Tumor Promotion by Phorbol Esters in Skin: Evidence for a Memory Effect

Abstract. *By means of a two-stage promotion protocol in mouse epidermis with 12-O-tetradecanoylphorbol-13-acetate as first-stage promoter and 12-O-retinoylphorbol-13-acetate as second-stage promoter, the effects of the former that are critical and obligatory for tumor promotion were shown to be irreversible in nature for at least 8 weeks. The reversibility of tumor promotion was related to the second stage of promotion, reflecting the reversibility of epidermal hyperplasia induced by 12-O-tetradecanoylphorbol-13-acetate.*

For studies on the mechanism of chemical carcinogenesis, a two-stage protocol in mouse skin provides an excellent model (*1*). Skin tumors can be induced by a combination of two essentially nontumorigenic manipulations, that is, the sequential application of a subthreshold dose of a carcinogen, most commonly 7,12-dimethylbenz[*a*]anthracene (DMBA; initiation phase) followed by repetitive applications of tumor promoters such as 12-*O*-tetradecanoylphorbol-13-acetate (TPA; promotion phase). Under such conditions, primarily benign tumors are produced (*1, 2*). Long-term treatment is required for the production of some carcinomas (*1, 2*). A feasible interpretation of such results is that a genetic change induced by the carcinogen remains latent unless it is phenotypically expressed in the course of promotion. Whereas initiation is irreversible, that is, remains manifest even when promoter treatment is delayed for several months (*3*), promotion is thought to be reversible because it is abolished by extending the time interval between promoter applications (*3*). Here we report on experiments indicating that the effects critical and obligatory for promotion in mouse epidermis may last for at least 2 months.

Tumor promoters of the phorbol ester type exert strong inflammatory effects and induce epidermal hyperproliferation (*1, 4*) (Table 1). Certain TPA analogs, such as 12-*O*-retinoylphorbol-13-acetate (RPA) (*5, 6*), 12-*O*-(2-*cis*,4-*trans*,6,8)tetradecatetraenoylphorbol-13-acetate (Ti_8) (*7, 8*), and mezerein (*9, 10*), all carrying conjugated double bonds in the long-chain fatty acid, show the same irritant and mitogenic efficacy as TPA but are much less promoting (Table 1) (*6, 7, 9*), indicating that cell proliferation and inflammation are probably necessary but not sufficient conditions of promotion (*4*). Compounds of this type have been called "incomplete promoters" since they strongly amplify the promoting efficacy of TPA (Table 2) (*6, 11*). They allow the process of tumor promotion in skin to be divided into two stages [a protocol originally reported by Boutwell (*3*)], each of which is by itself insufficient to promote tumor development—that is, one (or two) applications of TPA followed by long-term treatment with an incomplete promoter (Table 2) (*6*). This means that the effects critical and obligatory for promotion can be brought about by a single TPA application and that the function of the second stage is probably stimulation of tumor growth during the course of prolonged epidermal hyperplasia.

The two-stage protocol of promotion implies a more permanent nature of the events involved in the first stage of promotion, with the proliferative and hyperplasiogenic processes of the second stage being more transient in nature. Such a view is supported by the observation that there is no significant loss in tumor response when the time interval between TPA application and growth stimulation by an incomplete promoter such as RPA is increased (Fig. 1, A and B). The latency period and the kinetics of the appearance of tumors remain essentially constant. Control experiments

Table 1. Comparison of the irritant, mitogenic, and tumor-promoting activities of phorbol esters and mezerein in the epidermis of NMRI mice in vivo. The irritant activity was determined by means of the mouse ear assay (*26*). Ear reddening was evaluated 24 hours after administration of the phorbol esters. The equimitogenic dose was calculated from the maximum mitogenic activities induced by 10 nmole of TPA, Ti_8, RPA, or mezerein in mouse epidermis. The equipromoting doses were derived from tumor promotion experiments by using various doses of TPA, Ti_8, RPA, and mezerein. Tumors were initiated in female NMRI mice (7 weeks old; 16 animals per group) with a single dose of 100 nmole of DMBA; 1 week later the mice received twice weekly applications of various doses of TPA, Ti_8, RPA, and mezerein, each dissolved in 0.1 ml of acetone, or of acetone (0.1 ml; control). At the end of the experiments, 94 percent or more of the mice were alive. Tumor-promoting activity was measured as the ratio of the number of tumors to the number of survivors. All tumor-promoting experiments were performed at least twice and yielded similar results.

Compound	Irritant activity* (nmole/ear)	Equimitogenic dose (nanomoles per animal)	Equipromoting dose per application (nanomoles per animal)
TPA	0.016	10	1
Ti_8	0.03	9	10
RPA	0.04	6	> 10
Mezerein	0.03	9	10

*Median effective dose in 24 hours.

Table 2. Two-stage tumor promotion in NMRI mouse skin. Female NMRI-mice (7 weeks old; 16 per group) were treated with 100 nmole of DMBA. One week later treatment with TPA (in 0.1 ml of acetone) or acetone (0.1 ml) was started. After one or two applications of acetone or TPA, treatment was continued with RPA (in 0.1 ml of acetone, twice weekly) or with acetone alone for up to 18 weeks. At the end of the experiments ≥ 94 percent of the animals were alive. All experiments were repeated at least twice and yielded similar results.

Treatment						Tumor development after 18 weeks	
First			Second				
Compound	Concentration (nmole)	Applications (No.)	Compound	Concentration (nmole)	Applications (No.)	Rate (%)	Yield
TPA	20	1	Acetone		35	0	0
TPA	20	2	Acetone		34	0	0
Acetone		2	RPA	10	34	6	0.2
TPA	20	1	RPA	10	35	69	4.0
TPA	20	2	RPA	10	34	87	5.5
TPA	10	2	TPA	10	34	100	8.4

in which long-term treatments of initiated skin are carried out with either TPA or RPA alone show that the time between the initiation by DMBA and the beginning of the phorbol ester treatment does not influence the effects of either the full promoter TPA or of the almost nonpromoting hyperplasiogen RPA (data not shown).

These results indicate that the change induced by TPA in mouse epidermis is permanent for at least 2 months. This is of interest since it has been shown that within 24 hours the epidermis is cleared of 90 percent of the amount of TPA applied (*12*). The pleiotypic response caused by TPA in epidermis is also of much shorter duration (*1*). This response includes an early increase in the prostaglandin E content in mouse epidermis [peak activity at 10 minutes (*13*)] and of phospholipid synthesis (*14*), induction of ornithine decarboxylase activity (peak activity at 4 to 5 hours) and the resultant accumulation of polyamines (*7, 15*), as well as desensitization to epidermal G_1 chalone and β-adrenergic stimulation of adenosine 3′,5′-monophosphate (cyclic AMP) formation (*16*). Moreover, TPA stimulates epidermal DNA synthesis (with peaks at 18, 30, and 42 hours) and mitotic activity, finally leading to epidermal hyperplasia which reaches its maximum after about 36 hours (*17*). After 7 to 10 days, the numbers of nucleated cell layers in the initiated as well as the noninitiated interfollicular epidermis have returned to control values (*18*), indicating a complete reversibility of the pleiotypic effects of TPA. The TPA-induced symptoms of inflammation such as erythema, edema, hypertrophy of epidermal cells, and leukocyte infiltration are also fully reversible (*19*).

When promotion is carried out in the classical manner—that is, by long-term treatment with TPA—an increase of the time interval between TPA applications up to about 14 days is sufficient to almost eliminate the tumor-promoting effect (*3*). Our results indicate that this reversibility of promotion is probably related to the second stage of promotion—that is, is solely due to the reversibility of epidermal hyperplasia—whereas the critical alterations induced in the first stage are probably irreversible. This finding is supported by the observation that the tumor response in a two-stage promotion experiment is greatly diminished when the time interval between RPA applications in the second stage is increased to 2 weeks.

The apparent irreversibility of those responses to TPA which are thought to be critical and obligatory for promotion is consistent with the idea that in the first stage of promotion the (benign) tumor phenotype of the initiated cell is being expressed and that the second stage is only necessary to make the papillomas visible. It is well known that phenotypic expression can be an irreversible process. This implies that the memory effect in skin tumor promotion by phorbol esters is due to a permanent alteration of the genetic readout in initiated cells, whereas normal epidermal cells which are genetically or otherwise not predisposed for such phenotypic changes respond in a reversible manner.

An irreversibility of TPA-dependent events has also been demonstrated in the JB6 mouse epidermal cell line, where TPA and other skin tumor promoters induce a change of the preneoplastic into the transformed phenotype (*20, 21*). The recent finding that this effect is independent of the mitogenic properties of the promoter (*22*) is apparently incompatible with our findings—that is, that in vivo a mitogenic component is involved in both the first and second stage of promotion in adult mouse skin. In the classical approach to two-stage skin tumorigenesis in vivo, growth stimulation has to be maintained because the reversibly growing papillomas would otherwise not become visible. However, transformed JB6 cells obtained by TPA treatment express irreversibly an anchorage-independent growth pattern (*20*). Thus the TPA effect in the JB6 system probably reflects a later stage of tumor development—that is, the progression from a more benign to a more malignant autonomous state, rather than the promotion of initiated cells to predominantly benign tumor cells, as observed in vivo.

Slaga has shown (*23*) that in Sencar mice glucocorticoid hormones inhibit both the first and the second stage of promotion. The inhibitory effect of fluocinolone acetonide is likely to result from its antimitotic activity (*24*) impairing the hyperplasiogenic components of both first and second stages of promotion. However, retinoic acid, which does not inhibit epidermal hyperproliferation induced by TPA (*25*), inhibited only the second stage in the Sencar mouse, possibly indicating a nonhyperplasiogenic component in the second stage. Nevertheless, the mechanism by which repeated doses of vitamin A acid exhibit an inhibitory action is not known.

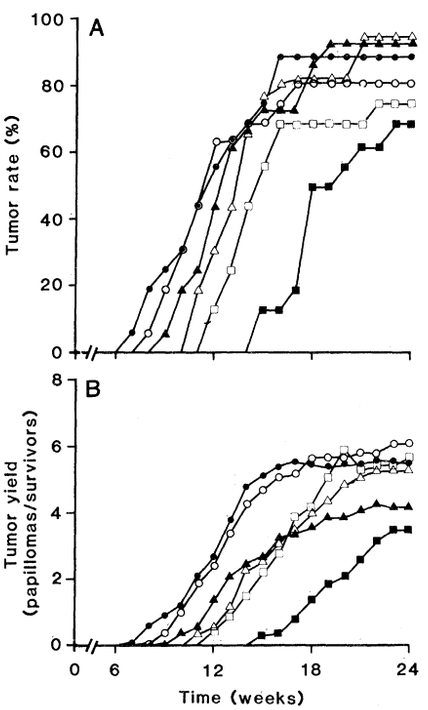

Fig. 1. Two-stage promotion in mouse skin: the effect of increasing the time interval between the first stage and the second stage. The tumor response is expressed as (A) the tumor rate (ratio of number of tumor bearers to number of survivors, shown as percentages), and as (B) tumor yield (ratio of papillomas to survivors). Female NMRI mice (7 to 8 weeks old; 16 per group) were treated with 100 nmole of DMBA. During the following week the mice were treated twice with TPA (620 nmole in 0.1 ml of acetone). Beginning with the second week of promotion the treatment was continued with (●) RPA (10 nmole in 0.1 ml of acetone) twice weekly for 23 weeks or with acetone (0.1 ml) twice weekly for (○) 1 week, (▲) 2 weeks, (△) 4 weeks, (□) 6 weeks, or (■) 8 weeks, each treatment being followed by twice weekly applications of RPA (10 nmole in 0.1 ml of acetone) for 21, 19, 17, or 15 weeks, respectively. At the end of the experiments 94 percent or more of the animals were alive. All experiments were repeated at least twice and yielded similar results.

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Morphological Basis of Long-Term Habituation and Sensitization in *Aplysia*

Abstract. *The morphological basis of the persistent synaptic plasticity that underlies long-term habituation and sensitization of the gill withdrawal reflex in* Aplysia californica *was explored by examining the fine structure of sensory neuron presynaptic terminals (the critical site of plasticity for the short-term forms of both types of learning) in control animals and in animals whose behavior had been modified by training. The number, size, and vesicle complement of sensory neuron active zones were larger in animals showing long-term sensitization than in control animals and smaller in animals showing long-term habituation. These changes are likely to represent an anatomical substrate for the memory consolidation of these tasks.*

Since the work of Ramón y Cajal at the turn of the century, it has often been suggested that learning and memory produce structural changes at the synapse (*1*). Although many investigators have reported alterations in synaptic morphology after experimental manipulation (*2*), the functional significance of these changes has been difficult to assess because the contribution of the synapses to the learning process has not been known. In recent years, the tractable nervous systems of higher invertebrates have proven useful for correlating changes in cellular function with learning (*3*). One such model system has been the gill and siphon withdrawal reflex of *Aplysia*, in which several forms of learning (short- and long-term and nonassociative and associative) have been studied to advantage on both the cellular and molecular level. This reflex undergoes two simple forms of nonassociative learning—habituation and sensitization—that can exist in a short-term form lasting minutes to hours (*4*) and in a long-term form lasting more than 3 weeks (*5*). The biophysical and biochemical mechanisms of short-term habituation and sensitization are known to involve changes in synaptic effectiveness produced by modulation of the Ca^{2+} current at a common locus—the presynaptic terminals of identified mechanoreceptor sensory neurons (*6*). Less well characterized are the morphological mechanisms that underlie habituation and sensitization, particularly their long-term form. For both habituation and sensitization the critical site of plasticity, the synapses between sensory neurons and follower cells, is shared by the short- and long-term forms (*7*, *8*).

We exploited the cellular specificity of the gill withdrawal reflex to explore the morphological basis of long-term habituation and sensitization in *Aplysia*. Using horseradish peroxidase (HRP) to label the presynaptic terminals (varicosities) of sensory neurons and serial reconstruction to analyze synaptic contacts, we compared the fine structure of identified sensory neuron synapses in control and behaviorally modified animals. Our results indicate that learning can modulate long-term synaptic effectiveness by altering the number, size, and vesicle complement of synaptic active zones.

Aplysia californica (70 to 100 g) were trained for long-term habituation or for long-term sensitization (*5*, *9*). To maximize the chances of detecting morphological differences, only those animals that demonstrated the most profound behavioral changes after training (< 10 percent of the day 1 score for habituated animals and > 200 percent for sensitized animals) were used for subsequent electron microscopic analysis. Within 48 hours of behavioral testing all animals were anesthetized by injection of $MgCl_2$ and the abdominal ganglion was removed and desheathed in seawater containing high Mg^{2+} (220 m*M*) and low Ca^{2+} (1 m*M*). This solution was washed out and the ganglion was bathed in seawater with normal concentrations of Ca^{2+} (10 m*M*) and Mg^{2+} (55 m*M*) for a minimum of 30 minutes before the cells were impaled (*7*). Individual mechanoreceptor sensory neurons were identified (*10*) and intrasomatically pressure-injected with HRP (type VI, Sigma) at a concentration of 20 mg/ml in distilled water. After approximately 2 hours the ganglia were fixed, histochemically processed, and embedded (*11*). Serial thin sections (0.1 μm; 500 to 1000 per set) were made through a region containing labeled sensory neuron processes in each ganglion. Every HRP-labeled profile in each section was photographed and sensory neuron varicosities were then completely reconstructed and analyzed through a

Table 1. Number of varicosities, number of active zones, and ratio of active zones to varicosities in control and behaviorally modified animals. Analysis of variance shows that the means are significantly different [$F(2, 3) = 79.8$, $P < .01$)]. Moreover, individual comparisons (Studentized range tests) show that the mean for habituated animals (12 percent) is significantly less than the mean for control animals (41 percent) ($P < .05$) and that the mean for sensitized animals (65 percent) is significantly greater than that for control animals ($P < .05$).

Group	Varicosities	Active zones	Ratio of zones to varicosities (%)*
Habituated			
Animal 1 (four cells)	63	8	13
Animal 2 (one cell)	48	5	10
Control			
Animal 3 (two cells)	47	17	36
Animal 4 (one cell)	46	21	46
Sensitized			
Animal 5 (two cells)	59	39	66
Animal 6 (four cells)	48	31	65

*(Number of active zones/number of varicosities) (100).