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TUMOR-PROMOTING PHORBOL ESTERS INTERFERE WITH THE VASOACTIVE INTESTINAL PEPTIDE RECEPTOR/EFFECTOR SYSTEM IN RAT PROSTATIC EPITHELIAL CELLS

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Pretreatment of rat prostatic epithelial cells with the tumor-promoting phorbol ester 4ß-phorbol 12-myristate 13-acetate resulted in a decrease of both the potency of vasoactive intestinal peptide (VIP) upon the stimulation of cyclic AMP accumulation and the affinity of the receptors of this peptide. These effects were dose-dependent and could be reproduced by other stimulators of protein kinase C (PKC). Thus, it is conceivably that phosphorylation of VIP receptors by PKC regulates VIP receptor function in the prostate gland.

The binding of extracellular stimuli to the cell surface leads to the subsequent issuing of intracellular signals that mobilize response mechanisms. In addition to the extensively studied, second messenger-generating adenylate cyclase system, the receptor-mediated stimulation of the breakdown of membrane polyphosphoinositides appears to constitute another quite general pathway for regulation of cellular metabolism (1). This second pathway involves the generation of diacylglycerol and inositol triphosphate that seem to act as second messengers in the activation of calcium-dependent kinases and the phospholipid-dependent kinase (protein kinase C, PKC), respectively (2). There is increasing evidence supporting the interrelationship between the cyclic AMP messenger system and the phosphatidylinositide, diacylglycerol-Ca^{2+} messenger system (3). Current data suggest that the stimulation of PKC can regulate hormone action upon adenylate cyclase by either altering the interaction of the stimulatory guanine nucleotide regulatory protein G_s with the catalytic subunit C, or phosphorylating the α subunit of the inhibitory guanine nucleotide regulatory protein G_i or the receptors (4).

Tumor-promoting phorbol esters like 4ß-phorbol 12-myristate 13-acetate (PMA) belong to the most potent co-carcinogens currently known and mimic endogenously produced diacylglycerol in activating PKC (5). Conflicting

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Abbreviations: VIP, vasoactive intestinal peptide; PKC, protein kinase C; PMA, 4ß-phorbol 12-myristate 13-acetate; PDD, 4α-phorbol 12,13-didecanoate; PDB, 4ß-phorbol 12,13-dibutyrate; DOG, 1,2-dioleoyl glycerol.

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results have appeared in the study of hormone-dependent cyclic AMP production in cells treated with phorbol esters since both potentiation (4,6) and inhibition (4,7) have been observed. In the present investigation, phorbol esters were used as a probe to examine the presence of the PKC system and its relation with the cyclic AMP system in the prostate gland. Since the neuropeptide vasoactive intestinal peptide (VIP) is a well established stimulator of cyclic AMP in rat prostatic epithelial cells (8) acting through specific membrane receptors (9), we have studied the effect of PMA upon the VIP receptor/effector system in this cell preparation.

MATERIALS AND METHODS

Materials. Rat VIP (Peninsula) was radioiodinated to a specific activity of about 250 Ci/g (9). PMA, 4α-phorbol 12,13-didecanoate (PDD), 4ß-phorbol 12,13-didecanoate (PDB) and 1,2-dioleoyl glycerol (DOG) (Sigma) were prepared in ethanol; the final concentration of this vehicle was 1% and it had no effect in control experiments (not shown).

Animals and cell isolation. Epithelial cells were isolated from the ventral lobes of the prostate of male Wistar rats (250-300 g) by a slight modification (9) of a previously reported method (10). Cell protein concentration was determined (11) using bovine serum albumin as a standard.

Binding conditions. Unless otherwise indicated, the cells were treated with phorbol ester at 10^{-5} M in a saline medium for 15 min at 25ºC and washed. The binding assay of [^{125}I]VIP was conducted as in (9) by incubating the cells (1 mg protein/ml) with 45 pM tracer for 60 min at 15ºC in a total volume of 0.5 ml of a medium containing 35 mM Tris-HCl buffer (pH 7.5), 50 mM NaCl, 1.4% bovine serum albumin and 1 mg/ml bacitracin in the absence or presence of unlabelled VIP. Cell-bound radioactivity was measured after centrifugation, and results were expressed as specific binding, i.e. total minus nonspecific (about 30% of the total) binding as determined in the presence of 10^{-6} M VIP.

Cyclic AMP studies. Cell incubation was carried out as described in (8). After treatment with phorbol ester, the cells were incubated in a medium similar to that of binding experiments but supplemented with a phosphodiesterase inhibitor (0.2 mM 3-isobutyl-1-methylxantine). The reaction was stopped after 60 min at 15ºC by adding 2.5 ml methanol. After centrifugation the supernatant was evaporated for determination of cyclic AMP (12).

Statistical analysis. The data are expressed as the mean ± S.E.M. of the indicated number of triplicate determinations. Statistical significance was analyzed by Student's t test. For Scatchard analysis (13), the binding data were treated with the LIGAND computer program (14).

RESULTS AND DISCUSSION

As shown in Fig.1, pretreatment of rat prostatic epithelial cells with PMA, a tumour-promoting phorbol ester, resulted in a dose-dependent PMA-induced decrease of the stimulatory effect of 10^{-8} M VIP upon cyclic AMP accumulation after a subsequent 1 h incubation, without affecting basal values. A maximal response (20-25% inhibition) was elicited at 10^{-5} M PMA. The effect was observed throughout the whole time interval (up to 30 min) of preincubation studied (data not shown). A 15-min preincubation with 10^{-5} M PMA has also been chosen to study the effect of phorbol esters on neurotransmitter-stimulation of cyclic AMP in rat brain slices (15).

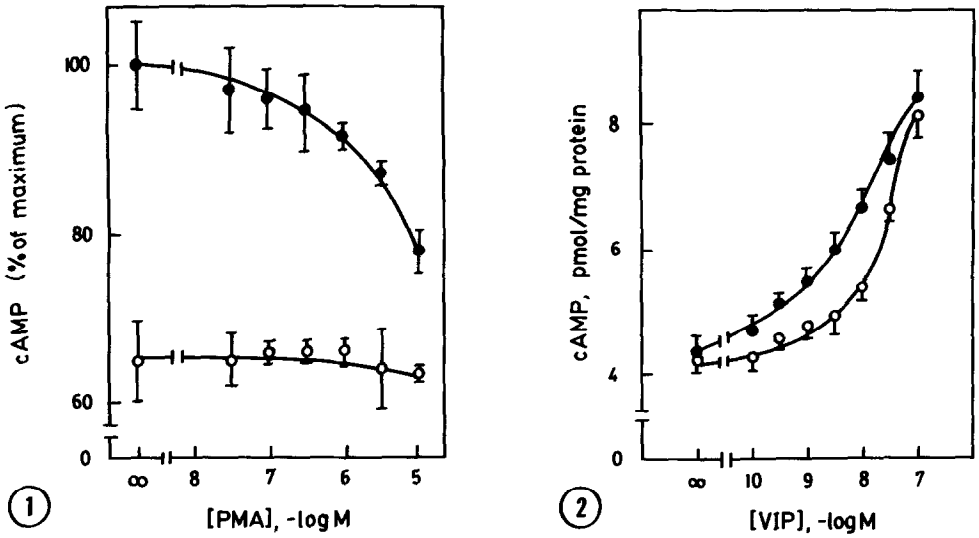

Figure 1. Basal (○) and VIP-stimulated (●) cyclic AMP levels in rat prostatic epithelial cells after cell pretreatment with increasing doses of PMA. Incubations were carried out as described in Methods. VIP concentration was 10^{-8} M. Results are the mean ± S.E.M. of four triplicate experiments.

Figure 2. Concentration-response curves of VIP-stimulated cyclic AMP accumulation in control (●) and PMA-treated (○) rat prostatic epithelial cells. Results are the mean ± S.E.M. of six triplicate experiments.

As shown in Fig. 2, PMA pretreatment of rat prostatic epithelial cells resulted in a decrease of the potency of VIP upon cyclic AMP accumulation since the EC_{50} values for VIP were 7.0 ± 1.5 and 30.0 ± 2.1 nM in control and PMA-treated cells, respectively ($P < 0.01$). However, the efficiency of VIP was not modified after cell exposure to PMA since similar maximal values of VIP-stimulated cyclic AMP levels were observed in both conditions. PMA also attenuated VIP-stimulated cyclic AMP production in the HT29 cell line which is derived from a human colon adenocarcinoma but the parameter affected was the efficiency and not the potency of the neuropeptide (7). On the other hand, the same phorbol ester enhanced VIP-induced cyclic AMP accumulation in rat vascular smooth muscle cells (6). Similar complex patterns of PMA modulation of cyclic AMP have been reported in various cell preparations for other hormones and neurotransmitters such as ß-adrenergic agonists (4,16), parathyroid hormone (16) or growth-hormone releasing factor (17). PMA-induced modification of cyclic AMP phosphodiesterase is unlikely to be involved in the present observations since the incubation medium included an inhibitor of this enzyme. Indeed, phorbol esters have been shown to inhibit cyclic AMP phosphodiesterase activity in intact cells (18).

As indicated in Table 1, another tumor-promoting phorbol ester such as PDB or the diacylglycerol DOG were similar to PMA in the inhibition of

Table 1. Effect of pretreatment of rat prostatic epithelial cells with various phorbol esters on the subsequent [^{125}I]VIP binding and VIP stimulation of cyclic AMP

Condition	^{125}I VIP binding (% of maximum)	cyclic AMP formation (% of maximum)
Control	100	100
PMA	78.7 ± 3.1*	76.2 ± 5.4*
PDB	82.0 ± 1.7*	81.7 ± 4.8*
PDD	95.0 ± 6.5	106.1 ± 12.8
DOG	74.7 ± 8.2*	76.2 ± 11.6*

Cell pretreatment was carried out for 15 min at 25ºC at 10^{-5} M phorbol ester. After washing, the binding of [^{125}I]VIP or the stimulation of cyclic AMP production by 10^{-8} M VIP were studied as described in Methods. Data are mean ± S.E.M. of six triplicate experiments.
*Statistically significant at $P < 0.05$ from control.

VIP-induced cyclic AMP production. However, a non-tumor-promoting phorbol ester such as PDD which does not activate PKC (5) had no significant effect. These results suggest that the action of PMA upon the cyclic AMP system involves PKC rather than nonspecific effects on membrane lipids.

The specific binding of [^{125}I]VIP to rat prostatic epithelial cells was also curtailed by pretreatment of the cells with PMA (Fig. 3). The effect was again dependent on the dose of phorbol ester so that a 20% inhibition could be observed at 10^{-5}M PMA. In agreement with cyclic AMP results, the inhibitory action of PMA upon [^{125}I]VIP binding was observed at each time point up to 30 min (data not shown).

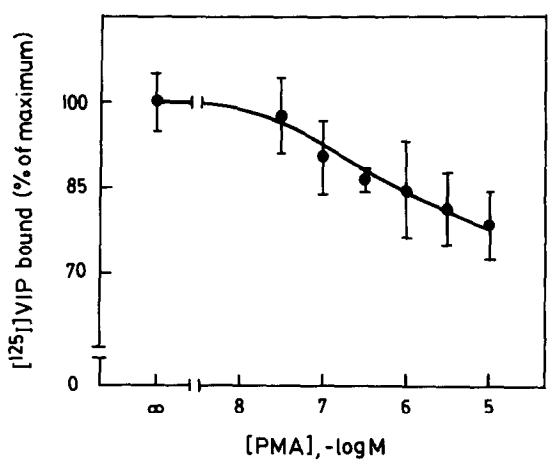

Figure 3. Effect of pretreatment of rat prostatic epithelial cells epithelial cells with increasing PMA concentrations on the subsequent binding of [^{125}I]VIP. Incubation conditions are described in Methods. Results are the mean ± S.E.M. of four triplicate experiments.

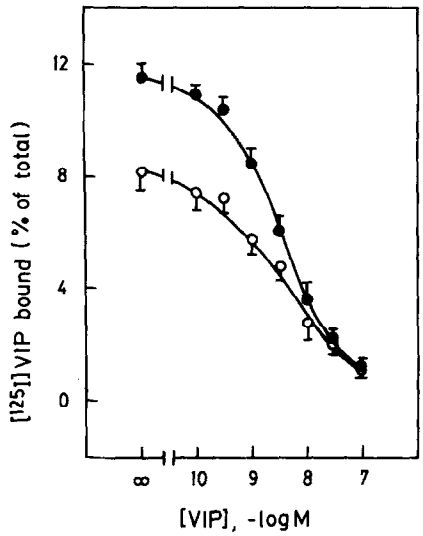

Figure 4. Competitive inhibition of [^{125}I]VIP binding by unlabeled VIP in control (●) and PMA-treated (○) rat prostatic epithelial cells. Results are the mean ± S.E.M. of six triplicate experiments.

When incubation of cells with [^{125}I]VIP were performed in the presence of varying concentrations of unlabeled VIP, the binding inhibition in PMA-treated cells could be only observed at low concentrations of the neuropeptide (Fig. 4). The analysis of the data by the method of Scatchard (13) resulted in concave upward curves (not shown) that were interpreted in terms of two classes of VIP receptors possessing different affinities, as previously discussed (9). The inhibitory action of PMA upon VIP binding appears to be due to changes in the affinity of VIP for its receptors since the binding capacities did not differ significantly whereas the K_d values were 1.95 ± 0.13 and 2.54 ± 0.18 nM ($P < 0.05$) for the high-, and 35.6 ± 6.7 and 55.9 ± 8.1 nM ($P < 0.05$) for the low-affinity sites in control and PMA-treated cells, respectively. This PMA-induced decrease of VIP binding affinity correlates well with the observed loss of the potency of the neuropeptide upon the stimulation of cyclic AMP. A previous report described a loss of VIP receptors in HT29 cells after PMA pretreatment (7) whereas diminished affinity values were observed after phorbol-ester treatment in binding studies with epidermal growth factor (19), insulin (20) or insulin-like growth factor (21). Specificity studies suggested that present observations on PMA inhibition of VIP binding resulted from a direct activation of PKC since PMA, PDB and DOG were again similarly active whereas PDD lacked of significant effects.

A number of membrane receptors appear to be phosphorylated upon cell treatment with tumor-promoting phorbol esters so that PKC involvement results in changes of receptor functionality or subcellular distribution

(5). Then, it could be thought that PMA-activated PKC in rat prostatic epithelial cells leads to phosphorylation of VIP receptors with a corresponding decrease of affinity that results in a diminished potency of the neuropeptide upon cyclic AMP production. Whether or not this is the actual mechanism responsible for the observed results deserves further studies but the present findings represent the first demonstration of the potential interaction between PKC and the adenylate cyclase system in the regulation of receptor-mediated responses in the prostate gland.

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REFERENCES

1. Hollenberg, M.D. (1986) Experientia 42, 718-727.
2. Rasmussen, H., Apfeldorf, W., Barret, P., Takuwa, N., Zawalich, W., Kreutter, D., Park, S., and Takuwa, Y. (1986) In Phosphoinositides and Receptor Mechanisms (J.W. Putney, Ed.), pp. 109-147. A.R. Liss, New York.
3. Enna, S.J., and Karbon, E.W. (1987) Trends Pharmacol. Sci. 8, 21-24.
4. Johnson, J.A., Goka, T.J., and Clark, R.B. (1986) J. Cyclic Nucleot. Phosphor. Res. 11, 199-215.
5. Thomopoulos, P. (1985) Ann. Endocrinol. 46, 307-312.
6. Nabika, T., Nara, Y., Yamori, Y., Lovenberg, W., and Endo, J. (1985) Biochem. Biophys. Res. Commun. 131, 30-36.
7. Bozou, J.C., Couvineau, A., Rouyer-Fessard, C., Laburthe, M., Vincent, J.P., and Kitabgi, P. (1987) FEBS Lett. 211, 151-154.
8. Carmena, M.J., and Prieto, J.C. (1983) Biochim. Biophys. Acta 763, 414-418.
9. Prieto, J.C., and Carmena, M.J. (1983) Biochim. Biophys. Acta 763, 408-413.
10. Jung-Testas, I., Groyer, M.T., Bruner-Lorand, J., Hechter, O., Baulieu, E.E., and Robel, F. (1981) Endocrinology 109, 1287-1289.
11. Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. (1951) J. Biol. Chem. 193, 265-275.
12. Gilman, A.C. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 305-312.
13. Scatchard, G. (1949) Ann. N.Y. Acad. Sci. 51, 660-672.
14. Munsen, P., and Rodbard, D. (1980) Anal. Biochem. 107, 220-239.
15. Karbon, E.W., Shenolikar, S., and Enna, S.J. (1986) J. Neurochem. 47, 1566-1575.
16. Pines, M., Santora, A., and Spiegel, A. (1986) Biochem. Pharmacol. 35, 3639-3641.
17. Cronin, M.J., and Canonico, P.L. (1985) Biochem. Biophys. Res. Commun. 129, 404-410.
18. Irvine, F., Pyne, N.J., and Houslay, M.D. (1986) FEBS Lett. 208, 455-459.
19. Lee, L.S., and Weinstein, I.B. (1978) Science 202, 313-315.
20. Grunberger, G., Iacopetta, B., Carpentier, J.L., and Gorden, P. (1986) Diabetes 35, 1364-1370.
21. Rouis, M., Thomopoulos, P., Postel-Vinay, M.C., Testa, U., Guyda, H.J., and Posner, B.I. (1984) Mol. Physiol. 5, 123-130.