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INFLAMMATORY, TUMOR INITIATING AND PROMOTING ACTIVITIES OF POLYCYCLIC AROMATIC HYDROCARBONS AND DITERPENE ESTERS IN MOUSE SKIN AS COMPARED WITH THEIR PROSTAGLANDIN RELEASING POTENCY IN VITRO

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SUMMARY

Release of prostaglandin E_2 (PGE_2) in cultured peritoneal macrophages of NMRI mice by skin irritant tumor initiators and promoters was investigated. Initiators of the polycyclic aromatic hydrocarbon type, e.g., DMBA, caused slight irritation on the mouse ear but even relatively high doses did not stimulate PGE_2-release to any measurable extent within 4 h after administration in vitro. Apparently there is no correlation between irritation and initiating activity in mouse skin and PGE_2-release in macrophages. On the other hand, promoters of the diterpene ester type, e.g., TPA, were strong irritants on the mouse ear. Even low doses of these compounds stimulated PGE_2-release from macrophages dramatically within 1 h after administration in vitro. Moreover, a good correlation was established between irritant and promoting activity in mouse skin and PGE_2-release in macrophages of a series of tigliane, ingenane and daphnane type diterpene derivatives. These results suggest that also in mouse skin PGE_2-release may occur following exposure of the target cells to promoters of the diterpene ester type resembling one of the most early molecular events of promotion. This event could initiate both skin irritation and cell proliferation.

Abbreviations: BP, benzo[*a*]pyrene; DMBA, 7,12-dimethyl-benz[*a*]anthracene; FCS, fetal calf serum; MC, 20-methylcholanthrene; MDCK, MC-transformed canine kidney; 4-*O*-MeTPA, 4-*O*-methyl-12-*O*-tetradecanoylphorbol-13-acetate; 4α-PDD, 4α-phorbol-12,13-didecanoate; PDD, phorbol-12,13-didecanoate; PGE_2, prostaglandin E_2; TPA, 12-*O*-tetradecanoylphorbol-13-acetate.

INTRODUCTION

Prostaglandins are assumed to be involved in both the development of inflammatory symptoms [5] and the growth of tumors [11]. On the other hand, tumor initiators, i.e., polycyclic aromatic hydrocarbons, and initiation promoters, i.e., the diterpene esters which are effective in the 2-stage model system of mouse skin carcinogenesis [7], are known also to cause skin irritation when applied locally. In particular, the active principles of the diterpene ester type derived from tigliane, ingenane or daphnane, which occur in plant species of the *Euphorbiaceae* and *Thymelaeaceae* families, are strong irritants and at the same time the most potent promoters known in tissues of experimental animals (for terminology and recent review see [7]). Evidence recently presented shows that temporary exposure to initiators followed by exposure to certain promoters derived from tigliane might be responsible for the high rate of esophageal cancer in Curacao [10].

Promoters of the diterpene ester type may exert their effects at the molecular level via receptor sites on or within cell membranes [7]. It therefore appeared useful to compare the irritant activities of tumor initiators and of initiation promoters, together with their initiating or promoting activities in vivo, with their putative abilities to release prostaglandins from an appropriate cellular system in vitro. Unstimulated macrophages of the peritoneal cavity of mice of the same strain used to assay irritating, initiating and promoting activities were chosen to measure PGE_2-release.

MATERIALS AND METHODS

The aromatic hydrocarbons (see Table 1) were obtained from commercial sources and were purified by recrystallisation. The diterpene derivatives (obtained by isolation or partial synthesis, see Table 3) were purified by thin-layer chromatography prior to use. Local irritant (ear) and initiating or promoting activities (back skin) were determined by standard methods [9] on NMRI mice (Zentralinstitut fur Versuchstiere, Hannover/G.F.R.).

Unstimulated macrophages were obtained by washing the peritoneal cavity of NMRI mice (Tierzuchtanstalt Tuttlingen/G.F.R.) with 2.5 ml Ringer's solution containing 10 U heparin/ml. The cells were washed once and then resuspended in an adequate volume of Eagle's medium as modified by Dulbecco and supplemented with 10% fetal calf serum (FCS) to give a concentration of 1×10^6 cells/ml. Two ml of this suspension were seeded in 35-mm Petri dishes and cultivated overnight at 37° C. Sixteen hours later non-adherent cells were washed off and fresh medium containing 5% FCS was added. This medium also contained either the compound under investigation dissolved in DMSO (final concentration, 0.5%) or the solvent as control. The culture fluids were separated 4 h later and assayed for their PGE_2 content using a direct radioimmunoassay as previously described [6]. Samples from all the incubation media which had not been in contact with macrophages were also assayed to exclude non-specific

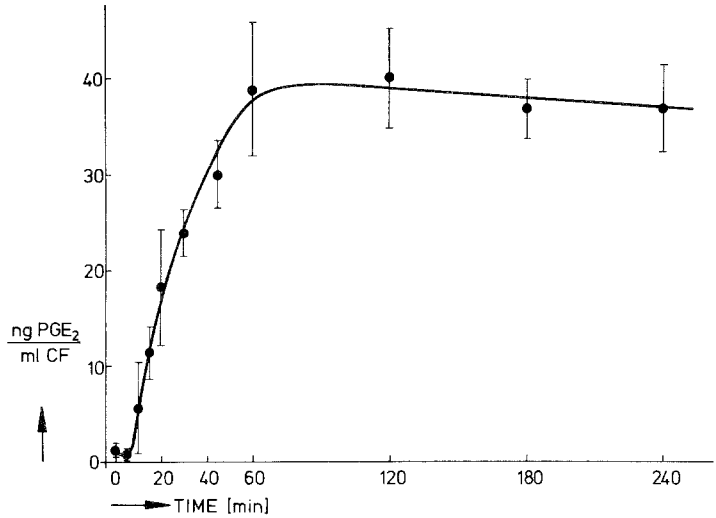

Fig. 1. Time course of PGE_2-release from macrophages in response to addition of TPA. TPA was added at 0-time, final concentration in the dishes 10^{-8} mol/l. Samples from 5 culture fluids (CF) were obtained as described in Materials and Methods. The data given represent means (5 cultures each) and standard deviations. In a control group the PGE_2-concentration did not rise above 10 ng/ml during the whole observation time.

interactions with the radioimmunoassay and/or the possibility that PGE_2 had been added with these solutions in measurable amounts. The time course of PGE_2-release by TPA was measured differently. Fine polyethylene tubes were inserted into 5 culture dishes. These enabled 50 μl samples to be withdrawn from the incubations at the time intervals shown in Fig. 1. The results obtained were analyzed using the Student's t-test.

Samples of the cell layers remaining from the cultures assayed for PGE_2 content as described above were incubated with a 0.2% (w/v) solution of methylene blue in Ringer's solution for 5 min and the viability of the cells checked microscopically. The percentage of dead (blue) macrophages was estimated by counting 100 cells/dish. Other cell layers were fixed with 2.5% formaldehyde in Ringer's solution, and photographs were taken with a phase contrast microscope.

RESULTS

The irritant potency of initiating and non-initiating aromatic hydrocarbons and the lowest concentration of these compounds causing a significant ($P < 0.01$) increase in PGE_2-release are recorded in Table 1. The non-initiator anthracene caused irritation only at very high doses as compared with promoters, e.g., TPA (see Table 3). The initiators DMBA and BP were about 10 times more active as irritants as was anthracene. However, none of these aromatic hydrocarbons released PGE_2 significantly at 10^{-6} mol/l. Hence, at this dose

TABLE 1

SKIN IRRITANT AND INITIATING ACTIVITY OF AROMATIC HYDROCARBONS IN VIVO AND THEIR CAPABILITY TO RELEASE PGE_2 IN VITRO

Compound	Irritation and initiation		Ref.	PGE_2-release activity (lowest concentration causing significant ($P < 0.01$) release) (mol/l)
	ID_{50}^{24} (mmol/ear)[a]	Initiating activity (relative potency)[c]		
Anthracene	6.6×10^{-4}	0	d	—[e]
DMBA	2.5×10^{-5}	++++	(7)	—[e]
BP	5.6×10^{-5}	++	d	—[e]
MC	n.t.[b]	++	d	—[e]

[a] Irritant dose 50 (ID_{50}) on the mouse ear read 24 h after administration.
[b] n.t., not tested.
[c] Measured in the initiation/promotion standard experiment with TPA as promoter
[d] Schmidt and Hecker, unpublished.
[e] Not significant at 10^{-6}; 4 h incubation.

TABLE 2

EFFECT OF DIFFERENT CONCENTRATIONS OF TPA AND PDD ON PGE_2-RELEASE FROM MACROPHAGES

Concn.	ng PGE_2/ml culture fluid recovered after 2 h (solvent only 4.80 ± 1.16[a])	
	TPA	PDD
10^{-6} M	>25.0	22.3 ± 0.9[b]
10^{-7} M	24.1 ± 3.0[b]	16.8 ± 2.2[b]
10^{-8} M	18.0 ± 1.2[b]	4.2 ± 0.5[c]
10^{-9} M	6.1 ± 1.8[c]	NT

[a] Mean ± S.D. of 5 cultures.
[b] $P < 0.01$.
[c] $P > 0.05$.

level, no correlation is apparent between their irritant and initiating activity in vivo and their PGE_2-releasing activity in vitro (Table 1).

In contrast, the well-established tigliane type irritants and promoters TPA and PDD released PGE_2 from mouse peritoneal macrophages within 4 h and at levels between 10^{-7} and 10^{-8} mol/l (Table 2). The effect was dose dependent

Fig. 2. Morphological changes of macrophages induced by 10^{-8} mol/l TPA (A) in comparison to a solvent control (B). TPA or the solvent were added 30 min before cells were fixed and photographs taken under phase contrast. TPA treated cells have flattened on the bottom of the Petri dish, showing many large vacuoles. Note that the number of cells and the magnification is the same for both pictures.

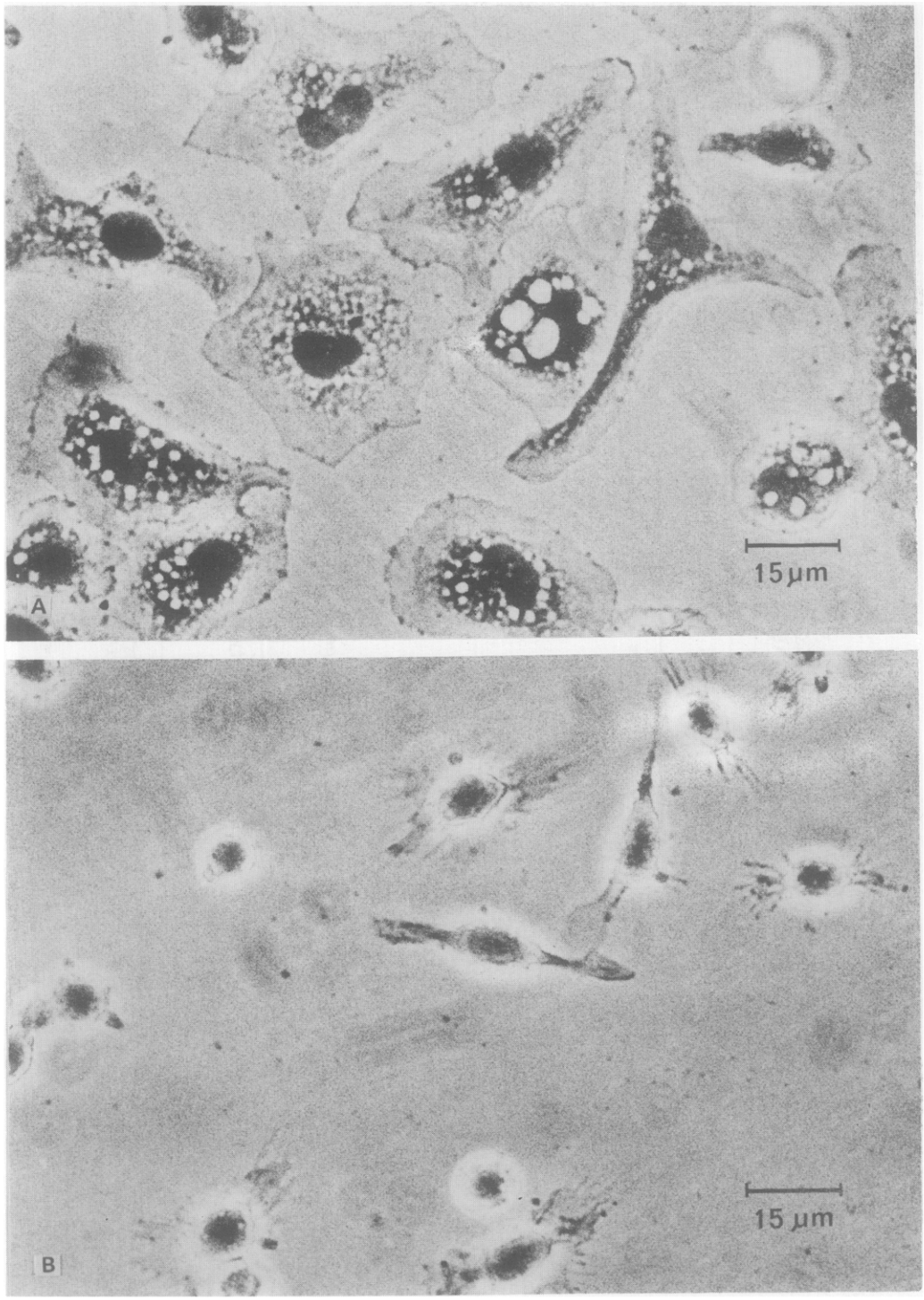
15 µm
A
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TABLE 3

SKIN IRRITATION AND PROMOTING EFFECTS OF TIGLIANE, INGENANE AND DAPHNANE TYPE DITERPENE DERIVATIVES IN VIVO AND THEIR CAPABILITY TO RELEASE PGE_2 IN VITRO

Compound	Irritation ID_{50}^{24} (mmol/ear)[a]	Promoting activity (relative potency)[b]	Ref.	PGE_2-releasing activity; lowest concentration causing significant ($P < 0.01$) release (mol/l)
	Tigliane type			
TPA	1.6×10^{-8}	++++	[7]	10^{-8}
TPA-6β,7β-oxide	1.6×10^{-7}	+++	[7]	10^{-7}
4-*O*-MeTPA	2.3×10^{-6}	(+)	[7]	10^{-6}
1,2-dihydroTPA-6β,7β-oxide	$>1.6 \times 10^{-4}$	0	[16]	—[d]
PDD	1.0×10^{-8}	+++	[7]	10^{-7}
4α-PDD	$>1.5 \times 10^{-4}$	0	[7]	—[d]
Phorbol-12,13-dibenzoate	2.4×10^{-7}	(+)	[14]	10^{-7}
Phorbol-12,13-diacetate	1.5×10^{-6}	(+)	[15]	10^{-6}
Phorbol	$>2.7 \times 10^{-4}$	0	[9]	—[d]
	Ingenane type			
Ingenol-3-hexadecanoate	1.7×10^{-7}	+++	[c]	10^{-6}
Ingenol	$>2.9 \times 10^{-4}$	0	[c]	—[d]
	Daphnane type			
Hippomane factors $M_1 + M_2$ (M_x)	2.0×10^{-8}	+++	[1]	10^{-8}
Pimelea factor P_2	3.0×10^{-9}	+++	[20]	10^{-7}

[a] Irritant dose 50 (ID_{50}) on the mouse ear read 24 h after administration.
[b] Measured in the initiation/promotion standard experiment with DMBA as initiator.
[c] Opferkuch and Hecker, unpublished results.
[d] Not significant at 10^{-6} mol/l.

and was inhibited by the well-known inhibitor of prostaglandin synthesis indomethacin (10^{-7} mol/l, data not shown). This release did not reflect cellular death as measured by dye exclusion; no significant increase in dead cells could be observed even at the highest concentrations (10^{-6} mol/l) of the compounds tested.

The time course of PGE_2-release by TPA is given in Fig. 1. An increase of PGE_2-release was observed after a short lag-period of 5—10 min. This process continued for approx. 1 h, after which the PGE_2 concentration remained steady for at least 3 h. The release process coincided with morphological changes of the macrophages as exemplified by a 30-min treatment in Fig. 2. Five minutes after addition of TPA, the macrophages spread on the culture dish and started to develop vacuoles which became visible under phase contrast after 5—10 min. These vacuoles appeared to develop from segments of the endoplasmic reticulum which fuse with other cell organelles as shown by transmission electron microscopic pictures (Kälin and Brune, in preparation). The vacuoles increased in size and number for 1—2 h, but after 3—4 h they began to disappear. Similar findings with regard to rapid vacuolisation by TPA in cell culture systems have been reported with polymorphonuclear leukocytes [19] and platelets [4].

In further experiments various irritant and promoting or non-irritant and non-promoting derivatives of tigliane, ingenane and daphnane type diterpenes, structurally related to TPA [7] were tested, and the lowest concentrations causing a significant ($P < 0.01$) increase in PGE_2-release in vitro was determined. As can be seen in Table 3, the biological activities of the diterpene derivatives are rigidly dependent on particular chemical structures: for example, TPA is highly active as an irritant on the mouse ear and as an initiation promoter on the back skin of mice, whereas, in the same tissue, its diterpene parent alcohol phorbol does not exhibit any of these activities [7]. A similar relation also holds true for ingenol-3-hexadecanoate and its diterpene parent alcohol ingenol. In the daphnane series both diterpene esters assayed exhibited extremely low ID_{50}^{24}s as compared with TPA. In addition, they were potent promoters in vivo and release PGE_2 very effectively in vitro. Their diterpene parent alcohol resiniferonol [7] is not yet available for testing. Altogether, the response elicited in these assays reveals a good correlation between irritation and promotion and the PGE_2-releasing activities (Table 3).

DISCUSSION

Stimulation of PG-release from macrophages without phagocytosis, as demonstrated in this study is a comparatively rare event. Thus, for example, adrenalin, bradykinine, carbachol, chloroquine, histamine, glucocorticoids and many other substances are inactive in this respect even at concentrations of 10^{-5} mol/l (Brune et al., unpublished data).

Tumor initiation has been shown to be a relatively rapid process, taking place in mouse skin in vivo most likely within the first 24 h after, e.g., intra-

gastric administration of DMBA [8]. Initiators of the solitary aromatic hydrocarbon type were suspected to possess initiation promoting capabilities ([3] and references cited therein) and were irritant to mouse skin. Yet, in macrophages even relatively high concentrations of the hydrocarbons did not stimulate PGE_2-release up to 4 h after administration. A stimulation of PGE_2-release by solitary carcinogens, including aromatic hydrocarbons, was reported in MC-transformed canine kidney cells (MDCK) by Levine [12]. However, the exposure time used was 24 h, which probably gave enough time for metabolic activation of the assayed hydrocarbons to occur. Also, transformed cells are likely to show different sensitivities toward chemicals as compared with the normal macrophages used in our system.

In contrast to the initiators, promoters of the diterpene ester type switched on PGE_2-release in macrophages very rapidly and at low doses. A good correlation was found between irritant and promoting activities in vivo and PGE_2-release in vitro with these compounds. Similarly, Levine and Hassid [13] reported that TPA and PDD, but not 4α-PDD, are potent stimulators of PGE_2-release in their system of MDCK. The differences in the cell type used, particularly in the experimental conditions (e.g., the lower cell concentration employed in their system which resulted in a much larger dose/cell ratio) may explain why these authors observed a significant stimulation of PGE_2-release by TPA and PDD at concentrations even lower than those used in our system.

For TPA (and PDD) conclusive evidence exists that these molecules do not require metabolic activation to exert irritant and promoting activity in mouse epidermis in vivo [7]. Structure-activity relationships of irritants and promoters of the diterpene ester type [7] in mouse skin and biochemical evidence obtained with TPA [e.g., 2,17] strongly indicate that the cell membrane is a primary subcellular target for these compounds. Therefore it may be assumed that rapid and specific release of PGE_2 by the diterpene esters, as demonstrated here in macrophages, may also take place in the epidermal cells of the target tissue. If so, prostaglandin release would be one of the earliest molecular events known to follow exposure of the target cells to irritants and promoters of this type. This may be taken as a strong indication that one or more crucial receptor(s) are located in the cell membrane. One may speculate, for example, that some diterpenes are able to activate the phospholipase-cycloxygenase system of macrophages and epidermal cells in vitro and in vivo, and thus cause prostaglandin release along with the development of inflammatory symptoms in vivo [5]. PG-release in addition may be involved in the development of cell proliferation and tumors (for detailed discussion of the role of PGs in cancer see [11]. Alternatively, irritation and promoting activity might represent the end of a number of interactions of the diterpene esters with different receptors. One such interaction may initiate PG-release, while another may switch resting cells from the G_1- to the S-phase and mitosis, thus triggering hyperplasia. In this context a recent paper of Verma et al. [18] is noteworthy. These authors demonstrated that indomethacin and other inhibitors of PG-synthesis inhibit the induction of mouse epidermal ornithine decarboxylase caused by TPA.

This inhibition was completely counteracted by treatment with prostaglandins E_1 and E_2 but not with prostaglandins $F_{1\alpha}$ and $F_{2\alpha}$. Also prostaglandin release was suggested to be the start of a chain of molecular events in the induction by TPA of lymphoblastoid cells transformed by herpesviruses to produce Epstein—Barr virus, herpesvirus saimiri and herpesvirus papio [21] from the corresponding persistent viral genomes.

The observations reported here may offer a start for further elucidation of the basic differences in the molecular mechanisms of the processes of irritation, initiation and promotion, aiming at a better understanding of the biochemical mechanisms involved in chemical carcinogenesis. Moreover, our results suggest PGE_2-release from macrophages as an easy, sensitive and reliable screening method for substances that exhibit promoting activity and which may thus be classified as second order carcinogenic risk factors [7].

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