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Rottlerin Sensitizes Colon Carcinoma Cells to Tumor Necrosis Factor-related Apoptosis-inducing Ligand-induced Apoptosis via Uncoupling of the Mitochondria Independent of Protein Kinase C[1]

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ABSTRACT

Signaling pathways involved in survival responses may attenuate the apoptotic response to the cytotoxic tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) in human colon carcinomas. In six lines examined, three were sensitive (GC_3/c1, VRC_5/c1, HCT116), HT29 demonstrated intermediate sensitivity, and RKO and HCT8 were resistant to TRAIL-induced apoptosis. Calphostin c [an inhibitor of classic and novel isoforms of protein kinase C (PKC)] sensitized five of six cell lines to TRAIL, whereas Go6976, (inhibitor of classic PKC isoforms), did not influence TRAIL sensitivity. Rottlerin, an inhibitor of novel isoforms of PKC, specifically PKCδ, sensitized five of six cell lines to TRAIL-induced apoptosis, suggesting that PKCδ may be involved in the mechanism of TRAIL resistance. Transfection of HCT116 with a proapoptotic cleaved fragment of PKCδ or an antiapoptotic full-length PKCδ did not influence the sensitivity of HCT116 to TRAIL. Furthermore, the incubation of HCT116 or RKO with phorbol myristate acetate for 16 h, which down-regulated the expression of novel PKC isoforms, also did not influence sensitivity to TRAIL either in the absence or presence of rottlerin. However, after 15-min incubation with rottlerin, mitochondrial membrane potential ($\Delta\psi$m) was dramatically reduced in RKO cells, and, in cells subsequently treated with TRAIL, rapid apoptosis was evident within 8 h. Calphostin c, but not Go6976, also caused a decrease in $\Delta\psi$m. In RKO, rottlerin induced the release of cytochrome *c*, HtrA2/Omi, Smac/DIABLO, and AIF from the mitochondria, potentiated in combination with TRAIL, with concomitant caspase activation and down-regulation of XIAP. In HT29, the release of proapoptotic factors was demonstrated only when rottlerin and TRAIL were combined, and Bcl-2 overexpression inhibited this release and the induction of apoptosis. TRAIL-induced apoptosis was not influenced by rottlerin or Bcl-2 overexpression in type I (GC_3/c1) cells. Data suggest that rottlerin affects mitochondrial function independent of PKCδ, thereby sensitizing cells to TRAIL, and that mitochondria constitute an important target in overcoming inherent resistance to TRAIL in colon carcinomas.

INTRODUCTION

TRAIL,[3] a type II transmembrane protein, is a cytotoxic ligand belonging to the TNF family of ligand and receptor pairs, which include FasL/Fas and TNF/TNFR1. TRAIL binds to four membrane-bound receptors (DR4, DR5, DcR1, DcR2). DR4 and DR5 contain a functional death domain and transmit an apoptotic signal via their 80-amino-acid intracellular death domains (1–3), which recruit adaptor proteins. However DcR1, which lacks an intracellular domain (4, 5), and DcR2, containing a truncated death domain (6, 7), constitute decoy receptors that sequester the ligand but are incapable of initiating an apoptotic signal. Unlike FasL and TNF, which have restricted tissue distribution, TRAIL is constitutively expressed in a wide variety of cells and tissues (8, 9). However few normal cell populations are sensitive to TRAIL (10, 11), in contrast to diverse types of malignant and transformed cell lines that are highly sensitive to the ligand (11). Although the TRAIL signaling pathway(s) remain to be fully elucidated, the receptor complexes formed after ligation of TRAIL are closer in composition to complexes formed with Fas (11, 12) than with TNFR1 (11). After ligation of TRAIL to DR4 or DR5 and trimerization of the receptor, FADD is recruited to the DISC followed by procaspase-8 (13–15). Death receptor signaling is considered to occur either via a type I pathway involving activation of large amounts of caspase-8 at the DISC followed by direct activation of downstream effector caspases, or via a type II pathway that requires mitochondrial involvement leading to caspase activation and a feedback amplification loop in the induction of apoptosis (16).

TRAIL resistance in malignant cells has not generally correlated with the relative levels of expression of DR4 and DR5 or the decoy receptors (17), suggesting the involvement of alternate mechanisms. PKC isoforms have been implicated in the mechanism of attenuation of death receptor-induced apoptosis (18–27). PKC constitutes a family of serine-threonine kinases comprising 12 different isoforms, which have been classified into three major groups based on their structures and on their activation mechanisms: conventional (α, β_I, β_{II}, γ), novel (δ, ϵ, η, θ), and atypical (ζ, υ, λ β Ref. 19). PKCs play critical roles in cell proliferation, differentiation, neoplastic transformation, and apoptosis. The classic α, β_I, (18), novel δ (20–24), ϵ (19, 25), or θ (25, 26) and atypical ζ (27) PKC isoforms have demonstrated a role in the regulation of death receptor-induced apoptosis. Activation of PKC by phorbol esters has delayed Fas-mediated apoptosis (28), whereas the inhibition of PKC has enhanced Fas-induced (28, 29) and TRAIL-induced (19) cell death. A requirement for the induction of apoptosis in the presence of PKC (PKCδ or PKCθ) appears to be cleavage of the protein to a catalytically active fragment, mediated by caspase-3 (20, 26).

In the present investigation, we have demonstrated that inhibitors of novel isoforms of PKC sensitized human colon carcinoma cell lines to TRAIL-induced apoptosis. In particular, rottlerin, which has demonstrated selective action in the inhibition of PKCδ activity (30–34), was highly effective in sensitizing cell lines to TRAIL, suggesting that PKCδ may play a critical role in attenuating TRAIL-induced apoptosis. However, studies demonstrated that the modulation of PKCδ, either by genetic regulation or by PMA treatment, did not influence TRAIL-induced apoptosis, indicating that the mechanism of rottlerin action was independent of PKCδ. More recently, rottlerin has been found to act in a PKCδ-independent manner by acting as an uncoupler of oxidative phosphorylation, thereby disrupting the $\Delta\psi$m (35, 36) in a manner similar to classic mitochondrial uncouplers such as carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP; Ref. 35). We subsequently demonstrated in human colon carcinoma cell lines that rottlerin induced a significant loss in $\Delta\psi$m within 15 min, and accel-

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[3] The abbreviations used are: TNF, tumor necrosis factor; TRAIL, TNF-related apoptosis-inducing ligand; TNFR, TNF receptor; DISC, death-inducing signaling complex; PKC, protein kinase C; PMA, phorbol myristate acetate; GFP, green fluorescence protein; FACS, fluorescence-activated cell sorting; MOI, multiplicity/multiplicities of infection; $\Delta\psi$m, mitochondrial membrane potential; IAP, inhibitor of apoptosis protein; dFBS, dialyzed fetal bovine serum.

erated the onset of TRAIL-induced apoptosis in TRAIL-resistant lines. These data demonstrate that rottlerin disrupts mitochondrial function independent of PKC and suggest that the mitochondria may constitute an important target in the sensitization of human colon carcinoma cells to TRAIL.

MATERIALS AND METHODS

Cell Lines. The HT29, HCT8, and HCT116 human colon carcinoma cell lines were obtained from American Type Culture Collection. GC_3/c1 and VRC_5/c1 were established as reported previously (37), and RKO was obtained from Dr. Michael Kastan, St. Jude Children's Research Hospital, Memphis, TN. Cells were maintained in the presence of folate-free RPMI 1640 containing 10% dFBS and 80 nM (6*RS*)5-methyltetrahydrofolate.

Production of Recombinant Human TRAIL. The cDNA of the extracellular domain of TRAIL corresponding to amino acids 114–281 was subcloned into the pET17/b (Novagen) bacterial expression vector and expressed in the BL21(DE3)pLysE (Novagen) bacterial host. After induction of TRAIL expression using isopropyl-β-thio-galactosidase (IPTG; 1 mM), bacterial pellets were harvested, and TRAIL was purified after passage through a nickel column (Ni-NTA) followed by a size exclusion column (Amersham), according to published procedures (38).

Apoptosis Assays. Cells were plated at a density of 150,000–200,000 cells/well in 12-well plates and, after overnight attachment, were treated with TRAIL (2–100 ng/ml) either in the absence or presence of calphostin c [an inhibitor of both classic (α, β, γ) and novel (δ, ϵ, η, θ) PKC isoforms; 0.1–0.25 μM; Ref. 39], or Go6976 (inhibitor of classic PKC isoforms; 1–20 μM; Ref. 40), or the PKCδ inhibitor rottlerin (1–10 μM) for up to 24 h. GC_3/c1 cells were pretreated for 2 h with rottlerin (2–10 μM) and subsequently cotreated with TRAIL (0.5–2 ng/ml) for 16 h. RKO cells were also treated with TRAIL (5–50 ng/ml) simultaneously and after 2 h pretreatment with the caspase inhibitors z-VAD-fmk (50 μM), z-DEVD-fmk (20 μM), or the control z-FA-fmk (20 μM; Enzyme Systems Products) for 24 h before the determination of the extent of apoptosis. Both the floating cells and the attached cells were pooled after trypsinization, were fixed in 70% ethanol, and were stored at −20° before analysis. Apoptotic cells were detected as a sub-G_1 fraction after propidium iodide staining and analysis using a Becton Dickinson FACScan (41). Alternatively, the extent of apoptosis was determined by Annexin-V-phycoerythrin (Alexis) and 17-aminoactinomycin-D (7-AAD; Molecular Probes) using a double staining procedure (42).

Transfection of PKCδ Constructs. A full-length PKCδ cDNA (PKCδFL), a PKCδ catalytically active fragment (PKCδCF), or a PKCδ kinase-inactive fragment [PKCδCF(K-R)] cloned into a modified pSVβ plasmid (Clontech) containing GFP were kind gifts from Dr. Donald Kufe, Dana-Farber Cancer Institute, Harvard Medical School, Boston, MA (24). HCT116 cells were plated at a density of 20×10^6 cells in T-175 flasks and, after overnight attachment, were transfected with 37.5 μg of the respective cDNAs in the presence of 111 μl of fugene (Roche Diagnostics) for 48 h. Cells were subsequently sorted by FACS for expression of GFP-containing cells that also contained the respective cDNA, subsequently plated at a density of 150,000 cells/well in 12-well plates, and treated with TRAIL (2 ng/ml) for an additional 16 h. The extent of apoptosis was subsequently determined by FACS analysis of the sub-G_1 compartment after propidium iodide staining.

Determination of $\Delta\psi$m. RKO was plated at a density of 150,000 cells/well in 12-well plates and, after overnight attachment, were treated with rottlerin (10 μM), calphostin c (0.25 μM), or Go6976 (20 μM) for 15 min. Cells were subsequently incubated in either the absence or the presence of TRAIL (50 ng/ml) for 4–16 h, followed by incubation for 15 min at 37° with $DiOC_6$ (2 μM), centrifuged at 200 $\times$ *g* for 5 min, resuspended in 0.5 ml of PBS, and analyzed by FACS for fluorescence (FL1) intensity (43).

HT29 Isogenic Cell Lines. The retroviral expression vector pMSCV-I-GFP (expressing GFP) was a kind gift from Dr. Jill M. Lahti and Dr. Vincent J. Kidd (St. Jude Children's Research Hospital) and has been described previously (44); and pMSCV-Bcl-2 (expressing human Bcl-2 protein) was kindly provided by Dr. John Cleveland (St. Jude Children's Research Hospital). Retroviral supernatants were prepared as described previously (45). HT29 cells were incubated overnight in a 50% mixture of RPMI 1640 and supernatant in the presence of Polybrene (8 μg/ml; Sigma). After replacement of this medium with fresh viral supernatants and culture medium, HT29 cells were incubated at 37° for an additional 48 h. The viral-transferred cells were sorted by expression of GFP using FACS, and stable GFP-positive cells were selected. The expression of Bcl-2 was confirmed by Western blotting.

Cellular Fractionation. RKO, HT29GFP, or HT29/Bcl-2 were plated at a density of 5×10^6 cells in T-162 flasks and allowed to proliferate for 3 days before treatment and then lysis and cellular fractionation into mitochondrial, nuclear, and cytosolic fractions using the ApoAlert cellular fractionation kit (Clontech) according to the manufacturer's directions.

Western Analysis. Western analyses were conducted as described previously (16, 46). Primary antibodies to the novel PKC isoforms PKCδ, PKCϵ, PKCθ, and PKCη, Bcl-2, and Bid were from Becton Dickinson, caspase-3 from Santa Cruz Biotechnology, and caspases-8 and -9 from MBL. The secondary antibody was HRP-conjugated sheep antimouse IgG1 (Amersham). Expression of novel PKC isoforms was also determined in RKO and HCT116 cell extracts after treatment with PMA (100 nM) for 16 h. Release of cytochrome *c*, HtrA2/Omi, Smac/DIABLO, or AIF from the mitochondria and expression of XIAP or c-IAP1 were determined by Western analysis. Primary antibodies were: cytochrome *c* (Clontech), HtrA2/Omi (a generous gift from Dr. Emad Alnemri, Thomas Jefferson University, Philadelphia, PA), Smac/DIABLO (MBL), AIF and XIAP (Santa Cruz Biotechnology), and c-IAP1 (Alexis Biochemicals). Secondary antibodies were sheep-antimouse Ig-HRP or donkey antirabbit Ig-HRP (Amersham).

Adenoviral Transduction of Bcl-2. Subcloning of a Bcl-2 cDNA into the pAVS6.DNA adenoviral vector (Genetic Therapy, Gaithersburg, MD) and amplification of Bcl-2-Adv or the vector alone (EV-Adv) have been described previously (47). GC_3/c1 cells were plated as described were and transduced with the Bcl-2-Adv or EV-Adv (MOI = 10) for 48 h before exposure to TRAIL (0–2 ng/ml) for 16 h. Apoptosis was determined by FACS analysis of the sub-G_1 compartment, as described.

RESULTS

Calphostin c but not Go6976 Sensitizes Human Colon Carcinoma Cell Lines to TRAIL. In a panel of six human colon carcinoma cell lines, calphostin c (0.1–0.25 μM), an inhibitor of both classic and novel isoforms of PKC, sensitized VRC_5/c1, HCT116, HT29, RKO, and HCT8 to TRAIL-induced apoptosis (Fig. 1). GC_3/c1 was highly sensitive to TRAIL alone in the absence of calphostin c and, hence, could not be sensitized to TRAIL-induced apoptosis in the presence of calphostin c. In VRC_5/c1, HCT116, RKO, or HCT8, at TRAIL concentrations that were relatively noncytotoxic, apoptosis was increased to >60% in the presence of calphostin c 24 h after the initiation of treatment. Similarly in HT29, calphostin c increased TRAIL-induced apoptosis to >43%. In contrast, in HT29 and in TRAIL-resistant RKO and HCT8, Go6976 (5–20 μM), an inhibitor of classic PKC isoforms, did not potentiate the cytotoxic activity of TRAIL (Fig. 2), suggesting that the inhibition of classic isoforms of PKC are not involved in attenuating the sensitivity of human colon carcinoma cell lines to TRAIL-induced apoptosis. However, because calphostin c sensitized colon carcinoma cell lines to TRAIL-induced apoptosis, we subsequently examined whether novel isoforms of PKC may be involved.

Human Colon Carcinoma Cell Lines Express PKCδ and PKCϵ. Of the four novel PKC isoforms, colon carcinoma cell lines expressed predominantly the PKCδ and PKCϵ isoforms, as determined by Western analysis (Fig. 3).

Rottlerin Sensitizes Cell Lines to TRAIL-induced Apoptosis. Rottlerin, an inhibitor of the novel PKC isoform PKCδ (30–34), was subsequently evaluated for the ability to sensitize human colon carcinoma cell lines to TRAIL-induced apoptosis during 24 h coincubation (Fig. 4). Five of the six lines (except for GC_3/c1) were sensitized to TRAIL (10–100 ng/ml) in the presence of rottlerin (1–10 μM), with the percentage of cells undergoing apoptosis increasing to >60% in VRC_5/c1, HCT116, RKO, and HCT8, and to >43% in HT29. Rot-

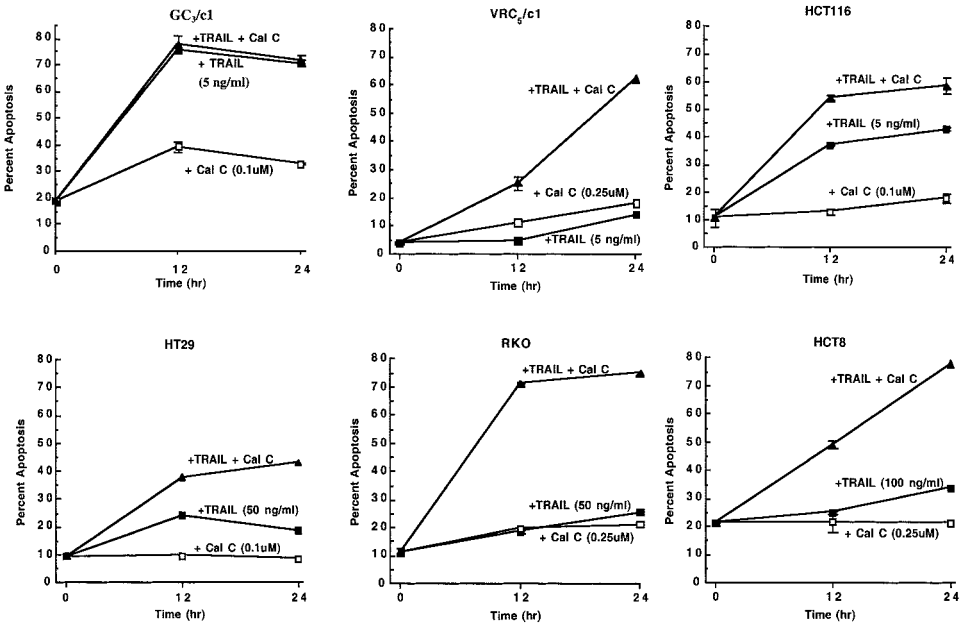

Fig. 1. Calphostin c (*Cal C*) sensitizes five of six human colon carcinoma cell lines to TRAIL-induced apoptosis. Cells were pretreated with calphostin c (0.1–0.25 μM) for 4 h before treatment with TRAIL (5–100 ng/ml) for up to 24 h. Apoptosis was determined by annexin V staining as described in "Materials and Methods." Results are the mean ± SD of duplicate determinations.

tlerin alone (control; 1–10 μM) did not induce apoptosis in any cell line.

Lack of a Role for PKCδ in Influencing TRAIL-induced Apoptosis. To elucidate the role of PKCδ in modulating the sensitivity of human colon carcinoma cell lines to TRAIL, we transfectedHCT116 with an antiapoptotic full-length PKCδ cDNA (PKCδFL), a proapoptotic PKCδ catalytically active fragment PKCδCF, or a PKCδ kinase-inactive fragment [PKCδCF(K-R); Ref. 44; Fig. 5). HCT116 transfected with the vector alone was slightly more sensitive to TRAIL than was nontransfected HCT116 cells. Of interest was that neither PKCδFL nor PKCδCF influenced the sensitivity of HCT116 to TRAIL. Subsequently, HCT116 and RKO cells were treated with PMA (100 nM) for 16 h, and the effect on expression of novel PKC isoforms was examined by Western analysis (Fig. 6*C*). Both PKCδ and PKCϵ were down-regulated in the presence of PMA. When HCT116 (Fig. 6*A*) and RKO (Fig. 6*B*) were treated with PMA for 16 h to down-regulate the expression of PKCs and were subsequently treated with TRAIL for 24 h, no effect on TRAIL-induced apoptosis was detected, and PMA alone was nontoxic. Furthermore, when these cells were treated with PMA for 16 h followed by rottlerin (10 μM) and TRAIL for 24 h, sensitization to TRAIL-induced apoptosis was still obtained.

Rottlerin and Calphostin c but not Go6976 Cause Rapid Loss in $\Delta\psi$m in RKO Cells. In addition to its effects on PKC, rottlerin has been reported to be capable of disrupting the $\Delta\psi$m (35, 36). To elucidate whether rottlerin may initiate a loss in $\Delta\psi$m in human colon carcinoma cell lines and to further elucidate the mechanism by which rottlerin sensitizes colon carcinoma cells to TRAIL-induced apoptosis, we treated RKO cells with rottlerin (10 μM) for 4–16 h in the absence or presence of TRAIL (50 ng/ml), and we examined the effect on $\Delta\psi$m by FACS analysis after cellular staining with $DiOC_6$ (Fig. 7). Under these conditions, rottlerin dramatically reduced the $\Delta\psi$m from 519 to a mean fluorescence intensity of 4.3 at 4 h. Additional experiments demonstrated that this change in $\Delta\psi$m occurred within 15 min of rottlerin treatment (data not shown). Similarly, treatment of RKO with calphostin c (0.25 μM) for 4–16 h also induced loss in $\Delta\psi$m, to a mean fluorescence intensity of 331 at 4 h and 299 at 16 h (Fig. 7). In contrast, 4–16 h pretreatment with Go9776 (20 μM), which had not demonstrated any influence on TRAIL-induced apoptosis in RKO (Fig. 2), did not induce a loss in $\Delta\psi$m in RKO cells (Fig. 7). TRAIL alone did not influence $\Delta\psi$m in RKO at the times examined. When RKO cells were pretreated with rottlerin (10 μM) for 15 min and subsequently treated with TRAIL (50 ng/ml), rapid apoptosis was induced within 8 h, and the cells became highly sensitive to TRAIL (Fig. 8).

Rottlerin + TRAIL Induce Release of Proapoptotic Factors from the Mitochondria and Caspase Activation in RKO Cells. In addition to the effects of rottlerin on the induction of loss in $\Delta\psi$m, rottlerin (10 μM; 15 min) ± TRAIL induced cytochrome *c* release and the release of HtrA2/Omi from the mitochondria at 8 h (Fig. 9*A*). The release of Smac/DIABLO and AIF was detected earlier, at 6 h after treatment with rottlerin ± TRAIL (Fig. 9*A*). Only when rottlerin was combined with TRAIL was decreased expression of XIAP (but not c-IAP1) determined, 8 h after TRAIL treatment (Fig. 9*A*). In addition,

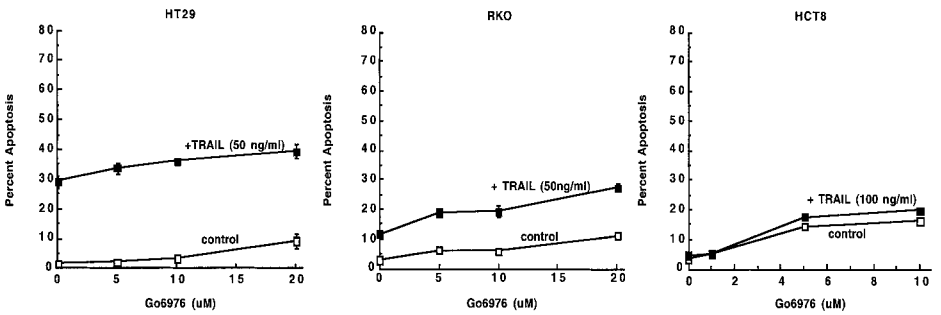

Fig. 2. Go6976 does not sensitize human colon carcinoma cell lines to TRAIL-induced apoptosis. HT29, RKO, and HCT8 cell lines were treated simultaneously with Go6976 (1–20 μM) in either the absence or the presence of TRAIL (50–100 ng/ml) for a period of 24 h. The extent of apoptosis was determined by propidium iodide staining and analysis of the sub-G_1 fraction using a Becton Dickinson FACScan, as described in "Materials and Methods." Data are the mean ± SD of duplicate determinations at each Go6976 concentration.

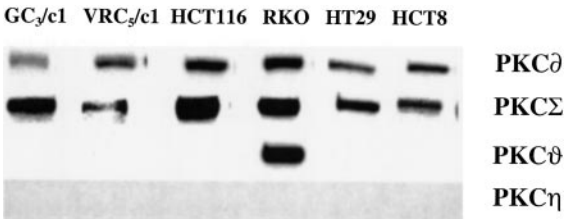

Fig. 3. Colon carcinoma cell lines express the novel PKC isoforms PKCδ and PKCε. Expression of novel PKC isoforms was determined by Western analysis.

the activation of caspases-8, -3, and -9 and cleavage of Bid were determined only when rottlerin and TRAIL were combined (Fig. 9*B*), and apoptosis induced by rottlerin + TRAIL at 18 h was inhibited by inhibitors of caspase activation (Fig. 9*C*). Delayed cell death (36%) induced by rottlerin treatment alone (10 μM) was observed at 48 h but not at 24 h (data not shown). This may explain the earlier release of proapoptotic factors from the mitochondria at 6–8 h (Fig. 9*A*) in the absence of caspase cleavage (Fig. 9*B*) or the induction of apoptosis (Fig. 4).

Bcl-2 Overexpression Inhibits the Release of Proapoptotic Factors into the Cytosol in HT29 Cells. HT29 cells were transfected with pMSCV-I-GFP (HT29GFP) or pMSCV-I-GFP-Bcl-2 (HT29Bcl-2), and cells containing GFP were selected by FACS analysis. In HT29GFP, 19–30% apoptosis was obtained after 24-h exposure to increasing concentrations of TRAIL (5–50 ng/ml; Fig. 10*A*). Overexpression of Bcl-2 protected cells from TRAIL-induced apoptosis. After preincubation with rottlerin (10 μM) for 15 min, TRAIL (10 ng/ml) induced apoptosis in 70% of HT29GFP cells, which was reduced to 24% in the presence of Bcl-2 overexpression (HT29Bcl-2; Fig. 10*B*). After cellular fractionation of HT29 or HT29/Bcl-2 cells at 6 h after treatment with rottlerin for 15 min (10 μM), TRAIL (50 ng/ml) for 6 h, or rottlerin (15-min pretreatment) and coincubation with TRAIL (6 h), cells treated with rottlerin + TRAIL but not rottlerin alone demonstrated release of HtrA2/Omi, Smac/DIABLO, AIF, and cytochrome *c* into the cytosol with corresponding reduced expression of both XIAP and c-IAP1 (Fig. 10*C*). C-IAP2 was not expressed either in RKO or HT29 cell lines.

Rottlerin Does Not Potentiate TRAIL-induced Apoptosis in Type I Cells. In contrast to type II cells (RKO, HT29), TRAIL-induced apoptosis occurred rapidly in type I cells (GC_3/c1), in which after treatment with TRAIL (50 ng/ml), 78% apoptosis was induced in 2 h (Fig. 11*A*). Pretreatment of GC_3/c1 cells with rottlerin (2–10 μM) for 2 h failed to sensitize cells to TRAIL-induced apoptosis after 16-h

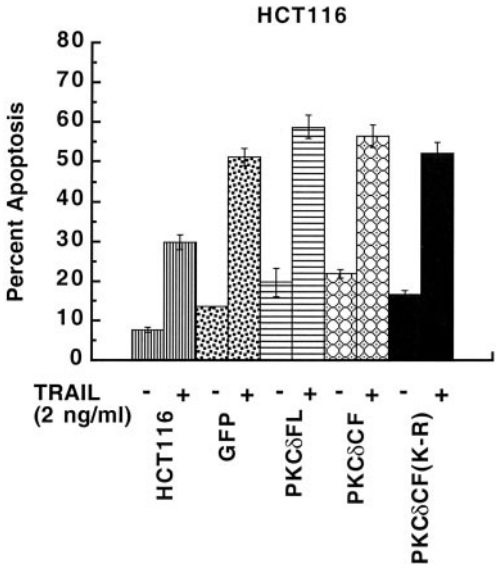

Fig. 5. Transfection of PKCδ constructs does not influence TRAIL-induced apoptosis in HCT116. HCT116 cells were transfected for 48 h with an antiapoptotic full-length PKCδ cDNA (PKCδFL), a proapoptotic PKCδ catalytically active fragment (PKCδCF), or a PKCδ kinase-inactive fragment [PKCδCF(K-R)] that had been cloned into a modified pSVβ plasmid (Clontech) containing GFP(44), as described in "Materials and Methods." Cells were sorted by FACS for expression of GFP-containing cells that also contained the respective cDNA, were subsequently plated in 12-well plates, and were treated with TRAIL (2 ng/ml) for an additional 16 h. The extent of apoptosis was determined as a sub-G_1 compartment by FACS analysis using duplicate determinations per point.

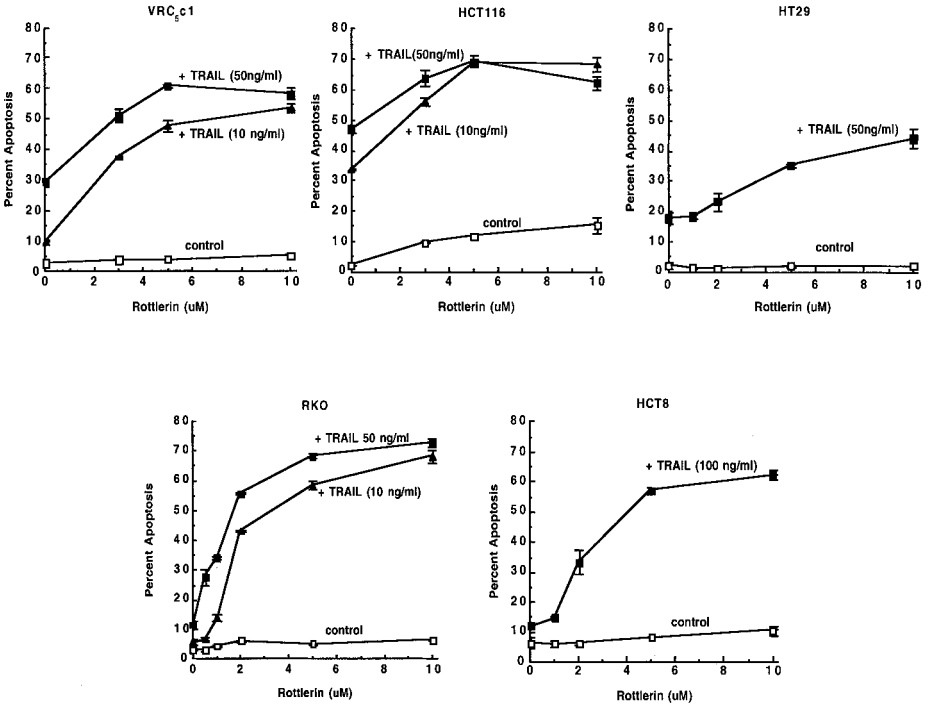

Fig. 4. Rottlerin sensitizes human colon carcinoma cell lines to TRAIL-induced apoptosis. Cells were pretreated with rottlerin (1–10 μM) for 2 h and were subsequently incubated in either the absence or the presence of TRAIL (10–100 ng/ml) for 24 h. The extent of apoptosis was determined by propidium iodide staining and analysis of the sub-G_1 fraction by FACS, as described in "Materials and Methods." Data are the mean ± SD of duplicate determinations at each rottlerin concentration.

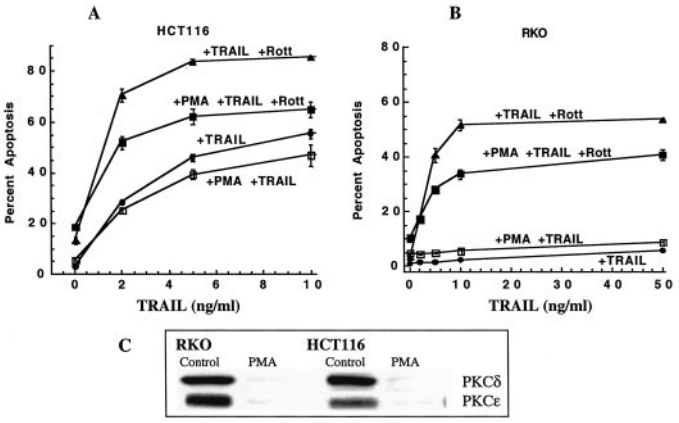

Fig. 6. *A*, HCT116 or *B*, RKO cells were treated with PMA (100 nM) for 16 h before pretreatment with rottlerin (*ROTT*; 10 μM) for 2 h in either the absence or the presence of TRAIL (2–50 ng/ml) for an additional 16 h. Apoptosis was determined by FACS analysis after propidium iodide staining. Data represent the mean ± SD of duplicate determinations. *C*, down-regulation of novel PKC isoforms was also determined in RKO and HCT116 cell extracts by Western analysis after treatment with PMA (100 nM) for 16 h.

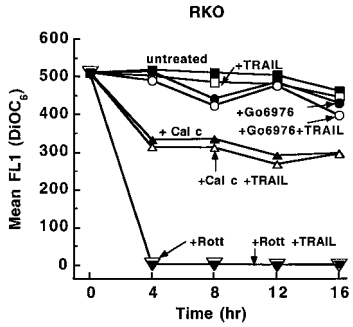

Fig. 7. Rottlerin and calphostin c but not Go6976 induce loss in $\Delta\psi$m in RKO cells. Cells were incubated with rottlerin (*ROTT*; 10 μM), calphostin c (*Cal c*; 0.25 μM), or Go6976 (20 μM) for 4–16 h in either the absence or the presence of TRAIL (50 ng/ml) for 4–16 h, followed by incubation with $DiOC_6$ (2 μM) for 15 min before an analysis of loss in $\Delta\psi$m by FACS. *FL1*, fluorescence intensity.

exposure to TRAIL (0.5–2 ng/ml; Fig. 11*B*). Furthermore, overexpression of Bcl-2 after transduction of cells with a Bcl-2 adenovirus (MOI = 10) also did not protect cells from TRAIL-induced apoptosis (Fig. 11*C*).

DISCUSSION

PKC has demonstrated a role in the attenuation of apoptosis induced by ligation of death receptors of the TNFR superfamily, such that several PKC inhibitors have sensitized cells to Fas- or TRAIL-induced apoptosis. Thus, the general PKC inhibitor bisindoylmaleimide VIII sensitized to Fas-mediated apoptosis human astrocytoma 1321N1 cells and Molt4 cells that were devoid of apoptotic responses induced by anti-Fas antibody in the absence of the compound (48). Inhibition of classic isoforms of PKC by Go6976 has also enhanced anti-Fas-mediated apoptosis in Jurkat cells (49). PKC is considered to promote survival responses via the activation of nuclear factor-κB (30, 50). Phosphorylation by PKC isoforms can inhibit the function of inhibitor of nuclear factor-κB (IκBα) in the activation of nuclear factor-κB (51), and can negatively regulate TNF family receptors (18, 52). Furthermore, PKC isoforms have mediated these effects by phosphorylation and inactivation of Bad (25, 26), inhibition of DISC formation, FADD recruitment, and caspase-8 activation (49), or inhibition of death receptor expression (18, 52). PKC also functions in type II cells that require amplification via the mitochondria in induction of an apoptotic response, and not in type I cells (53).

In the present study, human colon carcinoma cell lines demonstrated a wide spectrum of sensitivity to TRAIL, demonstrating exquisite sensitivity (GC_3/c1) or innate resistance (RKO, HCT8) to the ligand. The high degree of TRAIL-induced apoptosis obtained in five of the six cell lines when TRAIL was coincubated with calphostin c, which inhibits both classic and novel isoforms of PKC, and the lack of effect of Go6976, which inhibits only the classic isoforms of PKC, suggested that novel PKCs may be important in the attenuation of TRAIL-induced apoptosis in this cell type. In this regard, a similar degree of sensitization to TRAIL-induced apoptosis in the presence of the PKCδ inhibitor, rottlerin, and the expression of PKCδ in each cell line, suggested that this isoform of PKC may be important in influencing TRAIL-induced apoptosis. However, transfection of PKCδ

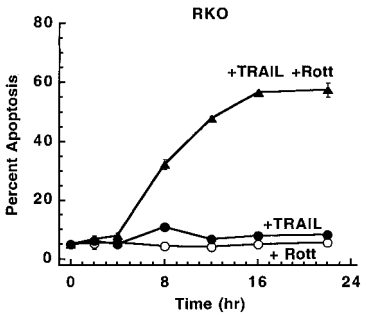

Fig. 8. RKO cells were pretreated with rottlerin (*Rott*; 10 μM) for 15 min before the addition of TRAIL (50 ng/ml) for periods up to 24 h. Apoptosis was determined, in duplicate, as a sub-G_1 fraction after propidium iodide staining and FACS analysis.

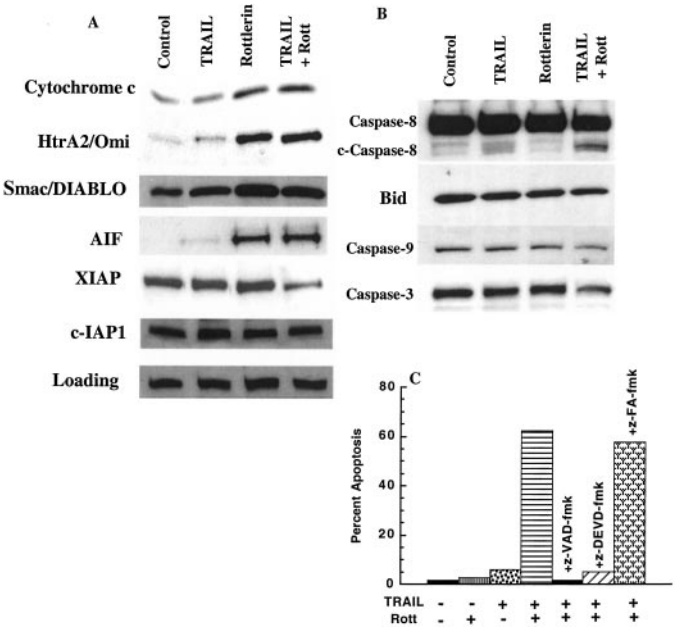

Fig. 9. *A*, RKO cells were treated with rottlerin (10 μM) for 15 min, TRAIL (50 ng/ml) for 6–8 h, or pretreated with rottlerin for 15 min before TRAIL for an additional 6–8 h. Cells were disrupted and separated into mitochondrial and cytosolic fractions, and the release of proapoptotic factors from the mitochondria into the cytosol, or expression of XIAP and c-IAP1, was determined by Western analysis as described in "Materials and Methods." Expression of all of the proteins were determined at 8 h after TRAIL treatment, except for Smac/DIABLO and AIF, which were determined at 6 h. *B*, RKO cells were treated as in *A*, and the activation of caspases-8, -9, and -3, and the cleavage of Bid were determined by Western analysis after treatment with TRAIL (50 ng/ml) for 8 h. *C*, RKO cells were pretreated with rottlerin (*Rott*; 10 μM) for 15 min and subsequently treated in either the absence or the presence of TRAIL (50 ng/ml) ± the caspase inhibitors z-VAD-fmk (50 μM) or z-DEVD-fmk (20 μM), or the control z-FA-fmk (20 μM). Apoptosis was analyzed at 18 h.

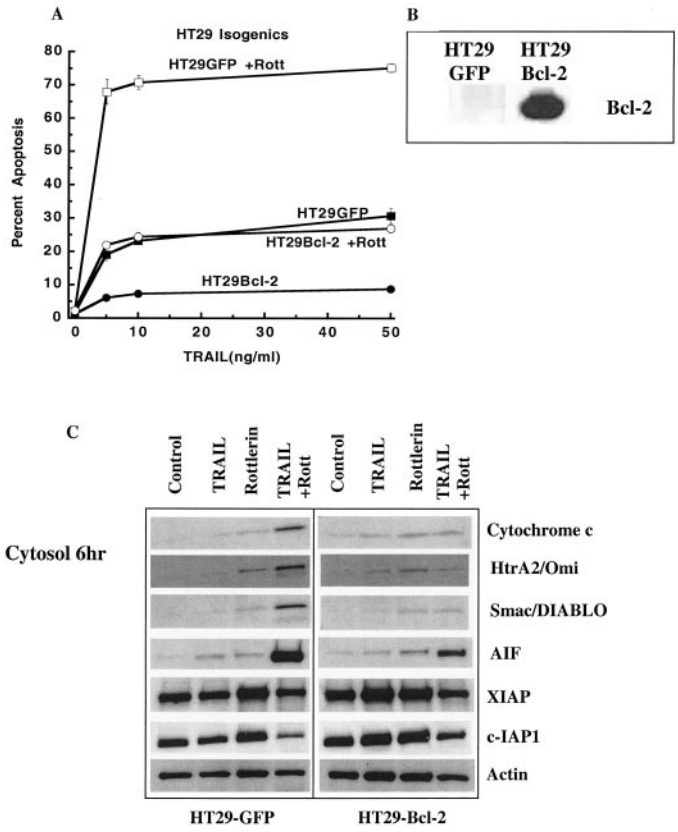

Fig. 10. HT29 isogenic cell lines HT29GFP or HT29 overexpressing Bcl-2 (HT29Bcl-2) were treated with TRAIL (5–50 ng/ml) in either the absence or the presence of rottlerin (10 μM) pretreatment for 15 min. Apoptosis was analyzed, in duplicate, at 18 h as a sub-G_1 fraction by FACS. *B,* expression of Bcl-2 was examined by Western analysis as described in "Materials and Methods." *C,* HT29 or HT29-Bcl-2 cells were treated with rottlerin (10 μM) for 15 min or TRAIL (50 ng/ml) for 6 h or were pretreated with rottlerin for 15 min before TRAIL for an additional 6 h. Cells were disrupted and separated into mitochondrial and cytosolic fractions, and the release of proapoptotic factors from the mitochondria into the cytosol, or expression of XIAP and c-IAP1, were determined by Western analysis as described in "Materials and Methods."

constructs with both proapoptotic and antiapoptotic function did not influence the sensitivity of HCT116 to TRAIL. In addition, when novel PKC isoforms were down-regulated after prolonged administration of PMA in either RKO or HCT116, no sensitization to TRAIL was obtained, and cells continued to be sensitized to TRAIL-induced apoptosis by rottlerin. These data, therefore, suggested that the mechanism of rottlerin-induced sensitization to TRAIL was independent of PKC.

PKCδ translocates to several compartments within cells including the mitochondria (54, 55) and has been reported to play multiple roles in apoptosis involving distinct roles both upstream and downstream of the mitochondria (32, 56, 57), as well as contributing to the loss of $\Delta\psi$m caused by agents that induce apoptosis (58). PKCδ has also initiated a cell death pathway in keratinocytes that involves direct interaction with mitochondria and alterations of mitochondrial function (55), and has amplified ceramide formation via mitochondrial signaling in prostate cancer cells (21). However in the present study, no direct role of PKCδ in influencing TRAIL-induced apoptosis was demonstrated in colon carcinoma cells. Rottlerin was originally identified as an inhibitor of PKCδ (30–34) and, in immunokinase assays, specifically inhibited the activity of PKCδ (31). However, the inhibition of PKCδ activity by rottlerin may be secondary to its effects at the level of the mitochondria, resulting in the inhibition of tyrosine phosphorylation of PKCδ (35). It was recently reported that rottlerin acts as a mitochondrial uncoupler independent of its effects as an inhibitor of PKCδ (35). In the RKO cells examined, rottlerin induced a dramatic loss in $\Delta\psi$m, which was clearly independent of its role as an inhibitor of PKCδ. Of interest was that calphostin c, which also sensitized five of six colon carcinoma cell lines to TRAIL-induced apoptosis, also induced loss of $\Delta\psi$m in RKO cells, in contrast to Go6976, which did not sensitize RKO to TRAIL and did not induce loss in $\Delta\psi$m. These data suggest that disruption of the $\Delta\psi$m plays a major role in TRAIL signaling and the subsequent induction of apoptosis. Of interest was the exquisite sensitivity of GC_3/c1 to TRAIL, which was not further sensitized in the presence of either calphostin c or rottlerin. This is consistent with a type I signaling mechanism for TRAIL in GC_3/c1, in which apoptosis commenced rapidly, at 2 h; and, in addition, overexpression of Bcl-2 did not protect cells from TRAIL-induced apoptosis, further indicating the requirement for mitochondrial involvement in rottlerin-induced sensitization to TRAIL-induced apoptosis in type II cells.

Decreased $\Delta\psi$m results in changes in the inner mitochondrial membrane function accompanied by an increase in outer membrane permeability, leading to the release of soluble intermembrane proteins that promote cell death into the cytosol (43), including cytochrome *c* (59, 60), HtrA2/Omi (61–63), Smac/DIABLO (64, 65), and AIF (66, 67). Cytochrome *c* forms a multimeric complex with Apaf-1 in an ATP-requiring reaction, subsequently recruiting caspase-9, followed by activation of caspase-9 and activation of downstream caspases (68, 69). Several members of the IAP family including XIAP, c-IAP1, and c-IAP2 are potent direct inhibitors of caspases-3, -7 and -9 thereby blocking enzymatic activity, XIAP being the most potent (70). Mitochondrial release of HtrA2/Omi (61–63) or Smac/DIABLO (64, 65) destabilizes these complexes, leading to the quenching of the caspase-inhibitory function of IAPs. Furthermore, the apoptosis-inducing-factor AIF may play a role in caspase-independent cell death (43, 67) and activate DNases (71). In RKO cells, rottlerin treatment, either alone or in combination with TRAIL, caused the release of cyto-

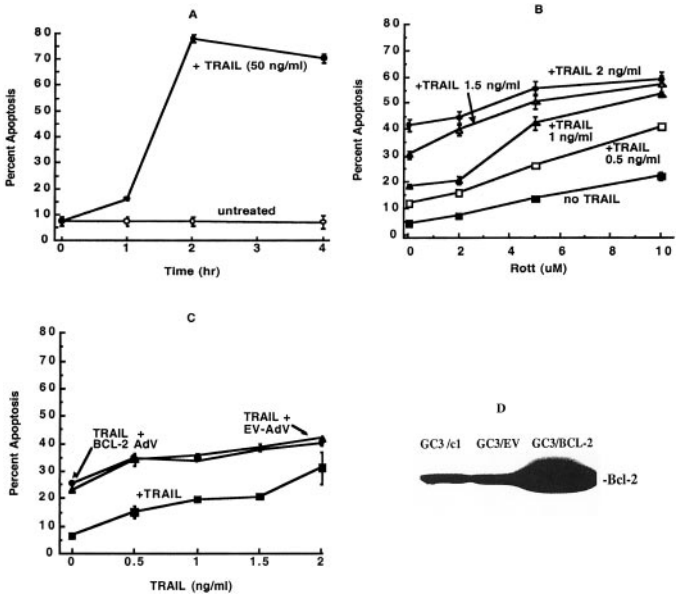

Fig. 11. **A.** GC_3/c1 cells were treated with TRAIL (50 ng/ml) and apoptosis, were determined as a sub-G_1 compartment by FACS analysis, and were determined for up to 4 h after treatment. Data are the mean ± SD of duplicate determinations. *B,* GC_3/c1 cells were pretreated with rottlerin (2–10 μM; 2 h) followed by cotreatment with TRAIL (0.5–2 ng/ml) for 16 h before determination of the extent of apoptosis (mean ± SD of duplicate determinations). *C,* GC_3/c1 cells were transduced with EV-Adv or Bcl-2-Adv (MOI = 10) for 48 h before treatment with TRAIL (0.5–2 ng/ml) for 16 h, followed by analysis of the extent of apoptosis (mean ± SD of two determinations per point). *D,* expression of Bcl-2, determined by Western analysis, in GC_3/c1, GC_3/EV (vector control), or GC_3/Bcl-2 (overexpressing Bcl-2).

chrome *c*, HtrA2/Omi, Smac/DIABLO, and AIF into the cytosol. XIAP was also down-regulated, and apoptosis was significantly enhanced only when rottlerin and TRAIL were combined, apoptosis occurring as early as 8 h after the initiation of TRAIL treatment. This appeared to be mediated by activation of a classic type II signaling pathway after release of proapoptotic factors with subsequent activation of caspases-8, -9, and -3 and cleavage of Bid. Cell death induced by rottlerin alone was not detected until considerably later (48 h; data not shown). The induction of apoptosis in RKO cells induced by rottlerin in combination with TRAIL could be completely inhibited by the caspase inhibitors z-VAD-fmk and z-DEVD-fmk. Further evidence of apoptosis induced by rottlerin + TRAIL requiring mitochondrial involvement was demonstrated in the HT29 isogenic cell line HT29Bcl-2, in which overexpression of Bcl-2 inhibited TRAIL-induced apoptosis in both the absence and the presence of rottlerin, consistent with the effect of Bcl-2 in inhibiting apoptosis induced by mitochondrial uncoupling (72). In addition, the release of cytochrome *c*, HtrA2/Omi, Smac/DIABLO, and AIF into the cytosol, and reduced expression of the IAP protein c-IAP1 (but not XIAP) occurred only when TRAIL and rottlerin were combined, and these effects were also inhibited in the presence of Bcl-2 overexpression.

In summary, we have demonstrated that rottlerin is effective in sensitizing human colon carcinoma cell lines to TRAIL-induced apoptosis via direct effects at the level of the mitochondria, prevented by inhibitors of caspase activation, and inhibited by Bcl-2, independent of its effects as an inhibitor of the novel PKC isoform PKCδ. The finding that TRAIL resistance can be overcome by initially inducing changes in the $\Delta\psi$m in TRAIL-resistant cells followed by administration of TRAIL suggests that the mitochondria may constitute an important target in sensitizing human colon carcinoma cells to therapeutic approaches involving cytotoxic ligands of the TNF family. Additional studies on the relationship between loss in $\Delta\psi$m and events leading to the induction of apoptosis, therefore, appear warranted.

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