Biochemical Pharmacology, Vol. 43, No. 4, pp. 865–872, 1992.
Printed in Great Britain.

0006-2952/92 $5.00 + 0.00

ACTIVATION OF HUMAN LEUKEMIA PROTEIN KINASE C BY TUMOR PROMOTERS AND ITS INHIBITION BY *N*-TRIFLUOROACETYLADRIAMYCIN-14-VALERATE (AD 32)

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(*Received* 19 *July* 1991; *accepted* 4 *October* 1991)

Abstract—*N*-Trifluoroacetyladriamycin-14-valerate (AD 32), a lipophilic, DNA non-binding analog of Adriamycin® (ADR), was found to be a potent inhibitor of the membrane-bound enzyme, protein kinase C (PKC). PKC was isolated and purified from human leukemia ML-1 cells, and the enzyme activity was shown to be activated by the tumor promoters 12-*O*-tetradecanoylphorbol-13-acetate (TPA) and phorbol-12,13-dibutyrate (PDBu). AD 32, nevertheless, inhibited the activation of PKC by TPA or PDBu. The IC_{50} values for AD 32 inhibition of PKC activation were 0.85 μM for TPA and 1.25 μM for PDBu. Under the same assay conditions, ADR demonstrated much higher IC_{50} values: 550 μM for TPA and >350 μM for PDBu. The inhibition of PKC by AD 32 was further shown to be competitive in nature; AD 32 inhibited the binding of [^{3}H]PDBu to PKC. Therefore, AD 32 competes with the tumor promoter for the PKC binding site and prevents the latter from both interacting with the phospholipid and binding to PKC. These effects of AD 32 were reproduced *in situ*; incubation of human leukemia ML-1 cells with TPA showed an increased phosphorylation of cellular proteins, and the TPA-induced protein phosphorylation was inhibited by the addition of AD 32 to the cultured cells.

Adriamycin® (ADR‖) is an anthracycline antineoplastic agent currently in widespread and routine clinical use for the treatment and maintenance of a large number of solid tumors as well as acute leukemias and malignant lymphomas [1]. The use of ADR, however, is complicated by a profile of untoward side-effects that include acute myelosuppression, cardiac toxicity, gastrointestinal toxicity, alopecia and stomatitis [1, 2]. *N*-Trifluoroacetyladriamycin-14-valerate (AD 32), an analog of ADR, has been developed for clinical trials, especially for patients previously unresponsive to ADR, for it was shown that in animal model studies, AD 32 demonstrated therapeutic superiority and remarkably less toxicity compared to ADR [3–5]. Furthermore, the highly lipophilic nature of AD 32 makes it easier for tumor mass penetration and hence ideal for its use as an intracavitary agent in specialized tumor studies [6].

Drug–DNA binding and its resultant inhibition of DNA and RNA polymerase activities have been considered to be the principal mechanism for the cytotoxic action of ADR and its related anthracyclines [1, 2]. AD 32, however, does not bind to DNA [7, 8]. AD 32 was found not to affect the function of DNA polymerase; it inhibits RNA polymerase activity at drug concentrations 100 to 200 times higher than those of ADR used to inhibit the same enzyme [7, 9]. The inhibition of RNA polymerase by AD 32 or its water-soluble derivative AD 143 (which is a potent RNA polymerase inhibitor) was shown to result from an interaction between the drug and the enzyme RNA polymerase [7, 10, 11].

DNA single-strand breaks and the associated DNA–protein cross-links have been observed with both ADR and AD 32 treatment [12–14]. However, the treatments of cells with caffeine, an agent that blocks DNA repair [15], lessens ADR and AD 32 cytotoxicity while increasing DNA damage [12, 16]. This observation precludes the DNA breakage as an explanation for ADR or AD 32 cytotoxicity [2]. Therefore, the biochemical mechanism by which AD 32 exerts its remarkable antitumor activity remains to be determined.

The lipophilic nature of AD 32 prompted us to study its effect on cell membrane activities. The present report demonstrates that AD 32, at submicromolar concentrations, inhibited the activities of protein kinase C (PKC), a membrane-bound enzyme. AD 32-induced inhibition of PKC activity may involve a hydrophobic interaction between the drug and the PKC activators, the tumor-promoting phobol esters TPA or PDBu.

MATERIALS AND METHODS

Materials. TPA, PDBu, diolein [as diacylglycerol (DAG)] and phosphatidylserine (PS) were from

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‖ Abbreviations: ADR, Adriamycin®; PKC, protein kinase C; PS, phosphatidylserine, DAG, diacylglycerol; TPA, 12-*O*-tetradecanoylphorbol-13-acetate; PDBu, phorbol-12,13-dibutyrate; AD 32, *N*-trifluoroacetyladriamycin-14-valerate; AD 143, *N*-trifluoroacetyladriamycin-14-*O*-hemiadipate; NP 40, Nonidet P40; and TCA, trichloroacetic acid.

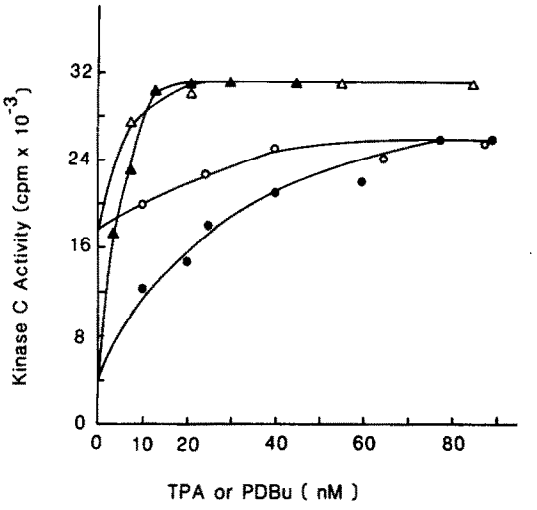


Fig. 1. Effect of TPA or PDBu on PKC activity. Human leukemia PKC was assayed at different concentrations of TPA (△, ▲) or PDBu (○, ●). The assay conditions were as described under Materials and Methods except that the system contained either 95 μM EGTA (▲, ●) or 5 μM EGTA (△, ○).

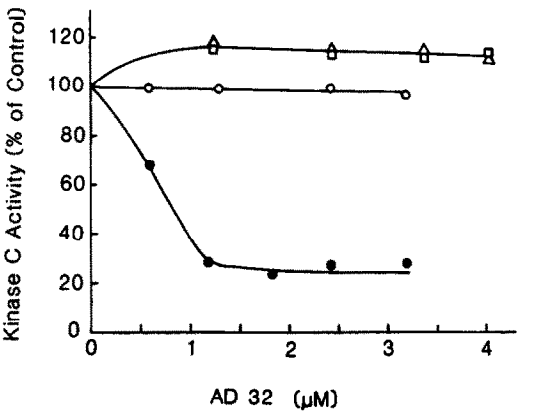


Fig. 2. Effect of AD 32 on TPA-activated PKC activities. Human leukemia PKC was assayed in the presence of TPA and various concentrations of AD 32, and the amount of [^{32}P]phosphate incorporated into H1 histone was determined by procedures described under Materials and Methods. AD 32 (●) or vehicle (NP 40, ○) was preincubated (5 min at 0°) with TPA. The other components of the reaction mixture were added at the end of this preincubation period to initiate the reaction, and H1 histone phosphorylation continued at 30° for 15 min. Alternatively, TPA was preincubated with PS (5 min at 0°) before exposure to AD 32 (△) or NP 40 (□). The concentrations of NP 40 used (○, □) were identical to those present in the corresponding AD 32 preparations (●, △). AD 32 at 3 μM contained 0.015% NP 40. The results shown here, as well as in all the other figures, have been reproduced in three to four independent experiments using different PKC or AD 32 preparations. The remaining enzyme activity resistant to AD 32 inhibition includes the basic (not simulated by TPA) PKC activity. The control (100%) activity presented here was 7,980 ± 790 cpm.

Sigma. ADR was obtained from Adria Laboratories, Inc. AD 32 was prepared as described [3]. Carrier-free $^{32}PO_4$ was from ICN Radiochemicals and [γ-^{32}P]ATP was provided by Dr. D. A. Walsh of the University of California, Davis, CA.

Preparation of human leukemia cells. Human leukemia ML-1 cells [17] were grown in RPMI 1640 (Gibco) with 7.5% fetal bovine serum. The cultures were incubated at 37° in a CO_2 incubator. Cell numbers were counted with a hemacytometer and viability was estimated by trypan blue dye exclusion.

Preparation of human leukemia PKC. PKC was isolated and purified from human leukemia ML-1 cells (5×10^9 cells) by DEAE-cellulose and phenyl-Sepharose column chromatographies, as we described previously [18]. The purified enzyme was free from cAMP-dependent kinase; its activity was dependent upon phospholipid/Ca^{2+} concentrations and stimulated by DAG [18].

PKC assays. The standard PKC assay system contained 200 μg/mL H1 histone, 50 μM [γ-^{32}P]ATP (250–500 cpm/pmol), 10 mM $MgCl_2$, 20 mM Tris–HCl (pH 7.6), 1 mM dithiothreitol, 0.05 mM $CaCl_2$, 16 μg/mL PS, 1.2 μg/mL DAG, 90 μM ethyleneglycolbis(aminoethylether)tetra-acetate(EGTA) and enzyme. When the phorbol esters were used instead of DAG, the reaction mixture contained 13.6 nM TPA or 60 nM PDBu. The reaction volume was 0.05 mL and incubation was for 15 min at 30°. After incubation, the reaction was terminated by trichloroacetic acid (TCA) precipitation; the radioactivity was collected on Whatman GF/C filters and counted in a scintillation counter.

In situ *phosphorylation. In situ* phosphorylation of ML-1 cells using carrier free $^{32}PO_4$ in the presence of TPA and/or AD 32 and the subsequent preparation of the cytoplasmic fraction of the cells

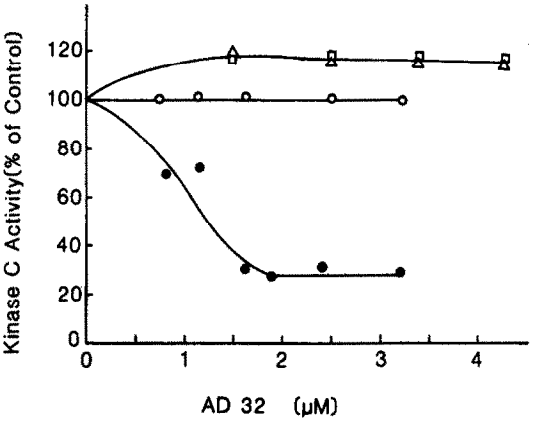


Fig. 3. Effect of AD 32 on PDBu-activated PKC activities. The experiments were performed under conditions described in Fig. 2, except that PDBu was used instead of TPA. Similar to the experimental conditions in Fig. 2, AD 32 at 3 μM contained 0.015% NP 40. Prior to the initiation of the phosphorylation reaction, components were preincubated (5 min at 0°) as follows: (●) PDBu with AD 32; (○) PDBu with NP 40; (△) PDBu with PS for the assay system containing AD 32; and (□) PDBu with PS for the assay system containing NP 40. The control (100%) activity presented here was 9,920 ± 470 cpm.

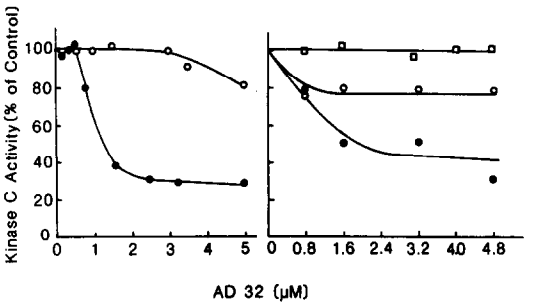


Fig. 4. Interaction of AD 32 with DAG or PS and its effect on PKC activities. The experiments were performed under conditions described in Fig. 2, except that DAG was used instead of TPA. Similar to the experimental conditions in Fig. 2, AD 32 at 3 μM contained 0.015% NP 40. Prior to the initiation of the phosphorylation reaction, components were preincubated (5 min at 0°) as follows: (Left panel) (●) DAG with AD 32; and (○) DAG with NP 40. The control (100%) activity presented here was 10,500 ± 880 cpm. (Right panel) (●) PS with AD 32; (○) PS with NP 40; and (□) PS with DAG before exposure to AD 32. The control (100%) activity presented here was 6,840 ± 1,040 cpm. Note that in the absence of PS (or DAG), the activity of DAG (or PS) was slightly sensitive to high concentrations of NP 40.

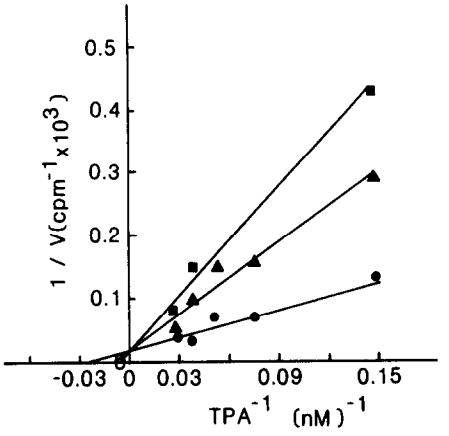


Fig. 6. Effect of increasing concentrations of TPA on the inhibition of PKC activity by AD 32 (Lineweaver–Burk plot). Human leukemia PKC was assayed with increasing concentrations of TPA in the presence of the following concentrations of AD 32 (▲) 0.5 μM; (■) 1.0 μM; and (●) no AD 32 added. AD 32 or NP 40 (as controls, ●) was preincubated (5 min at 0°) with TPA before the phosphorylation reaction was initiated. The ordinate (1/V) represents the reciprocal of the amount of [^{32}P]-phosphate incorporated into H1 histone per 0.05 mL reaction mixture per 15 min at 30°.

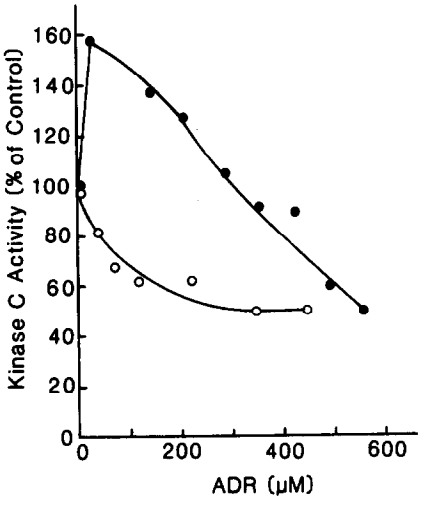


Fig. 5. Effect of ADR on TPA- or PDBu-induced PKC activation. Human leukemia PKC was assayed in the presence of TPA (●) or PDBu (○) and various concentrations of ADR. ADR or H_2O (as control) was preincubated (5 min at 0°) with TPA (●) or PDBu (○) before phosphorylation reaction was initiated. Other assay conditions are described under Materials and Methods. The control (100%) activities were 11,100 ± 30 cpm (TPA) and 12,900 ± 1,800 cpm (PDBu).

were done according to the procedure described by Kreutter *et al.* [19]. Trichloroacetic acid (TCA) was added to the isolated cytoplasmic fraction to a final concentration of 7% and the mixture was left overnight at 4°. After centrifugation, the pellet was washed with cold 100% ethanol, dried under vacuum, and resuspended in buffer B [18] to remove soluble proteins. The insoluble portion of the pellet was washed once with 100 μL of a solution containing 0.15 M Tris–HCl, pH 8.0, 0.2 M EDTA and 0.3% Triton X-100, and finally solubilized with 50 μL of a solution containing 1% sodium dodecylsulfate (SDS) and 0.2 N NaOH. After neutralization, the protein content of each sample was determined and an aliquot (30 μg protein) of each sample was subjected to SDS/polyacrylamide gel electrophoresis, a procedure described by Laemmli [20]. Results were analyzed by autoradiography on Kodak XR-5 film. Alternatively, the isolated cytoplasmic fraction of the cells after *in situ* phosphorylation was subjected to two-dimensional gel electrophoresis according to the method described by O'Farrell [21]. For isoelectric focusing in tube gels, Bio · Rad ampholytes were used in the following concentrations: pH 3–10, 0.4% and pH 5–7, 1.6%. The isoelectric focusing gel was layered on an SDS/polyacrylamide gel (12% separation gel) for the second dimension electrophoresis.

RESULTS

TPA or PDBu activation of human leukemia PKC. The activity of PKC from human leukemia cells was stimulated by the tumor promoting phorbol esters TPA and PDBu (Fig. 1). The enzyme had a greater basal activity and responded to the phorbol esters to a lesser extent when the assay system contained a higher amount of calcium or a lower amount of EGTA, which is a Ca^{2+}-chelating agent (Fig. 1).

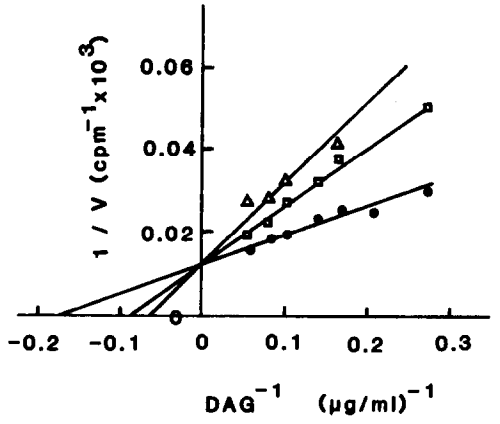
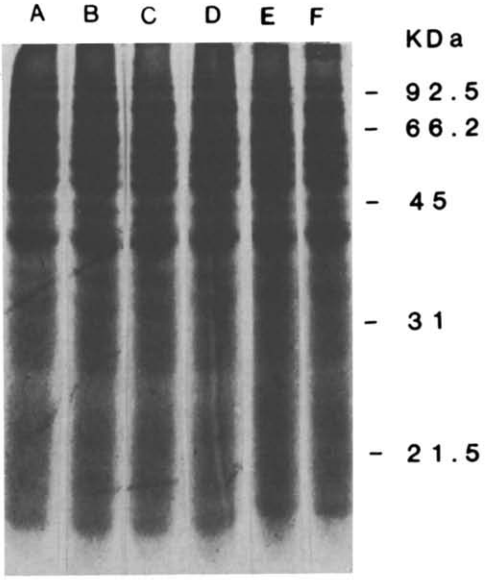


Fig. 7. Effect of increasing concentrations of DAG on the inhibition of PKC activity by AD 32 (Lineweaver–Burk plot). The assay conditions were similar to those described in Fig. 6 except that DAG was used in place of TPA and that AD 32 was used at 1 μM (□) or 2 μM (△). The closed circle (●) indicates that no AD 32 was present.



When the Ca^{2+} concentration remained the same (50 μM), TPA and PDBu caused a 7.5- and 6-fold stimulation, respectively, of PKC activity in the presence of 95 μM EGTA, and a 1.7- and 1.4-fold stimulation, respectively, in the presence of 5 μM EGTA (Fig. 1). The activities of PKC reached a plateau at 20 nM TPA or 75 nM PDBu (Fig. 1). These results were consistent with the notion [22, 23] that TPA or PDBu mimics the effects of endogenously

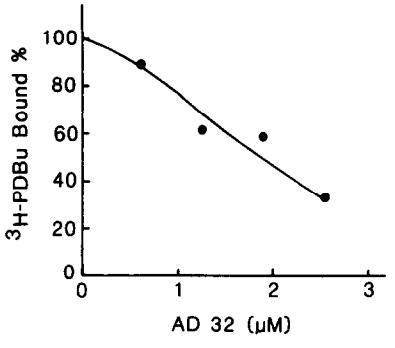


Fig. 8. Competition for [^{3}H]PDBu binding by AD 32. [^{3}H]-PDBu binding to human leukemia PKC was determined in the presence of various concentrations of AD 32 using procedures described elsewhere [29]. The assay system contained 20 mM Tris–HCl, pH 7.5, 50 μM Ca^{2+}, 10 mM Mg^{2+}, 10 μM ATP, 100 μg/mL PS, 10 nM [^{3}H]PDBu ± 5 μM unlabeled PDBu and PKC, in a total volume of 250 μL. AD 32 was preincubated with [^{3}H]PDBu ± unlabeled PDBu for 5 min at 0° prior to the addition of other components of the assay mixture. The assay mixture was filtered through Whatman DE81 filters. The filters were washed with 20 mM Tris–HCl, pH 7.5, containing 0.5 mM Ca^{2+} and 20% methanol and then counted in a scintillation counter. The results reported are the counts obtained in the absence of unlabeled PDBu minus the counts obtained in the presence of unlabeled PDBu. The net control (100%) activity presented here was 3250 cpm.

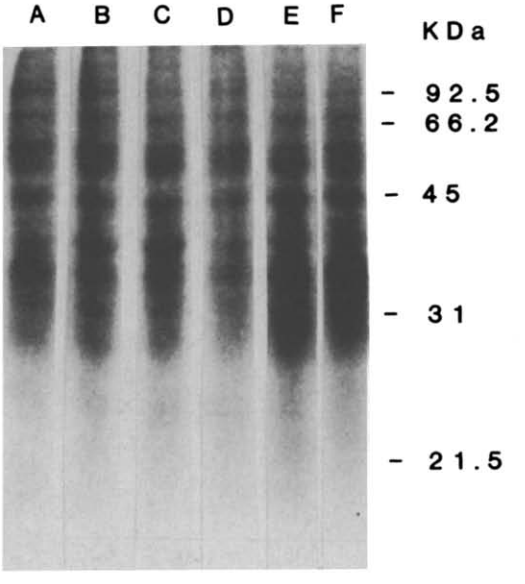


Fig. 9. Effect of TPA or AD 32 on ML-1 protein phosphorylation *in situ*. Cells grown in phosphate-free RPMI 1640 were incubated at 37° with carrier-free $^{32}PO_4$ for 45 min and then TPA or AD 32 for an additional 1.5 hr. Cells were lysed and centrifuged to remove nuclei. Cytoplasmic proteins were isolated as described under Materials and Methods and electrophoresed in a 10% polyacrylamide gel. After staining with Coomassie Blue, the proteins were autoradiographed. [Top] Coomassie Blue staining of the gel. Each lane contained 30 μg of cytoplasmic protein. [Bottom] autoradiograms of the same gel showing protein bands with different amounts of phosphorylation. Key: (A) control; (B) 10.67 nM TPA; (C) 0.1% DMSO; (D) 1.38 μM AD 32 (in DMSO); (E) 10.67 nM TPA and 0.1% DMSO, added separately to the cells; and (F) 10.67 nM TPA and 1.38 μM AD 32 (in DMSO), added separately to the cells. Molecular weights of protein standards are indicated on the right.

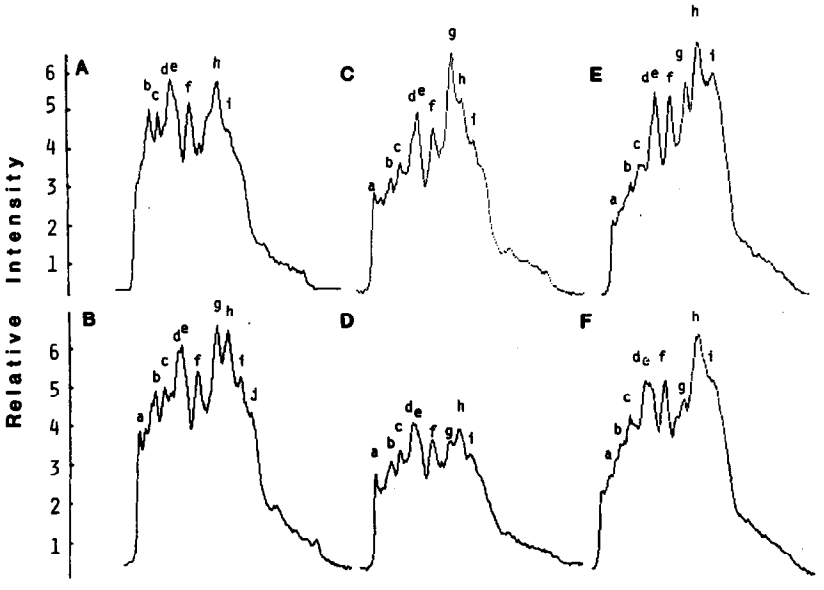


Fig. 10. Differential phosphorylation of ML-1 proteins in response to TPA or AD 32. Densitometry scanning of the autoradiogram presented in Fig. 9 showed relative intensities of phosphorylation. Graphs A through F correspond to lanes A through F of Fig. 9. The molecular masses (in kDa) for a through j are (a), 96; (b) 92; (c) 74; (d) 58; (e) 54; (f) 44; (g) 37; (h) 35; (i) 31; and (j) 28. Densitometry scanning of the film was done using a Zeineh Soft Lazer Scanning Densitometer, model SL-504-XL, and a Zeineh 1-D Autostepover/Videophoresis software program (Biomedical Instruments Inc., Fullerton, CA).

produced DAG in activating PKC, that is, these phorbol esters associate with lipid lamellae (or micelles) and dramatically increase the affinity of PKC for calcium [22, 24].

Effect of AD 32 on TPA- or PDBu-activated PKC activities. To determine the effect of AD 32 on TPA- or PDBu-activated PKC activities, human leukemia PKC was assayed in the presence of various concentrations of AD 32 at nearly saturated concentrations of TPA or PDBu (13.6 nM TPA or 60 nM PDBu). The results as shown in Figs. 2 and 3 indicate that the TPA- or PDBu-activated PKC activities were highly susceptible to AD 32 inhibition. The IC_{50} values for this inhibition were determined to be 0.85 μM for TPA and 1.25 μM for PDBu. In these experiments, the nonionic solubilizer Nonidet P40 (NP 40) was used as the solvent for AD 32. Under the assay conditions used, NP 40 had no effect on PKC activities (Figs. 2 and 3).

Site of action of AD 32 on PKC activation. It has been suggested that PKC activators interact with phospholipids and alter the microenvironment of the phospholipids in the membrane bilayer, and this interaction precedes PKC activations [25]. To show the site at which AD 32 acts in the *in vitro* PKC activation system, AD 32 was added to the reaction mixture before or after the interaction between TPA (or PDBu) and PS, a phospholipid, took place. The results showed that preincubation of AD 32 (5 min at 0°) with TPA (or PDBu) before it was added to the reaction mixture caused the inhibition. On the other hand, preincubation of TPA (or PDBu) with PS (5 min at 0°) before exposure to AD 32 prevented the inhibition (Figs. 2 and 3). This phenomenon was also true with DAG, the endogenous PKC activator (Fig. 4). These data suggest that AD 32 may act on a site on TPA (PDBu or DAG) which would normally be occupied by PS during PKC activations. Furthermore, the data in Fig. 4 show that AD 32 may also act on PS, although to a much lesser extent, to prevent PS from interacting with the PKC activator, in this case, the DAG.

Effect of ADR on TPA- or PDBu-induced PKC activation. ADR, the parental compound of AD 32, was shown previously to inhibit DAG-activated PKC activity with an IC_{50} value of 150–200 μM [26, 27]. The degree of inhibition increases when ADR is complexed with iron [28] or when it is preincubated with DAG at 37° for a length of time prior to the initiation of PKC reactions [27]. Preincubation at a lower temperature (e.g. 0°) or for a time period shorter than 1 hr produces little effect on the inhibition [27], indicating a temperature-dependent but slow-acting hydrophobic interaction between ADR and DAG [27, 29]. This observation was also true for the effect of ADR on TPA- or PDBu-activated PKC activities. As with the AD 32 inhibition, ADR was preincubated at 0° with either TPA or PDBu for 5 min, and then assayed for its inhibitory activity on TPA- or PDBu-activated enzyme activity. The results (Fig. 5) show that in comparison with AD 32 inhibition, ADR inhibited PKC with much higher IC_{50} values: 550 μM for TPA and >350 μM for PDBu.

Competition of AD 32 with TPA or PDBu for PKC binding. It has been suggested that PKC consists of a hydrophilic catalytic domain and a hydropholic phorbol ester- or diacylglycerol-binding

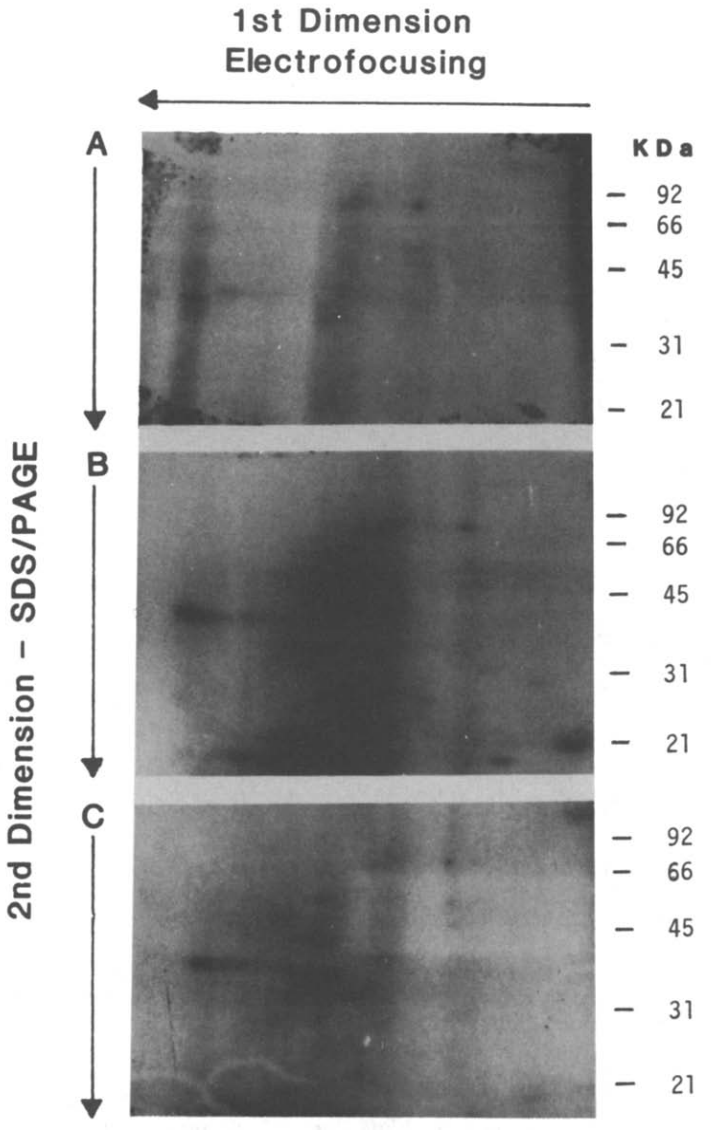


Fig. 11. Autoradiograph of ML-1 phosphoproteins after separation with two-dimensional gel electrophoresis. Cells were incubated with carrier-free $^{32}PO_4$ and then TPA or AD 32 as described in Materials and Methods. Cells were lysed and centrifuged to remove nuclei. Proteins (15 μg) were electrophoresed in two dimensions and autoradiographed. Key: (A) control (0.1% DMSO); (B) 10.67 nM TPA and 0.1% DMSO, added separately to the cells; and (C) 10.67 nM TPA, 1.38 μM AD 32 and 0.1% DMSO, added separately to the cells. Molecular weights of protein standards are indicated on the right.

domain [30, 31]. If the interaction between AD 32 and phorbol esters (or DAG) was the cause of PKC inactivation as it was revealed from the preincubation (order of addition) experiments (Figs. 2–4), one would expect that the interaction would also interfere with the normal binding of the PKC activators to PKC. AD 32 was mixed with various concentrations of TPA (Fig. 6) or DAG (Fig. 7), and the mixtures were then added to the protein phosphorylation system to determine PKC activities. It was found that AD 32 was a competitive inhibitor of PKC with regard to TPA (Fig. 6) or DAG (Fig. 7): the inhibition could be reversed by increasing the concentrations of TPA or DAG. In addition, with a direct filter binding assay [29], AD 32 was found to compete with [^{3}H]PDBu for its binding to PKC (Fig. 8). These results further support the hypothesis that AD 32, after association with TPA (or DAG), will prevent the latter, or a putative TPA (or DAG)–PS complex, from binding to the PKC molecule.

ML-1 protein phosphorylation. Treatment of human leukemia ML-1 cells with 10.67 nM TPA for 1.5 hr resulted in the increased phosphorylation of approximately ten cytoplasmic proteins or polypeptides with the molecular mass of 96K, 92K, 74K, 58K, 54K, 44K, 37K, 35K, 31K, and 28K, respectively (Fig. 9, lanes A and B). Treatment with 0.1% dimethyl sulfoxide (DMSO) (the solvent for

AD 32) under the same conditions produced little effect on ML-1 phosphorylation except for the 37 kDa polypeptide, which showed an increased phosphorylation (Fig. 9, lane C). Treatment with 1.38 μM AD 32 (in DMSO), however, inhibited ML-1 phosphorylations including the phosphorylation of the 37 kDa polypeptide (Fig. 9, lane D). Inhibition of the phosphorylation of the 37 kDa as well as the 28–31 kDa polypeptides was also seen with ML-1 cells that had been treated with both TPA and AD 32 (Fig. 9, lanes E and F). The other seven polypeptides, which showed increased phosphorylations in response to TPA, were less susceptible to AD 32 inhibitions. These studies were quantified by densitometer scanning of the radioautoradiograms of the phosphorylated samples and the results are shown in Fig. 10. A two-dimensional gel electrophoresis analysis of ML-1 phosphoproteins after TPA/AD 32 treatment is presented in Fig. 11. The results again showed that TPA activated protein phosphorylations (Fig. 11, A and B), and that TPA-activated phosphorylations were reduced markedly if, following TPA treatment, the cells were also treated with AD 32 (Fig. 11C).

DISCUSSION

Tumor promoters are compounds which by themselves are not carcinogenic but which induce tumors in animals previously exposed to a subthreshold dose of a carcinogen. The identification of protein kinase C as the major target for the phorbol esters suggests that the phorbol esters, by substituting for endogenously produced DAG, are acting on one of the major signal transduction mechanisms within cells [24, 25]. However, because of their greater stability and potency, the phorbol esters are better compounds than DAG for studies of antipromoters [32] or inhibitors which block the signal transduction system. Our studies demonstrate that AD 32, one of the antitumor agents, may indeed be considered as an effective antipromoter.

AD 32 is a lipophilic, DNA non-binding analog of ADR. It has been demonstrated that AD 32 is superior to ADR as an anticancer agent in both experimental leukemia and solid tumor models. However, certain mechanistic properties so far reported for this drug, such as its ability to inhibit RNA synthesis and to produce DNA lesions, are not sufficiently different from those of ADR to account for its greater antitumor activity. The present studies show that the lipophilic nature of AD 32 makes it a potent inhibitor of the cytoplasmic and membrane-bound enzyme PKC. These studies also show that AD 32 interacts preferentially with the tumor-promoting phorbol esters or DAG, the main regulatory molecules of PKC [24, 25]. TPA, PDBu or DAG has a domain which represents the binding site for the drug, perhaps a hydrophobic region [29]. As a result, AD 32 prevents TPA (PDBu or DAG) from binding to and activating PKC. By this mechanism, the action of AD 32 is considered highly selective—it has no effect on the enzymes of other kinases which do not depend upon the tumor promoters or DAG for activity. As an example, we have found that AD 32 has no inhibitory activity on the cAMP-dependent protein kinase or on protein kinase L, an unidentified kinase [18, 29], both from ML-1 cells (data not shown). Among all the anthracycline antibiotics and their derivatives that we have studied, inhibition of PKC at submicromolar drug concentrations is also a property unique to AD 32. Neither ADR, its parental compound, nor AD 143, its most closely related anthracycline, has such a specificity and potency.* Previous studies on the uptake and intracellular localization of anthracyclines have shown that unlike ADR which appears in both the nuclear and cytoplasmic portions of the cells, AD 32 accumulates almost exclusively in the cytoplasm or extra-nuclear fraction [33]. Since PKC is predominantly of a cytoplasmic origin [24], it may represent the major target for AD 32 action.

Acknowledgements—We thank Dr. D. A. Walsh for his supply of [γ-^{32}P]ATP and Ann Chuang for her technical assistance in the densitometry studies. This work was supported by Grant HHHERP 88 from the Hawaii Heptachlor Research and Education Foundation and Grants DA 05901 and CA 37082 from the Department of Health and Human Services.

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