Vol. 140, No. 1, 1986 BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
October 15, 1986 Pages 267-272

LACK OF DIRECT EFFECT OF 2,3,7,8-TETRACHLORODIBENZO-P-DIOXIN (TCDD) ON PROTEIN KINASE C ACTIVITY IN EL4 CELLS

Caroline M. Kramer, Julianne J. Sando and Michael P. Holsapple*

Department of Pharmacology, University of Virginia, Charlottesville, VA

and *Department of Pharmacology and Toxicology, Medical College of Virginia, Richmond, VA

Received September 2, 1986

SUMMARY: In vivo administration of TCDD produces an increase in the level of Protein Kinase C in the hepatic plasma membrane. We have studied the direct effects of TCDD on cultured EL4 thymoma cells, which contain a large amount of Protein Kinase C and respond to phorbol esters with rapid translocation of the kinase to the membrane, followed by growth inhibition, adherence to substrate and production of Interleukin 2. TCDD (10-1000 nM) did not compete with ^{3}H-phorbol dibutyrate for binding to cytosolic Protein Kinase C, and had no effect on Protein Kinase C activity In vitro. TCDD did not stimulate translocation of Protein Kinase C to the membrane, and did not affect phorbol ester-stimulated translocation. TCDD did not inhibit EL4 cell growth or affect phorbol ester induced growth inhibition, and failed to stimulate production of Interleukin 2. Thus, TCDD does not appear to activate Protein Kinase C in EL4 cells.

Many of the in vivo effects of the environmental contaminant, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) , are similar to those of tumor promoting phorbol esters. These effects include enhanced cellular proliferation and differentiation (1,2), tumor promotion in mouse skin (3-5) and induction of gene expression (6,8-11). In vivo administration of TCDD results in reduced binding of insulin, glucagon, con A and epidermal growth factor to their receptors (12,13). Similarly, phorbol esters cause a reduction in affinity of insulin and epidermal growth factor receptors for their ligands, presumably via PKC-mediated receptor phosphorylation (14). The phorbol ester receptor, now shown to be identical with PKC (15,16), is found in the cytosolic fraction

The following abbreviations were used: TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; PKC, Protein Kinase C; PDB, phorbol 12,13-dibutyrate; PMA, phorbol 12-myristate, 13-acetate; PS, phosphatidylserine; DO, diolein; IL-2, interleukin 2.

0006-291X/86 $1.50

(17), as is the putative TCDD receptor (18). These observations led to speculation that TCDD might exert at least some of its effects via PKC (2).

In this investigation we have studied the effects of TCDD on PKC activity *in vivo* and *in vitro*, using the EL4 cell which has been used as a model for the study of PKC (7,17,20). EL4 is a mouse T helper lymphoma derived from a 9,10-dimethylbenzanthracene treated C57BL/6N mouse (21). Since lymphocytes are second only to brain in relative content of PKC (22), it is not surprising that EL4 cells contain a large amount of PKC (7). Treatment of EL4 cells with phorbol esters results in rapid apparent movement of PKC from the cytosol to the membrane, followed by adherence of cells to substrate, growth inhibition and production of IL-2 (7,23,27). In addition, EL4 cytosol contains abundant PKC/phorbol ester receptors and therefore is useful for measuring direct effects of agents on PKC. We have used this well defined system to measure the effects of TCDD on PKC.

METHODS

Competition for ^{3}H-PDB binding was measured as described (17). PKC activity was measured by incubating cytosol (0.4 mg/ml) with 0.8 mg/ml crude histone, 75 mM MgAc, 0.1 or 0.5 mM $CaCl_2$, 5 mM ATP containing 2.5 uCi ^{32}P-ATP, and PS, DO, PMA and TCDD as indicated, in a final volume of 150 ul, for 3 min. at 30 C. Reactions were stopped by spotting onto phosphocellulose paper and processed as described (20). Translocation of PKC was measured as described (7). Growth inhibition was measured by an 8 hour pulse of ^{3}H-thymidine (2 Ci/mmol) 18 hours after treatment of cells with TCDD or PMA. Cells were harvested onto filter paper, washed and counted. IL-2 was assayed as described (23), and units were calculated as the inverse of the dilution which gave a half maximal response.

RESULTS AND DISCUSSION

The ability of PDB or TCDD to bind to cytosolic PKC was assessed by competition with ^{3}H-PDB for cytosolic binding sites in the presence of calcium and PS. As shown in Fig. 1, PDB (1 uM) but not TCDD (10-1000 nM) was able to compete for ^{3}H-PDB binding sites, indicating that TCDD does not bind to the same site as PDB. The batch of TCDD used in these studies was shown to be biologically active in the nM range in other studies (24). In order to assess whether TCDD may activate PKC by a mechanism independent of the PDB binding site, we examined the direct effect of TCDD on PKC activity (Tables 1 and 2). While PMA and DO were capable of activating PKC in cytosol in the presence of

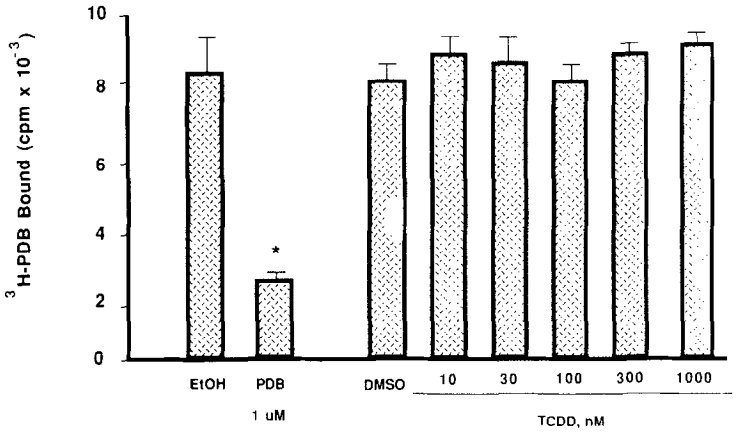

Figure 1. Competition by TCDD for ^{3}H-PDB Binding. EL4 cytosol (0.8 mg/ml) was incubated for 2 hours on ice with 40 nM ^{3}H-PDB, 75 mM MgAc, 0.5 mM $CaCl_2$, 1.2 mg/ml bovine serum albumin, 96 ug/ml PS, and PDB, TCDD or vehicle as indicated. Bars represent means with standard errors of triplicate determinations. *p 0.01 vs. EtOH (t-test). PDB was prepared in EtOH, TCDD in DMSO.

calcium and PS, TCDD (3-300 nM) was unable to increase PKC activity alone or with either PS or DO, or with a suboptimal combination of PS and DO, at 0.1 or 0.5 mM calcium, nor did it affect basal kinase activity. To determine whether TCDD could affect PKC in intact EL4 cells, we measured translocation of PKC

Table 1. PROTEIN KINASE C ACTIVITY. EL4 cytosol (0.4 mg/ml) was incubated for 3 min. at 30 C with 0.8 mg/ml crude histone, 75 mM MgAc, 0.5 mM ^{32} $CaCl_2$, 5 mM ATP containing 2.5 uCi P-ATP, and PS, DO and TCDD as indicated. Results are means with standard errors of a representative experiment of 2-3 done in triplicate

		PROTEIN KINASE ACTIVITY (^{32}P-histone, cpm x 10^{-3})				
		PS (ug/ml)		DO (ug/ml)		PS (48 ug/ml) +
addition	control	48	96	1.6	3.2	DO (1.6 ug/ml)
none	5.30 ± 0.16	10.08 ± 0.23*	11.7 ± 0.4*	5.19 ± 0.32	5.26 ± 0.23	12.1 ± 1.1*
TCDD 3 nM	5.37 ± 0.10		12.6 ± 0.2*		4.84 ± 0.02	13.2 ± 1.5*
10 nM	5.18 ± 0.14		12.6 ± 0.4*		4.78 ± 0.06	13.0 ± 0.7*
30 nM	5.45 ± 0.13		11.9 ± 0.9*		4.67 ± 0.20	13.3 ± 0.6*
100 nM	5.39 ± 0.12		12.2 ± 0.6*		5.03 ± 0.14	13.1 ± 0.3*
300 nM	5.35 ± 0.28		12.6 ± 0.2*		4.84 ± 0.12	12.8 ± 0.4*
DMSO	5.13 ± 0.26		11.7 ± 0.1*		4.71 ± 0.08	12.2 ± 1.0*
PS (96 ug/ml)					11.40 ± 0.40*	

*p 0.01 vs. DO alone.

TABLE 2. PROTEIN KINASE C ACTIVITY: CALCIUM DEPENDENCE. EL4 cytosol (0.4 mg/ml) was incubated at 30 C for 3 min. with 0.8 mg/ml crude histone, 75 mM MgAc, 0.1 or 0.5 mM $CaCl_2$, 5 mM ATP containing 2.5 uCi ^{32}P-ATP and PS, DO and TCDD as indicated. Results are means with standard errors of a representative experiment of 2-3 done in triplicate

	PKC ACTIVITY (^{32}P-histone, cpm x 10^{-3})
0.1 mM $CaCl_2$	
PS (15 ug/ml)	2.92 ± 0.39
+ DO (3.2 ug/ml)	5.98 ± 0.13
+ PMA (50 nM)	4.52 ± 0.16
+ TCDD (100 nM)	2.91 ± 0.16
0.5 mM $CaCl_2$	
PS (15 ug/ml)	12.4 ± 0.40
+ DO (3.2 ug/ml)	15.6 ± 0.50
+ PMA (50 nM)	14.8 ± 1.00
+ TCDD (100 nM)	14.0 ± 0.70

which presumably results in PKC activation (20). While PDB produced the expected loss in cytosolic and gain in membrane associated PKC, TCDD did not affect PKC localization or alter that response to PDB (Fig. 2). Therefore, in EL4 cells TCDD does not mimic early responses of phorbol esters involving PKC.

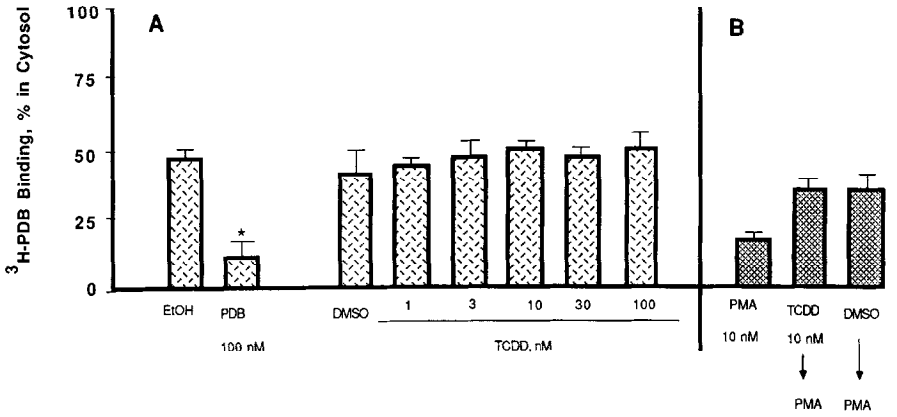

Figure 2. Effect of TCDD on Translocation of Protein Kinase C. EL4 cells (10^8 in 10 ml each condition) were treated with 100 nM PDB, TCDD or vehicle for 30 min. (A), or pretreated with 10 nM TCDD or DMSO for 15 min. before 15 min. incubation with 10 nM PDB (B). Cells were then fractionated into crude cytosol and membrane fractions by Dounce homogenization followed by centrifugation at 100,000 x g, and ^{3}H-PDB binding was measured in both fractions. Bars represent means of triplicate determinations from one of four similar experiments. *p 0.05 vs. control.

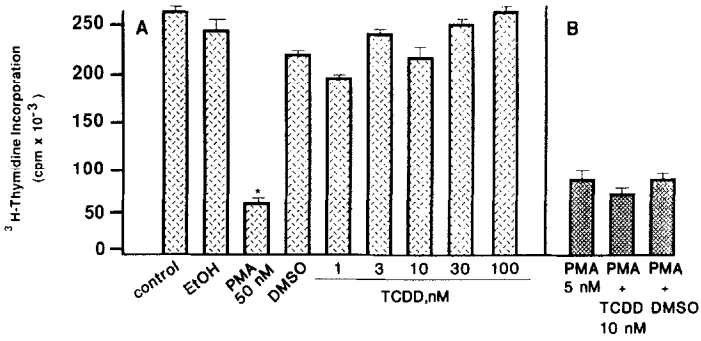

Figure 3. Effect of TCDD on Growth Inhibition. EL4 cells ($2x10^6$/ml) were incubated with 50 nM PMA, TCDD or vehicle (A) or incubated simultaneously with 5 nM PMA and 30 nM TCDD or DMSO (B) for 18 hours, then proliferation was measured as ^{3}H-thymidine incorporation during an 8 hour pulse. Bars represent means from one experiment performed in triplicate, with standard errors. *p 0.01 vs. EtOH (t-test).

Recently, Bombick et al. have reported an increase in PKC activity after long term (48 hour) incubation of hepatocytes with TCDD (25). Therefore, we measured two long term responses of EL4 cells to phorbol esters, growth inhibition and induction of IL-2 synthesis. TCDD (1-100 nM) failed to mimic the growth inhibitory effect of PMA (Fig. 3) and did not induce synthesis of IL-2 (not shown). Therefore, these results do not support an interpretation that TCDD can directly stimulate PKC activity. These results are in agreement with those of Clark et al. (26) in which con A-stimulated IL-2 production by C57Bl/6 mouse spleen cells *in vitro* was not affected by prior *in vivo* administration of TCDD. These results are not necessarily in disagreement with those of Bombick, et al. (25). In their studies, induction of synthesis of PKC was not ruled out. Induction of several hepatic enzymes by TCDD has been well documented (8-11),and in the case of PKC may be an intriguing explanation for tumor promotion by TCDD. In this light, our results clearly eliminate the hypothesis that TCDD produces the increase in hepatic PKC activity by a direct effect on PKC. Whether the similarities between TCDD and the phorbol esters in liver and other systems result from TCDD induction of PKC synthesis remains to be determined. However, our results support the hypothesis that effects of TCDD on PKC are not direct.

REFERENCES

1. Blumberg, P.M. (1980) Crit. Rev. Toxicol. 8,153-234.
2. Knutson, J.C. and Poland, A. (1984) Molecular and Cellular Approaches to Understanding Mechanisms of Toxicity, A.H. Tashjian, ed., Harvard School of Public Health.
3. Poland, A., Palen, D., and Glover, E. (1982) Nature 300, 271-273.
4. Kociba, R.J., Keyes, D.G., Beyer, J.E., Carreon, R.M., Wade, C.E., Dittenber, D.A., Kalnins, R.P., Frauson, L.E., Park, C.N., Barnard, S.D., Hummel, R.A., and Humiston, C.G. (1978) Toxicol. Appl. Pharmacol. 46, 279-303.
5. Furstenberger, G., Berry, D.L., Sorg, B., and Marks, F. (1981) Proc. Nat. Acad. Sci. 78, 7722-7726.
6. Jones, P.B.C., Galeazzi, D.R., Fisher, J.M., and Whitlock, J.P. (1985) Science 227, 1499-1502.
7. Kraft, A.S., Anderson, W.B., Cooper, H.L., and Sando, J.J. (1982) J. Biol. Chem. 257, 13193-13196.
8. Lucier, G.W., McDaniel, O., and Hook, G.E.R. (1975) Biochem. Pharmacol. 24, 325-334.
9. Poland, A. and Glover, E. (1973) Mol. Pharmacol. 9, 736-747.
10. Nebert, D.W., Goujon, F.M., and Gielen, J.E. (1972) Nature New Biology 236, 107-110.
11. Dietrich, R.A., Bludeau, P., Roper, M. and Schmuck, J. (1978) Biochem. Pharmacol. 27, 2343-2347.
12. Matsumura, F., Brewster, D.W., Madhukar, B.V., and Bombick, D.W. (1984) Arch. Env. Contamin. Toxicol. 13, 509-515.
13. Madhukar, B.V., Brewster, D.W., and Matsumura, F. (1984) Proc. Nat. Acad. Sci. 81, 7407-7411. 14. Cochet, C., Gill, G.N., Meisenhelder, M., Cooper, J.A., and Hunter, T. (1984) J. Biol. Chem. 259, 2553-2558.
15. Kikkawa, U., Takai, Y., Tanaka, Y., Miyake, R. and Nishizuka, Y. (1983) J. Biol. Chem. 258, 11442-11445.
16. Parker, P., Stabel, S. and Waterfield, M.D. (1983) EMBO 3, 953-959.
17. Sando, J.J., and Young, M.C. (1983) Proc. Nat. Acad. Sci. 80, 2642-2646.
18. Gasiewicz, T.A. and Neal, R.A. (1982) Anal. Biochem. 124, 1-11.
19. Hirota, K. Hirota, T., Aguilera, G. and Catt, K. (1985) J. Biol. Chem. 260, 3243-3246.
20. Kramer, C.M. and Sando, J.J., (1986) Cancer Res. 46, 3040-3045.
21. Gorer, P.A.(1950) Brit. J. Cancer, 4, 372.
22. Kuo, J.F., Anderson, R.G.G., Wise, B.C., Mackerlova, L., Salomon, D., Brackett, N.L., Katoh, N., Shoji, M. and Wrenn, R.W. (1980) Proc. Nat. Acad. Sci. 77, 7039-7043.
23. Farrar, J.J., Fuller-Farrar, J., Simon, P.L., Hilfiker, M.L., Stadler, B.M., and Farrar, W.L. (1982) J. Immunol. 125, 2555-2558.
24. Holsapple, M.P., Dooley, R.K., McNerney, P.J., and McKay, J.A. (1986), Immunopharmacology, In press.
25. Bombick, D.W., Madhukar, B.V., Brewster, D.W., and Matsumura, F. (1985) Biochem. Biophys. Res. Comm. 127, 296-302.
26. Clark, D.A., Sweeney, G., Safe, S., Hancock, E., Kilburn,, D.G., and Gauldie, J. (1983) Immunopharmacology 6, 143-153.
27. Sando, J.J., Hilfiker, M.L., Piacentini, M.J., and Laufer, T.M. (1982) Cancer Res. 42, 1676-1680.