete disruption of the Golgi architecture, which has been reported to occur at much higher cal-C concentrations [20,21,48].

**Discussion**

The present study provides several new insights into the previously reported anticancer activity of the photoactivatable perylenequinone, cal-C. First, we show that the tendency of cal-C to cause rapid cytoplasmic vacuolization, initially reported only in breast cancer cells [22], extends to several other cancer cell lines and thus represents a general characteristic of this compound. Second, we establish that the vacuoles induced by cal-C arise from dilated ER cisternae. Third, we
show that early vacuolization of the ER is accompanied by a perturbation of ER → Golgi protein trafficking and a concurrent induction of the ER stress response. Finally, we demonstrate a very close temporal correlation between ER vacuolization, induction of the stress response, and the early onset of caspase-dependent apoptosis. On the basis of these observations, we suggest a mechanism to explain the potent cytotoxic effects of cal-C in neoplastic cells. Specifically, we postulate that cal-C initially inhibits proteins with phorbol ester/DAG binding sites that control vesicular trafficking out of the ER. The resulting block in protein and membrane export leads to the distention of the ER and to the accumulation of misfolded or aggregated proteins, which trigger an ER stress response. Unabated ER stress, probably compounded by additional effects of cal-C related to inhibition of PKC, finally results in activation of a caspase-mediated apoptotic death pathway.

In the original report describing cal-C–mediated vacuolization and killing of parental and drug-resistant MCF-7 cell lines, cleavage of PARP and mitochondrial cytochrome c release were not observed, suggesting a nonapoptotic mechanism of cell death [22]. However, in our studies, we clearly observed PARP cleavage, caspase activation, increased staining with annexin V, and protection with z-VAD. One possible explanation for this discrepancy may lie in the methods used for harvesting the cells. In our studies, all of the PARP and caspase assays were done with pooled attached and floating cells to avoid losing detached cells that accounted for most of the apoptotic population (Figure 1E). Our findings agree with numerous reports indicating that cal-C triggers caspase-dependent apoptosis in other types of cells [12,13,17,36].

Our observation that STS, a potent inhibitor of PKC, did not stimulate ER vacuolization or induction of CHOP expression strongly suggests that the initial effects of cal-C on ER function are related to the interaction with targets other than PKC. One possibility, raised by the work of Sciorra et al. [19], is that the effects of cal-C on protein export are due to binding to the catalytic domain of phospholipase D1, a known regulator of ER → Golgi trafficking [52]. An alternative possibility is that the effects of cal-C could be mediated by disruption of calcium homeostasis. For example, in acute lymphoblastic leukemia cells, the cytotoxic effect of cal-C correlates with rapid calcium mobilization from intracellular stores and seems to be independent from inhibition of PKC [17]. A third possibility, suggested by the early work of Fabbri et al. [48], is that cal-C disrupts vesicular protein export from the ER through the interaction with novel DAG/phorbol ester-binding

Figure 8. Export of an endogenous type I membrane glycoprotein (β-APP) from the ER is impaired in MCF-7 cells treated with cal-C. Control- and cal-C–treated MCF-7 cells were labeled with [35S]methionine for 30 minutes. One dish from each condition was harvested immediately (pulse), and the other set was chased for 2 hours in medium containing excess unlabeled methionine. β-APP was immunoprecipitated as described in the Materials and Methods section. (A) The pulse and chase samples were run on separate gels and subjected to fluorography. The images are representative of results obtained from three separate cultures. (B) The bar graph shows the densitometric ratio of mature (135 kDa) to immature (115 kDa) β-APP determined in the pulse and chase samples. The results are expressed as mean (± SD) determined from three separate cultures. The asterisk denotes a significant increase at \( P = .01 \). (C) Western blot showing total mature and immature β-APP. Cells maintained with or without 50 nM cal-C were harvested 30 minutes or 2 hours after light activation. Equal amounts of the whole-cell lysates were subjected to immunoblot analysis for β-APP with tubulin as a loading control. (D) MCF-7 cells were incubated with cal-C or DMSO for 2 hours after light activation and then processed for immunofluorescence detection of the Golgi apparatus using an antibody against GM130. The immunofluorescent images were merged with the phase-contrast images of the fixed cells.
proteins that have yet to be identified. Finally, we cannot completely rule out the possibility that ER vacuolization triggered by cal-C is related to a general increase in cellular ROS [16], which could lead to protein misfolding in the ER. However, with respect to the latter possibility, we note that addition of the ROS inhibitor, N-acetylcysteine, at concentrations as high as 50 mM, did not prevent ER vacuolization (data not shown).

As noted previously, STS did not mimic the effects of cal-C on ER vacuolization and stress response. Nevertheless, it was clearly able to induce caspase 9 activation and apoptosis. Therefore, although the disruption of ER morphology and protein export by cal-C is probably due to the inhibition of a non-PKC target, the subsequent apoptosis could be related to composite effects of cal-C mediated through both direct inhibition of PKC and PKC-independent ER stress. We found that cleavage of procaspase 9 (Figure 7A) occurs very soon after photoactivation of cal-C, implying early mitochondrial involvement in the apoptotic response. This could be triggered by the rapid induction of the mitochondrial permeability transition, as noted in previous studies with STS [53,54]. At the same time, the close temporal correlation of caspase 9 activation and PARP cleavage with swelling of the ER and activation of JNK, PERK, and CHOP could support a model wherein apoptosis induced by cal-C is driven by unmitigated ER stress. These mechanisms need not be mutually exclusive. In fact, the probable multifactorial nature of the death pathway induced by cal-C is supported by our observation that interference with one key effector of the ER stress response (e.g., CHOP), offered only partial protection from cell death (Figure 5).

The exact sequence of molecular events that occurs between the onset of ER stress and the activation of a mitochondrial apoptotic response remains incompletely understood [55–57]. One possibility is that apoptosis is mediated by down-regulation of Bcl-2 and Bcl-XL [58], with concomitant oligomerization of Bax or Bad on the ER or mitochondria [59]. This scenario would fit with a study in glioma cells showing that cal-C stimulates rapid integration of Bax into mitochondrial membranes, followed by cytochrome c release [36]. The early spike in activation of JNK in cells treated with cal-C (Figure 4A) raises a second possibility that mitochondrial injury might be caused by the ability of STS to stimulate cleavage of Bid [60] or activation of other BH3-only Bcl-2 family members such as Bim or Bmf [61]. Finally, it is conceivable that ER stress caused by cal-C could trigger apoptosis through local activation of an initiator caspase in the ER. Procaspase 12 has been reported to localize in the ER and undergo cleavage in response to ER stress in rodent cells [62]. However, a specific requirement for caspase 12 for subsequent apoptosis remains controversial [38,45,63]. Caspase 12 is truncated or inactive in human breast tissue [64], and the related caspase 4 has been proposed to function as an alternate ER stress–sensitive initiator of apoptosis [43,44]. We did not observe a consistent increase in caspase 4 activation during the early period when cal-C triggered caspase 9 activation and PARP cleavage. Thus, it seems unlikely that caspase 4 plays a key role as an initiator caspase in the apoptotic response induced by cal-C. Caspase 7 is traditionally regarded as an executioner caspase that functions downstream from proapoptotic signals emanating from the mitochondria [40]. However, it has also been proposed to function as an ER-localized initiator caspase that can respond to ER stress by promoting cleavage of procaspase 12 [42] and, by inference, caspase 4. We observed the activation of caspase 7 together with activation of caspase 9 (Figure 7) and cleavage of PARP (Figure 1) within 1 to 2 hours after photoactivation of cal-C, well before increased cleavage of procaspase 4. This timing would be most consistent with caspase 7 acting as an executioner caspase in concert with activation of caspase 9.

The findings of the present study could have important implications for the design of new cancer therapeutic strategies aimed at exploiting the ER stress response. Bortezomib, which causes accumulation of misfolded proteins in the ER by blocking the activity of the 26S proteasome, has already been approved for the treatment of multiple myeloma and is being evaluated for efficacy against other malignancies [65–67]. Other ER stress inducers have shown promise in preclinical trials. For example, 2,5-dimethyl-4-oxazoline, which is inactive against cyclooxygenase-2 but causes ER stress by increasing intracellular free calcium [68], has proven to be effective in reducing the growth and survival of several different types of cancer in cell culture and animal models [69,70]. In addition to their intrinsic antineoplastic activity, there is some evidence that ER stress inducers may be useful in potentiating the effects of established therapeutic agents that trigger cell death through other mechanisms (e.g., DNA damage) [71–73]. Most recently, several studies have suggested that combining different drugs that induce ER stress through distinct mechanisms may yield synergistic anticancer activity [74–76]. The results of the present study raise the prospect that cal-C may be a useful addition to the arsenal of agents that can be applied to promote death in tumor cells by inducing morphologic and functional perturbation of the ER or by triggering mitochondrial death pathways independent of direct DNA damage. In this respect, cal-C could offer a unique advantage because its activity depends on photoexcitation, allowing for the possibility of restricting cytotoxicity to localized tumor sites. We suggest that this concept merits further evaluation in preclinical studies.

Acknowledgments

The authors thank Jean Overmeyer for helpful advice and discussions throughout these studies.

References


