EFFECTS OF PHORBOL-12,13-DIESTERS ON PROSTAGLANDIN PRODUCTION AND PHOSPHOLIPASE ACTIVITY IN CANINE KIDNEY (MDCK) CELLS

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SUMMARY

Two tumor promoting phorbol-12,13-diesters, 12-O-tetradecanoyl-phorbol-13-acetate and phorbol-12,13-didecanoate, at concentrations of 10^{-9} to 10^{-10} M, stimulated prostaglandin production by dog kidney (MDCK) cells cultured in serum-supplemented medium. The non-tumor producing phorbol diester, 4α-phorbol-12,13-didecanoate, at a concentration of 10^{-7} M, had no effect. The two biologically active phorbol diesters, but not the non-tumor promoting analog, stimulated deacylation of the cellular phospholipids of MDCK cells radioactively labelled with [^{3}H]arachidonic acid. Most of the arachidonic acid was converted into prostaglandins.

INTRODUCTION

Among the substances that stimulate cells to produce prostaglandins are the mitogens thrombin (1), serum (2) and epidermal growth factor (3). The increased prostaglandin production results from stimulated deacylation of cellular phospholipids. We now report that phorbol diesters which are mitogens (4-6) and tumor promoters (7), but not the phorbol diester that is inactive biologically (7), stimulate dog kidney cells (MDCK) to deacylate cellular phospholipids and to produce prostaglandins. Recently, it has been shown that this dog kidney cell line is stimulated to produce prostaglandins and to deacylate phospholipids by metabolically activated polycyclic hydrocarbon carcinogens and aflatoxin B_1 (8,9).

MATERIALS AND METHODS

The dog kidney (MDCK) cells were grown in Eagle's minimal essential medium containing 2 mM l-glutamate and supplemented with 10% (v/v) fetal bovine serum and penicillin-streptomycin. Cells were plated at 2 x 10^5 cells per dish and incubated for 24 hours in serum-supplemented medium with

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and without increments of the phorbol diesters. After the medium was removed, and the cells were washed twice, 2 ml of serum-supplemented medium lacking the phorbol esters were added and the cells were incubated for another 24 hours. Medium from the second 24 hour incubation was assayed serologically for $PGF_{2\alpha}$ and PGE_2 with anti-$PGF_{2\alpha}$ and anti-PGE_2 (anti-$PGF_{2\alpha}$ reacts 0.1% with PGE_2 and anti-PGE_2 reacts 0.01% with $PGF_{2\alpha}$; but the antisera do not identify the prostaglandins as either monoenoic or dienoic). Duplicate dishes were used for each reagent and each experiment was repeated several times. The values obtained for prostaglandins per cell were similar for each reagent in all experiments. The MDCK cells were labelled with [^{3}H]arachidonate in their cellular lipids as previously described (9,10). The cells were extracted with 2:1 chloroform:methanol and the extract chromatographed on thin-layer silica gel G (E. Merck, Darmstadt, Germany). Analysis of the radioactive materials in the cells after extraction with 2:1 chloroform:-methanol showed that 69% of the radioactivity was associated with phospholipids, 15.0% with triglycerides, 11% with unidentified materials, 3.4% with prostaglandins, and 1.4% with free arachidonic acid. The solvent system, described by Marinetti (11), 2,6-dimethyl-heptanone:acetic acid:-saline (80:40:6), was used to develop the chromatograms. The radioactive compounds released by the cells into the medium were extracted according to Thomas *et al* (12). [^{3}H]Arachidonic acid, [^{3}H]$PGF_{2\alpha}$, and [^{3}H]PGE_2 were purchased fron New England Nuclear (Boston, MA.).

The phorbol diesters, 12-O-tetradecanoyl-phorbol-13-acetate (TPA), phorbol-12,13-didecanoate (PDD), and 4α-phorbol-12,13-didecanoate (4α-PDD) were purchased from Consolidated Midland Corporation, Brewster, New York.

RESULTS

When MDCK cells were incubated in the presence of TPA or PDD (0.001 μg/ml) the content of $PGF_{2\alpha}$ and PGE_2 in the medium increased. TPA and/or PDD were not required throughout the incubation. If cells were incubated at 2 x 10^5 per dish for 24 hours with TPA or PDD, washed twice and incubated in medium lacking TPA or PDD for a further 24 hours, the cells continued to produce increased levels of prostaglandins. Table I shows that the cells incubated with the tumor promoting phorbol diesters, TPA and PDD for 24 hours, produced increased levels of prostaglandins during the second 24 hour incubation in medium lacking the phorbol analogs, while cells incubated with the phorbol diester that lacks tumor promoting activity, 4α-PDD, even at a 1000-fold higher concentration did not. The MDCK cells were not killed by treatment with 1.0 ng/ml of TPA or PDD as 98% of the cells after the second 24 hour incubation excluded trypan blue. However, the number of cells as measured by direct count was less with increments of the promoters. When tested under identical experimental conditions i.e., incubation of 2 x 10^5

TABLE I

EFFECT OF PHORBOL-12,13-DIESTERS ON PROSTAGLANDIN PRODUCTION BY MDCK CELLS[a]

EFFECTOR	ng/ml	PGE_2 fg/cell	$PGF_{2\alpha}$
No addition	---	3.2	10.5
TPA	1.0*	115	131
	0.4	35.6	58.8
	0.1	9.8	18.5
	0.08	6.2	12.4
	0.02	4.1	9.0
	0.01	3.3	11.0
PDD	1.0*	30.1	45.4
	0.4	13.6	21.1
	0.1	7.7	20.1
	0.08	3.8	9.6
	0.01	3.6	18.8
	0.01	5.2	11.0
4α-PDD	100	4.5	9.7
	10	4.2	12.2

[a] MDCK cells, 2 x 10^5 per 60 mm dish, were incubated with increments of the phorbol-12,13-diesters in serum-supplemented medium for 24 hours, washed twice, and incubated for a second 24 hours in serum-supplemented medium lacking the effectors. The medium from the second 24 hour incubation was assayed serologically. At the end of the second 24 hour incubation, the MDCK cells were removed from the dish after treatment with trypsin, counted and tested for viability.

* Viability, as judged by trypan blue exclusion, was 98%.

cells per 60 mm dish for 24 hours in serum-supplemented medium containing TPA and PDD at 10 ng/ml followed by two washes and a second 24 hour incubation in serum-supplemented medium lacking the phorbol diesters, prostaglandin production by methylcholanthrene transformed fibroblasts (1,2) was also elevated, but only 1½ to 2-fold.

Stimulation of prostaglandin production by cells can be demonstrated not only by serologic analyses of the conditioned medium but also by release of radioactive compounds from the cells labelled with [^{3}H]arachidonic acid (9,10). With the use of the latter procedure, it was shown that cellular phospholipase activity plays a major role in prostaglandin biosynthesis.

TABLE II

RADIOACTIVE COMPOUNDS IN CONDITIONED MEDIA OF RADIOACTIVELY LABELLED MDCK CELLS AND PHORBOL DIESTERS

	Radioactivity (cpm)[a]			
COMPOUND	MEM (+)[b]	MEM (+) with TPA (0.25 ng/ml)	MEM (+) with PDD (1.0 ng/ml)	MEM (+) with 4α-PDD (10 ng/ml)
Phospholipids	281	718	457	144
Prostaglandin $F_{2\alpha}$	568	7088	5224	528
Prostaglandin E_2	300	5507	3996	302
Arachidonic Acid	3429	4725	6387	3922
Triglycerides	122	214	241	33
Unidentified*	1508	8170	5781	1018
TOTAL	6208**	26422**	22086**	5947**

[a] 1.6 ml radioactive medium (out of a total of 2 ml) was extracted and chromatographed as described by Hassid and Levine (9). Incubation was for 20 hours.

[b] MEM (+) represents serum-supplemented medium.

* Scatter throughout TLC.

** Cell number initially was 0.64 x 10^6/60 mm dish but cells were not counted after the 20 hour incubation.

When the radioactively labelled cells were cultured for 14 hours in serum-supplemented medium in the presence of increments of TPA, increased quantities of radioactivity were found in the conditioned medium, even at a concentration of 125 pg/ml (2×10^{-10} M). Most of the identified radioactive materials released from MDCK cells growing in serum-supplemented medium alone corresponded to arachidonic acid, $PGF_{2\alpha}$ and PGE_2 (the ratio of arachidonate to $PGF_{2\alpha}$ plus PGE_2 was 4:1). After 20 hours of incubation in the presence of TPA (250 pg/ml) or PDD (1.0 ng/ml), the radioactivity released was increased around 4-fold, whereas the radioactivity released by cells incubated in the presence of 4α-PDD (10 ng/ml) was equal to that of the control serum-supplemented medium (Table II). The released radioactivity in the conditioned medium as a result of incubation with TPA and PDD was due mainly to increased prostaglandins E_2 and $F_{2\alpha}$ (14.5-fold with TPA and 10.6-fold with PDD); the arachidonic acid that had been made available by increased deacylation of the cellular phospholipids was efficiently converted to the prostaglandins by the prostaglandin synthetase system.

DISCUSSION

The following experimental observations led us to test for the effects of the tumor promoting phorbol diesters on MDCK cells: 1) epidermal growth factor at 10^{-9} and 10^{-10} M concentrations stimulates prostaglandin production and phospholipase activity in MDCK cells (3); 2) epidermal growth factor is cocarcinogenic for epidermal carcinogenesis (13); 3) MDCK cells are stimulated to produce prostaglandins by carcinogenic aromatic hydrocarbons and the potent carcinogen aflatoxin B_1 (8) and also have increased phospholipase activity after incubation with benzo(a)pyrene (9); 4) some carcinogens can act as both carcinogens (initiators) and promoters (14); 5) epidermal growth factor retards terminal differentiation of cultured human epidermal keratinocytes (15) and 6) the tumor promoting phorbol diesters inhibit differentiation in Friend erythroleukemia cells (16,17) and myogenesis in chick

embryo muscle cells (18). That TPA and PDD, but not 4α-PDD, were so effective at stimulating prostaglandin production by MDCK cells (2×10^{-10} M TPA stimulated prostaglandin production) was not unexpected.

The sum of the radioactive prostaglandins and arachidonate released by cells cultured in serum-supplemented medium exceeds the sum of the cellular prostaglandins and unesterified arachidonate (9,10). The stimulated release of radioactive arachidonic acid and prostaglandins by TPA and PDD resulted from increased deacylation of the phospholipids and was characterized by the high efficiency of conversion of the arachidonic acid into the prostaglandins. Most likely, TPA and PDD perturb the plasma membrane in such a way that the phospholipase and prostaglandin cyclooxygenase molecular complexes are brought closer together. Whether this coupled phospholipase-cyclooxygenase reaction is occurring in the cell membrane, where the TPA has been reported to act (4,19-21), or in the endoplasmic reticulum is not known. Alternatively, since TPA also stimulates increased phospholipid turnover, as measured by incorporation of choline (22) and [^{32}P] (23) into phospholipids, the efficient conversion of the released arachidonic acid into prostaglandins may reflect increased reacylation of the released arachidonic acid.

The findings that TPA stimulated deacylation of phospholipids and release of arachidonic acid suggests an alternative explanation for the potent aggregation of platelets by TPA (24).

If the stimulation of MDCK's prostaglandin synthesis can be attributed to that caused by promoters and initiators, this cell line and this system will be useful to screen for promoting and initiating compounds. In addition, MDCK cells may prove to be useful in establishing the relationships, if any, between phospholipase activity and/or arachidonic acid metabolism and differentiation.

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