Proc. Natl. Acad. Sci. USA
Vol. 78, No. 12, pp. 7722–7726, December 1981
Medical Sciences

Skin tumor promotion by phorbol esters is a two-stage process

(carcinogenesis)

G. FÜRSTENBERGER, D. L. BERRY*, B. SORG, AND F. MARKS

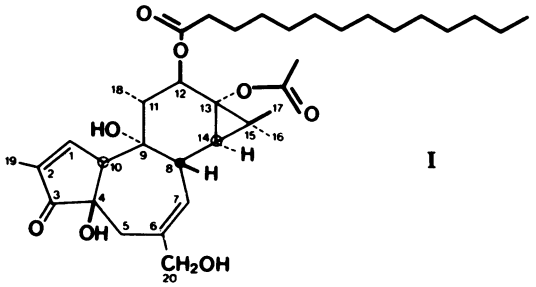
German Cancer Research Center, Institute of Biochemistry, D-6900 Heidelberg, Federal Republic of Germany

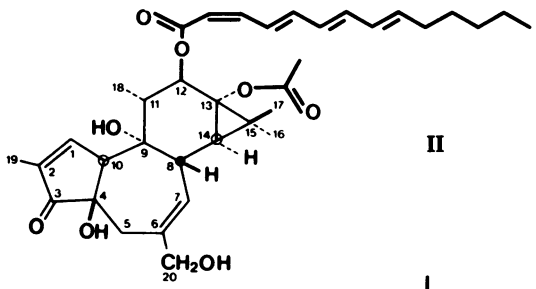
Communicated by Charles Heidelberger, July 20, 1981

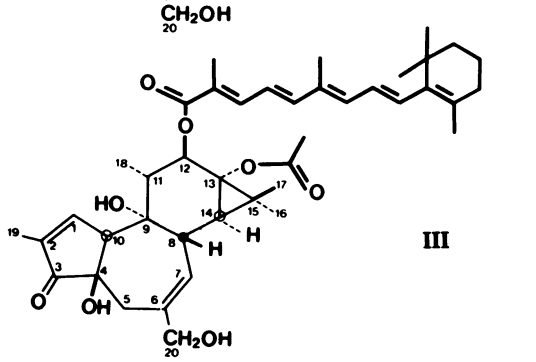
ABSTRACT **In the semisynthetic compound phorbol 12-retinoate 13-acetate (PRA), the antipromoting principle of vitamin A acid is combined with the structure of a phorbol ester tumor promoter. In skin of NMRI mice, a single topical application of PRA induces skin inflammation, epidermal proliferation, and sustained hyperplasia to a similar extent and apparently along the same pathway as an equimolar dose of the strong tumor promoter phorbol 12-myristate 13-acetate (PMA). The mitogenic effects of both PRA and PMA are mediated by prostaglandin E synthesis. However, in mouse skin initiated with 7,12-dimethylbenz[*a*]anthracene, PRA does not promote tumor development, even at a high dose. Under continuous PRA treatment, however, one to four applications of PMA (insufficient by itself to promote tumor growth) gave a strong tumor response. Thus, it can be demonstrated that the effects necessary for tumor promotion can be brought about by a single application of PMA and that the subsequent chronic hyperproliferation of epidermis is probably necessary only to make the tumors visible. By using the nonpromoting irritant mitogen PRA, the concept of two-stage tumor promotion can thus be strongly supported. Furthermore, in the NMRI mouse, PRA is a much more potent second-stage promoter than mezerein, recently reported to be an incomplete promoter in the Sencar mouse.**

In mouse skin, tumor promotion can be brought about by a single application of a subthreshold dose of a carcinogen followed by repetitive applications of a noncarcinogenic promoter. One of the most potent skin tumor promoters is phorbol 12-myristate 13-acetate (PMA, **I**). Although the promoting effect of PMA seems to be inseparably linked to inflammatory effects and epidermal hyperproliferation (1, 2), the mechanism of action of phorbol ester tumor promoters is still a mystery because irritant skin mitogens have been described that induce epidermal hyperproliferation as effectively as PMA without being potent tumor promoters. These include the weakly promoting phorbol ester-type compounds phorbol 12-tetradecatetra-2,4,6,8-enoate 13-acetate† (**II**; refs. 3 and 4) and mezerein (5, 6) and the nonpromoting ionophore A23187 (7). Thus, the induction of cellular proliferation is possibly a necessary but certainly not a sufficient condition for tumor promotion. A similar conclusion has been drawn from studies of tumor promotion in cell cultures (8, 9).

There is an increasing body of evidence supporting the theory that skin tumor promotion has at least two separate stages. It was reported, for example, that turpentine, a nonpromoting irritant skin mitogen, accomplished in a so-called "propagation stage" the tumor-promoting effect of a limited number of croton oil treatments (conversion stage; ref. 10). Although other investigators were apparently unable to confirm this result (11, 12), the concept of two-stage promotion has recently gained support after it was observed that only a few PMA applications (which, by themselves have no, or only a weak, promoting effect) are sufficient to promote tumor development when followed by long-term treatment with the irritant skin mitogen mezerein (13). Unfortunately, mezerein itself exhibits some tumor-promoting efficacy so that PMA and mezerein could be misinterpreted as acting in an additive manner.







By introducing a nonpromoting phorbol ester, we are now able to confirm the concept of two-stage promotion. As suggested by experiments with the unsaturated PMA analogue (**II**) (3, 4), the tumor-promoting potency of PMA can be diminished without impairing the irritant and mitogenic properties by the introduction of double bonds into the long-chain fatty acid residue. The tumor-promoting efficacy is completely abolished when the side chain is replaced by the retinoyl residue. The

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

Abbreviations: PMA, phorbol 12-myristate 13-acetate; PRA, phorbol 12-retinoate 13-acetate.

* Present address: U.S. Department of Agriculture, Western Regional Research Laboratory, Berkeley, CA 94710.

† The *trans* configuration of double bonds 6,7 and 8,9 is not definitely proved.

resulting phorbol 12-retinoate 13-acetate (PRA, III) is a non-promoting skin mitogen and the most powerful "second-stage promoter" currently known.

MATERIALS AND METHODS

Materials. 7,12-Dimethylbenz[*a*]anthracene, indomethacin, and prostaglandins E_2 and $F_{2\alpha}$ were purchased from Sigma. PMA was kindly provided by E. Hecker (German Cancer Research Center, Heidelberg). [*methyl*-^{3}H]Thymidine (specific activity, 20 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels) was obtained from New England Nuclear.

PRA was obtained by partial synthesis and purified by preparative high-pressure liquid chromatography; its structure was proved by spectroscopic means (unpublished results). Mezerein was isolated from seeds of *Daphne mezereum* and characterized spectroscopically (14). All compounds were tested for purity by thin-layer or high-pressure liquid chromatography before use and during the time of tumor-promotion experiments.

Animals and Treatments. Female albino mice (strain NMRI; 7 to 8 weeks old) were used in all experiments. The back skin of the animals was shaved by an electric clipper 3 to 4 days before beginning the biochemical experiments or 7 days before beginning the tumor-promotion experiments. Only those animals that did not show regrowth of hair were used.

PMA, PRA, mezerein, indomethacin, and prostaglandins E_2 and $F_{2\alpha}$ were dissolved in acetone (0.1 ml) and topically applied to the shaved area by a micropipette. For pulse labeling of epidermal DNA, 30 μCi of [*methyl*-^{3}H]thymidine in 0.3 ml of 0.9% NaCl was injected intraperitoneally. The animals were killed 1 hr later by cervical dislocation. For processing of the dissected skin, isolation of epidermal DNA, and measurement of radioactivity, see ref. 15.

For the tumor-promotion experiments, animals (16 per group) received single topical applications of 100 nmol of 7,12-dimethylbenz[*a*]anthracene (16) followed, beginning 1 week later, by twice-weekly applications of the promotion regimen. The number and incidence of papillomas were counted weekly.

RESULTS

PRA as an Irritant Skin Mitogen. A single application of the semisynthetic phorbol ester PRA to mouse skin *in vivo* stimulated the incorporation of [*methyl*-^{3}H]thymidine into epidermal DNA and provoked epidermal hyperplasia as effectively as an equimolar dose of PMA (Figs. 1 and 2). The effect was dose-dependent. As shown in Fig. 3, the proliferative effect of PRA was inhibited by previous treatment of the skin with the cyclooxygenase inhibitor indomethacin (17). This inhibition could be reversed by prostaglandin E_2, but not by prostaglandin $F_{2\alpha}$, applied simultaneously with PRA (Fig. 3). The test for irritant activity using the mouse ear assay gave an irritant dose affecting 50% of test animals (ID_{50}) of 0.04 nmol per ear as compared with 0.01 nmol per ear for PMA (16).

PRA as an Incomplete Promoter. The results of tumor promotion experiments with PMA using the standard dose of 3.08 μg (5 nmol) and with PRA in various doses are given in Table 1. Whereas PMA exerted a strong promoting effect, PRA did not produce any tumors when tested in doses of 1.72–6.88 μg (2.5–10 nmol) over a period of 24 weeks. Using a two-stage promotion protocol, PRA was shown to be a potent "second-stage promoter." The results of these experiments are shown in Fig. 4. One to four applications of 12.3 μg of PMA (20 nmol) each did not promote tumor development when followed by two applications per week of acetone over for 14–17 weeks. However, when the PMA treatment was followed by repetitive applications of PRA (6.88 μg; 10 nmol), a significant tumor response

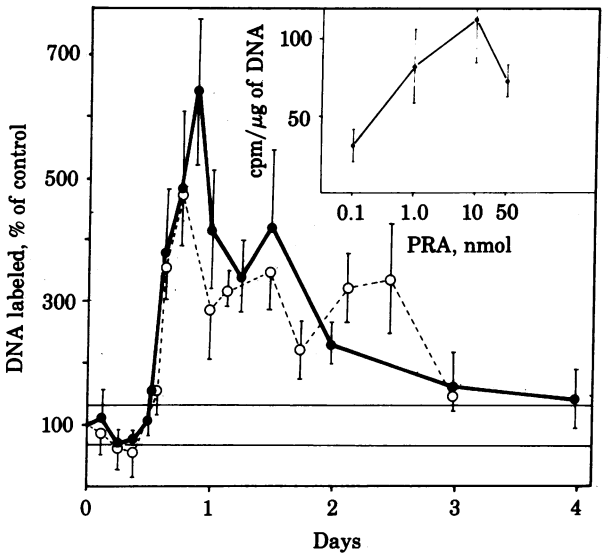


FIG. 1. Time course of DNA labeling in mouse epidermis *in vivo* after a single topical application of 10 nmol PRA (●) or 10 nmol PMA (○). Control animals (═) received acetone instead of phorbol ester. (*Inset*) Dose–response curve for the effect of PRA on DNA labeling measured after 18 hr. Results represent mean ± SEM for 10 mice; control, 45.7 ± 11.4 cpm/μg of DNA; $n = 40$.

was observed. At a given dose, the tumor-promoting efficiency depended on the number of PMA applications. Even a single PMA treatment followed by continuous PRA applications exhibited a strong tumor-promoting effect (Fig. 4). At a given number of PMA applications, the tumor-promoting efficiency

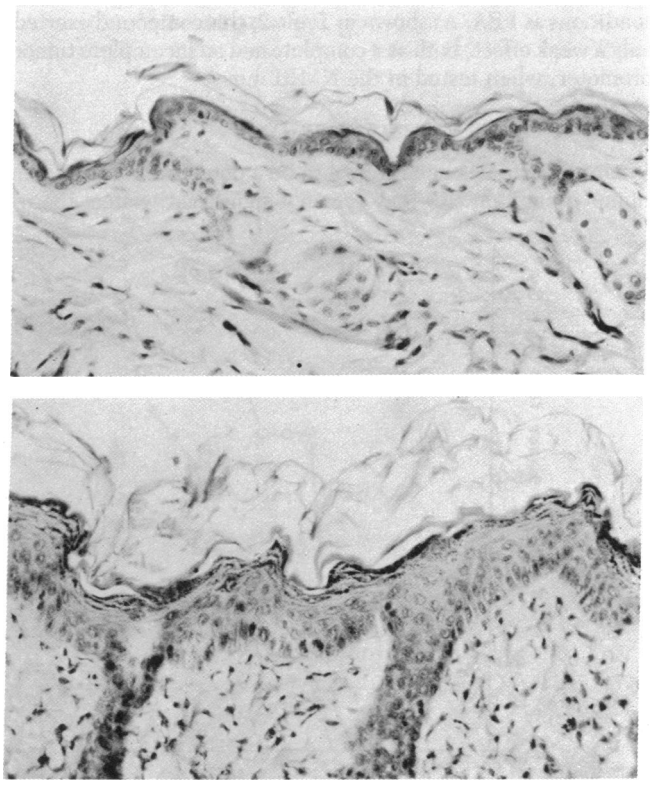


FIG. 2. Effect of PRA on mouse skin. (*Upper*) Control. (*Lower*) Forty-eight hours after topical application of 10 nmol of PRA. Sections (5 μm) of skin were prepared, stained with hematoxylin/eosin, and photographed. (×280.)

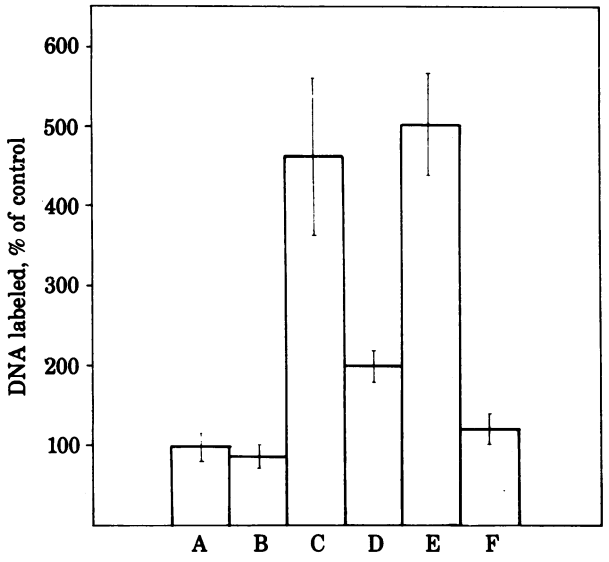


FIG. 3. Effect of prostaglandins E_2 and $F_{2\alpha}$ on epidermal DNA synthesis after treatment with indomethacin/PRA. Mice were treated with 0.1 ml of acetone or 1.1 μmol of indomethacin in 0.1 ml of acetone 1 hr before the application of 0.1 ml of acetone, 10 nmol of PMA in 0.1 ml of acetone, or 10 nmol of PMA/10 μg of prostaglandin E_2 or $F_{2\alpha}$ in 0.1 ml of acetone and killed 21 hr after PMA treatment. Results are mean ± SEM; n = 10. A, Control; B, indomethacin treated; C, PRA treated; D, indomethacin/PRA treated; E, indomethacin, PRA/prostaglandin E_2 treated; F, prostaglandin E_2 treated.

depended on the PMA dose (Table 2). As shown in Fig. 5, the effect of PRA as a second-stage promoter was also dose dependent.

Mezerein, which has been reported to be a second-stage promoter in the Sencar mouse (13), was tested under the same conditions as PRA. As shown in Table 2, this compound exerted only a weak effect, both as a complete and an incomplete tumor promoter, when tested in the NMRI mouse.

DISCUSSION

Vitamin A acid has been shown to be a potent inhibitor of skin tumor promotion (18) that does not significantly affect the irritant and mitogenic effects of phorbol ester tumor promoters (ref. 19; unpublished results). PRA, which combines the structure of the anti-tumor-promoter vitamin A acid with the structure of a phorbol ester in one and the same molecule, does not exhibit any tumor-promoting efficacy but induces skin irritation and epidermal hyperplasia to the same degree and along the same pathway as PMA (7, 20, 21). This includes mediation of the mitogenic effect by prostaglandin E synthesis and its inhibition by indomethacin (Fig. 3), induction of early phospholipid turnover, induction of ornithine decarboxylase activity, and development of catecholamine and G_1 chalone refractoriness (unpublished results).

These results confirm the conclusion based on earlier observations that tumor promotion is not due solely to prostaglandin-mediated hyperproliferation (hyperplastic transformation) of epidermis (7, 22). Moreover, the vitamin A acid analogue of PMA provides an appropriate negative control compound for experiments on the biological effects of phorbol ester tumor promoters because, as an irritant mitogen, it probably has the same mechanism of action as PMA without being a promoter. In contrast, 4-*O*-methylphorbol 12-myristate 13-acetate, which is currently used as a negative control, does not evoke skin in-

Table 1. Tumor-promoting activities of phorbol esters

		Tumor formation					
		After 12 weeks		After 18 weeks		After 24 weeks	
Ester	Dose, nmol	Rate, %	Yield	Rate, %	Yield	Rate, %	Yield
PMA	5	100	4.6	100	7.6	100	7.4
PRA	2.5	0	0	0	0	0	0
	5	0	0	0	0	0	0
	10	0	0	0	0	0	0

Seven-week old female NMRI mice (16 per group) were treated with a single dose of 100 nmol of 7,12-dimethylbenz[a]anthracene (in 0.1 ml of acetone). One week later, treatment with phorbol ester was started. The phorbol ester was applied twice a week in 0.1 ml of acetone. At the end of the experiment, ≥94% of the animals were alive. Tumor-promoting activity was measured as rate (no. of tumor-bearing animals per no. of survivors) and as yield (no. of tumors per no. of survivors).

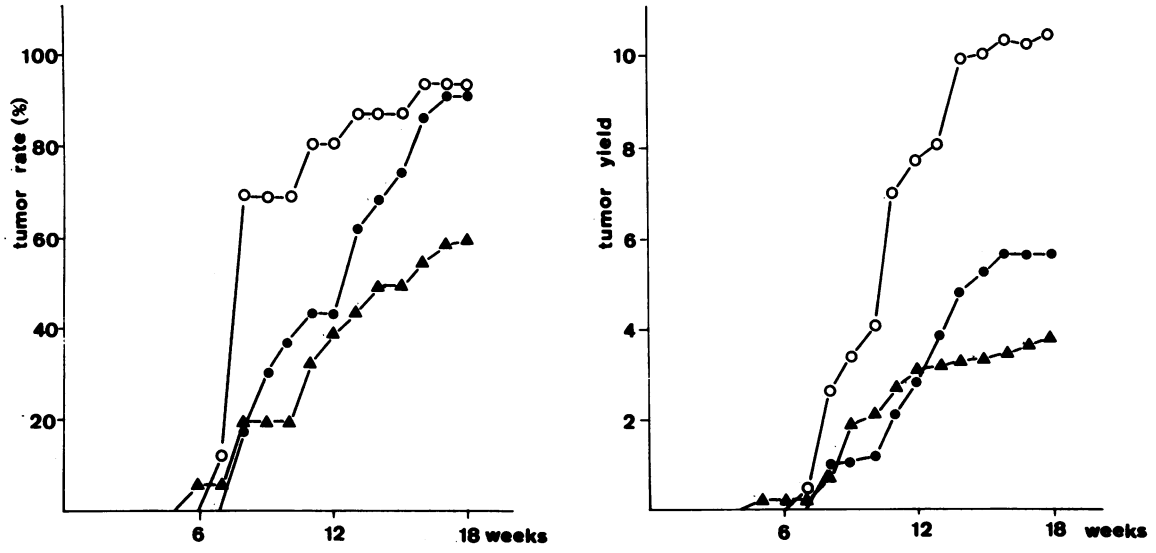


FIG. 4. Two-stage tumor promotion after treatment of mouse skin with 100 nmol of 7,12-dimethylbenz[a]anthracene. One week later, twice-weekly applications of 20 nmol of PMA were started. After one (▲), two (●), or four (○) PMA treatments, the mice received 10 nmol of PRA in acetone twice weekly for the rest of the 18-week experiment. At the end of the experiment all of the mice were alive. Tumor-promoting activity was measured as rate (no. of tumor-bearing animals per no. of survivors) and as yield (no. of tumors per no. of survivors).

Table 2. Two-stage tumor promotion in NMRI mouse skin

Treatment		Tumor formation					
		After 12 weeks		After 15 weeks		After 18 weeks	
First	Second	Rate, %	Yield	Rate, %	Yield	Rate, %	Yield
PMA (4 times; 20 nmol)	Acetone (32 times)	0	0	0	0	0	0
Acetone (4 times)	PRA (32 times; 10 nmol)	0	0	0	0	0	0
PMA (4 times; 20 nmol)	PRA (32 times; 10 nmol)	82	8.0	88	10.6	94	10.8
PMA (4 times; 10 nmol)	PRA (32 times; 10 nmol)	60	1.9	67	3.3	80	5.2
PMA (4 times; 5 nmol)	PRA (32 times; 10 nmol)	25	0.8	40	1.6	54	2.4
PMA (4 times; 10 nmol)	Mezerein (32 times; 8 nmol)	13	0.1	13	0.1	20	0.3
Mezerein (4 times; 8 nmol)	Mezerein (32 times; 8 nmol)	0	0	7	0.7	7	0.7

Female NMRI mice (16 per group) were treated with 100 nmol of 7,12-dimethylbenz[*a*]anthracene. One week later, treatment with PMA and mezerein (in 0.1 ml of acetone each) or acetone (0.1 ml) was started. The compounds were applied twice a week for 2 weeks. Beginning with the third week of promotion the treatment was continued with PRA or mezerein (in 0.1 ml acetone each) or with acetone up to 18 weeks. At the end of the experiment, ≥94% of the mice were alive. Tumor-promoting activity was measured as rate (no. of tumor-bearing animals per no. of survivors) and as yield (no. of tumors per no. of survivors).

flammation and induces epidermal hyperproliferation via a mechanism different from that of PMA (22).

Thus, our experiments support the concept of two-stage promotion proposed by Boutwell (10). Considering the results, it may be assumed that effects probably obligatory and critical for tumor promotion can be brought about by a *single* application of PMA and that the subsequent long-term induction of epidermal hyperproliferation may be necessary only to make the tumors, which are mainly reversibly growing benign papillomas, visible to the naked eye. The second stage of tumor promotion in mouse skin may be a feature of the assay system rather than a fundamentally important step.

The events involved in the first stage of promotion are still entirely unknown, presumably because they are camouflaged by the strong pleiotropic effects of PMA. It has been proposed, mainly for theoretical reasons, that the first stage may include expression of the tumor phenotype in the course of a functional reprogramming (metaplasia; refs. 2 and 23). This has been postulated to be necessary because initiation has occurred in a tissue (i.e., epidermis) that, under normal conditions, is thought to be invariably committed to a special kind of function and, thus, cannot spontaneously express the functional alterations induced by the carcinogen.

In our hands, PRA has been found to be a much more powerful second-stage promoter than mezerein, which has been found recently to be an incomplete promoter in the Sencar mouse (13). Moreover, mezerein shows weak full-promoter efficiency and (at least in NMRI mice) toxic effects when repeatedly applied, which make the interpretation of two-stage promotion experiments carried out with this compound somewhat difficult. Our results do not characterize mezerein as a second-stage promoter in NMRI mice. Whether or not the lack of tumor-promoting potency of PRA is due to its intrinsic vitamin A-like activity remains to be established. Such an assumption, however, seems to be inconsistent with the observation that the second stage of tumor promotion induced by mezerein in the Sencar mouse is inhibited by vitamin A acid (19).

The concept of two-stage promotion (and the availability of a pure second-stage promoter such as PRA) may have important implications in that it not only facilitates the investigation of the promotion-specific effects of PMA but, in addition, may enable investigators to decide whether an effect of a phorbol ester

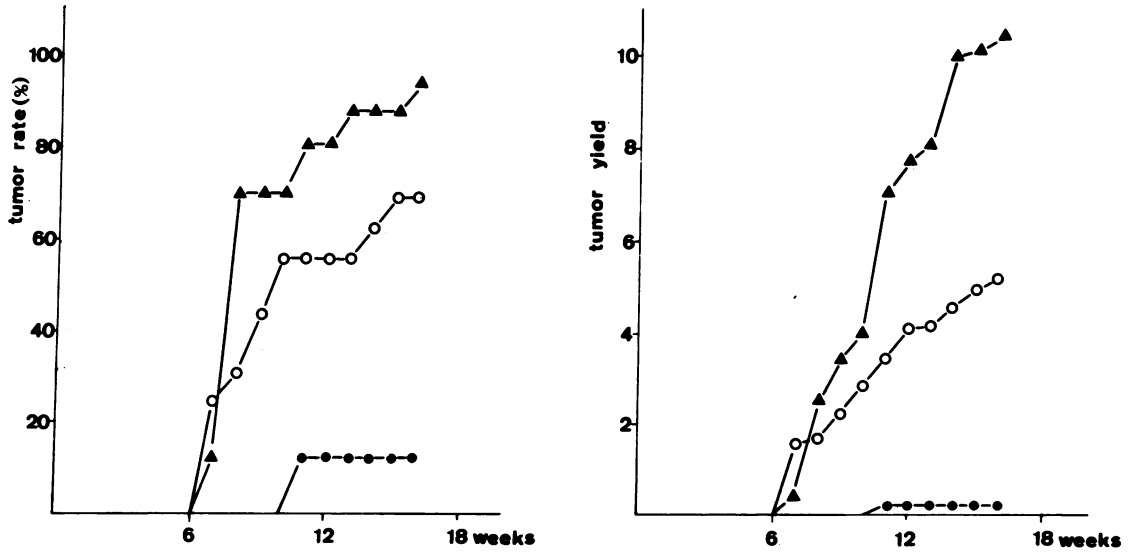


FIG. 5. Dose–response relationship in second-stage promotion by PRA. Beginning 1 week after treatment with 100 nmol of 7,12-dimethylbenz[*a*]anthracene, mice were treated twice weekly with 20 nmol of PMA for 2 weeks. Starting on the third week of promotion, the mice received twice-weekly applications of 2.5 (●), 5 (○), or 10 (▲) nmol doses of PRA. At the end of the experiment, ≥94% of the animals were alive. Tumor-promoting activity was measured as rate (no. of tumor-bearing animals per no. of survivors) and as yield (no. of tumors per no. of survivors).

measured in a given biological system is related to tumor promotion or merely reflects the pleiotropic effects of PMA as a skin mitogen. Because of the large amount of data on the biological actions of PMA, this question is important for experimental cancer research.

1. Slaga, T. J., Sivak, A. & Boutwell, R. K., eds. (1978) *Carcinogenesis, Mechanisms of Tumor Promotion and Cocarcinogenesis* (Raven, New York), Vol. 2.
2. Marks, F., Berry, D. L., Bertsch, S., Fürstenberger, G. & Richter, H. (1982) in *Carcinogenesis, Cocarcinogenesis, and the Biological Effects of Tumor Promoters*, eds. Hecker, E., Fusenig, N. E., Marks, F., Kunz, W. & Thielmann, H. W. (Raven, New York), Vol. 7, pp. 331–346.
3. Fürstenberger, G. & Hecker, E. (1977) *Experientia* **33**, 986–989.
4. Marks, F., Bertsch, S. & Fürstenberger, G. (1979) *Cancer Res.* **39**, 4183–4188.
5. Fürstenberger, G. & Hecker, E. (1972) *Planta Med.* **22**, 241–266.
6. Mufson, R. A., Fischer, S. M., Verma, A. K., Gleason, G. L., Slaga, T. J. & Boutwell, R. K. (1979) *Cancer Res.* **39**, 4791–4795.
7. Marks, F., Fürstenberger, G. & Kownatzki, E. (1981) *Cancer Res.* **41**, 696–702.
8. Mondal, S. & Heidelberger, C. (1976) *Cancer Res.* **36**, 2254–2260.
9. Mondal, S., Brankow, D. W. & Heidelberger, C. (1980) *Cancer Res.* **40**, 334–338.
10. Boutwell, R. K. (1964) *Prog. Exp. Tumor Res.* **4**, 207–250.
11. Raick, A. N. & Burdzy, K. (1973) *Cancer Res.* **33**, 2221–2230.
12. Slaga, T. J., Bowden, G. T. & Boutwell, R. K. (1975) *J. Natl. Cancer Inst.* **55**, 983–987.
13. Slaga, T. J., Fischer, S. M., Nelson, K. & Gleason, G. L. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 3659–3663.
14. Schildknecht, H., Edelmann, G. & Maurer, H. (1970) *Chemiker Zeitung* **94**, 347–351.
15. Krieg, L., Kühlmann, I. & Marks, F. (1974) *Cancer Res.* **34**, 3135–3146.
16. Hecker, E. (1971) in *Methods in Cancer Research*, ed. Busch, H. (Academic, New York), Vol. 6, pp. 439–484.
17. Flower, R., Gryglewski, R., Cedro, K. H. & Vane, J. R. (1972) *Nature (London) New Biol.* **238**, 104–106.
18. Verma, A. K., Shapas, B. G., Rice, H. M. & Boutwell, R. K. (1979) *Cancer Res.* **39**, 419–425.
19. Slaga, T. J., Klein-Szanto, A. J. P., Fischer, S. M., Weeks, G. E., Nelson, K. & Major, S. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 2251–2254.
20. Fürstenberger, G. & Marks, F. (1978) *Biochem. Biophys. Res. Commun.* **84**, 1103–1111.
21. Fürstenberger, G. & Marks, F. (1980) *Biochem. Biophys. Res. Commun.* **92**, 749–756.
22. Fürstenberger, G., deBravo, M., Bertsch, S. & Marks, F. (1979) *Res. Commun. Chem. Pathol. Pharmacol.* **24**, 533–541.
23. Marks, F., Bertsch, S., Grimm, W. & Schweizer, J. (1978) in *Carcinogenesis, Mechanisms of Tumor Promotion and Cocarcinogenesis*, eds. Slaga, T. J., Sivak, A. & Boutwell, R. K. (Raven, New York), Vol. 2, pp. 97–116.