Biochemical Pharmacology, Vol. 40, No. 10, pp. 2377–2380, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

SHORT COMMUNICATIONS

Effects of tumor promoters on adenylate cyclase activity in melanoma cells in culture

(*Received* 14 *February* 1990; *accepted* 27 *June* 1990)

Tumor-promoting phorbol esters such as phorbol-12-myristate-13-acetate (PMA) after a short treatment (within 1 hr) stimulate [1–5] or inhibit [6–10] adenylate cyclase (AC) responses to β-adrenoceptor agonists, depending upon the type of cells in culture. Since phorbol esters after a short treatment activate protein kinase C (PKC) [11] and the activated PKC is translocated from the cytosol to the plasma membrane [12–15], a role of PKC in the regulation of AC activity has been proposed. It has been reported that a prolonged treatment (18 hr or more) with phorbol esters causes PKC deficiency in some cells, but not in others [14, 16, 17]. The effect of a short or long treatment with phorbol esters on basal or melanocyte-stimulating hormone (MSH)-stimulated AC activity in melanoma cells has not been investigated. We now report that a short treatment of murine B-16 melanoma cells (within 1 hr) with a tumor-promoting phorbol ester, PMA, and a non-phorbol ester compound, mezerein, enhanced basal and MSH-stimulated AC activities *in vitro*, whereas a prolonged treatment (24 hr or more) inhibited them. These compounds also stimulated or inhibited sodium fluoride (NaF)- and forskolin-stimulated AC activity, depending upon the treatment time.

Materials and Methods

Murine B-16 melanoma cells were used for this study. This is an original B-16 clone and was initially supplied to us by Dr. Helson who was then at the Memorial Sloan-Kettering Cancer Center, New York. The procedures for culturing melanoma cells have been described previously [18]. The doubling time of melanoma cells under the growth conditions given in Ref. 18 was about 24 hr. During the course of our investigation, we observed that the basal AC activity increased as a function of time in culture; however, this change was not apparent within a few months.

PMA, 4α-phorbol 12,13-didecanoate (4α-PD), mezerein and forskolin (Sigma, St. Louis, MO) were dissolved in dimethyl sulfoxide (DMSO). NaF was dissolved in water, whereas α-MSH was dissolved in phosphate-buffered saline. All solutions were stored at −20°. The efficacy of MSH, NaF and forskolin decreases if they are stored at 4°.

Cells (0.5×10^6) were plated in large tissue culture dishes (100 mm), and agents were added 24 hr later. The agents and growth medium were changed 2 days after treatment, and the AC activity was assayed 3 days after treatment. One set of control cultures was untreated, whereas another set of control cultures received the same amount of solvent as the test cultures. On the day of the experiment, fresh growth medium was changed, and the cultures were further incubated for 15 min before using them for the enzyme assay. Since the pH of the growth medium affects enzyme activity [19] and the pH of the medium becomes acidic after 3 days of treatment, the change of growth medium prior to the assay of AC activity was considered essential. Cells were washed twice with 5 mL of cold buffer containing 2 mM Tris–HCl and 2 mM ethyleneglycolbis(aminoethyl-ether)tetra-acetate (EGTA) using a Potter–Elvehjem tissue homogenizer at 3000 rpm. A portion of the homogenate was used to determine the protein by the method of Lowry *et al.* [20], and the remaining homogenate was used for assaying AC activity. The AC activity was determined by measuring the conversion of [α-^{32}P]ATP to [^{32}P]cAMP and isolating the products by the method of Salomon *et al.* [21] as modified by Minneman *et al.* [22] and adapted by ourselves [23]. In the present study, cells were removed in Tris–HCl buffer containing multiple protease inhibitors (4 μg/mL antipain, 10 units/mL aprotinin, 2 μg/mL pepstatin, 0.3 mM phenylmethylsulfonyl fluoride (PMSF), 0.3 mM benzamidine, 20 μg/mL soybean trypsin inhibitor and 4 μg/mL leupeptin). The remaining procedures were similar to those described previously [21–23].

The significant difference between mean values of control and experimental groups was determined, using a one-tailed Student's *t*-test at a 0.05 level of significance.

Results and Discussion

Basal and MSH-stimulated AC activities *in vitro* were increased more by PMA treatment for 15 min than by the solvent treatment (Table 1). The stimulatory effects of PMA on AC activity were similar whether the treatment time was 15, 30 or 60 min (data not shown). The stimulatory effect of PMA was most pronounced at a concentration of 10^{-7} M; this change was not observed at a concentration of 10^{-9} M (data not shown). 4α-PD, an inactive analog of phorbol ester, did not affect basal or MSH-stimulated AC activities in melanoma cells (data not shown). AC activity *in vitro* was stimulated more by a non-phorbol tumor promoter, mezerein [24], than by the solvent treatment (Table 2). The stimulatory effect of mezerein was most pronounced at a concentration of 10^{-7} M and after 15 min of treatment, whereas a concentration of 10^{-9} M was ineffective. The addition of PMA or mezerein (10^{-7} M and 10^{-8} M) directly into the AC reaction mixture did not alter basal or MSH-stimulated AC activity. NaF [25, 26] and forskolin [27] act directly on G-proteins and the catalytic subunit of AC respectively. NaF-stimulted AC activity was enhanced by both PMA and mezerein, whereas forskolin-stimulated AC activity was increased by PMA only (Tables 1 and 2).

PMA and mezerein treatment for 24 hr or more reduced basal and MSH-, NaF- and forskolin-stimulated AC activities in melanoma cells in culture when compared to the solvent-treated control (Table 3). The extent of inhibition was similar whether the enzyme activity was measured 1 day or 3 days after treatment. PMA or mezerein concentrations of 10^{-7} M and 10^{-8} M were equally effective; however, a lower concentration (10^{-9} M) was ineffective (data not shown). The solvent treatment slightly, but significantly, enhanced basal AC activity *in vitro*.

The present study shows that two structurally different tumor promoters, PMA and mezerein, enhanced basal and MSH-stimulated AC activity in B-16 melanoma cells 15 min after treatment, whereas 4-α-PD, an inactive analog of phorbol ester, did not. These results are consistent with previous studies performed on other mammalian cells in which phorbol esters stimulated AC actiity [1–5]. PMA treatment also increased NaF- and forskolin-stimulated AC activities in melanoma cells.

Table 1. Effect of a short PMA treatment of melanoma cells on adenylate cyclase activity *in vitro*

Treatment	Adenylate cyclase activity (pmol/min/mg protein)			
	Basal	MSH (1 μM)	NaF (2 mM)	Forskolin (100 μM)
Control	251 ± 6	709 ± 24	443 ± 5	3749 ± 23
DMSO (0.001%), 15 min	282 ± 20*	805 ± 29	459 ± 24	3687 ± 47
PMA 10^{-8} M, 15 min	352 ± 15†	966 ± 22†	512 ± 21*	3953 ± 70†
PMA 10^{-7} M, 15 min	431 ± 8†	1275 ± 14†	718 ± 15†	4444 ± 61†

Cells (0.5×10^6) were plated in 100-mm tissue culture dishes, and phorbol-12-myristate-13-acetate (PMA) was added after 4 days of plating. Adenylate cyclase activity was determined in the 16,500 *g* pellet. Each value is the mean ± SE of an average of three samples. Each experiment was repeated three times, and similar changes were observed in treated groups when compared to controls.

* Significantly different ($P < 0.05$) from control.

† Significantly different ($P < 0.05$) from solvent-treated control.

Table 2. Effect of a short mezerein treatment of melanoma cells on adenylate cyclase activity *in vitro*

Treatment	Adenylate cyclase activity (pmol/min/mg protein)			
	Basal	MSH (1 μM)	NaF (2 mM)	Forskolin (100 μM)
Control	92 ± 4	311 ± 4	146 ± 6	1840 ± 10
DMSO (0.001%), 15 min	95 ± 2	322 ± 14	163 ± 5	1823 ± 31
Mezerein 10^{-8} M, 15 min	92 ± 3	329 ± 11	154 ± 5	1869 ± 12
Mezerein 10^{-7} M, 15 min	154 ± 11*	394 ± 10*	251 ± 14*	1840 ± 15
DMSO (0.001%), 1 hr	98 ± 3	378 ± 6†	153 ± 4	2020 ± 7†
Mezerein 10^{-8} M, 1 hr	108 ± 2†	371 ± 3†	176 ± 6*	1908 ± 29
Mezerein 10^{-7} M, 1 hr	144 ± 9*	420 ± 9*	239 ± 4*	1992 ± 13†

Cells (0.5×10^6) were plated in 100-mm tissue culture dishes, and mezerein was added 4 days after plating. Adenylate cyclase activity was determined in the 16,500 *g* pellet. Each value is the mean ± SE of an average of three samples. Each experiment was repeated three times, and similar changes were observed in treated groups when compared to controls.

* Significantly ($P < 0.05$) different from solvent-treated control.

† Significantly ($P < 0.05$) different from control.

Table 3. Effect of a long PMA and mezerein treatment of melanoma cells on adenylate cyclase activity *in vitro*

Treatment	Adenylate cyclase activity (pmol/min/mg protein)			
	Basal	MSH (1 μM)	NaF (2 mM)	Forskolin (100 μM)
Control	234 ± 4	660 ± 6	350 ± 8	2745 ± 36
Solvent (0.001%), 3 days	288 ± 3*	698 ± 36	423 ± 5*	3108 ± 50*
PMA 10^{-7} M, 1 day	60 ± 4†	198 ± 9†	313 ± 3†	2244 ± 31†
PMA 10^{-7} M, 3 days	67 ± 3†	157 ± 7†	330 ± 13†	2113 ± 43†
Mezerein 10^{-7} M, 3 days	75 ± 2†	162 ± 5†	357 ± 5†	2136 ± 56†

Cells (0.5×10^6) were plated in 100-mm tissue culture dishes, and phorbol-12-myristate-13-acetate (PMA) or mezerein was added 24 hr after plating for a 3-day treatment and 3 days after plating for a 1-day treatment. Adenylate cyclase activity was determined in the 16,500 *g* pellet. Each value is the mean ± SE of an average of three samples. Each experiment was repeated three times, and similar changes were observed in treated groups when compared to controls.

* Significantly ($P < 0.05$) different from untreated control.

† Significantly ($P < 0.05$) different from solvent-treated control.

The present study has demonstrated for the first time that the treatment of melanoma cells with PMA or mezerein for a prolonged period of time (24 hr or more) markedly decreases basal and MSH-stimulated AC activities. They also inhibit NAF- and forskolin-stimulated AC activities. Further studies are needed to determine if an increase (short-term) or decrease (long-term) in basal levels of AC underlies the effects of PMA and mezerein in melanoma cells.

Tumor-promoting phorbol esters, which activate PKC, have been shown to stimulate or inhibit β-adrenoceptor agonist stimulated AC activity *in vitro*, depending upon the type of cells in culture. In this study, a short treatment (within 1 hr) of murine B-16 melanoma cells with PMA and mezerein increased basal and MSH-stimulated AC activities *in vitro*, whereas a prolonged treatment (24 hr or more) inhibited them. PMA treatment also enhanced or inhibited NaF- and forskolin-stimulated AC activities depending upon the treatment time.

Acknowledgements—This work was supported by the Samuel Freeman Charitable Trust and the John Shafroth Memorial Fund.

Center for Vitamins and Cancer Research
Department of Radiology
School of Medicine
University of Colorado
Health Sciences Center
Denver
CO 80262, U.S.A.

KEDAR N. PRASAD*
JUDITH EDWARDS-PRASAD

REFERENCES

1. Bell JD and Brunton L, Enhancement of adenylate cyclase activity in S49 lymphoma cells by phorbol esters. *J Biol Chem* **261**: 12036–12041, 1986.
2. Sibley DR, Jeffs RA, Daniel K, Nambi P and Lefkowitz RJ, Phorbol diester treatment promotes enhanced adenylate cyclase activity in frog erythrocytes. *Arch Biochem Biophys* **244**: 373–381, 1986.
3. Hollingsworth EB, Sears EB and Daly JW, An activator of protein kinase C (phorbol-12-myristate-13-acetate) augments 2-chloroadenosine-elicited accumulation of cyclic AMP in guinea pig cerebral cortical particulate preparations. *FEBS Lett* **184**: 339–342, 1985.
4. Naghshineh S, Noguchi M, Huang K-P and Londos C, Activation of adipocyte adenylate cyclase by protein kinase C. *J Biol Chem* **261**: 14534–14538, 1986.
5. Choi EJ and Toscano JWA, Modulation of adenylate cyclase in human keratinocytes by protein kinase C. *J Biol Chem* **263**: 17167–17172, 1988.
6. Garte SJ and Belman S, Tumour promoter uncouples β-adrenergic receptor from adenyl cyclase in mouse epidermis. *Nature* **284**: 171–173, 1980.
7. Sibley DR, Nambi P, Peters JR and Lefkowtiz RJ, Phorbol diesters promote β-adrenergic receptor phosphorylation and adenylate cyclase desensitization in duck erythrocytes. *Biochem Biophys Res Commun* **121**: 973–979, 1984.
8. Kelleher DJ, Pessin JE, Ruoho AE and Johnson GL, Phorbol ester induces desensitization of adenylate cyclase and phosphorylation of the β-adrenergic receptor in turkey erythrocytes. *Proc Natl Acad Sci USA* **81**: 4316–4310, 1984.
9. Kassis S, Zaremba T, Patel J and Fishman PH, Phorbol esters and β-adrenergic agonists mediate desensitization of adenylate cyclase in rat glioma C6 cells by distinct mechanisms. *J Biol Chem* **260**: 8911–8917, 1985.
10. Meurs H, Kauffman HF, Timmermans A, van Amsterdam FTM, Koëter GH and de Vries K, Phorbol 12-myristate 13-acetate induces beta-adrenergic receptor uncoupling and non-specific desensitization of adenylate cyclase in human mononuclear leukocytes. *Biochem Pharmacol* **35**: 4217–4222, 1986.
11. Nishizuka Y, Perspective on the role of protein kinase C in stimulus–response coupling. *J Natl Cancer Inst* **76**: 363–370, 1986.
12. Donnely TE, Sittlen R and Scholar EM, Relationship between membrane bound protein kinase C activity and calcium-dependent proliferation of Balb/C3TC cells. *Biochem Biophys Res Commun* **126**: 741–747, 1985.
13. Nel AE, Wooten MW, Landreth GE, Goldschmidt-Clermont PJ, Stevenson HE, Miller PJ and Galbraith RM, Translocation of phospholipid/Ca^{2+}-dependent protein kinase in B-lymphocytes activated by phorbol ester or cross-linking of membrane immunoglobulin. *Biochem J* **233**: 145–149, 1986.
14. Kitajima Y, Inoue S, Nagao S, Nagata K, Yaoita H and Nozawa Y, Biphasic effects of 12-*O*-tetradecanoylphorbol-13-acetate on the cell morphology of low calcium-grown human epidermal carcinoma cells: Involvement of translocation and down regulation of protein kinase C. *Cancer Res* **48**: 964–970, 1988.
15. Thomas TP, Talwar HS and Anderson WG, Phorbol ester-mediated association of protein kinase C to the nuclear fraction in NIH 3T3 cells. *Cancer Res* **48**: 1910–1919, 1988.
16. Rodriguez-Pena A and Rozengurt E, Disappearance of Ca^{2+}-sensitive phospholipid-dependent protein kinase activity in phorbol ester treated 3T3 cells. *Biochem Biophys Res Commun* **120**: 1053–1059, 1984.
17. Hovis JG, Stumpo DJ, Halsey DL and Blackshear PJ, Effect of mitogens on ornithine decarboxylase activity and messenger RNA level in normal and protein kinase C-deficient NIH-3T3 fibroblasts. *J Biol Chem* **261**: 10380–10386, 1986.
18. Prasad KN and Edwards-Prasad J, Effect of tocopherol (vitamin E) acid succinate on morphological alterations and growth inhibition in melanoma cells in culture. *Cancer Res* **42**: 550–554, 1982.
19. Eagle H, Some effects of environmental pH on cellular metabolism and function. In: *Control of Proliferation in Animal Cells* (Eds. Clarkson B and Baserga R), pp. 1–12. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1974.
20. Lowry OH, Rosebrough NJ, Farr AL and Randall RJ, Protein measurement with the Folin phenol reagent. *J Biol Chem* **193**: 265–275, 1951.
21. Salomon Y, Londos C and Rodbell M, A highly sensitive adenylate cyclase assay. *Anal Biochem* **54**: 541–548, 1974.
22. Minneman KP, Hegstrand LR and Molinoff PB, The pharmacological specificity of *beta*-1 and *beta*-2 adrenergic receptors in rat heart and lung *in vitro*. *Mol Pharmacol* **16**: 21–23, 1979.
23. Sahu SN, Edwards-Prasad J and Prasad KN, Alpha tocopheryl succinate inhibits melanocyte-stimulating hormone (MSH)-sensitive adenylate cyclase activity in melanoma cells. *J Cell Physiol* **133**: 585–589, 1987.
24. Slaga TJ, Klein-Szanto AJP, Fischer SM, Weeks CE, Nelson K and Major S, Study on the mechanism of action of antitumor-promoting agents: Their specificity in two-stage promotion. *Proc Natl Acad Sci USA* **77**: 2251–2254, 1980.
25. Gilman AG, G-proteins: Transducers of receptor-generated signals. *Annu Rev Biochem* **56**: 615–649, 1987.

* Correspondence: Dr. Kedar N. Prasad, Center for Vitamins and Cancer Research, Department of Radiology, School of Medicine, University of Colorado Health Sciences Center, 4200 East Ninth Ave., Box A-031, Denver, CO 80262.

26. Rodbell M, The role of hormone receptors and GTP-regulatory protein in membrane transductions. *Nature* **284**: 17–22, 1980.

27. Seamon KB, Vaillancourt R, Edwards M and Daly JW, Binding of [^{3}H]forskolin to rat brain membranes. *Proc Natl Acad Sci USA* **81**: 5081–5085, 1984.

Biochemical Pharmacology, Vol. 40, No. 10, pp. 2380–2382, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

Substrate specificity of guinea pig liver flavin-containing monooxygenase for morphine, tropane and strychnos alkaloids

(*Received* 23 *April* 1990; *accepted* 30 *July* 1990)

Flavin-containing monooxygenase (FAD-monooxygenase) localized in many tissues of mammals catalyses oxidation of hetero atoms of a wide variety of sulfur and nitrogen containing xenobiotics [1, 2]. This enzyme has been purified from the liver of hogs [3], rats [4], mice [5, 6] and rabbits [6, 7] and the lung of mice [6] and rabbits [6, 8]. A current interest of many workers in the field of FAD-monooxygenase enzymology is the multiplicity of this enzyme. Recently, we have purified and characterized two distinct forms of FAD-monooxygenase from guinea pig liver [9].

N-Oxidation is a common metabolic pathway of alkaloids. For example, morphine [10], oxycodone [11], atropine [12] and strychnine [13] are metabolized to the *N*-oxide in the animal bodies. FAD-monooxygenase is ar important candidate of the enzyme responsible for the *N*-oxidation of these alkaloids. Although substrate specificity of FAD-monooxygenase has been widely studied especially for sulfur-containing compounds, the information for FAD-monooxygenase-dependent oxygenation of alkaloids is very limited. In the present study, we determined catalytic parameters of a purified guinea pig liver FAD-monooxygenase for oxidation of morphine, tropane and strychnos alkaloids. These results showed interesting structural requirements for the substrate.

Materials and Methods

Chemicals. Nalorphine hydrochloride and naloxone hydrochloride were donated by the Ministry of Health and Welfare of Japan and the National Institute on Drug Abuse, U.S.A., respectively. Other materials used were of the highest quality commercially available. All drugs examined as the substrate were *l*-form.

Purification of guinea pig liver FAD-monooxygenase. FAD-monooxygenase was purified from guinea pig liver microsomes by the method described elsewhere [9]. This method gave two distinct forms (FMO-I and FMO-II), which are distinguishable from the molecular weights, peptide mappings, amino terminal sequences, immunochemical natures and substrate specificities [9]. FMO-II was shown to exist in larger amounts than did FMO-I in the guinea pig liver microsomes [9]. Because of the small quantity of FMO-I isolated, this isozyme was unavailable for enzyme assays in the present study.

Assays. FAD-monooxygenase activity was assayed by measuring absorbance decrease at 340 nm due to substrate-dependent NADPH oxidation. The reaction was carried out at 37° in a final volume of 1 mL of mixture containing a substrate, 0.125 mM NADPH, enzyme (FMO-II 8.9 μg

Table 1. Kinetic constants of guinea pig liver flavin-containing monooxygenase for morphine congeners

Substrate	K_m* (μM)	V_{max}* (nmol/min/mg protein)	V_{max}/K_m
Morphine	3390 ± 790	257 ± 33	0.06
Codeine	460 ± 54	386 ± 20	0.86
Ethylmorphine	331 ± 41	418 ± 19	1.34
Thebaine	234 ± 18	422 ± 10	1.78
Oxymorphone	UD	>154†	—
Oxycodone	7100 ± 3450	665 ± 263	0.11
Nalorphine	UD	>138†	—
Naloxone	UD	>164†	—
Thiourea	54 ± 1	365 ± 5	7.4

UD, unable to determine.

* The values represent optimal values ± SD which were calculated by a curve fitting program of Michaelis–Menten equation.

† These values were obtained at a substrate concentration of 10 mM.