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EFFECTS OF MULTIPLE PHORBOL MYRISTATE ACETATE TREATMENTS ON CYCLIC NUCLEOTIDE LEVELS IN MOUSE EPIDERMIS

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SUMMARY

The basal levels of 3',5' adenosine monophosphate and 3',5' guanosine monophosphate were measured in mouse epidermis after initiation with 7,12 dimethylbenzanthracene and 1,2,10 or 20 skin treatments with the tumor promoter phorbol myristate acetate. Slight but significant decreases in cAMP and dramatic (5-10 fold) increases in cGMP were found after multiple treatments with the promoter. The cyclic nucleotide levels found in isolated solid tumors closely paralleled these changes.

INTRODUCTION

The role of cyclic nucleotides in skin tumor promotion by phorbol ester has been investigated by a number of laboratories. Although early reports (1,2) indicated marked effects of PMA[1] on basal cAMP levels in mouse epidermis, Mufson et al. (3) have shown some of these results to be artifactual. No changes in either cyclic nucleotide were seen up to 18 hr after a single PMA treatment (3). We have recently confirmed the data of Mufson et al., although we did observe a 2 fold increase in cGMP levels 36 hr after a single treatment (4). The ratio of cGMP/cAMP also doubled at 36 hr, and remained high up to 72 hr after treatment (5). The persistence of a high value for cGMP/cAMP ratios 3 days after treatment may be of significance with respect to tumor promotion. Van Duuren (6) and others have observed that

[1] Abbreviations: PMA,-Phorbol Myristate Acetate; cAMP-3',5' Adenosine Monophosphate; cGMP-3',5' Guanosine Monophosphate DMBA-7,12 Dimethylbenzanthracene.

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maximum promoting activity of PMA is obtained with a frequency of 2-3 weekly applications. We therefore decided to examine the effects of multiple treatments of PMA on cyclic nucleotide levels and ratios in mouse epidermis. It should be noted that all previous reports on PMA action vis a vis cyclic nucleotides involved a single treatment with PMA, which is not sufficient to produce tumors.

MATERIALS AND METHODS

Phorbol Ester Treatment. Female Swiss Ha/ICR mice purchased from Sprague Dawley (Madison, Wis.) were shaved at 6-8 weeks of age. Animals which showed wounding or hair regrowth were not used. Mice were given an initiating dose of 25 µg DMBA in 0.2 ml acetone to the back skin. Control groups were also initiated. One week after initiation PMA treatments of 10 µg/0.2 ml acetone (16 nmole) began. Applications of PMA supplied by Dr. P. Borchert were administered on Monday, Wednesday and Friday at approximately 9 A.M. Each of four groups of mice (20 animals/group)were treated with either 1,2,10 or 20 applications of PMA. Groups receiving 10 and 20 treatments were on test for 21 and 42 days respectively from the start of PMA painting. From each group of 20 animals 5 mice were sacrificed at either 2,36,72 or 120 hr after the last treatment. Three groups of controls (5 animals/group) were treated with either 2,10 or 20 applications of 0.2 ml acetone. One group of 10 animals was treated continuously until the appearance of tumors (approximately 3 months).

Preparation of Epidermis. Animals were sacrificed by cervical dislocation between 9-10 A.M. Immediately after sacrifice animals were plunged into liquid nitrogen for 30 seconds and the epidermis was scraped off the frozen back skin as described previously (4,5). Solid tumors (3-8 mm diameter) were excised off the frozen back skin with a scalpel. Tissue was homogenized for 1 min in a Polytron P-10 at top speed in 6% trichloroacetic acid. Homogenates were filtered through 0.45 µM Millipore filters and filtrates were extracted 3 times with 5 volumes of ether.

Cyclic Nucleotide Assay. Cyclic AMP and cGMP were assayed by radioimmune assay as described previously (4) using antisera and [^{125}I] labelled antigens purchased from Collaborative Research (Waltham, Mass.). Samples for cGMP assay were purified by column chromatography as described by Murad et al. (7). The acetylation procedure of radioimmune assay (8) was used for cGMP determination. Specificity of the assay for both cyclic nucleotides was demonstrated by the loss of at least 90% of competing material after 2 hr incubation of samples with cyclic nucleotide phosphodiesterase (Sigma, St. Louis, Mo.).

Epidermis homogenates were assayed for DNA by the fluorometric assay (9) using diaminobenzoic acid (Aldrich, Milwaukee, Wisc.).

RESULTS AND DISCUSSION

The basal levels of cAMP and cGMP from control epidermis were 0.269 ± 0.019 pmole/μg DNA and 4.38 ± 0.51 fmole/μg DNA respectively. Figures 1 and 2 show the effects of multiple PMA treatments on cyclic nucleotide levels in mouse epidermis.

Although a single treatment of PMA did not affect cAMP levels up to 120 hr as previously reported (3,4,5) multiple treatments did produce significant ($p < 0.05$) decreases (Fig. 1) at 5 days after the second treatment (62% of control) and at 3 and 5 days after 10 treatments (57% and 50% of control) respectively. The solid skin tumors also had significantly lower cAMP levels than controls (62%).

The return to near control levels after 20 treatments reverses this trend and is difficult to explain. At this point many mice had developed papillomas which were harvested along with the rest of the epidermis. A brief increase in cAMP has been implicated in the mechanism of cell proliferation by mitogens (10) and our results might be representative of a proliferative signal operating during the early stages of tumor formation. Separate assays for cAMP in isolated papillomas and in "normal" epidermis after 20 PMA treatments are required to clarify these results.

Figure 2 illustrates the effect of multiple PMA treatments on cGMP levels in mouse epidermis. Starting with the 2nd treatment cGMP levels are dramatically raised from 5-10 fold. This elevation is seen up to at least 5 days following the last treatment. The average increase of cGMP in all multiple treatment groups was 7.07 ± 0.50 fold over control. The solid tumors showed a 7.42 ± 1.32 fold increase over control. Ratios of cGMP/cAMP were calculated for each animal and aver-

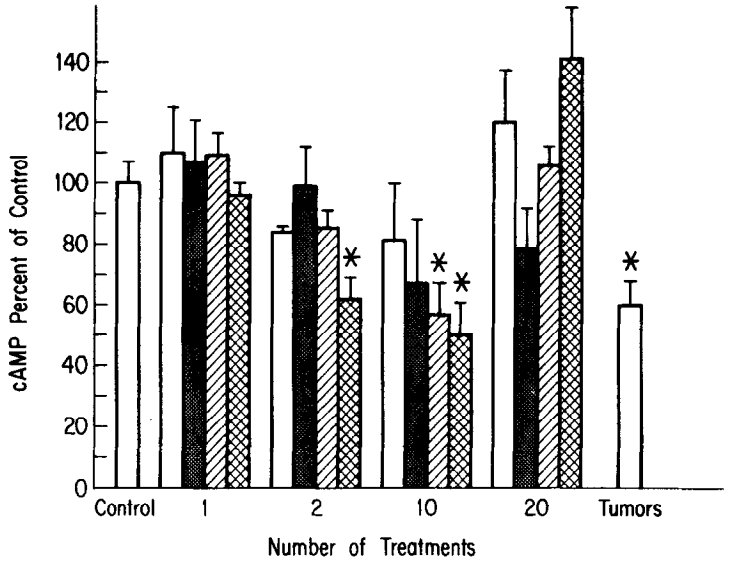

Figure 1 Cyclic AMP levels (and standard errors) after multiple PMA treatments. Animals were sacrificed at 2, 36, 72 or 120 hr after the last treatment. Each treatment group contained 5 mice. Statistically significant ($p<0.05$ by T-test) decreases from control are indicated by asterisk.

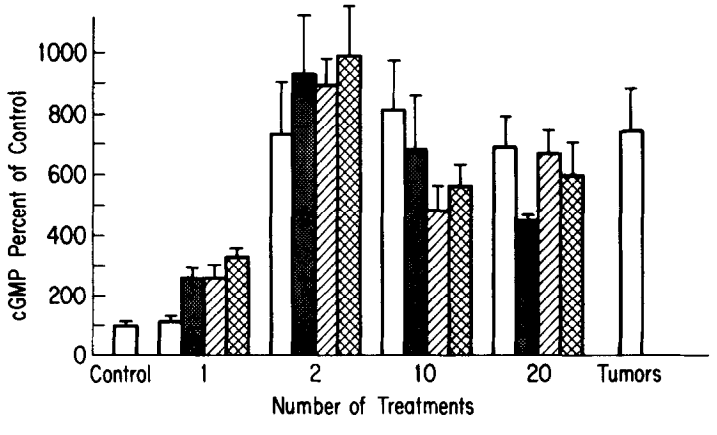

Figure 2 Cyclic GMP levels (and standard errors) after multiple PMA treatments. Legend same as for Figure 1. All values except for 2 hr after 1 treatment are statistically significant ($p<0.05$) increases over control.

aged in each group. These ratios were fairly constant after 2, 36 and 72 hr but further increased by approximately 50% at 120 hr. The average ratios for 2 and 10 treatments were respectively 11.43 $\pm$ 1.53 fold and 11.41 $\pm$ 1.31 fold higher than control. The cGMP to cAMP ratio in the solid tumors was 11.59 $\pm$ 2.09 fold higher than control. Thus, the values for the cyclic nucleotide levels and ratios found in the isolated solid tumors are very close to those found in the skin after 2 or more treatments.

Although neither cyclic nucleotide appears to be involved in the early biochemical events following a single PMA administration (3,4) the data presented here are consistent with an important role for the cyclic nucleotides in tumor promotion. The high ratio of cGMP/cAMP found in mouse epidermis during continuous PMA treatments may be necessary for development of neoplasia or may be a result of other cellular stimulating effects of PMA. Elevated cGMP or cGMP/cAMP ratios have been found in a number of proliferating and neoplastic systems such as psoriasis (11) human colon adenocarcinoma (12) and rat hepatoma (13). Goldberg and coworkers have postulated a crucial role for increased cGMP/cAMP ratios in cellular growth stimulation (14).

It should be noted that the results described here were from initiated mice, unlike those from previous reports (1-5). Some evidence for a different response to croton oil by initiated and uninitiated mouse epidermis has been obtained by Frankfort and Raitcheva (15).

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