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TRANSIENT ENHANCEMENT OF MULTIDRUG RESISTANCE BY THE BILE ACID DEOXYCHOLATE IN MURINE FIBROSARCOMA CELLS *IN VITRO*

CATHERINE A. O'BRIAN,* DOMINIC FAN, NANCY E. WARD, ZHONGYUN DONG, LARA IWAMOTO, KRISHNA P. GUPTA, LAURA E. EARNEST and ISAIAH J. FIDLER

Department of Cell Biology, The University of Texas M.D. Anderson Cancer Center, Houston, TX 77030, U.S.A.

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Abstract—Recent studies have implicated protein kinase C (PKC) activation in drug resistance *in vitro*. PKC can be activated directly by phorbol-ester tumor promoters as well as by the bile acid deoxycholate. In this report, we demonstrate that deoxycholate, at concentrations that are chronically present in the lumen of the colon *in vivo*, mimicked phorbol-ester tumor promoters by protecting Adriamycin® (ADR)-sensitive and multidrug-resistant (MDR) murine fibrosarcoma UV-2237M cells from ADR cytotoxicity. Deoxycholate also enhanced the resistance of the MDR cell line UV-2237M-ADR^R to the cytotoxic effects of vincristine and vinblastine. In contrast to cytotoxic drug-selected MDR phenotypes, deoxycholate-induced drug resistance was transient and required continuous exposure to the bile acid. The protein kinase inhibitor H7 completely reversed the protection against ADR cytotoxicity conferred on UV-2237M-ADR^R cells by deoxycholate, providing evidence that deoxycholate exerts its protective effects by a mechanism that involves stimulation of protein phosphorylation and not merely by detergent effects on membrane permeability. PKC consists of a family of at least seven isozymes with distinct modes of activation and substrate specificities. We previously reported that MDR UV-2237M cell lines contain higher levels of PKC activity than the parental ADR-sensitive UV-2237M cell line (O'Brian *et al.*, *FEBS Lett* **246**: 78–82, 1989). The present report shows that PKC-III is a major PKC isozyme in ADR-sensitive and MDR UV-2237M cell lines. Thus, the resistance to ADR induced by the phorbol esters in UV-2237M cell lines provides strong evidence that PKC-III activation confers protection against ADR on ADR-sensitive and MDR UV-2237M cell lines. Furthermore, since deoxycholate is an endogenous molecule in the colonic epithelium, our finding that physiological concentrations of deoxycholate can render cells more resistant to chemotherapeutic drugs *in vitro* may have implications for the biology and therapy of intestinal cancers.

Resistance to anticancer drugs presents a major limitation to successful cancer chemotherapy [1–3]. *In vitro* selection of drug-resistant cells from a drug-sensitive population by exposure to cytotoxic natural products such as Adriamycin® (ADR)† generally results in the emergence of a multidrug resistant (MDR) phenotype. MDR cells are resistant to the selecting agent and are cross-resistant to diverse anticancer drugs, including anthracyclines and Vinca alkaloids [1–3]. The most common feature of the MDR phenotype is overexpression of P-glycoprotein, a membrane protein believed to function as an ATP-driven pump that transports drugs out of cells [1–3]. Recent evidence also implicates the tumor promoter receptor protein kinase C (PKC) in MDR phenotypes [4–7].

* Address reprint requests to: Dr. Catherine A. O'Brian, Department of Cell Biology, The University of Texas, M.D. Anderson Cancer Center, 1515 Holcombe Blvd., Box 173, Houston, TX 77030.

† Abbreviations: ADR, Adriamycin®; cAMP, adenosine 3′,5′-cyclic monophosphate; MDR, multidrug resistant; PBS, phosphate-buffered saline; PDBu, phorbol dibutyrate; PDE, phosphodiesterase; PKC, protein kinase C; PS, phosphatidylserine; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; VCR, vincristine; and VLB, vinblastine.

The murine fibrosarcoma cell lines UV-2237M-ADR^R, and UV-2237M-rev are ADR-resistant cell lines selected from the ADR-sensitive parental cell line UV-2237M by culturing the cells in the presence of ADR. These fibrosarcoma cell lines express different degrees of ADR resistance [8], which correlate directly with PKC activity levels [6, 8]. Consistent with this observation in murine fibrosarcoma cells, elevated PKC activity has also been reported in an MDR human breast MCF-7 cell line [5] and in an ADR-resistant HL60 cell line [9]. A role for PKC in drug resistance is also supported by our finding that the potent PKC inhibitor H7 partially reverses ADR resistance in UV-2237M cell lines, whereas HA-1004, which is a structural analogue of H7 that only weakly inhibits PKC, does not [6].

Phorbol-ester tumor promoters are highly selective activators of PKC [7, 10]. Subsequent to *in vitro* treatment with phorbol-ester tumor promoters, MCF-7 cells [5], human KB cells [4], and chronic lymphocytic leukemia cells [11] have been reported to have increased resistance to anticancer drugs, thus implicating PKC activation in drug resistance *in vitro*. Furthermore, phorbol esters inhibit ADR uptake in drug-sensitive and MDR P388 cells [12]. The bile acid deoxycholate is an endogenous activator of PKC in the colonic epithelium [7, 13, 14].

Deoxycholate stimulates PKC activity by direct effects on the enzyme [7, 13] and by enhancing phospholipase C-catalyzed production of diacylglycerol, a potent PKC activator [7, 14, 15]. In this report, we show that deoxycholate mimicked phorbol-ester tumor promoters by enhancing ADR resistance in an ADR-selected MDR UV-2237M cell line and by endowing ADR-sensitive UV-2237M cells with ADR resistance at deoxycholate concentrations that are chronically present in the lumen of the colon *in vivo*. Furthermore, we report that deoxycholate also enhanced vincristine (VCR) and vinblastine (VLB) resistance in the MDR UV-2237M cell line.

PKC consists of a family of at least seven isozymes with distinct modes of activation and substrate specificities [7, 10]. We report that PKC-III is a major PKC isozyme in ADR-sensitive and MDR UV-2237M cells. Thus, the protection against ADR cytotoxicity exerted by the phorbol esters in the UV-2237M cell lines provides evidence for a role for PKC-III activation in the ADR resistance of murine UV-2237M fibrosarcoma cells *in vitro*, and the protection against ADR, VCR, and VLB cytotoxicity exerted by deoxycholate suggests that the bile acid may serve as an environmental factor that contributes to the drug resistance of intestinal cancers *in vivo*.

MATERIALS AND METHODS

Cell lines. The parental UV-2237 fibrosarcoma was induced in a C3H/HeN (mammary tumor virus-negative) mouse by chronic exposure to ultraviolet-B radiation [16]; the UV-2237M cell line was isolated from several spontaneous lung metastases produced by the parental subcutaneous tumor [17]. Both lines are sensitive to the cytotoxic effects of ADR. UV-2237M-ADR^R cells were selected from UV-2237M cells by their ability to grow in medium containing 1 μg/mL ADR. The doubling times for the UV-2237M and UV-2237M-ADR^R cells are 20.8 and 31.7 hr, respectively. These lines have been maintained in medium containing ADR at the concentrations used for selection [8]. UV-2237M-rev cells were derived from UV-2237M-ADR^R cells grown in medium without ADR for 4 months. UV-2237M-rev cells exhibit an intermediate degree of ADR resistance [8]. P-glycoprotein is overexpressed in UV-2237M-ADR^R cells, but is not expressed in parental UV-2237M cells according to Western blot analysis [8, 18]. The ADR-resistant phenotypes expressed by these cells were stable during the course of this study. The cell lines were maintained on plastic in Eagle's minimum essential medium supplemented with 5% fetal bovine serum, sodium pyruvate, non-essential amino acids, L-glutamine, and vitamins (5% CMEM). The cell lines were confirmed to be free of mycoplasma, reovirus type 3, murine pneumonia virus, mouse adenovirus, murine hepatitis virus, lymphocyte choriomeningitis virus, ectromelia virus, and lactate dehydrogenase virus (assayed by Microbiological Associates, Walkersville, MD). Each cell line was passaged at least once in the absence of ADR and then cultured for 24 hr (in the absence of ADR) to allow cell attachment prior to experiments. The MDR phenotype of the UV-2237M-ADR^R cells (Table 1) and the level of PKC activity in the cells were stable for 3 weeks, when the cells were cultured in the absence of ADR.

Reagents. ADR was purchased from Adria (Columbus, OH). [^{3}H]Vincristine sulfate, [^{14}C]ADR, and [γ^{32}P]ATP were from the Amersham Corp. (Arlington Heights, IL), and phosphocellulose paper, grade p81, was from Fisher Scientific (Houston, TX). Deoxycholate, chenodeoxycholate, cholate, tetrazolium, PDBu, TPA, ATP, histone IIIS, leupeptin, phenylmethylsulfonyl fluoride, PS, Triton X-100, bovine brain calmodulin (>40,000 units/mg protein, 98% pure), *Crotalus atrox* venom 5'-nucleotidase (200–500 units/mg), and a kit for the measurement of inorganic phosphate were purchased from the Sigma Chemical Co. (St. Louis, MO). Bovine heart calmodulin-deficient cAMP-PDE was purchased from Boehringer Mannheim (Indianapolis, IN). Tissue culture reagents were purchased from GIBCO (Grand Island, NY). Protein concentration assay solution and hydroxylapatite resin were from BioRad Laboratories (Richmond, CA). Vinblastine sulfate was from Cetus (Emeryville, CA), vincristine sulfate was from Quid Pharmaceuticals (Indianapolis, IN), and actinomycin D was from Merck, Sharp & Dohme (West Point, PA). 5-FU (5-fluorouracil) was from Roche Laboratories (Nutley, NJ).

Measurement of the sensitivities of parental and ADR-selected UV-2237M cell lines to ADR and other cytotoxic agents. Parental and ADR-selected UV-2237M cell lines were harvested in their exponential growth phase by a 2-min treatment with 0.25% trypsin/0.02% EDTA (w/v). Single-cell suspensions with a viability of >95% (trypan blue exclusion) were seeded into 96-well microculture plates at 2500 cells/38 mm^2 well and incubated for 24 hr at 37° in a humidified atmosphere containing 5% CO_2. Cells were then incubated in medium containing different concentrations of reagents such as ADR, vincristine, and TPA, as indicated in Results. After a 96-hr incubation, the number of viable cells was determined by a colorimetric tetrazolium procedure, as described in detail previously [6, 19]. This entailed the addition of 40 μL tetrazolium dye at 2.5 mg/mL in PBS to each well at the end of the assay period. Cells were incubated with the dye for 2 hr at 37° to allow the dye to react with mitochondrial dehydrogenases in the viable cells. Unreacted dye and medium were

Table 1. Multidrug resistant phenotypes of ADR-selected UV-2237M cell lines

Drugs	Parent IC_{50} (μM)	Rev IC_{50} (μM)	ADR^R IC_{50} (μM)
Adriamycin	0.61	1.8	14.3
Actinomycin-D	0.0014	0.0018	0.014
Vinblastine	0.0027	0.0036	0.173
Vincristine	0.0049	0.0158	0.242

The IC_{50} values represent drug concentrations eliciting a 50% reduction in cell number after 96 hr. These values were derived from cells exposed to three log concentrations of each drug. SD < 10%. Parent = UV-2237M; Rev = UV-2237M-rev; and ADR^R = UV-2237M-ADR^R.

then removed, dimethyl sulfoxide (100 μL) was added to each well, and the absorbance at 570 nm was measured with a microplate scanning spectrophotometer [6, 19].

Drug accumulation assays. Drug accumulation assays in UV-2237M and UV-2237M-ADR^R cells were based on previously described protocols [20]. Stock solutions of [^{14}C]ADR and [^{3}H]VCR were prepared in 0.9% saline to a final concentration of 100 μM. Their respective specific activities were 25 mCi/mmol and 220 mCi/mmol, and they were diluted in CMEM containing HEPES (10 mM, pH 7.5) prior to their addition to cells. UV-2237M and UV-2237M-ADR^R cells were pipetted into 24-well plates (5×10^5 cells/well), preincubated with/without deoxycholate or TPA, and washed with assay medium (CMEM–10 mM HEPES, pH 7.3) at 37°. Then the cells were incubated with the radiolabeled drugs for 2 hr at 37°, washed rapidly, and detached with trypsin. Cell suspensions were harvested and counted in 15 mL of scintillation fluid [20].

Isolation of UV-2237M PKC isozymes. To isolate PKC isozymes from the cell lines UV-2237M and UV-2237M-ADR^R, cell lysates were subjected sequentially to DEAE ion-exchange [6] and hydroxylapatite chromatographies [21]. Approximately 2×10^7 subconfluent cells were lysed with 1% Triton X-100 in buffer A (20 mM Tris–HCl, 5 mM EDTA, 5 mM ethyleneglycolbis(aminoethylether)tetra-acetate (EGTA), 0.25 mM phenylmethylsulfonyl fluoride, 10 μg leupeptin/mL, 20 μg soybean trypsin inhibitor/mL, 15 mM 2-mercaptoethanol, pH 7.5) by stirring at 4° for 1 hr. The cell lysate (10 mL) was filtered through glass wool and loaded onto a 1-mL DEAE Sephacel column equilibrated in buffer A. The column was washed with 3 mL buffer A, and PKC was eluted with 3 mL buffer A containing 0.2 M NaCl. The PKC-containing eluant was diluted with 3 mL buffer B (20 mM potassium phosphate, 0.5 mM EDTA, 0.5 mM EGTA, 0.25 mM phenylmethylsulfonyl fluoride, 100 μg leupeptin/mL, 15 mM 2-mercaptoethanol, pH 8.3) and loaded onto a 3-mL hydroxylapatite column equilibrated in buffer B. The column was washed with 6 mL buffer B and eluted with a 50-mL linear gradient of 20–230 mM potassium phosphate at pH 8.3; 1.5-mL fractions were collected.

The PKC activity of each fraction was determined as previously described [22], by subtracting the phosphotransferase activity between [γ^{32}P]ATP and histone IIIS observed in the presence of Ca^{2+} from the activity observed 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 PS/mL (or none), 6 μM [γ^{32}P]ATP (4000–6000 cpm/pmol), 0.67 mg histone IIIS/mL, and PKC. Reactions proceeded for 10 min at 30° and were terminated on phosphocellulose paper [22].

Calmodulin assay. The activation of cAMP-PDE by calmodulin was assayed as previously described [23, 24]. The cAMP-PDE-catalyzed production of 5′-AMP from cAMP was coupled to the 5′-nucleotidase-catalyzed production of P_i from 5′-AMP. P_i formation was measured using a kit from the Sigma Chemical Co. Reaction mixtures (0.9 mL) contained 40 mM Tris–HCl, 40 mM imidazole, 5 mM Mg^{2+}-acetate, 500 μM $CaCl_2$ (or 100 μM EGTA), 0.3 units 5′-nucleotidase/mL, 0.01 units cAMP-PDE/mL, and 5 units calmodulin/mL at pH 7.5. Reactions were initiated by the addition of 1.2 mM cAMP (final concentration), proceeded for 30 min at 30°, and were terminated with 100 μL of 55% trichloroacetic acid [23, 24].

RESULTS

Table 1 shows a direct correlation between ADR resistance and multidrug resistance among murine fibrosarcoma UV-2237M cell lines with different degrees of resistance to ADR. These data extend our reported observation of a direct correlation between the level of PKC activity and the degree of ADR resistance among these tumor cell lines [6]. To test the possibility that PKC activation contributes to ADR resistance in these cells, we next examined the effects of the highly selective PKC activator PDBu on ADR resistance in UV-2237M-ADR^R cells. Figure 1A shows that continuous exposure to 2 nM PDBu enhanced ADR resistance in UV-2237M-ADR^R cells. These results could not be attributed to down-regulation of PKC by PDBu, since control studies with UV-2237M-ADR^R cells exposed to 2 nM PDBu for 96 hr indicated that they still contained 85 ± 9% of the PKC activity observed in unexposed UV-2237M-ADR^R cells. Similarly, the phorbol ester TPA (2 nM) enhanced ADR resistance without down-regulating PKC (data not shown). Furthermore, a 1-hr exposure to 2 nM PDBu reduced the growth inhibition achieved by 4.3 μM ADR from 14 ± 2% to 4 ± 1% ($P < 0.013$), indicating that even a short-term exposure to the phorbol ester could enhance the ADR resistance of UV-2237M-ADR^R cells.

The colonic epithelium is chronically exposed to deoxycholate and related bile acids at concentrations exceeding 100 μM [25]. We have reported that deoxycholate and eight other bile acids can stimulate purified rat brain PKC activity [13], and Craven *et al.* [14] have demonstrated that deoxycholate causes PKC activation in isolated colonic crypts. To examine the possibility that deoxycholate contributes to drug resistance in cells exposed to bile acids *in vivo*, we determined the effect of continuous exposure to deoxycholate on ADR resistance in UV-2237M-ADR^R cells. The data shown in Fig. 1A demonstrate that 50 μM deoxycholate was as effective as the phorbol ester PDBu in enhancing ADR resistance in UV-2237M-ADR^R cells. The observed reversal of ADR cytotoxicity by deoxycholate could not be ascribed to alterations in the uptake of 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl-tetrazolium bromide (MTT) into the mitochondria by deoxycholate, since deoxycholate had only minor effects on the cell survival observed in the absence of ADR (Fig. 1A). Control studies indicated that the reversal of ADR cytotoxicity by deoxycholate did not involve down-regulation of PKC, since UV-2237M-ADR^R cells retained 84 ± 3% of their original PKC activity after a 96-hr exposure to 50 μM deoxycholate. To test whether the enhancement of ADR resistance in

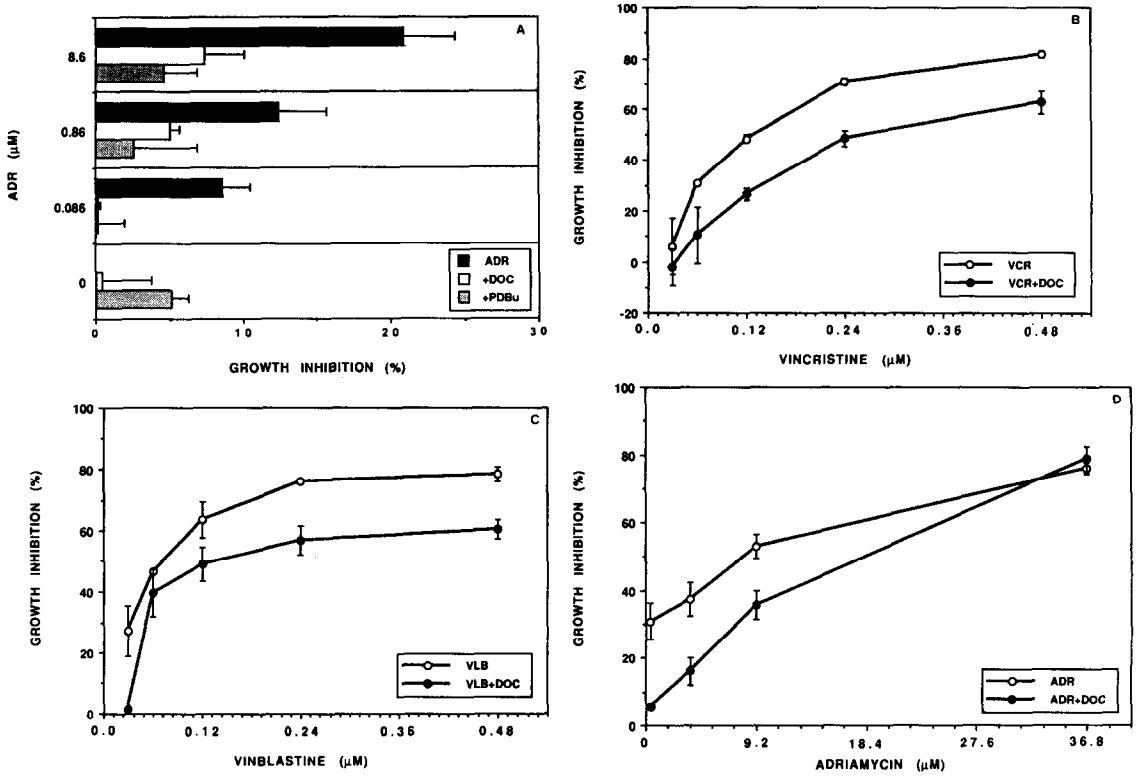

Fig. 1. Enhancement of drug resistance in UV-2237M-ADR^R cells by phorbol-ester tumor promoters and the bile acid deoxycholate. (A) UV-2237M-ADR^R cells were exposed to 2 nM PDBu, 50 μM deoxycholate (DOC) and ADR, as indicated, throughout a 96-hr incubation period. At the end of the incubation period, the number of viable cells was determined using a colorimetric tetrazolium assay. "Growth inhibition" represents the reduction in cell number elicited by the indicated compounds. For experimental details, see Materials and Methods. Values are means ± SD derived from quadruplicate assays. (B) UV-2237M-ADR^R cells were exposed to VCR at the indicated concentrations in the continuous presence or absence of 50 μM DOC over a 96-hr incubation period. For further details, see (A). (C) UV-2237M-ADR^R cells were exposed to VLB at the indicated concentrations in the continuous presence or absence of 50 μM DOC over a 96-hr incubation period. For further details, see (A). (D) UV-2237M-ADR^R cells were exposed to ADR at the indicated concentrations in the continuous presence or absence of 50 μM DOC over a 96-hr period. For details, see (A).

UV-2237M-ADR^R cells by deoxycholate could be attributed to stimulation of protein phosphorylation, we determined the effects of the protein kinase inhibitor H7 [6, 7] on the action of deoxycholate. We found that, under conditions where deoxycholate significantly reduced the cytotoxicity of ADR in the UV-2237M-ADR^R cells, the co-incubation of deoxycholate and 20 μM H7 eliminated the protective effects of deoxycholate (Fig. 2). Similarly, the enhancement of ADR resistance mediated by PDBu appeared to be reversed completely by 20 μM H7 (Fig. 2), providing further evidence that PKC activation induced the resistance observed with PDBu. We also examined the ability of deoxycholate to enhance the multidrug resistance phenotype of the UV-2237M-ADR^R cells. Panels B and C of Fig. 1 show representative data indicating that deoxycholate enhanced resistance to VCR and VLB across a broad range of Vinca alkaloid concentrations. The results shown in panels B and C of Fig. 1 were reproducible, and the enhancements of VCR ($P = 0.01$, $N = 4$) and VLB ($P = 0.045$, $N = 4$) resistance achieved by deoxycholate were statistically significant. Figure 1D shows that deoxycholate also enhanced ADR resistance at ADR concentrations as high as 9.2 μM which caused >50% cytotoxicity when administered alone. In contrast with the effects of deoxycholate on VCR and VLB cytotoxicity, however, the bile acid had no effect on ADR cytotoxicity at an ADR concentration that elicited >70% cytotoxicity (Fig. 1D). Deoxycholate increased the IC_{50} of VCR from 0.13 to 0.29 μM, and it increased the IC_{50} of VLB from 0.086 to 0.20 μM in the UV-2237M-ADR^R cells. The IC_{50} of ADR was increased from 8.1 to 18.4 μM by deoxycholate. Thus, deoxycholate ostensibly enhanced the MDR phenotype of the UV-2237M-ADR^R cell line. In contrast, deoxycholate did not influence the sensitivity of the UV-2237M-ADR^R cells to either m-AMSA (amsacrine) ($P > 0.250$, $N = 3$) or 5-FU ($P > 0.63$, $N = 3$).

In the next set of studies, we examined the effects of PDBu and deoxycholate on the sensitivity of parental UV-2237M cells to ADR. We found that continuous treatment of parental ADR-sensitive UV-2237M cells with 2 nM PDBu or 10 μM

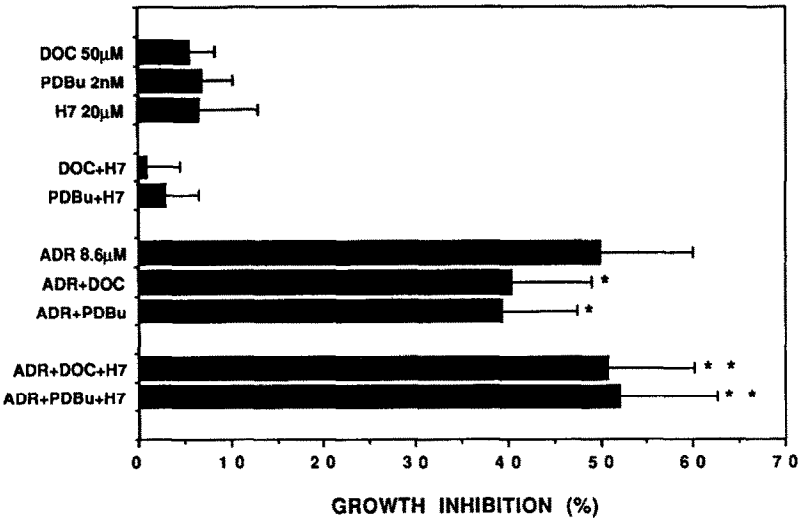

Fig. 2. Reversal by H7 of the enhanced ADR resistance induced by deoxycholate and PDBu in UV-2237M-ADR^R cells. Experimental conditions are described in the legend to Fig. 1. For further details, see Materials and Methods. Values are means ± SEM of four experiments. Key: (*) $P < 0.05$ vs ADR, $N = 4$; (**) $P < 0.039$ vs ADR + DOC or ADR + PDBu, $N = 4$.

deoxycholate produced resistance to ADR (Fig. 3). However, neither PDBu nor deoxycholate down-regulated PKC under these conditions. Subsequent to a 96-hr exposure to 2 nM PDBu or 10 μM deoxycholate, UV-2237M cells contained 100 ± 4 and 101 ± 12% of the PKC activity present in untreated UV-2237M cells respectively. Taken together, the results presented in Figs 1 and 3 suggest that exposure of drug-resistant cells to bile acids *in vivo* may enhance their drug resistance, and exposure of drug-sensitive cells to bile acids *in vivo* may endow them with a drug-resistant phenotype.

To determine whether bile acid-induced ADR resistance was reversible, we preincubated UV-2237M cells with 50 μM deoxycholate for 96 hr and then cultured the cells in medium in the absence of deoxycholate for various times. Cell survival assays indicated that a 96-hr preincubation with deoxycholate offered transient protection against ADR in cells exposed to ADR immediately after removal of deoxycholate. Under these conditions, deoxycholate enhanced cell survival in the presence of ADR approximately 25%. This deoxycholate-induced ADR resistance disappeared when cells preincubated with deoxycholate were incubated in deoxycholate-free medium for 24 hr prior to exposure to ADR (data not shown). Furthermore, a short-term exposure to deoxycholate (<2 hr) during the 96-hr cytotoxicity assay failed to modulate ADR sensitivity (data not shown). Therefore, in contrast to drug-resistance phenotypes selected by cytotoxic drugs, bile acid-induced ADR resistance appears to be a transient phenomenon.

Next we examined the abilities of deoxycholate and the phorbol ester TPA to alter the rates of [^{14}C]ADR and [^{3}H]VCR accumulation in UV-2237M-ADR^R and UV-2237M cells. We found that deoxycholate (3–100 μM) had no effect on the extent of accumulation of either 0.4 μM [^{3}H]VCR or 0.4 μM [^{14}C]ADR observed in UV-2237M-ADR^R and UV-2237M cells after a 2-hr incubation, regardless of whether or not cells were pretreated with deoxycholate for up to 96 hr and whether or not deoxycholate was also present during the 2-hr incubation (for experimental details, see Materials and Methods) (data not shown). In analogous studies, we determined that TPA also failed to alter the accumulation of [^{14}C]ADR and [^{3}H]VCR under these experimental conditions (data not shown). These results provide evidence that the mechanism of enhanced ADR and VCR resistance mediated by deoxycholate and phorbol esters in UV-2237M cell lines is independent of P-glycoprotein. Furthermore, deoxycholate did not induce P-glycoprotein expression in UV-2237M cells according to studies employing a monoclonal antibody to P-glycoprotein (FITC-C219, pglyco CHECK, Centocor, Inc.) and FACS analysis (data not shown).

Since our previous studies with MDR UV-2237M cells implicated PKC activity in their drug-resistance phenotypes [6], we examined the PKC isozyme compositions of ADR-sensitive UV-2237M and MDR UV-2237M-ADR^R cells. Hydroxylapatite chromatography resolves rat brain PKC isozymes I, II, and III into three peaks [21, 26]. PKC-II represents two closely related forms of PKC [10]. In our hydroxylapatite PKC isozyme separation system (see Materials and Methods), baseline separation was achieved between rat brain PKC-III, which eluted at 130 mM phosphate, and rat brain PKC-I and PKC-II, which eluted at 70 and 90 mM phosphate respectively (data not shown). The elution positions of DEAE-purified rat brain PKC activity observed in our hydroxylapatite chromatographic system were similar to those previously reported for rat brain PKC-I (70 mM), PKC-II (90 mM) and PKC-III (140 mM) [26]. Studies with NIH-3T3 PKC-III indicate that murine PKC-III coelutes with rat brain PKC-III during hydroxylapatite chromatography [27]. When we partially purified PKC from murine

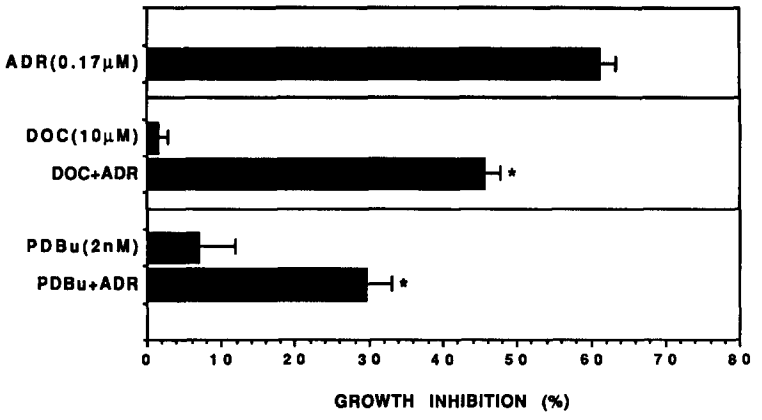

Fig. 3. Conferral of an ADR-resistance phenotype on parental UV-2237M cells by PDBu and deoxycholate. Cells were exposed to deoxycholate (DOC), ADR, and PDBu, as indicated, throughout a 96-hr incubation period. At the end of the incubation period, the number of viable cells was determined using a colorimetric tetrazolium assay. "Growth inhibition" represents the reduction in cell number elicited by the indicated compounds. For experimental details, see Materials and Methods. Values are means ± SEM of four experiments. Key: (*) $P < 0.05$ vs ADR.

UV-2237M cell lysates by DEAE chromatography and subjected the DEAE-purified PKC preparation to hydroxylapatite chromatography, we observed a single peak of PKC activity at the elution position corresponding to PKC-III (130 mM phosphate) (Fig. 4A). No PKC activity was observed at the elution positions corresponding to PKC-I and PKC-II. Similarly, a single peak of PKC activity was observed at 130 mM phosphate, when DEAE-purified UV-2237M-ADR^R PKC was chromatographed on hydroxylapatite resin (Fig. 4B), indicating the presence of PKC-III. In confirmation of our chromatographic profiles, we found that UV-2237M and UV-2237M-ADR^R cell lysates both reacted with a monoclonal antibody that is specific for PKC-III (Seikagaku America Inc., Rockville, MD) according to ELISA assays, and the amount of immunoreactive material per mg protein in the UV-2237M-ADR^R cell lysate was about twice as much as that in the UV-2237M cell lysate (data not shown).

PKC-ζ differs from PKC-I, PKC-II, and PKC-III in its chromatographic properties on DEAE ion-exchange resin [28]. PKC-II and PKC-III can be eluted from DEAE-cellulose with 0.05 M NaCl, and PKC-I can be eluted with 0.1 M NaCl [29]. In contrast, 0.38 M NaCl is required to elute PKC-ζ during DEAE ion-exchange chromatography [28]. We chromatographed UV-2237M and UV-2237M-ADR^R cell lysates on DEAE-Sepharose (3 mL) using a linear gradient of 0.0 to 0.6 M NaCl in buffer A (30 mL). In each case, we observed a single peak of PKC activity at 0.04 M NaCl (data not shown), indicating the absence of PKC-ζ in the cell lysates and supporting our previous evidence (Fig. 4) that PKC-III is a major PKC isozyme in UV-2237M and UV-2237M-ADR^R cells. These results indicate that the enhancement of drug resistance mediated by TPA, PDBu, and deoxycholate in drug-sensitive and MDR UV-2237M cell lines does not involve PKC-ζ, PKC-I, or either form of PKC-II.

Since the regulatory properties of PKC isozymes differ [10], it was important to determine whether TPA and deoxycholate could stimulate the activity of PKC-III isolated from UV-2237M and from UV-2237M-ADR^R cells. Figure 5 shows that TPA could stimulate the activity of the UV-2237M-ADR^R PKC-III preparation in the presence of PS or deoxycholate, but that the PKC activity could not be stimulated by TPA, PS, or deoxycholate alone. In addition, deoxycholate enhanced the activation of PKC-III by TPA plus PS (Fig. 5) and by 1 mM Ca^{2+} plus PS (data not shown). Similar regulatory properties were observed with purified rat brain PKC-III and PKC-III purified from UV-2237M cells by hydroxylapatite chromatography (data not shown). Thus, PKC-III activation is implicated in the antagonism of ADR cytotoxicity mediated by phorbol esters in UV-2237M and UV-2237M-ADR^R cells, and may also be involved in the multidrug resistance mediated by deoxycholate.

A positive role for calmodulin in multidrug resistance is suggested by the partial reversal of multidrug resistance by a number of calmodulin inhibitors [2], although the lack of specificity of the calmodulin inhibitors has precluded their use in a direct examination of whether calmodulin is involved in multidrug resistance. Since calmodulin interacts with a wide variety of hydrophobic drugs [30], we tested deoxycholate for stimulatory effects on calmodulin. In assays of calmodulin-dependent cAMP-PDE activity, we found that deoxycholate and related bile acids inhibited the activation of cAMP-PDE by calmodulin (Fig. 6). The inhibitory activities of the bile acids could not be attributed simply to Ca^{2+} chelation, since 500 μM Ca^{2+} was present in the reaction mixtures. These results show that deoxycholate does not enhance drug resistance by directly activating calmodulin. Since calmodulin is a PKC inhibitor [31], calmodulin antagonists may augment the net activity of activated PKC in intact cells by reversing calmodulin-mediated inhibition of PKC, although they could not replace the role of PKC activators by this mechanism. Thus, our results suggest that the direct stimulation of PKC activity

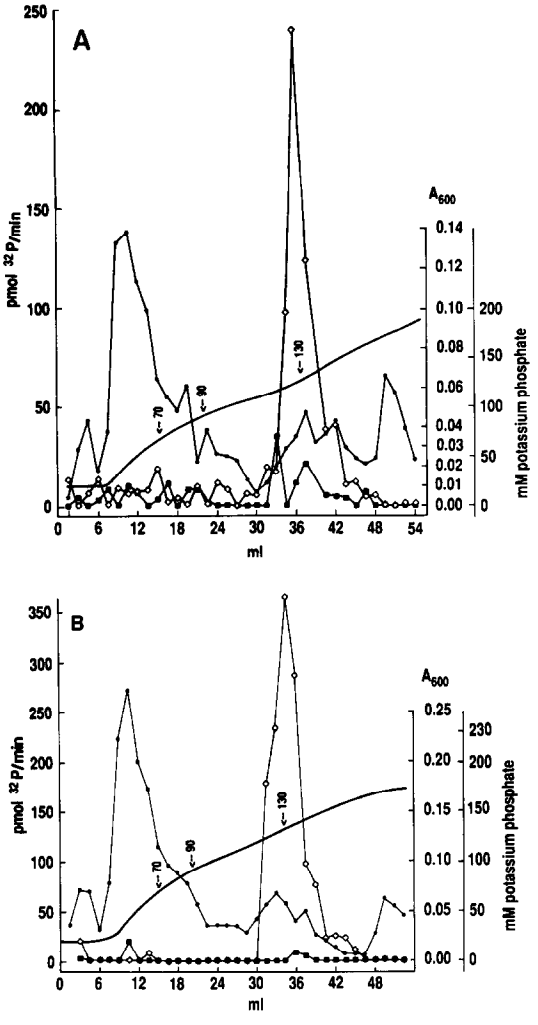

Fig. 4. Hydroxylapatite chromatography of UV-2237M and UV-2237M-ADR^R PKC. (A) PKC was partially purified from UV-2237M cells by DEAE ion-exchange chromatography and was then subjected to hydroxylapatite chromatography, as described in Materials and Methods. The elution of PKC activity from a 3-mL hydroxylapatite column by a 20–230 mM potassium phosphate gradient is illustrated. Key: (◇) protein kinase activity observed in the presence of 1 mM Ca^{2+} and 30 μg/mL PS; (■) protein kinase activity observed in the presence of 1 mM Ca^{2+}; and (●) A_{600} observed by incubating 25 μL of each fraction with 200 μL of BioRad Protein Assay Solution; 70, 90 and 130 indicate the elution positions of rat brain PKC-I, PKC-II and PKC-III respectively. Picomoles ^{32}P per minute represent the picomoles ^{32}P incorporated into histone IIIS per minute per milliliter. (B) PKC was partially purified from UV-2237M-ADR^R cells by DEAE chromatography and was then chromatographed on a hydroxylapatite column. For further details, see (A) and Materials and Methods.

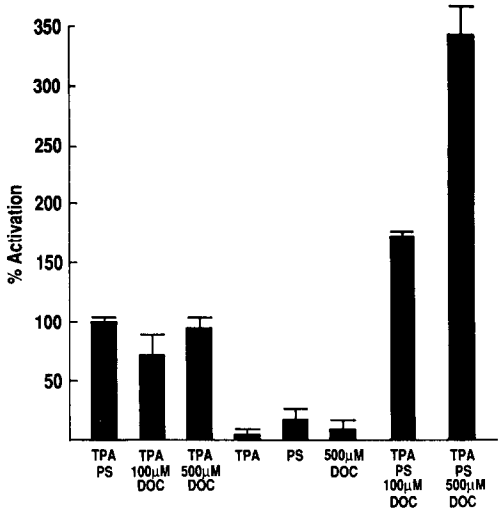

Fig. 5. Activation of UV-2237M-ADR^R PKC-III by TPA, PS and deoxycholate. UV-2237M-ADR^R PKC-III was isolated by DEAE and hydroxylapatite chromatographies (Fig. 4B). The concentrations of TPA and PS employed in PKC assays were 100 nM and 30 μg/mL respectively. All reaction mixtures contained 1 mM EGTA and lacked added Ca^{2+}. PKC assays were done under standard conditions (see Materials and Methods), except that the histone concentration was reduced to 0.3 mg/mL to ensure complete solubilization of deoxycholate (DOC) in the reaction mixtures. Bars represent the mean percent PKC activity observed in triplicate assays (±SD). One hundred percent activity represents 4.3 pmol ^{32}P incorporated into histone IIIS per reaction mixture. This experiment was reproducible in its entirety.

by deoxycholate may be augmented by its inhibition of calmodulin.

DISCUSSION

While it is clear that P-glycoprotein plays a central role in multidrug resistance *in vitro* [1–3], emerging evidence also implicates PKC activity in the MDR phenotypes of cultured cells. An elevated level of PKC activity has been observed in MDR human breast MCF-7 cells [5, 32], and we have demonstrated a direct correlation between the level of PKC activity and the degree of multidrug resistance among four murine fibrosarcoma UV-2237M cell lines [6] (Table 1). In addition, we have shown that the PKC inhibitor H7 partially reverses the ADR-resistance phenotypes of the MDR UV-2237M cell lines [6]. Complementary studies reported here show that, at a concentration of 2 nM, the highly selective PKC activator PDBu endowed drug-sensitive UV-2237M cells with ADR resistance (Fig. 3) and enhanced ADR resistance in an ADR-selected MDR UV-2237M cell line (Fig. 1). Taken together, these results provide strong evidence that PKC plays an important role in drug resistance in these tumor cell lines.

PKC consists of a family of at least seven isozymes, which have subtly different modes of activation and substrate specificities and may also have distinct biological functions [7, 10]. In this report, we demonstrated that the drug-sensitive cell line UV-2237M and the ADR-selected MDR cell line UV-2237M-ADR^R express PKC-III but not PKC-I, PKC-II (which represents two closely related isozymes), or PKC-ζ. The chromatographic properties of PKC-δ and PKC-ε are not well-characterized [10], and it remains to be determined whether these isozymes are expressed in the UV-2237M cell lines. It is clear,

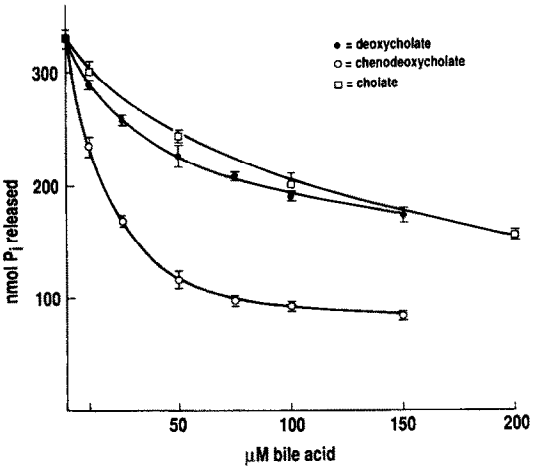

Fig. 6. Inhibition of calmodulin-mediated cAMP-PDE activation by deoxycholate and other bile acids. Calmodulin-dependent cAMP-PDE activity was measured by subtracting the basal cAMP-PDE activity observed in the presence of 100 μM EGTA plus calmodulin from the activity observed with 500 μM Ca^{2+} plus calmodulin. In control experiments, we determined that the bile acids had no effect on basal cAMP-PDE activity. "nmol P_i released" represents the calmodulin-dependent production of P_i from cAMP by the coupled activities of cAMP-PDE and 5'-nucleotidase. In control experiments, we determined that the bile acids had no effect on 5'-nucleotidase activity. For further details, see Materials and Methods. Each data point is the mean of triplicate determinations (±SD). This experiment was reproducible in its entirety.

however, that PKC-III is a major PKC isozyme of drug-sensitive and MDR UV-2237M cells. It is not surprising that the PKC isozyme profiles of drug-sensitive and MDR UV-2237M cells appear to be similar, since phorbol-ester PKC activators protect both drug-sensitive and MDR UV-2237M cells from ADR cytotoxicity (Figs. 1A and 3). To determine whether PKC-III activation is consistently associated with phorbol ester-induced protection of diverse drug-sensitive and MDR mammalian cell lines from ADR cytotoxicity, it will be important to identify the PKC isozymes and the protective effects of phorbol esters against ADR cytotoxicity in other types of drug-sensitive and MDR cell lines. If activation of a particular PKC isozyme, such as PKC-III, were generally associated with protection of cells from certain cytotoxic drugs, the development of specific antagonists of that isozyme could perhaps provide an approach to sensitizing tumor cells to the cytotoxic drugs.

Bile acids such as deoxycholate can stimulate PKC by direct effects on the enzyme [7, 13], by stimulating production of diacylglycerol, an endogenous PKC activator [7, 14, 15], and by inhibiting calmodulin (Fig. 6), which has inhibitory effects against PKC [31]. The relevance of deoxycholate-mediated PKC activation in the colonic epithelium *in vivo* has been demonstrated by studies of PKC activation in isolated colonic crypts [14].

Since phorbol-ester PKC activators confer drug-resistance phenotypes on drug-sensitive MCF-7 cells [5] and UV-2237M cells (Fig. 3) and enhance drug resistance in MDR MCF-7 cells [5] and MDR UV-2237M cells (Fig. 1A), we tested whether deoxycholate could protect UV-2237M cells from ADR-mediated cytotoxicity. This was, in fact, the case. At deoxycholate concentrations that chronically occur in the lumen of the colon *in vivo* [25], this bile acid reduced the sensitivities of drug-sensitive and MDR UV-2237M cells to ADR (Figs. 1D and 3). We found that the deoxycholate-induced resistance to ADR in the UV-2237M-ADR^R cells was linked to resistance against the MDR drugs VCR and VLB. Deoxycholate and TPA stimulated the activities of isolated UV-2237M PKC III and UV-2237M-ADR^R PKC-III, suggesting that they protected the UV-2237M cell lines from ADR cytotoxicity by activating PKC-III. However, the evidence that deoxycholate may enhance ADR resistance by activating PKC-III cannot be considered conclusive, since deoxycholate is a detergent with multiple biochemical effects. We found that the enhancement of ADR resistance in UV-2237M-ADR^R cells mediated by deoxycholate could be antagonized by the protein kinase inhibitor H7. Our results provide strong evidence that deoxycholate enhances drug resistance through a mechanism that involves stimulation of protein phosphorylation rather than merely nonspecific detergent effects on membrane permeability. In further support of a mechanism of resistance other than modulation of cell membrane permeability, we found that neither deoxycholate nor phorbol esters altered the extent of accumulation of [^{14}C]ADR or [^{3}H]VCR in UV-2237M and UV-2237M-ADR^R cells, under conditions where they induced drug resistance. These results also provide evidence that the induction of drug resistance in UV-2237M and UV-2237M-ADR^R cells by deoxycholate and phorbol esters does not involve modulation of P-glycoprotein-mediated drug efflux. This distinguishes phorbol ester-mediated induction of drug resistance in UV-2237M cell lines from that observed in human breast cancer MCF-7 cell lines, since exposure to phorbol esters results in a reduction in ADR and VCR accumulation in the breast cancer cell lines [5]. Our results also suggest that deoxycholate and perhaps other bile acids can protect intestinal cells that come in contact with bile acids from toxic substances which may have physiological relevance.

Drugs that are highly active against the growth of colon cancer cell lines *in vitro* are often relatively ineffective *in vivo* [33, 34]. In fact, colon cancer is thought to have an intrinsic resistance to chemotherapy. One mechanism that may contribute to this resistance is elevated P-glycoprotein expression. The gene encoding P-glycoprotein and P-glycoprotein itself are expressed at relatively high levels in the normal colonic epithelium *in vivo* [35–38], and the gene for P-glycoprotein is generally elevated in untreated human colon tumors [39]. Our results suggest an additional mechanism for this intrinsic resistance. We hypothesize that the bile acid deoxycholate may serve as an environmental factor that contributes a potentially reversible mechanism of resistance to chemotherapy in cancer of the intestine.

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