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In Vitro Model for Intrinsic Drug Resistance: Effects of Protein Kinase C Activators on the Chemosensitivity of Cultured Human Colon Cancer Cells

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SUMMARY

We investigated the effects that phorbol ester and diacylglycerol protein kinase C (PKC) activators had on the chemosensitivity of the human colon cancer cell line KM12L4a to Adriamycin (ADR), vincristine (VCR), and vinblastine (VLB) and on the intracellular accumulation of those drugs. Exposure of the cells to the PKC activator phorbol-12,13-dibutyrate (PDBu) (15 nM) during a 96-hr *in vitro* chemosensitivity assay significantly reduced the sensitivity of KM12L4a cells to ADR, VCR, and VLB, but not to 5-fluorouracil. Because a 96-hr treatment with 15 nM PDBu did not down-regulate PKC activity in KM12L4a cells, activation of PKC appeared to be responsible for the observed protection conferred by PDBu. PDBu-induced alterations in drug accumulation may account for its protective effects against these cytotoxic drugs, because both PDBu and the phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate significantly reduced accumulation of [^{3}H]VCR and [^{14}C]ADR in the cultured human colon cancer cells. Unsaturated diacylglycerols are structural and functional analogues of phorbol ester PKC activators that are present in the lumen of the colon. We found that treatment of KM12L4a human colon cancer cells with the diacylglycerol 1-oleoyl-2-acetyl-*sn*-glycerol (OAG) significantly reduced [^{14}C]ADR and [^{3}H]VCR accumulation in the cells. The effects of OAG were dose dependent at physiological diacylglycerol concentrations and were completely reversed by the protein kinase inhibitor H7. OAG, which is rapidly metabolized in cultured cells, did not protect KM12L4a cells against the cytotoxic drugs in our 96-hr *in vitro* chemosensitivity assay. However, rapid metabolism of diacylglycerols should not limit their capacity to activate PKC in the colonic epithelium *in vivo*, because that tissue is chronically exposed to replenished supplies of unsaturated diacylglycerols in the intestinal tract. Our results provide evidence that unsaturated diacylglycerols may be environmental factors that contribute to the intrinsic drug resistance of colon cancer *in vivo* by reducing drug accumulation in the cancer cells.

Resistance of cancer cells to chemotherapeutic drugs presents a major barrier to successful cancer therapy (1–5). *In vitro* selection of drug-resistant cells from a drug-sensitive population by exposure to cytotoxic natural products commonly results in the emergence of a MDR phenotype. MDR cells are not only resistant to the selecting agent but are also cross-resistant to diverse anticancer drugs, including ADR, VCR, and VLB (1–5). In recent years, numerous studies have provided evidence that the MDR phenotype is intimately associated with overexpression of a 170-kDa membrane protein, Pgp, which apparently reduces the intracellular concentration of certain drugs by pumping them out of cells in an ATP-dependent manner (1–5). The central role of Pgp in MDR has been confirmed directly by the acquisition of MDR by drug-sensitive cells that were transfected with DNA from MDR cells or with cDNA encoding Pgp (*mdr*) (6–9).

PKC is a Ca^{2+}- and phospholipid-dependent protein kinase that can be directly activated by phorbol ester tumor promoters, such as TPA and PDBu, and by the second messenger diacylglycerol (10, 11). Evidence is now emerging that PKC may play a regulatory role in MDR. We have shown that the level of PKC activity correlates with the degree of MDR in a series of ADR-selected MDR murine fibrosarcoma UV-2237M cell lines (12). In addition, Fine *et al.* (13) recently reported that PDBu can induce a phenotype resembling MDR in human breast cancer MCF-7 cells. Under conditions in which PDBu activated but did not down-regulate PKC, PDBu endowed drug-sensitive MCF-7 cells with resistance against ADR and VCR and enhanced the resistance of an ADR-selected MDR MCF-7 cell line against these agents (13). Similarly, exposure to TPA conferred protection against VCR and several other cytotoxic

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ABBREVIATIONS: MDR, multidrug resistance (-ant); ADR, Adriamycin; VCR, vincristine; VLB, vinblastine; PDBu, phorbol dibutyrate; PKC, protein kinase C; Pgp, P glycoprotein; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MTT, 3-(4,5-dimethylthiezol-2-yl)-2,5-diphenyltetrazolium bromide; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N,N′,N′*-tetraacetic acid; 5-FU, 5-fluorouracil; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; OAG, 1-oleoyl-2-acetyl-*sn*-glycerol; PBS, phosphate-buffered saline; CMEM, complete minimum essential medium; PS, phosphatidylserine.

drugs on human KB cells (14). Protection of KB cells was not observed, however, with the PKC-activating diacylglycerol OAG or with exogenous phospholipase C, which increases intracellular diacylglycerol levels, and the authors concluded that PKC activation may not be sufficient to exert the protection observed with TPA (14). Phorbol ester-induced drug resistance may involve modulation of drug accumulation, because TPA and PDBu reduce ADR and VCR accumulation *in vitro* in drug-sensitive and MDR P388 and MCF-7 cells (13, 15). However, the effects of diacylglycerols on drug accumulation have not been reported for any cell line.

Colon carcinoma is a major cause of cancer mortality. The intrinsic resistance of colon tumors to commonly used anticancer drugs renders them generally unresponsive to chemotherapy (2). The relatively high level of expression of Pgp observed in the colonic epithelium *in vivo* is believed to play a role in this resistance (16). However, based on the observation that colon cancer cells are often highly sensitive to anticancer drugs *in vitro* but resistant to them when grown in the colonic epithelium,[1] we hypothesize that environmental factors may also contribute to the intrinsic resistance of colon tumors *in vivo*. Friedman *et al.* (17) recently reported that the colonic epithelium is chronically exposed to high concentrations of extracellular unsaturated diacylglycerols that are present in the lumen of the colon. Because unsaturated diacylglycerols include PKC activators that are structurally homologous to phorbol ester PKC activators (18), we hypothesized that unsaturated diacylglycerols could be environmental factors that contribute to the intrinsic resistance of colon tumors by activating PKC. To test this hypothesis, we measured the effects of TPA, PDBu, and OAG on the *in vitro* chemosensitivity of the human colon cancer cell line KM12L4a and on the accumulation of ADR and VCR in the cells. Our *in vitro* model provides the first evidence that exogenously added diacylglycerol can inhibit drug accumulation in cultured tumor cells. Our results suggest that chronic exposure of the colonic epithelium to extracellular diacylglycerol *in vivo* may contribute to the intrinsic resistance to chemotherapy commonly observed in intestinal cancers.

Materials and Methods

Cell culture. KM12L4a, a human colorectal carcinoma cell line established in our laboratory from a primary tumor (19, 20) was maintained as a monolayer culture in minimum essential medium supplemented with 5% fetal bovine serum, nonessential amino acids, vitamin A, and glutamine (CMEM).

Chemicals. The following reagents, purchased from the indicated companies, were used: ADR (Adria); VLB sulfate (Quid Pharmaceuticals); VCR sulfate (Eli Lilly & Co); 5-FU (Hoffmann-La Roche Inc), TPA, PDBu, MTT, OAG, ATP, histone IIIS, leupeptin, phenylmethylsulfonyl fluoride, PS, and Triton X-100 (Sigma Chemical Co.); H7 (Calbiochem); phosphocellulose paper, grade p81 (Fisher Scientific); protein concentration assay solution (Bio-Rad); tissue culture reagents (GIBCO); [γ-^{32}P]ATP (specific activity, >10 Ci/mmol), [^{3}H]VCR sulfate (specific activity, 6.67 Ci/mmol), and [^{14}C]ADR (specific activity, 50 mCi/mmol) (Amersham Corp); and [^{14}C]5-FU (specific activity, 56 mCi/mmol) (New England Nuclear).

Growth inhibitory assay. Growth inhibitory effects of cytotoxic drugs were assessed by using an MTT assay to determine the reduction in viable cell number elicited by the drugs, as previously described (12, 21). Previous studies indicate that measurements of *in vitro* tumor cell growth by the MTT assay correlate well with measurements of cellular protein, dye exclusion, and viable cell count. Thus, the MTT assay is suitable for measurements of cell growth and drug sensitivity (21). KM12L4a cells cultured on plastic were harvested by exposure to 0.25% trypsin/0.02% EDTA (w/v) and seeded into 96-well microculture plates, at a density of 5000 cells/well. After incubation in 5% CO_2 at 37° for 16 to 20 hr, the cells were incubated under these conditions with cytotoxic drugs for a 96-hr period. At the end of the incubation period, 40 μl of MTT (5 mg/ml in PBS) were added to each well, and the cells were incubated for 2 hr at 37° to allow complete reaction between the dye and the enzyme mitochondrial dehydrogenase in the viable cells. After removal of the unreacted dye and medium, 100 μl of dimethyl sulfoxide were added to each well, and the absorbance at 600 nm was measured with a microplate-scanning spectrophotometer. Cytostasis was calculated according to the following formula: cytostasis (%) = [1 − (A_{600} of experimental group/A_{600} of control group)] × 100. The IC_{50} was defined as the concentration of a drug that inhibited cell growth by 50%, compared with an untreated control. For experiments designed to elucidate the modulating effects of PKC activators and/or protein kinase inhibitors on cytostasis induced by cytotoxic drugs, the cells were first exposed to the activators and/or inhibitors for 1 hr and then incubated with the drugs for 96 hr in the presence of the activators and/or inhibitors. Control experiments, in which cells were exposed to PKC activators or inhibitors in the absence of cytotoxic drugs, were done in parallel.

Drug accumulation and efflux assays. Drug accumulation and drug efflux assays in KM12L4a cells were based on previously described protocols (22). Stock solutions of [^{14}C]ADR and [^{3}H]VCR were prepared in 0.9% saline to a final concentration of 100 μM, and their specific activities were 25 and 220 mCi/mmol, respectively. Before use, they were diluted in CMEM containing 10 mM HEPES (pH 7.3). KM12L4a cells in CMEM were pipetted into 24-well plates (201 mm^2/well) (Costar) at a density of 5 × 10^5 cells/well. After an overnight incubation, the medium was decanted, and the wells were washed once with assay medium (CMEM with 10 mM HEPES, pH 7.3) at 37°. For drug accumulation studies, the cells were then incubated with 0.1 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr at 37°, followed by four rapid washes with cold PBS (4°), and detached by the addition to each well of 0.5 ml of PBS with trypsin/EDTA. After incubation at 37° for 30 min, cell suspensions were harvested and then counted in vials containing 15 ml of scintillation fluid (ScintiVerse II; Fisher Scientific), using a Beckman LS1800 liquid scintillation counter. For drug efflux assays, the cells were preloaded with 0.2 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, followed by four rapid washes with PBS and incubation at 37° in 0.5 ml of fresh medium for the indicated lengths of time. Aliquots of the supernatants were removed and counted as described above. To examine the effects of PKC activators and/or protein kinase inhibitors on drug accumulation and efflux, the cells were pretreated with the modulators for 30 min and then incubated with radiolabeled drugs in the presence of the modulators.

Isolation and determination of PKC activity. PKC activity was isolated from KM12L4a cells by a previously described procedure (12). KM12L4a cells were seeded into 75-cm^2 flasks at a density of 1–2 × 10^6 cells/flask. Twenty-four hours later, the medium was decanted and the cells were cultured for 96 hr in fresh medium (10 ml/flask), with or without 15 nM PDBu. The cells were then harvested with a rubber policeman and lysed at 4° with 1% Triton X-100 in buffer A (20 mM Tris·HCl, 5 mM EDTA, 5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, 20 μg/ml soybean trypsin inhibitor, and 15 mM 2-mercaptoethanol, pH 7.5), and lysates were stirred at 4° for 1 hr. PKC activity was then partially purified from cell lysates by chromatography on a 0.5-ml DEAE 52 column equilibrated in buffer A. After the cell lysates were loaded onto the DEAE columns, each column was washed with 3 ml of buffer A, and the enzyme was eluted with buffer A containing 0.2 M NaCl, as previously described (12).

PKC activity was determined as described previously (23), by subtracting the phosphotransferase activity between [γ-^{32}P]ATP and histone IIIS observed in the presence of Ca^{2+} from the activity observed

[1] C. Wilmanns and I. J. Fidler, personal communication.

in the presence of Ca^{2+} and PS. PKC assay reaction mixtures (120 μl) contained 20 mM Tris·HCl, pH 7.5, 5 mM 2-mercaptoethanol, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 30 μg/ml PS (or none), 6 μM [γ-^{32}P]ATP (4000–6000 cpm/pmol), 0.67 mg/ml histone IIIS, and 5 μg of DEAE-purified cell lysate protein. Reactions proceeded for 10 min at 30° and were terminated as previously described (23). PKC activity levels represent the average ± standard deviation of triplicate assays of Ca^{2+}- and PS-dependent ^{32}P incorporation into histone IIIS. Protein concentrations were determined using the Bio-Rad protein assay solution, according to the manufacturer's instructions, with bovine serum albumin as the standard protein.

Statistical analysis. Statistical differences between various treated groups were calculated by paired Student's *t* test and expressed as *p* values.

Results

Effects of PDBu on the chemosensitivity of a drug-sensitive human colon cancer cell line. To address whether unsaturated diacylglycerol, an endogenous molecule in the lumen of the colon (17), could be an environmental factor that contributes to intrinsic drug resistance in human colon cancer, we first examined the effects of the phorbol ester PDBu on the *in vitro* sensitivity of the human colon cancer cell line KM12L4a to anticancer drugs, because PDBu and unsaturated diacylglycerol activate PKC by the same mechanism (18, 24, 25). We found that continuous exposure to PDBu protected KM12L4a cells from the cytotoxic effects of ADR, VCR, and VLB, but not 5-FU. As shown in Fig. 1, in a 96-hr assay system, continuous treatment with ADR, VCR, VLB, or 5-FU induced a dose-dependent inhibition of KM12L4a cell growth, and the addition of 15 nM PDBu to the cells 1 hr before incubation with the cytotoxic drugs significantly reduced the sensitivities of the cells to ADR, VCR, and VLB (Fig. 1, A, B, and C), whereas the sensitivity of the cells to 5-FU was unchanged (Fig. 1D). In the absence of PDBu, the IC_{50} values for ADR, VCR, and VLB were 40 ± 11, 1.5 ± 0.4, and 2.7 ± 0.8 ng/ml, respectively. Administration of 15 nM PDBu increased their IC_{50} values significantly ($p < 0.03$), resulting in IC_{50} values of 68 ± 15 ng/ml for ADR (six experiments), 3.4 ± 0.8 ng/ml for VCR (five experiments), and 6.8 ± 1.5 ng/ml for VLB (four experiments). PDBu reduced the sensitivities of the cells to all three of the drugs in a dose-dependent manner, at PDBu concentrations of 0 to 20 nM. PDBu exhibited approximately 50% of its maximal efficacy at concentrations of 6 nM (ADR), 4 nM (VCR), and 4 nM (VLB) (data not shown), i.e., with potencies comparable to its potency as a PKC activator (25). Control studies indicated that PDBu alone (0–20 nM) did not significantly affect cell growth (data not shown). In additional control experiments, we determined that a 2-hr exposure to PDBu (1.5–150 nM) did not alter the sensitivity of the cells to ADR, providing evidence that the reduction of the chemosensitivity of the cells to MDR-linked drugs by PDBu required prolonged activation of PKC (data not shown).

Effect of PDBu treatment on the level of PKC activity in KM12L4a cells. Prolonged exposure of mammalian cells to high concentrations of phorbol ester PKC activators generally results in down-regulation of PKC activity (26). Recent reports have shown that exposure to phorbol ester tumor promoters elicits the disappearance of several PKC isozymes at different rates in a variety of cell types, whereas up-regulation of specific PKC isozymes by exposure to phorbol esters has not been reported (27, 28). We found that the protective effects of PDBu that we observed were not due to down-regulation of PKC. When KM12L4a cells were incubated with 15 nM PDBu for 96 hr, there was only a minor change in the level of PKC activity. The total cellular PKC activities in the control and in PDBu-treated KM12L4a cells were 111 ± 10 and 99 ± 11 pmol of ^{32}P/min/mg of protein, respectively (for experimental details, see Materials and Methods). Thus, PDBu treatment resulted in only a minor loss of PKC activity.

Effects of the protein kinase inhibitor H7 on the chemosensitivity of KM12L4a cells. As an additional test of whether protein phosphorylation protects KM12L4a cells against the cytotoxicity of MDR-linked drugs, we examined the effects of H7, a protein kinase inhibitor that potently inhibits PKC (29), on the chemosensitivity of KM12L4a cells in the presence and absence of PDBu. As shown in Fig. 2, 10 μM H7 alone or in combination with 15 nM PDBu caused <10% inhibition of cell growth. The administration of H7 in the presence of PDBu resulted in chemosensitivities to ADR, VCR, and VLB that were comparable to those observed in the absence of H7 and PDBu (Fig. 2). Furthermore, H7 significantly increased the sensitivity of the cells to ADR, VCR, and VLB in the absence of PDBu (Fig. 2), indicating that inhibition of basal levels of protein phosphorylation can sensitize KM12L4a cells to the cytostatic effects of these drugs.

Effects of phorbol esters on drug accumulation and efflux. To examine whether the mechanism by which PDBu protected KM12L4a cells from the cytotoxic drugs could involve changes in drug uptake or drug efflux rates, drug accumulation was measured. In experiments shown in Fig. 3, the accumulation rates of both [^{14}C]ADR and [^{3}H]VCR consisted of a rapid phase with a duration of approximately 2 hr, followed by a slow phase. Coincubation with 50 nM TPA or 100 nM PDBu significantly reduced the extent of [^{14}C]ADR and [^{3}H]VCR accumulation (Fig. 3). Inhibition of drug accumulation by TPA and PDBu was dose dependent, and each phorbol ester exerted approximately 50% of its inhibitory effect at a concentration of 25 nM (Table 1). Consistent with our observation that PDBu had no effect on 5-FU cytotoxicity in KM12L4a cells (Fig. 1D), we found that PDBu also had no effect on [^{14}C]5-FU accumulation in the cells, under the experimental conditions employed with [^{3}H]VCR and [^{14}C]ADR (data not shown).

To test whether the inhibition of ADR and VCR accumulation by the phorbol esters could be related to phorbol ester-induced inhibition of ADR and VCR cytotoxicity, we compared the effects of H7 on these processes. In the experiments shown in Table 2, 50 nM TPA and 100 nM PDBu inhibited ADR accumulation by 52 and 36%, respectively; coincubation with 100 μM H7 reduced the respective degrees of inhibition to 5 and 14%. H7 also antagonized the phorbol ester-mediated reduction in VCR accumulation (Table 2). Thus, H7 had parallel effects against phorbol ester-induced changes in drug cytotoxicities and accumulation rates. However, the concentration of H7 required to antagonize the phorbol ester-mediated reduction in [^{14}C]ADR and [^{3}H]VCR accumulation (Table 2) was substantially greater than the concentration of H7 needed to alter the chemosensitivity of the KM12L4a cells to ADR and VCR in the presence of PDBu (Fig. 2). Although this discrepancy may arise from the fact that phorbol ester concentrations in the drug accumulation assays (Table 2) were higher than the phorbol ester concentrations employed in the growth inhibition assay (Fig. 2), the possibility that H7-induced

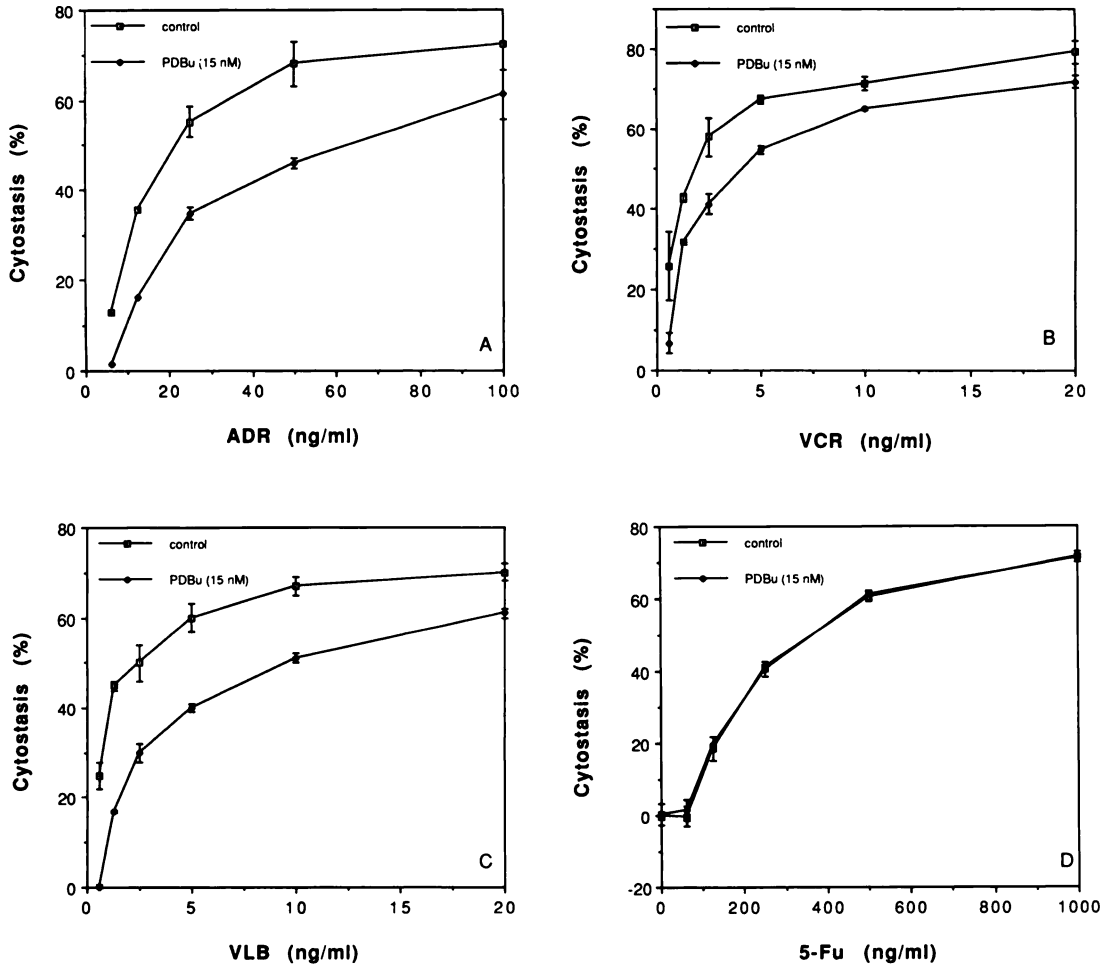

Fig. 1. Inhibitory effects of PDBu on cytostasis induced by anticancer drugs. KM12L4a cells were seeded at a density of 5×10^3 cells/well in a 96-well plate. After a 16- to 20-hr attachment period, the cells were treated with 15 nM PDBu for 1 hr and then incubated with various concentrations of drugs for 96 hr in the presence of PDBu. Viable cells were stained with MTT and read with a microspectrophotometer at 600 nm. For further details, see Materials and Methods. A, Cells exposed to ADR and PDBu; B, cells exposed to VCR and PDBu; C, cells exposed to VLB and PDBu; D, cells exposed to 5-FU and PDBu.

changes in drug accumulation do not entirely account for the observed alterations in drug sensitivity caused by H7 cannot be ruled out.

Because drug accumulation represents the sum of drug influx and efflux components (4, 5, 22), we next investigated the effects of phorbol esters on drug efflux rates. In control experiments, ADR and VCR were slowly but continuously effluxed from preloaded KM12L4a cells over a 2-hr period (for experimental details, see Materials and Methods). Coincubation with 100 nM TPA or 100 nM PDBu did not detectably alter the rates of [^{14}C]ADR and [^{3}H]VCR efflux (data not shown).

Effects of OAG on drug accumulation and efflux. In the next set of experiments, the effects of a diacylglycerol PKC activator, OAG, on drug accumulation and efflux rates were studied, in an attempt to correlate our findings concerning the effects of the phorbol esters on KM12L4a human colon cancer cells *in vitro* with effects of environmental factors that are present in the colon *in vivo* (17). Under the conditions used in the study of phorbol esters, 0–500 μM OAG induced a significant, dose-dependent reduction of [^{14}C]ADR and [^{3}H]VCR accumulation. At 500 μM, for example, OAG reduced ADR and VCR accumulation to 76 and 72% of the control, respectively (Table 3). As observed with phorbol esters, the inhibitory effects of OAG on both [^{14}C]ADR and [^{3}H]VCR accumulation could be completely overcome by H7 (Table 4). We also determined the effect of OAG on drug efflux. We found that, like PDBu, OAG did not detectably affect the efflux rates for [^{14}C]ADR or [^{3}H]VCR during a 2-hr assay period (Table 5). Finally, we tested whether OAG could induce resistance to the cytotoxic drugs in KM12L4a cells during a 96-hr incubation period. In contrast to the results with PDBu (Fig. 1), we found that OAG did not induce resistance to ADR, VCR, or VLB, under the experimental conditions employed in Fig. 1 (data not shown).

In order to determine whether rapid metabolism of OAG could account for its inability to induce drug resistance in KM12L4a cells, we measured the amount of diacylglycerol present over a 24-hr period at 37° in wells that initially contained 500 μM OAG in CMEM and KM12L4a cells. Diglyceride was measured by a diglyceride kinase assay (30), using a kit from Sphinx Pharmaceuticals Corp. (Durham, NC), according to the instructions. A linear standard curve was generated with OAG (60–1000 pmol in CMEM). In 1 hr, the amount of diacylglycerol fell by 20%. The amount of diacylglycerol was reduced to 25 and 5% of its original value after 4 and 24 hr, respectively.

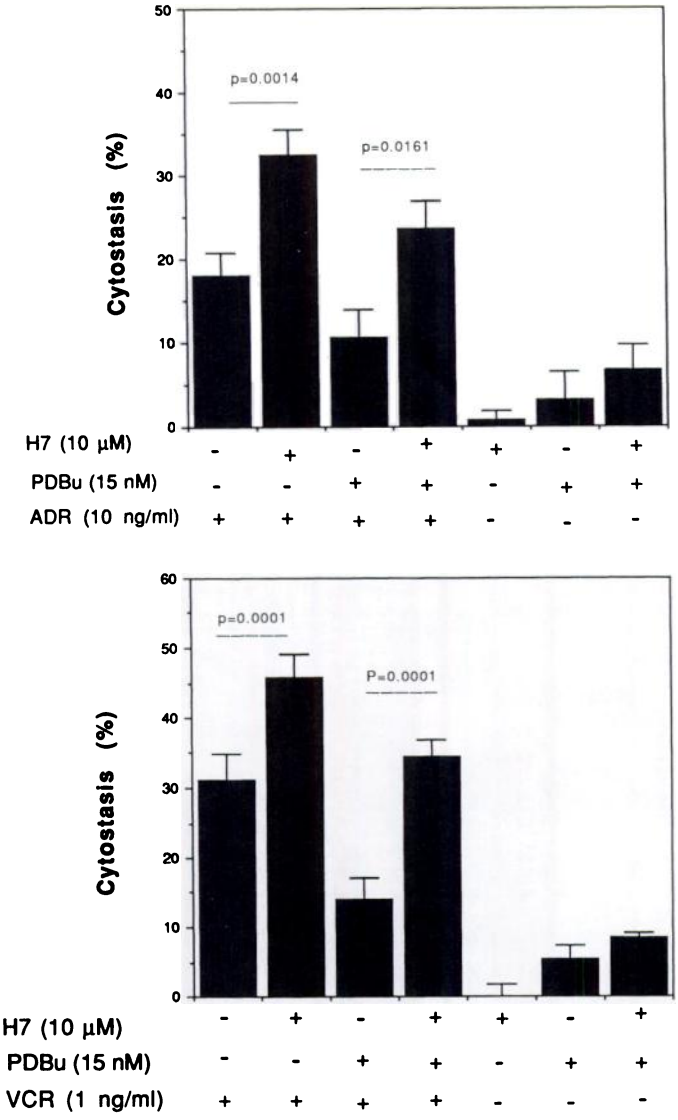

Fig. 2. Effect of H7 on the protective role of PDBu. KM12L4a cells in 96-well plates were exposed to H7 and PDBu, where indicated, for 1 hr and then incubated with various drugs for 96 hr in the continuous presence of H7 and/or PDBu. The viable cells were stained with MTT and read with a microspectrophotometer at 600 nm. For further details, see Materials and Methods. Top panel, Cells exposed to ADR, H7, and PDBu; Center panel, cells exposed VCR, H7, and PDBu; Bottom panel, cells exposed to VLB, H7, and PDBu.

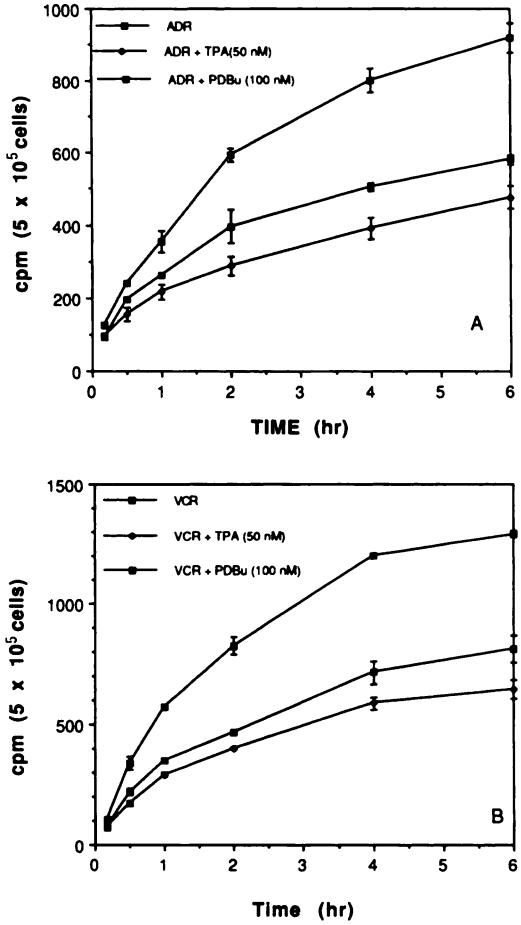

Fig. 3. Inhibitory effects of TPA and PDBu on ADR and VCR accumulation. KM12L4a cells were seeded at a density of 5×10^5 cells/well in 24-well plates. After a 16-hr attachment period, the cells were treated with 50 nM TPA or 100 nM PDBu for 30 min and then incubated with 0.1 μM [^{14}C]ADR (A) or [^{3}H]VCR (B) for the indicated lengths of time, in the presence of TPA or PDBu. The amount of [^{14}C]ADR or [^{3}H]VCR incorporated was determined as described in Materials and Methods.

Thus, rapid metabolism of OAG appears to account for its lack of effect on the drug resistance measured in KM12L4a cells.

Discussion

In the present studies, we demonstrate that treatment of the human colon cancer cell line KM12L4a with the phorbol ester PDBu reduced the sensitivity of the cells to ADR, VCR, and VLB (Fig. 1 and Table 1), under conditions that did not down-regulate PKC. Intrinsic drug resistance is a major obstacle to successful treatment of human colon cancer with chemotherapy. Although coincubation with PDBu protected KM12L4a cells from the cytotoxicity of ADR, VCR, and VLB, PDBu treatment had no effect on the cytotoxicity of 5-FU (Fig. 1). Thus, the spectra of drugs subject to PDBu-induced resistance, MDR (1–5), and the intrinsic resistance phenotypes observed in colon cancer *in vivo* appear to be related (31). Pgp is expressed at relatively high levels in the normal colonic epithe-

TABLE 1

Inhibitory effects of phorbol esters on ADR and VCR accumulation in KM12L4a cells

KM12L4a cells in 24-well plates were exposed to various concentrations of TPA or PDBu for 30 min and then incubated with 0.1 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, in the presence of TPA and PDBu. At the end of the incubation period, drug incorporation was measured. For further details, see the legend to Fig. 3.

Concentration of phorbol esters	Drug accumulation			
	TPA		PDBu	
	ADR[a]	VCR[b]	ADR[a]	VCR[b]
nM	*% of control*			
0	100	100	100	100
12	82 ± 7	65 ± 3	97 ± 4	87 ± 1
25	69 ± 5	57 ± 4	90 ± 4	82 ± 5
50	59 ± 7	49 ± 1	84 ± 6	73 ± 3
100	55 ± 2	45 ± 0	79 ± 7	63 ± 3

[a] Mean ± standard error of three experiments.
[b] Mean ± standard error of two experiments.

TABLE 2

Effects of H7 on the inhibition of ADR and VCR accumulation by phorbol esters

KM12L4a cells in 24-well plates were exposed to H7 and phorbol esters for 30 min and then incubated with 0.1 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, in the presence of H7 and phorbol esters. The drugs incorporated were then determined. For details, see the legend to Fig. 3 and Materials and Methods. One experiment, representative of four independent experiments, is shown.

Drug	Treatment	Drug accumulation			
		0 μM[a]	10 μM	30 μM	100 μM
		% of control			
[^{14}C]ADR	Medium	100	103	103	105
	TPA (50 nM)	48	53	62	95
	PDBu (100 nM)	64	69	75	86
[^{3}H]VCR	Medium	100	98	107	123
	TPA (50 nM)	40	45	52	82
	PDBu (100 nM)	64	63	79	95

[a] H7 concentration.

TABLE 3

Inhibitory effects of OAG on ADR and VCR accumulation

KM12L4a cells seeded in 24-well plates were exposed to the indicated concentrations of OAG for 30 min and then incubated with 0.1 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, in the presence of OAG. The amount of each drug incorporated into the cells was determined as described previously. For further details, see the legend to Fig. 3 and Materials and Methods.

OAG	Drug accumulation	
	[^{14}C]ADR[a]	[^{3}H]VCR[b]
μM	*% of control*	
0	100	100
63	96 ± 3	93 ± 2
125	84 ± 2	88 ± 3
250	81 ± 2	78 ± 2
500	76 ± 2	72 ± 2

[a] Mean ± standard error of four experiments.
[b] Mean ± standard error of five experiments.

lium and in certain colon tumors (16, 32, 33) and, therefore, appears to contribute to the intrinsic resistance observed in colon tumors; there is evidence, however, that environmental factors may also contribute to intrinsic resistance in the colon. The observation that colon cancer cells are often highly sensitive to anticancer drugs *in vitro* but very resistant to them *in vivo* (33)[1] supports a role for environmental factors in the intrinsic resistance phenotype. Recently, Friedman *et al.* (17) demonstrated that human colonic epithelium is chronically exposed to high concentrations (>500 μM) of unsaturated diacylglycerols *in vivo*. Because unsaturated diacylglycerols include structural and functional analogues of phorbol ester tumor promoters (18), we hypothesized that unsaturated diacylglycerols present in the lumen of the colon might include environmental factors that contribute to the intrinsic resistance of colon tumors. In this report, we tested this hypothesis *in vitro* by comparing the effects of phorbol esters and OAG on the drug responsiveness of KM12L4a human colon cancer cells. We found that the diacylglycerol OAG mimicked the effects of phorbol esters on the accumulation of MDR-linked drugs (Table 3) in cultured human colon cancer cells. However, OAG is rapidly metabolized in intact cells, and it failed to protect the cultured colon cancer cells against MDR-linked drugs in a 96-hr cytotoxicity assay. Because the colonic epithelium is exposed to constantly replenished supplies of unsaturated diacylglycerol derived from dietary fat *in vivo* (17), rapid metabolism of diacylglycerol in the epithelial cells should not prevent chronic activation of PKC by extracellular diacylglycerol in that tissue. Thus, our *in vitro* results provide evidence that unsaturated diacylglycerols may be environmental factors that contribute to the intrinsic drug resistance of colon cancer *in vivo* by reducing drug accumulation in the cancer cells. Furthermore, our observation that OAG antagonizes [^{14}C]ADR and [^{3}H]VCR accumulation in cultured tumor cells provides a new line of evidence that phorbol ester-induced drug resistance in cultured cancer cells (13–15) is a consequence of PKC activation.

Phorbol ester PKC activators reduce the chemosensitivities of P388 murine leukemia cells (15), human KB carcinoma cells (14), and parental and ADR-selected MDR human breast cancer MCF-7 cells (13) to MDR-linked drugs. In this report, we

TABLE 4

Antagonistic effects of H7 on the reduction of drug accumulation by OAG

KM12L4a cells in 24-well plates were exposed to H7 and/or OAG for 30 min and then incubated with 0.1 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, in the presence of H7 and/or OAG. The amount of drug incorporated into the cells was determined as described previously. For further details, see the legend to Fig. 3 and Materials and Methods. Data are mean ± standard deviation. This experiment was reproducible in its entirety.

OAG (500 μM)	Drug accumulation			
	[^{14}C]ADR		[^{3}H]VCR	
	Medium	H7 (10 μM)	Medium	H7 (10 μM)
	% of control			
−	100	108 ± 2	100	111 ± 5
+	77 ± 5	102 ± 3	76 ± 1	109 ± 1

TABLE 5

Effects of OAG on the efflux of [^{14}C]ADR and [^{3}H]VCR

KM12L4a cells in 24-well plates were preloaded with 0.2 μM [^{14}C]ADR or [^{3}H]VCR for 2 hr, followed by rapid washing with PBS and incubation at 37° for the indicated lengths of time. Aliquots of the supernatants were removed and counted. For further details, see Materials and Methods. Data are mean ± standard deviation. This experiment was reproducible in its entirety.

Time	Drug efflux			
	[^{14}C]ADR		[^{3}H]VCR	
	Medium	OAG (500 μM)	Medium	OAG (500 μM)
min	*cpm*			
10	107 ± 8	118 ± 5	190 ± 10	215 ± 47
30	131 ± 11	130 ± 1	257 ± 26	260 ± 18
60	182 ± 11	190 ± 11	429 ± 16	405 ± 0
120	259 ± 17	277 ± 1	671 ± 68	610 ± 43

show that PDBu also reduces the chemosensitivity of human colon cancer KM12L4a cells to MDR-linked drugs. Recently, Chambers *et al.* (35) demonstrated that purified PKC phosphorylates Pgp isolated from the MDR cell line KB-V1 and that phorbol esters inhibit verapamil-induced [^{3}H]VLB accumulation in the cells, providing evidence that phorbol ester-mediated PKC activation may reduce the chemosensitivity of cultured tumor cells to MDR-linked drugs by stimulating Pgp-mediated drug efflux. However, Kessel (15) has shown that phorbol esters inhibit the uptake of ADR by P388 cells but do not affect the rate of ADR efflux. Similarly, in this report we show that phorbol esters inhibit the accumulation of [^{14}C]ADR and [^{3}H]VCR in KM12L4a cells but do not alter the efflux rates of these drugs. Thus, the protection that phorbol esters provide P388 and KM12L4a cells against MDR-linked cytotoxic drugs cannot be accounted for by stimulation of Pgp-mediated drug efflux. Therefore, the phorbol ester-mediated protection appears to entail events that modulate the accumulation of MDR-linked drugs independently of Pgp. However, the phorbol ester concentrations needed to alter drug accumulation rates were considerably greater than those needed to alter the chemosensitivities of the cells. Thus, alterations in drug accumulation rates by phorbol esters may not entirely account for their protective effects against cytotoxic MDR-linked drugs in KM12L4a cells.

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