Decreased Phorbol Ester Receptor and Protein Kinase C in P388 Murine Leukemic Cells Resistant to Etoposide[1]

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ABSTRACT

A variant P388 murine leukemic cell resistant to 4'-demethylepipo-dophyllotoxin-9-(4,6-O-ethylidene)-β-D-glucopyranoside (etoposide) (VP-16-213) was cloned. The variant P388/VP-16 cell line was 159-fold resistant to 4'-demethylepipodophyllotoxin-9-(4,6-O-ethylidene)-β-D-glucopyranoside and showed cross-resistance to vincristine (18.9-fold) and Adriamycin (522.9-fold), determined by comparing the 50% inhibitory concentrations in a 48-h growth inhibition assay. To identify the possible role of Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) in this drug resistance, we studied the specific phorbol ester binding component and protein kinase C in the parent and drug-resistant sublines of P388 cells. The phorbol ester receptor, as expressed by the numbers of sites per cell, significantly decreased in P388/VP-16 (57.6% of control). Scatchard analysis revealed that the variant contained a single class of binding sites. However, no difference was observed in the dissociation constants (K_d), thereby suggesting much the same affinity of receptors between the two lines. Phorbol diester analogues inhibited [20-³H]phorbol-12,13-dibutyrate binding of both the variant and control cell lines, in a stereospecific manner and consistent with their binding potency. The activity of protein kinase C, which is related to the phorbol ester receptor, significantly decreased in the variant cell. The enzyme activity, particularly in the membrane fraction of P388/VP-16 cells, was remarkably decreased. These data suggest that the decrease in the specific phorbol diester receptor and protein kinase C in the variant cells might correlate with the pleiotropic drug resistance.

INTRODUCTION

VP-16-213[3] is a semisynthetic derivative of podophyllotoxin. The clinical efficacy of this compound has been shown against a spectrum of human tumors, including small cell lung cancer, testicular carcinoma, acute nonlymphocytic leukemia, and lymphomas (1). The development of resistance of tumor cells to anticancer agents is one of the major problems related to effective cancer chemotherapy. Resistance to VP-16-213 of a tumor cell is often associated with decreased drug accumulation, presumably the result of an enhanced drug efflux via a process shared by several classes of drug, including anthracycline, *Vinca* alkaloids, dactinomycin, and podophyllotoxin (2, 3). Calmodulin inhibitors (4) and calcium channel blockers (2–6) will reverse this drug resistance by reducing the efflux of the drug from the resistant cells. The calcium-calmodulin-dependent system is speculated to be a major mechanism linked to this resistance. However, no major difference was observed in the calmodulin content of these cells (5, 7).

Many of the intracellular Ca^{2+} actions appear to be mediated

by calcium-dependent protein phosphorylation (8). Two classes of calcium-dependent protein kinase have been identified, namely Ca^{2+}-calmodulin-dependent protein kinase (8) and Ca^{2+}-phospholipid-dependent protein kinase (protein kinase C) (9). A tumor-promoting phorbol diester such as TPA has profound effects on a variety of cellular functions and the phorbol diester receptor was reported to be copurified with protein kinase C (10). The effect of TPA is mediated via the activation of this enzyme (9). We now present evidence that the induction of the pleiotropic resistance of P388 murine leukemic cell to anticancer drugs such as etoposide is accompanied by down regulation of both the phorbol diester receptor and protein kinase C.

MATERIALS AND METHODS

Chemicals. Histone III-S, histone H-2B, TPA, PDB, phorbol-12,13-dibenzoate, phorbol-12,13-didecanoate, and 4β-phorbol were purchased from Sigma Chemical Co., St. Louis, MO, Phosphatidylserine (pig liver) was purchased from Serdary Research Laboratories, Inc. Chloroform was removed from this phospholipid by a stream of nitrogen, and the phospholipid was sonicated in water for 1 min to produce a suspension of 0.5 mg/ml. [20-³H]PDB (specific activity, 8.3 Ci/mmol) was purchased from New England Nuclear (Boston, MA). Stock solution (10 μM) was stored in −20°C. [γ-³²P]ATP was purchased from Amersham Japan, Ltd.

Tumor Cell Lines. P388 murine leukemic cells were maintained by serial i.p. passages in Shi: (C57BL × DBA)F₁ (hereafter called BDF₁) mice. P388/VP-16 cells were developed by treatment of BDF₁ mice bearing the P388 ascites cells with 8 mg VP-16-213/kg body weight on days 1 and 5 after i.p. implantation of 10⁶ P388 cells, as described by Wilkoff and Dulmadge (11). P388 and P388/VP-16 cells were collected on day 10 after passage. Erythrocytes were removed by Ficoll-Conray density gradient sedimentation. Cells were then washed with phosphate-buffered saline (0.8% NaCl/0.115% Na₂HPO₄/0.02% KH₂PO₄/0.02% KCl, pH 7.5), resuspended in RPMI 1640 medium supplemented with 10% FBS and 20 μM 2-mercaptoethanol, and used immediately for *in vitro* experiments.

Quantitation of Drug Effects. The growth-inhibitory effects of drugs were assessed by plating cells in multiwell dishes (Falcon 3047) at a final density of 1–2 × 10⁵/ml. Drugs were diluted with 0.9% NaCl solution and were less than 1% of the final volume of the cell suspension. The IC_{50} was defined as the concentration of drug that inhibits the 48-h cell growth by 50%, compared with untreated controls. The extent of increase in IC_{50} was determined by dividing the IC_{50} for the resistant cells by that for the controls.

Phorbol Ester Binding Assay. Phorbol ester binding was carried out as described by Sando et al. (12). Briefly, P388 and P388/VP-16 cells (1–3 × 10⁶ in RPMI 1640 + 10% FBS) were incubated with [20-³H] PDB (1–400 nM) and either vehicle or various unlabeled concentrations of phorbol esters. Incubation was carried out at 4 or 37°C for varying times, as indicated in the figures. After the incubation, bound [20-³H] PDB was separated from free PDB by rapid passage through glass fiber filters. Cells on the filters were washed with iced phosphate buffered saline, and the filters were counted in a liquid scintillation counter. Total or nonspecific binding is expressed as the mean of triplicate determinations carried out in the absence or presence of unlabeled PDB, respectively. Specific binding was determined by subtracting the

mean nonspecific binding from the total value measured at the same concentration of [20-³H]PDB.

Determination of Protein Kinase C Content. P388 and P388/VP-16 cells were prepared as described above. Protein kinase C was prepared from the cytosolic and particulate fractions, as described (13–15). Cell fractionation, protein kinase C preparation and kinase assay were carried out in the same day so as to avoid inactivation of the enzyme. Protein kinase C was assayed as described (14–16) and expressed as pmol of phosphorus-32 incorporated into lysine-rich histone type III-S over 1, 2, 3, and 5 min at 30°C in the presence of Ca^{2+} and PS. Ca^{2+}-dependent activity was subtracted from Ca^{2+}- and PS-dependent activity in calculating protein kinase C activity.

RESULTS

Resistance to Anticancer Drugs and PDB Binding Capacity. Table 1 shows the sensitivity of P388 and P388/VP-16 cells for VP-16-213, vincristine, and Adriamycin. By comparison with the parent P388 cells, P388/VP-16 cells were 159-fold resistant to VP-16-213 and showed cross-resistance to vincristine (18.9-fold) and to Adriamycin (522.9-fold).

The time course for specific binding of [20-³H]PDB to P388 or P388/VP-16 cells is shown in Fig. 1. In both cell lines, the binding reached the plateau within 15 min at 37°C and 60 min at 4°C. Nonspecific binding did not increase with prolonged incubation, at either temperature. Specific PDB binding was significantly lower in P388/VP-16 cells (Fig. 1). Nevertheless, the reduction in binding does not necessarily indicate a decrease in the number of binding sites, but rather may reflect a change in affinity. Therefore, we measured the capacities of P388 and P388/VP-16 cells to bind [20-³H]PDB. Fig. 2A shows the concentration dependency for [20-³H]PDB binding determined
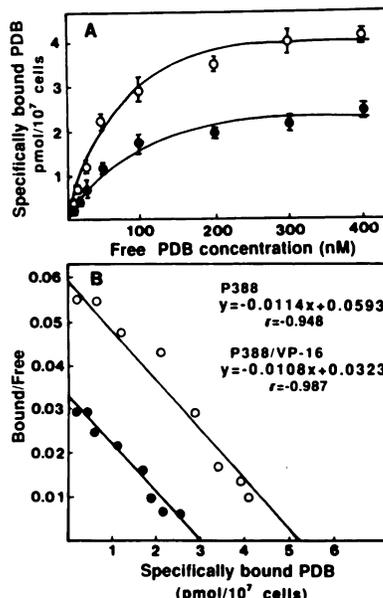
Table 1 *Sensitivity of cultured P388 and P388/VP-16 cells to VP-16-213*

Growth-inhibitory effects of VP-16-213, vincristine, and Adriamycin on P388 and P388/VP-16 cells. Cells were grown in RPMI 1640 medium supplemented with 10% FBS and 20 µM 2-mercaptoethanol in a humidified volume of 5% CO_2 and 95% air. The IC_{50} value for each cell line was determined by 48-h growth inhibition. The extent of resistance is defined as the IC_{50} value for the concentration of drugs producing 50% inhibition of the P388/VP-16 cell growth divided by that of the parent P388 cell line.

Drug	IC_{50} (nM)		Degree of re-sistance[b]
	P388[a]	P388/VP-16[a]	
VP-16-213	18.8	3000	159.3
Vincristine	1.60	30.3	18.9
Adriamycin	3.27	1710	522.9

[a] Diluted to a density of 1–2 × 10⁵/ml, were exposed to drugs for 48 h and then counted.
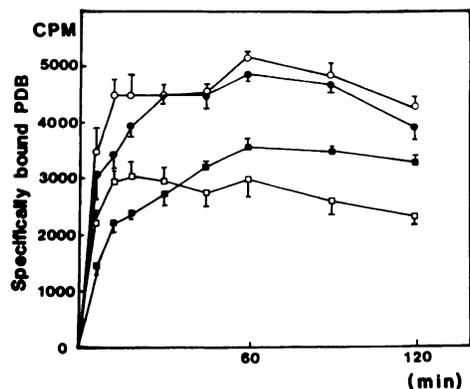[b] Calculated by dividing the IC_{50} value of P388/VP-16 cells by that of the parent P388 cells.

Fig. 1. Time course for specific binding of PDB to P388 and P388/VP-16 cells. P388 cells at 37°C (O) or 4°C (●) and P388/VP-16 cells at 37°C (□) or 4°C (■) were incubated with 50 nM [20-³H]PDB in the presence or absence of 50 µM nonradioactive PDB, and specific binding was determined at the indicated time. Nonspecific bindings were under 10% of total.
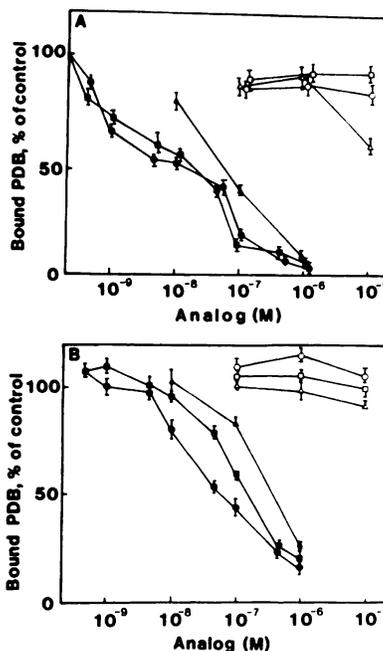
Fig. 2. Concentration dependency of [20-³H]PDB bindings. *A,* P388 (O) and P388/VP-16 (●) cells were incubated at 37°C for 15 min with varying concentrations of [20-³H]PDB in the presence or absence of 1000-fold excess of unlabeled PDB. Specific binding was determined by subtracting the nonspecific binding at each [20-³H]PDB concentration from the corresponding total value. *Points,* mean of triplicate samples. *B,* Scatchard plot of the data from *A.*

Table 2 *PDB receptor activity in P388 and P388/VP-16 cells*
Numbers of sites per cell and dissociation constants of phorbol ester receptor.

Cell line	PDB receptor sites/cell[a]	K_d (nM)[a]
P388	6.56 ± 0.84 × 10⁵	76.7 ± 17.3
P388/VP-16	3.78 ± 1.01 × 10⁵	76.9 ± 11.6

[a] Mean ± SD for four separate experiments.

Fig. 3. Displacement of [20-³H]PDB binding to (*A*) P388 and (*B*) P388/VP-16 cells by various phorbol esters. Cells were incubated with 50 nM [20-³H]PDB and vehicle (control) or at the presence of indicated concentrations of nonradioactive TPA (●), PDB (■), phorbol 12,13-dibenzoate (▲), phorbol 12,13-didecanoate (O), 4-O-methylphorbol-12-myristate-13-acetate (△), or 4β-phorbol (□) for 15 min at 37°C. Specific bindings were determined as described in "Materials and Methods."

Table 3 *Distribution of protein kinase C activity between cytosolic and particulate fractions*

Protein kinase C assay was done as described in "Materials and Methods" and expressed as pmol ^{32}P incorporated into lysine-rich histone type III-S over 1, 2, 3, and 5 min at 30°C in the presence of Ca^{2+} and PS. Ca^{2+}-dependent activity was subtracted from Ca^{2+}- and PS-dependent activity in calculating protein kinase C activity.

Cell line	Protein kinase C activity, ^{32}P incorporation (pmol/min/10⁶ cells)[a]		
	Cytosol	Membrane	Total
P388	2.17 ± 0.49	4.05 ± 0.81	6.21 ± 0.34
P388/VP-16	1.79 ± 0.10	2.35 ± 0.49	4.14 ± 0.45
P value[b]	NS[c]	<0.05	<0.01

[a] Mean ± SD for three different experiments.
[b] Student's t test.
[c] NS, not significantly different.

after 15 min at 37°C. Two hundred nM of PDB led to a saturation of the specific binding with both P388 and P388/VP-16 cells; nonspecific binding, determined in the presence of a 1000-fold excess of unlabeled PDB, was under 10%. Scatchard analysis of these data, shown in Fig. 2B, revealed linear plots indicative of a single class of receptor, in both cell lines. No difference was observed in the dissociation constants between the control and the variant cell lines, thereby suggesting that the affinity of receptor was much the same. However, there was a marked difference in the number of sites per cell: the drug-resistant cells contained only one-half of the binding sites compared with those of the parental (Table 2).

The specificity of [^{3}H]PDB binding to P388 and P388/VP-16 cells was determined by examining the relative abilities of various tumor promoters to displace labeled PDB from intact cells. As shown in Fig. 3, A and B, the specific [20-^{3}H]PDB binding to both cell types was inhibited by the biological active promoters TPA and PDB, whereas phorbol-12,13-dibenzoate was less effectively bound. Phorbol-12,13-didecanoate, 4-O-methylphorbol-12-myristate-13-acetate, and 4β-phorbol bound only weakly.

Protein Kinase C Activity. To determine if the lower phorbol diester receptor concentration of P388/VP-16 cells was due to the depletion of protein kinase C, the enzyme activity of cytosol and membrane fraction from the two cell lines was measured. The activity of protein kinase C significantly decreased in the P388/VP-16 cells, particularly in the membrane fraction, as shown in Table 3.

DISCUSSION

Calcium channel blockers (2–6) and calmodulin inhibitors (4) are effective in circumventing multidrug resistance, by inhibiting efflux of the drug from the cell. The Ca^{2+}-calmodulin complex was speculated to be involved in this efflux mechanism. Nair et al. (5) found no difference in the calmodulin content between Adriamycin-sensitive and -resistant P388 cells. These results agree well with those of earlier reported studies on vincristine and Adriamycin-resistant P388 cells by Tsuruo et al. (7). Beck et al. (6) found the same to be true for their CEM and vinblastine-resistant CEM cell lines and suggested that calmodulin may not be directly involved in multidrug resistance.

We found that the phorbol diester receptor is decreased in P388/VP-16 cells. Evidence was obtained that the down-regulated PDB receptor corresponds to a decrease in the surface density of receptor rather than a decrease in the affinity of the receptor for PDB.

As protein kinase C is closely related to the receptor for

phorbol esters (10), we then examined whether the down-regulated phorbol ester receptors in the drug-resistant cells are related to Ca^{2+}-phospholipid-dependent protein kinase. The activity of protein kinase C of P388/VP-16 cells significantly decreased, particularly in the membrane fraction. A decrease in the level of protein kinase C in the P388/VP-16 cells correlates with the decrease in phorbol ester receptor. The translocation of protein kinase C from the cytosol to the membrane after treatment with TPA also occurred in P388/VP-16 cells (data not shown).

Down regulation of the specific high-affinity phorbol ester receptor occurs in several cell systems after treatment with phorbol esters (16, 17) or another ligand (18). Collins and Rozengurt (16) found that prolonged treatment of Swiss 3T3 cells with PDB rendered them refractory to subsequent mitogenic stimulation by both PDB and vasopressin, with a decrease in the PDB receptor. Perrella et al. (17) reported that the HL60 phorbol ester-resistant variant R1B6, when grown in the presence of phorbol esters, had a reduced concentration of phorbol ester receptors, relative to parental cells, and could not be induced by phorbol esters to differentiate into macrophage-like cells. Hormones and polypeptide growth factors that affect cellular metabolism, cell division, and synthesis of specialized products do down regulate their receptors. The cells can become refractory to responsiveness following prolonged exposure to ligand, and this often correlates with a 50–75% decrease in the number of receptors for that ligand (19).

One hypothesis for multidrug resistance is that the phorbol ester receptor constitutes a regulator which inhibits the Ca^{2+}-calmodulin-dependent system. If such is the case, then the down regulation of the phorbol ester receptors following prolonged VP-16-213 treatment would eliminate the inhibition of phorbol esters on calcium-calmodulin-dependent activation of drug efflux. This suggested mechanism of regulation is the least complex explanation as to why calcium and calmodulin inhibitors can increase intracellular drug levels and restore the sensitivity of cells to the drug, despite the lack of difference in calmodulin contents between sensitive and resistant cells (4, 5, 7).

Although the physiological role of phorbol ester receptor and protein kinase C in P388 murine leukemic cell remains to be elucidated, the down regulation of specific PDB binding and protein kinase C reported in this communication may well be closely related to the mechanism of pleiotropic drug resistance.

ACKNOWLEDGMENTS

REFERENCES

1. O'Dwyer, P. J., Leyland-Jones, B., Alonso, M. T., Marsoni, S., and Witters, R. E. Etoposide (VP-16-213) current status of anticancer drug. N. Engl. J. Med., *312:* 692–700, 1985.
2. Yalowich, J. C., and Ross, W. E. Verapamil-induced augmentation of etoposide accumulation in L1210 cells in vitro. Cancer Res., *45:* 1651–1656, 1985.
3. Slater, L. M., Murry, S. L., Wetzel, M. W., Sweet, P., and Stupecky, M. Verapamil potentiation of VP-16-213 in acute lymphatic leukemia and reversal of pleiotropic drug resistance. Cancer Chemother. Pharmacol., *16:* 50–54, 1986.
4. Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. Increased accumulation of vincristine and Adriamycin in drug-resistant P388 tumor cells following incubation with calcium antagonists and calmodulin inhibitors. Cancer Res., *42:* 4730–4733, 1982.
5. Nair, S., Samy, T. S. A., and Krishan, A. Calcium, calmodulin, and protein content of Adriamycin-resistant and -sensitive murine leukemic cells by verapamil. Cancer Res., *46:* 229–232, 1986.
6. Beck, W. T., Cirtain, M. C., Look, A. T., and Ashmun, R. A. Reversal of

Vinca alkaloid resistance but not multiple drug resistance in human leukemic cells by verapamil. Cancer Res., *46:* 778–784, 1986.

7. Tsuruo, T., Iida, H., Kawabata, H., Tsukagoshi, S., and Sakurai, Y. High calcium content of pleiotropic drug-resistant P388 and K562 leukemia and Chinese hamster ovary cells. Cancer Res., *44:* 5095–5099, 1984.

8. Schulman, H. *In:* J. A. Nathason and J. W. Kebabian (eds.), Handbook of Experimental Pharmacology, 58/I, pp. 425–478. New York: Springer-Verlag, 1982.

9. Nishizuka, Y. The role of protein kinase C in cell surface signal transduction and tumor promotion. Nature (Lond.), *308:* 693–698, 1984.

10. Niedel, J. E., Kuhn, L. J., and Vandenbark, G. R. Phorbol diester receptor copurifies with protein kinase C. Proc. Natl. Acad. Sci. USA, *80:* 36–40, 1983.

11. Wilkoff, L. J., and Dulmadge, E. A. Resistance and crossresistance of cultured leukemia P388 cells to vincristine, Adriamycin, Adriamycin analogs, and actinomycin D. J. Natl. Cancer Inst., *61:* 1521–1524, 1978.

12. Sando, J. J., Hilfiker, M. L., Salomon, D. S., and Farrar, J. J. Specific receptors for phorbol esters in lymphoid cell populations: role in enhanced production of T-cell growth factor. Proc. Natl. Acad. Sci. USA, *78:* 1189–1193, 1981.

13. Kraft, A. S., and Anderson, W. B. Phorbol esters increase the amount of Ca^{2+}, phospholipid dependent protein kinase associated with plasma membrane. Nature (Lond.), *301:* 621–623, 1983.

14. Hidaka, H., Inagaki, M., Kawamoto, S., and Sasaki, Y. Isoquinolinesulfonamides, novel and potent inhibitors of cyclic nucleotide dependent protein kinase and protein kinase C. Biochemistry, *23:* 5036–5041, 1984.

15. Takai, Y., Kishimoto, A., Inoue, M., and Nishizuka, Y. Studies on a cyclic nucleotide-independent protein kinase and its proenzyme in mammalian tissues. J. Biol. Chem., *252:* 7603–7609, 1977.

16. Collins, M. K. L., and Rozengurt, E. Homologous and heterologous mitogenic desensitization of Swiss 3T3 cells to phorbol esters and vasopressin: role of receptor and postreceptor steps. J. Cell. Physiol., *118:* 133–142, 1984.

17. Perrella, F. W., Hellmig, B. D., and Diamond, L. Up regulation of the phorbol ester receptor-protein kinase C in HL-60 variant cells. Cancer Res., *46:* 567–572, 1986.

18. Jacken, S., Tashjian, A. H., Jr., and Blumberg, P. M. Characterization of phorbol ester receptors and their down-modulation in GH_4C_1 rat pituitary cells. Cancer Res., *41:* 2175–2181, 1981.

19. Catt, K. J., Harwood, J. P., Aguilera, G., and Dufau, M. L. Hormonal regulation of peptide receptors and target cell responses. Nature (Lond.), *280:* 109–116, 1979.