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PROTEIN KINASE C ACTIVITIES AND BINDINGS OF A PHORBOL ESTER TUMOR PROMOTER IN 41 CELL LINES*

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Summary The activities of protein kinase C (PKC) and the bindings to phorbol-12,13-dibutyrate (PDBu) of 41 cell lines were measured. The activities of PKC varied from 0.2 to 37 mU/10^6 cells in different cell lines, and in general were high in normal or untransformed cells and low in malignant, or transformed cells. The PDBu binding also varied considerably in different cell lines, and was again higher in normal or untransformed cells. In some cell lines, the binding was much higher at 4°C than at 37°C, suggesting rapid down-regulation of the binding. A correlation between PKC activity and PDBu binding was found only within certain cell types, i.e., epithelial cell lines derived from human tumors.

Introduction PKC is a phospholipid- and calcium-dependent protein kinase originally isolated by Nishizuka and his colleagues. DG, a product of signal-induced inositol phospholipid breakdown, greatly increases the affinity of PKC for phospholipids and calcium at a physiological level, indicating that PKC has a crucial role in signal transduction by a variety of growth factors and differentiation factors (1). Recent work has shown that there is a family of PKC genes, which may be expressed specifically depending on the type of tissue or cells (2).

PKC is the receptor for phorbol ester tumor promoters (3) and binding sites for phorbol esters have been co-purified with PKC activity

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Abbreviations: PKC, protein kinase C; SCC, squamous cell carcinoma; DG, diacylglycerol; PDBu, phorbol-12,13-dibutyrate; PS, phosphatidylserine; EB, extraction buffer consisting of 2 mM EDTA, 5 mM EGTA, 2 mM phenylmethylsultonyl fluoride, 5 mM 2-mercaptoethanol and 20 mM Tris-HCl, pH 7.5.

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from rat and mouse brains (4, 5). Transfection of PKC genes resulted in increase in binding of ^{3}H-PDBu in parallel with increase in expression of PKC activity (6). Thus, the pleiotropic actions of phorbol esters on cell growth, differentiation and carcinogenesis can be explained at least in part by their bindings to PKC, which result in its activation.

Recent progress in cellular and molecular biology largely depends on the use of cell lines. For example, A431 cell line, which contains a large number of binding sites for epidermal growth factor (7), has facilitated studies of this receptor. Similarly, cell lines with special characters of PKC should be useful. We, therefore, examined the activities of PKC and the binding to PDBu of 41 cell lines derived from normal and malignant tissues of experimental animals and humans. The results indicated considerable variations in the PKC activities and bindings of PDBu in different cell lines, with high values in normal or untransformed cells and low values in malignant or transformed cells.

Materials and Methods

Materials. The following chemicals were used: PDBu (L. C. Service Corp., MA); histone (type III-S), ATP and PS, diolein (Sigma, MO); [γ-^{32}P]ATP (specific activity, 20-40 Ci/mmol) and [20-^{3}H]PDBu (specific activity, 12.5-30.8 Ci/mmol) (New England Nuclear, MA).

Cell culture. The following culture media supplemented with 10% fetal calf serum were used: RPMI1640 (for P3-X63-Ag8U1, FM3A, U937, HL-60, Friend erythroleukemia cells, and TE-1, -2, -8, and -9); Dulbecco's modified Eagle's medium (for SUSM-1, SV-W1-38, and A431); Eagle's minimum essential medium (for other cell lines). All cells were grown at 37°C in an atmosphere of 5% CO_2 in air.

Preparation and assay of PKC. About 5 x 10^7 cells in the late log phase of growth were solubilized in EB containing 1% Triton X-100 for 30 min. The supernatant of centrifugation at 100,000 x g for 1 h was applied to a DEAE-cellulose (DE52, Whatman) column (1 ml). The eluate with 3 ml of 0.3 M NaCl in EB was mixed with 1.5 ml of EB saturated with ammonium sulfate, and the mixture was stood for 1 h, and then centrifuged at 15,000 x G for 20 min. The desalted solution through a PD-10 column (Pharmacia) was used for assay of PKC. Protein kinase activity was assayed by measuring in the reaction mixture consisted of 20 mM Tris-HCl (pH 7.5), 5 mM $MgSO_4$, 200 µg/ml histone, 10 µM [γ-^{32}P]ATP, and 50 µl of enzyme preparation in a final volume of 250 µl (8). PKC activity is defined as the difference between the ATP-histone phospho-transferase activity in reaction mixture containing 1 mM $CaCl_2$, 64 µM PS, and 1.3 µM diolein (Ca/PS/DG) and in that containing 1 mM EGTA but no Ca/PS/DG. After incubation for 3 min at 30°C, the reaction was stopped by adding 5 ml of 25% trichloroacetic acid, and radioactivity of acid-insoluble fraction was counted. One unit of PKC activity was defined as the amount of enzyme catalyzing incorporation of 1 nmol of phosphate per min from ATP into histone.

Assay of PDBu binding. Binding of [^{3}H]PDBu to intact cells was assayed as described previously (9) with a slight modification. Briefly, subconfluent cultures (about 3-8 x 10^5 attached cells/35-mm dish or 1-2 x 10^6 floating cells/1.5-ml microcentrifuge tube) were washed 3 times with PBS and incubated in 1 ml of assay buffer (1 mg/ml bovine serum albumin in PBS) containing 3 nM [^{3}H]PDBu in the presence and absence of a large

excess (30 μM) of unlabeled PDBu at 4°C or 37°C for 60 min. After incubation, the cells were washed 3 times with ice-cold assay buffer and solubilized in PBS containing 0.8% Triton X-100, 0.02% EDTA, and 0.25% trypsin. The radioactivity of the cell lysate was counted in a liquid scintillation counter. Specific PDBu binding is defined as the difference between the amounts of [^{3}H]PDBu bound in the presence and absence of unlabeled PDBu.

Results and Discussion

PKC activities. As summarized in Table 1, the PKC activity varied considerably in different cell lines, ranging from 0.2 to 37 mU/10^6 cells. In human cell lines, diploid cells showed higher activities than cell lines derived from malignant tumors: the activities of human diploid fibroblasts were 19.7 (JHU-1) or 37.1 mU/10^6 cells (dermal fibroblasts), whereas those of the transformed human fibroblast lines, SUSM-1, SV-WI-38 and KMST-6 were 12.2, 6.3 and 0.9 mU/10^6 cells, respectively. Similarly, human epidermal keratinocytes in primary culture had an activity of 20.0 mU/10^6 cells, whereas 14 epithelial cell lines derived from human tumors had activities ranging from 0.5 to 13.1 mU/10^6 cells (average, 5.2 mU; SD, 4.0). Consistent with the present observation, Guillem et al. (10) reported that PKC activities were reduced, to a similar extent to the present study, in human colon carcinomas when compared with their normal adjacent colon mucosa.

NIH/3T3, BALB/3T3 and C3H/10T1/2 cells derived from mouse embryos, which all possess the phenotype of sensitivity to contact inhibition and are widely used in studies on cell transformation, had moderate activities in the range of 9 to 16 mU/10^6 cells. Mouse JB6 and rat FRSK cell lines, in which two-stage transformation occurs as in BALB/3T3 and C3H/10T1/2 cell lines, also showed moderate activities.

These large variations may be mainly attributable to variations in activity of PKC itself or in specific expression of its subtypes. The activity may also be influenced by other factors, such as cell specific substrate specificity, the presence of inhibitors or activators and the conditions of cell growth. There are several recent reports of elevated levels of diacylglycerol, an endogenous activator of PKC, in cells transformed by ras-oncogene (11-13). We found that in ras-transformed rat fibroblasts, an elevated level of diacylglycerol caused intracellular translocation, activation and down-regulation of PKC (14).

PDBu binding. Binding of PDBu was also found to vary considerably in different cell lines (Table 1). Human diploid fibroblasts and epidermal keratinocytes contain a large number of binding sites for PDBu, i.e., 335 and 788 fmol/10^6 cells, respectively, at 37°C, these numbers being at least 10 times those in other cell lines. Transformable 3T3 and

Table 1. PKC activities and PDBu bindings of 41 cell lines

Cells	Origin	PKC activity[a]	PDBu binding[b] at 4°C	PDBu binding[b] at 37°C
Mouse				
NIH/3T3	Whole embryo (17)	8.7 (16.1-7.4)	55.8	49.4
BALB/3T3	Whole embryo (18)	12.0 (19.2-7.2)	91.8	46.3
C3H/10T1/2	Whole embryo (19)	15.9 (20.1-4.2)	43.1	25.7
JB6	Epidermis (20)	19.1 (34.7-15.6)	13.8	19.9
P3-X63-Ag8U1	Myeloma (21)	N.T.[c]	2.5	2.2
FM3A	Mammary carcinoma (22)	4.0 (19.1-15.1)	0.6	0.9
HemA	Mammary carcinoma[d]	1.8 (8.1-6.3)	8.5	7.2
Friend	Erythroleukemia (23)	N.T.	2.3	1.2
Rat				
FRSK	Fetal keratinocytes (24)	11.0 (16.8-5.8)	82.0	4.9
FRSK(T)	Tx[e] FRSK (25)	18.4 (35.2-16.8)	N.T.	N.T.
IAR-20	Adult liver (26)	N.T.	2.4	4.6
Ac2F	Adult liver (27)	1.0 (3.9-2.9)	1.1	1.9
Chinese hamster				
CHL	Lung (28)	13.2 (13.2-0)	4.8	21.1
V79	Lung (29)	26.1 (38.7-12.6)	15.7	13.5
CHO-K1	Ovary (30)	8.0 (8.7-0.7)	20.9	20.4
Human				
Dermal fibroblasts		37.1 (79.9-42.8)	N.T.	335
Epidermal keratinocytes		20.0 (25.2-5.2)	N.T.	788
JHU-1	Dermal fibroblasts (31)	19.7 (24.3-4.6)	70.1	37.7
SUSM-1	Tx fibroblasts (32)	12.2 (12.2-0)	10.6	11.2
SV-WI-38	Tx fibroblasts (33)	6.3 (11.4-5.1)	8.8	10.9
KMST-6	Tx fibroblasts (34)	0.9 (5.3-4.4)	45.2	41.4
A431	SCC of vulva (7)	0.7 (2.6-1.9)	3.2	0.2
HSC-1	SCC of skin (35)	5.6 (7.9-2.3)	5.9	5.4
HSC-2	SCC of mouth floor (36)	11.3 (19.9-8.6)	87.4	53.3
HSC-3	SCC of tongue (36)	4.1 (6.6-2.5)	4.9	2.7
HSC-4	SCC of tongue (36)	3.0 (6.1-3.1)	10.4	7.4
Ca9-22	SCC of gingiva (36)	3.8 (7.7-3.9)	15.7	12.6
NA	SCC of tongue (36)	13.1 (21.2-8.1)	13.4	23.4
TE-1	SCC of esophagus (37)	7.6 (14.2-6.6)	26.2	29.2
TE-2	SCC of esophagus (37)	1.4 (5.3-3.9)	2.8	1.8

a, mU/10^6 cells, average of duplicate measurements. The first values in parentheses indicate protein kinase activity in the presence of Ca/PS/DG, while the second values indicate that in the absence of Ca/PS/DG but with EGTA.

b, fmol/10^6 cells, average of duplicate measurements, except human dermal fibroblasts (4 measurements) and epidermal keratinocytes (6 measurements).

c, N.T., not tested.

d, unpublished cell line.

e, Tx, transformed.

f, obtained from Dr. T. Nishihira, Tohoku University.

g, obtained from Dr. S. Kondo, Tokyo Medical and Dental University.

Table 1. - Continued.

Cells	Origin	PKC activity[a]	PDBu binding[b] at	
			4°C	37°C
TE-8	SCC of esophagus[f]	0.5 (1.6-1.1)	6.0	0.9
TE-9	SCC of esophagus[f]	3.4 (9.0-5.6)	7.3	0
HeLa	Adenocarc. of cervix (44)	7.0 (19.0-12.0)	2.2	6.7
MCF-7	Mammary carcinoma (39)	1.8 (8.1-6.3)	8.2	5.4
EJ	Bladder carcinoma (40)	9.4 (15.0-5.6)	4.9	5.4
MIO-1	Malignant melanoma[g]	0.2 (1.9-1.7)	10.4	15.0
MIO-2	Malignant melanoma[g]	0.3 (69.3-69.0)	13.6	22.5
MIO-nu	Malignant melanoma[g]	N.T.	17.6	25.7
TE-85	Osteosarcoma (41)	1.3 (4.4-3.1)	7.9	12.5
U937	Histiocytic lymphoma (42)	7.9 (18.8-10.9)	1.0	0.3
HL-60	Promyelocyt.leukemia (43)	N.T.	0.9	0.4

C3H/10T1/2 cell lines bound moderate amounts of PDBu. In general, transformed cells and cell lines derived from tumors had lower activity for PDBu binding than normal and untransformed cells. This is not consistent with the previous observation of Shoyab and Todaro on mouse cell lines (15).

Down-regulation depends on temperature, occurring at 37°C, but not at 4°C, so differences in values at these two temperatures may indicate the occurrence and extent of down-regulation of PDBu binding (16). FRSK, A431 and TE-9 cells showed large differences in PDBu bindings at 4°C and 37°C, the values at 37°C being only 5.9, 6.3 and 0%, respectively, of those at 4°C. We demonstrated elsewhere that PKC activity and PDBu binding decreased 7 times faster in FRSK cells than in BALB/3T3 cells, and that this down-regulation was due to proteolytic cleavage of the PKC molecule (16). In contrast, human epidermal keratinocytes do not show down-regulation, although they contained a large number of binding sites for PDBu (9).

Correlation of PKC activities and PDBu bindings. As shown in Fig. 1, there was no significant correlation between the PKC activities and PDBu binding at 4°C, (correlation coefficient, $r = 0.39$). The distribution of values could be divided into the following four groups: A, cell lines with high PKC activity and low PDBu binding (e.g. V79, CHL and JB6); B, cell lines with high activities of both parameters (e.g. JHU-1, BALB/3T3 and C3H/10T1/2); C, cell lines with low PKC activity and high PDBu binding (KMST-6); and D, cell lines with low activities of both parameters (most human tumor cell lines).

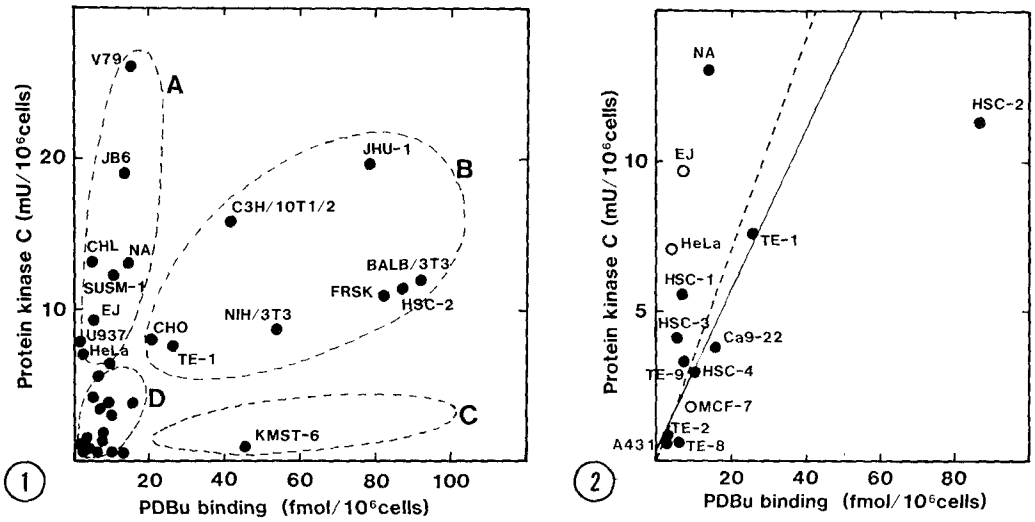

Fig. 1. Correlation of PKC activities and PDBu bindings at 4°C of the 33 cell lines. A, cell lines with high PKC activity and low PDBu binding; B, cell lines with high values of both parameters; C, cell lines with low PKC activity and high PDBu binding; D, cell lines with low values of both parameters. The names of cell lines in Groups A, B and C are indicated.

Fig. 2. Correlation of PKC activities and PDBu bindings at 4°C of 14 epithelial cell lines derived from human tumors (●, SCC; ○, other types of tumor). There is a significant correlation between these two parameters for epithelial tumor cell lines (dotted line; correlation coefficient, 0.58; $P < 0.05$). A higher level of correlation was obtained for SCC cell lines only (solid line; correlation coefficient, 0.62; $P < 0.05$).

This absence of a correlation may be due to the inclusion in the study of diverse cell lines derived from different species and cell types (epithelial or mesenchymal) and with different natures (normal or malignant). When examined the correlations within various types of cell lines, a significant correlation was obtained with epithelial cell lines derived from human tumors ($r = 0.58$; $P < 0.05$) (Fig. 2), but not with non-epithelial human tumor cell lines ($r = 0.52$; $P > 0.2$). A higher correlation coefficient was obtained when only cell lines derived from SCC were compared ($r = 0.62$, $P < 0.05$), especially for 4 cell lines of esophageal SCC ($r = 0.94$; $P < 0.1$). No significant correlation was found with other categories, such as cell lines from mice, rats or Chinese hamsters.

The availability of cell lines with PKC showing different characters, such as high or low activity, or rapid or slow down-regulation, should be useful for further studies on the mechanisms by which PKC regulates cell growth, differentiation and tumor promotion.

References

1. Nishizuka, Y. (1984) Nature 308, 693-698.
2. Nishizuka, Y. (1988) Nature 334, 661-665.
3. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R., and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.
4. Niedel, J. E., Kuhn, L. J., and Vandenbark, G. R. (1983) Proc. Natl. Acad. Sci. USA 80, 36-40.
5. Ashendel, C. L., Staller, J. M., and Boutwell, R. K. (1983) Cancer Res. 43, 4333-4337.
6. Knopf, J. L., Lee, M.-H., Sultzman, L. A., Kriz, R. W., Loomis, C. R., Hewick, R. M., and Bell, R. M. (1986) Cell 46, 491-502.
7. Fabricant, R. N., De Larco, J. E., and Todaro, G. J. (1977) Proc. Natl. Acad. Sci. USA 74, 565-569.
8. Chida, K., Hashiba, H., Sasaki, K., and Kuroki, T. (1986) Cancer Res. 46, 1055-1062.
9. Chida, K., and Kuroki, T. (1983) Cancer Res. 43, 3638-3642.
10. Guillem, J. G., O'Brian, C. A., Fitzer, C. J., Forde, K. A., LoGerfo, P., Treat, M., and Weinstein, I. B. (1987) Cancer Res. 47, 2036-2039.
11. Fleischman, L. F., Chahwala, S. B., and Cantley, L. (1986) Science 231, 407-410.
12. Preiss, J., Loomis, C. R., Bishop, W. R., Stein, R., Niedel, J. E., and Bell, R. M. (1986) J. Biol. Chem. 261, 8597-8600.
13. Lacal,J.C., Moscat,J., and Aaronson,S.A. (1987) Nature 330, 269-272.
14. Huang, M., Chida, K., Kamata, N., Nose, K., Kato, M., Homma, Y., Takenawa, T., and Kuroki, T. J. Biol. Chem. (in press)
15. Shoyab, M., and Todaro, G. J. (1980) Nature 288, 451-455.
16. Chida,K., Kato,N., and Kuroki,T. (1986) J.Biol.Chem. 261, 13013-13018.
17. Jainchill, J. L., Aaronson, S. A., and Todaro, G. J. (1969) J. Virol. 4, 549-553.
18. Aaronson,S.A., and Todaro,G.J. (1968) J. Cell. Physiol. 72, 141-148.
19. Reznikoff, C. A., Brankow, D. W., and Heidelberger, C. (1973) Cancer Res. 33, 3231-3238.
20. Colburn, N. H., Bruegge, W. F. V., Bates, J. R., Gray, R. H., Rossen, J. D., Kelsey, W. H., and Shimada, T. (1978) Caner Res. 38, 624-634.
21. Köhler, G., and Milstein, C. (1975) Nature 256, 495-497.
22. Nakano, N. (1966) Tohoku J., Exptl. Med. 88, 69-84.
23. Friend, C., Patuleia, M. C., De Harven, E. (1966) Natl. Cancer Inst. Monograph 22, 505-522.
24. Indo, K., and Miyaji, H. (1979) J. Natl. Cancer Inst. 63, 1017-1027.
25. Indo, K., and Miyaji, H. (1981) Gann Monograph Cancer Res.27, 145-158.
26. Montesano, R., Drevon, C., Kuroki, T., Vincent, L. S., Handleman, S., Sanford, K. K., DeFeo, D., Weinstein, I. B. (1977) J. Natl. Cancer Inst. 59, 1651-1658.
27. Tokiwa, T., Nakabayashi, H., Miyazaki, M., and Sato, J. (1979) In Vitro 15, 393-400.
28. Koyama, H., Utakoji, T., and Ono, T. (1970) Gann 61, 161-167.
29. Ford, D. K., and Yerganian, G. (1958) Natl. Cancer Inst. 21, 393-425.
30. Puck, T. T., Cieciura, S. J., and Robinson, A. (1958) J. Exp. Med. 108, 945-956.
31. Lin, S. L., Ts'o, P. O. P., Hollenberg, M. D. (1980) Biochem. Biophys. Res. Commun. 96, 168-174.
32. Namba, M., Nishitani, K., Fukushima, F., Kimoto, T., Utsunomiya, J., and Hayflick, L. (1981) Gann Monograph Cancer Res. 27, 221-230.
33. Stanbridge, E., Önen, M., Perkins, F. T., and Hayflick, L. (1969) Exp. Cell Res. 57, 397-410.
34. Namba, M., Nishitani, K., Hyodoh, F., Fukushima, F., and Kimoto, T. (1985) Int. J. Cancer 35, 275-280.
35. Kondo, S., and Aso, K. (1981) Br. J. Dermatol. 105, 125-132.
36. Kamata, N., Chida, K., Rikimaru, K., Horikoshi, M., Enomoto, S., and Kuroki, T. (1986) Cancer Res. 46, 1648-1653.

37. Nishihira, T., Kasai, M., Mori, S., Watanabe, T., Kuriya, Y., Suda, M., Kitamura, M., Hirayama, K., Akaishi, T., and Sasaki, T. (1979) Gann 70, 575-584.
38. Gey,G.O.,Coffman,W.D.,and Kubicek,M.T. (1952) Cancer Res.12, 264-265.
39. Brooks, S. C., Locke, E. R., and Soule, H. D. (1973) J. Biol. Chem. 248, 6251-6253.
40. Marshall, C. J., Franks, L. M., and Carbonell, A. W. (1977) J. Natl. Cancer Inst. 58, 1743-1751.
41. Rhim,J.S., Cho,H.Y., and Huebner,R.J. (1975) Int. J. Cancer 15, 23-29.
42. Sundström, C., and Nilsson, K. (1976) Int. J. Cancer 17, 565-577.
43. Collins,S.J.,Gallo,R.C.,and Gallagher,R.E. (1977) Nature 270, 347-349.