Stimulation of the Phosphorylation of Mouse Epidermal Histones by Tumor-promoting Agents

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SUMMARY

A single topical application of 0.017 μmole of the potent tumor promoter, 12-O-tetradecanoyl-phorbol-13-acetate, resulted in a stimulation of the phosphorylation of mouse epidermal histones with an early peak at 2 hr followed by peaks between 1 and 3 days after treatment. The effect of phorbol and two phorbol esters on histone phosphorylation was found to be related to their tumor-promoting activity as well as to their capacity to stimulate RNA and DNA synthesis. Stimulation of histone phosphorylation was slight following treatment with the weak promoter, phorbol-12,13-dibenzzoate, and nonexistent after treatment with phorbol, which has no tumor-promoting activity. A dose dependency relationship was established linking the ability of 12-O-tetradecanoyl-phorbol-13-acetate to stimulate histone phosphorylation with its ability to promote tumors.

Cycloheximide prevented the increase in histone phosphorylation caused by 12-O-tetradecanoyl-phorbol-13-acetate but had no effect on histone phosphorylation in control mice.

INTRODUCTION

Skin tumors can be induced in mice by a 2-step procedure. The 2nd step, called promotion, is accomplished by treatment with croton oil or with certain phorbol esters found in croton oil (2, 6, 11). Studies on the biochemistry of skin tumor promotion have shown a sequential activation of phosphatidylcholine, RNA, protein, and DNA synthesis followed by cell division in skin treated once with an appropriate tumor-promoting agent (4, 9, 15). This temporal metabolic and morphological response characteristic of gene activation occurs in a number of cell systems such as the salivary gland stimulated with isoproterenol (5), the regenerating liver (7), the uterus stimulated with 17β-estradiol (12), and lymphocytes stimulated with phytohemagglutinin (14).

It has been postulated that small chemical changes in histone molecules such as acetylation, methylation, oxidation of sulphydryl groups, and phosphorylation cause a change in DNA-histone interaction resulting in increased gene activation (1, 13). In an effort to observe any existing correlation between the phosphorylation of mouse epidermal histones, tumor promotion, and the increased epidermal macromolecular biosynthesis caused by phorbol esters, we have studied the effect and specificity of some phorbol esters on histone phosphorylation, both in the intact mouse and with skin preparations in vitro.

MATERIALS AND METHODS

Materials. Mice were female Charles River CD1 strain purchased from the Charles River Breeding Laboratories, Wilmington, Mass. Cycloheximide was purchased from the Sigma Chemical Co., St. Louis, Mo.; ATP-γ-32P (10 Ci/mmole) and H3PO4-32P (carrier free) were from New England Nuclear, Boston, Mass.; croton oil was from S. B. Penick and Co., New York, N. Y. Phorbol and phorbol esters were generously supplied by Dr. William M. Baird and Dr. John D. Scribner (synthesis described in Ref. 2).

Treatment of Mice. An area on the backs of mice (7 to 9 weeks old) approximately 2.5 x 4.5 cm was shaved with electric clippers 2 to 3 days prior to use. Mice wounded during shaving and mice in the hair regrowth stage were excluded from experimentation. The entire shaved area was evenly treated with either 0.1 ml of acetone or 0.1 ml of a solution of various compounds in acetone.

In Vivo Experiments. All mice were killed between 2 p.m. and 4 p.m. in order to decrease the influence of diurnal variation. One hr before killing, the mice were given i.p. injections with 500 μCi of H3PO4-32P. Immediately upon killing, a depilatory was applied to the backs of the mice, and after 5 min it was removed by rinsing with cold water. The skin was cut from the back of the mouse and placed in 55° water for 30 sec, followed by submersion in ice water for at least 30 sec (10). The skin was then placed epidermis-side-up on an ice-cold porcelain plate, and the epidermis was removed by scraping with a razor blade. This method yielded excellent separation of epidermis from dermis as verified by observation with the light microscope. The epidermal sheets were stored in liquid nitrogen until just prior to homogenization.

The tissue sample from each group of 5 mice was treated by the following procedure.2 The tissue was placed in 2.5 ml of

1 This work was supported in part by American Cancer Society Grant E-6M and by National Cancer Institute Grants CA-07175 and CA-05002.

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Received May 31, 1972; accepted October 11, 1972.

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ice-cold 0.05 M Tris-HCl buffer, pH 7.4, containing 0.25 M sucrose and 0.0014 M HgCl₂, and homogenized with 8 strokes in a Potter-Elvehjem homogenizer. The homogenate was filtered through 4 layers of cheesecloth and centrifuged for 10 min at 1000 X g. After the supernatant was decanted, the pellet was suspended in 2.5 ml of homogenizing medium, homogenized with 15 strokes in an ice-cold Dounce homogenizer, and then centrifuged for 10 min at 1000 X g. The pellet was washed in 5 ml of homogenizing medium. The constituents of epidermal tissue are such that nuclear preparations by the usual methods yield considerable cytoplasmic contamination. This procedure results in preparations composed of approximately 40% nuclear material as estimated by observation with the light microscope. The recovery of total epidermal DNA from the nuclei was 90%. The DNA was extracted from the tissue from duplicate groups of 5 mice by a modified Schmidt-Thannhauser procedure (16) and measured in triplicate by use of the diphenylamine reaction.

It was impractical to add additional steps in the nuclear isolation procedure because of the number of groups of mice used in these experiments. However, some experiments were performed for comparative purposes with the use of nuclei of a purer nature prepared by the procedure described under “In Vitro Experiments.”

Prior to the extraction of the epidermal histones, the pellet was washed in 5 ml of ice-cold 0.14 M NaCl with 0.01 M sodium citrate and 0.0014 M HgCl₂ followed by a wash with 5 ml of ice-cold 80% ethanol. The crude histone was then extracted with 2 ml of ice-cold 0.25 N HCl for 20 min at 0°. The supernatant was removed and saved, and a 2nd extraction was performed in the same manner. The pellets were combined, and 10 volumes of ice-cold acetone were added, and the samples were stored overnight at −20°.

In Vitro Experiments. The skins from the backs of each group of 10 topically treated mice were removed and placed in 25 ml of 0.1 M Tris-HCl buffer, pH 7.4, containing 0.008 M EDTA and 50 units of elastase. After incubation in elastase for 17 hr at 37°, each group of 10 skins was washed in 5 ml of Medium A, which contained 0.25 M sucrose, 0.025 M KCl, and 0.007 M 2-mercaptoethanol. After the supernatant was decanted, the pellet was suspended in 3 ml of Medium B which contained 1.7 M sucrose, 0.05 M Tris-HCl buffer (pH 7.4), 0.003 M MgCl₂, 0.025 M KCl, and 0.007 M 2-mercaptoethanol. The suspension was transferred to a centrifuge tube and underlaid with 1 ml of Medium B. The preparation was centrifuged in an SW 39 rotor for 30 min at 124,000 X g. The supernatant was decanted, and the inside of the tube was wiped clean. The pellet was suspended in an incubation medium containing 0.05 M Tris-HCl buffer (pH 7.4), 0.003 mM MgCl₂, 0.025 M KCl, and 0.007 M 2-mercaptoethanol and centrifuged for 10 min at 1200 X g. The wash was repeated once. This procedure yielded preparations consisting of 40 to 50% whole nuclei as estimated by the use of light microscopy. Ninety-five % of the total epidermal DNA was recovered from nuclei prepared as described above.

The pellet from each group was suspended to a volume of 2.0 ml in ice-cold incubation medium and divided equally into 2 tubes. These preparations were incubated with 25 μl of a solution of ATP-γ-32P (10 μCi) for 30 min at 37°. The tubes were placed immediately in ice, and 10 volumes of ice-cold acetone were added. The tubes were then centrifuged for 10 min at 1200 X g, and the pellets were washed twice with 5 ml of ice-cold 0.10 M NaCl with 0.01 M sodium citrate and twice with 5 ml of ice-cold 80% ethanol. The crude histone was extracted and precipitated as described in the in vivo experiments.

Cellulose Acetate Electrophoresis. The crude histone precipitates from both the in vivo and in vitro experiments were dried and subjected to cellulose acetate electrophoresis as described by Tidwell et al. (20) except that histone bands on cellulose acetate strips were dissolved in acetic acid instead of acetone:ethanol:acetic acid (3:3:1). The histones were electrophoretically separated into 2 main bands, and in most experiments these bands were treated separately. The slower migrating band comprised between 80 and 90% of the total histone, and the faster migrating band contained the remaining 10 to 20% of the histone.

RESULTS

Time Dependency. The effect of 1 topical administration of 12-O-tetradecanoyl-phorbol-13-acetate on the phosphorylation of epidermal histones was studied over varying periods of time after treatment. The mice were treated topically with either 0.1 ml of acetone or 10.5 μg (0.017 pmole) of 12-O-tetradecanoyl-phorbol-13-acetate in acetone. The results for time periods of 1 to 7 days after treatment are shown in Chart 1. Maximum phosphorylation of the electrophoretically faster migrating histones occurred 1 day after treatment (495% of control), while phosphorylation of the electrophoretically slower migrating histones occurred 3 days after treatment (395% of control). In 3 separate experiments the extent of phosphorylation at 1, 2, and 3 days only was studied, but it was difficult to ascertain, due to the inherent variations in these experiments, whether the peaks in histone phosphorylation actually occurred at 1 and 3 days or whether they coincided at some time between 1 and 3 days.

In addition to the increased phosphorylation observed between 1 and 3 days, a small but significant increase in total epidermal histone phosphorylation was observed 2 hr after 12-O-tetradecanoyl-phorbol-13-acetate administration, with the stimulation returning to control value by 4 hr after treatment (Chart 2). Radioactivity in the electrophoretically faster migrating histones was very low at these time points,
and essentially all of the activity found in the total histone was accounted for by phosphorylation of the slower migrating fraction.

Specificity. Phorbol, phorbol-12,13-dibenzoate, and 12-O-tetradecanoyl-phorbol-13-acetate have been described by Baird et al. (4) as essentially ineffective, weak, and very effective tumor promoters, respectively. The ability of these compounds to bring about a change in phosphorylation was studied, and the results are shown in Table 1. The mice were treated with 0.017 μmole of the appropriate compound and were observed after 24 hr. After treatment with 12-O-tetradecanoyl-phorbol-13-acetate, there was a dramatic increase in the phosphorylation of histones in both electrophoretic bands. Phorbol-12,13-dibenzoate (a weak promoter) caused an increased phosphorylation of histones in only the faster migrating band (210% of control), but the increase was only 14% of the increase found after 12-O-tetradecanoyl-phorbol-13-acetate treatment. Mice treated with phorbol (an essentially ineffective promoter) showed an inhibition in the phosphorylation of epidermal histones in contrast to the stimulation found after treatment with the effective tumor promoters. One application of 0.1 ml of 0.5% croton oil (a very effective promoter) caused a large increase in the phosphorylation of epidermal histones (438% of control) 24 hr after treatment.

Histone extracted from a purer preparation of epidermal nuclei than that used in the previous experiments would be expected to reveal a greater degree of phosphorylation if the phosphorylated histones were being extracted from the nuclei rather than from cytoplasmic contaminants. Table 2 presents data from an in vivo experiment with histones extracted from nuclei prepared by the procedure described under “In Vitro Experiments.” With this purer preparation, all specific activities were greater than those observed in all previous appropriate experiments in which the cruder nuclear preparation were used.

It was of interest to determine whether the ability of mouse epidermis to phosphorylate histones could be retained under in vitro conditions. For these experiments, groups of 10 mice were treated with 12-O-tetradecanoyl-phorbol-13-acetate or phorbol and a nuclear preparation was obtained and incubated with ATPγ32P as described under “In Vitro Experiments.” When nuclei were isolated and incubated at 24 hr after

<table>
<thead>
<tr>
<th>Compound</th>
<th>Histone (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Slow band</td>
</tr>
<tr>
<td>None</td>
<td>6,074</td>
</tr>
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<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
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</tr>
<tr>
<td>Phorbol-12,13-dibenzoate</td>
<td>5,729</td>
</tr>
<tr>
<td>Phorbol</td>
<td>3,745</td>
</tr>
</tbody>
</table>
Phosphorylation of Mouse Epidermal Histones

of both histone fractions following phorbol ester administration was completely prevented by 1 mg of cycloheximide applied to the same area of skin as the phorbol ester. Interestingly, the value for the slower migrating histone of the acetone control was not significantly changed by cycloheximide, while the 3.2-fold stimulation caused by phorbol ester was completely prevented by cycloheximide, yielding values well below those of the control.

Effect of Phorbol and Phorbol Esters on the Uptake of $^{32}$P into Mouse Epidermis. Groups of 4 mice were topically treated with 0.017 µmole of phorbol, phorbol-12,13-dibenoate, or 12-O-tetradecanoyl-phorbol-13-acetate and $^{32}$P uptake into the acid-soluble fraction of the epidermis was observed. Table 6 illustrates the increased uptake of $^{32}$P at 4, 8, and 24 hr after 12-O-tetradecanoyl-phorbol-13-acetate treatment. Table 6 also shows the effects of phorbol and phorbol-12,13-dibenoate on the uptake of $^{32}$P. These effects are similar to the effects of these compounds on epidermal histone phosphorylation and tumor induction. However, it appears unlikely that the stimulation of histone phosphorylation by 12-O-tetradecanoyl-phorbol-13-acetate is dependent only on this increased $^{32}$P uptake because the increased uptake is present at 4 and 8 hr as well as 24 hr after 12-O-tetradecanoyl-phorbol-13-acetate administration. Stimulation of histone phosphorylation was not found at 4 and 8 hr.

DISCUSSION

A correlation between histone phosphorylation and DNA and RNA synthesis has been demonstrated in regenerating epidermis (18). The effect of cycloheximide on the incorporation of $^{32}$P into mouse epidermal histones from phorbol-treated mice (Table 3).

In the in vitro experiments, epidermal tissue was separated from the dermis by incubation of the mouse skin in elastase at 2° for 17 hr. It has been reported by Segal et al. (17) that epidermal tissue separated by incubation with elastase and hyaluronidase at 37° for 30 min may result in degradation of f1, f3, and f2(a)2 histones. Although the incubation was at 2°, it is possible that histone-degrading enzymes were functional and that partial histone degradation resulted. However, the degree of stimulation of histone phosphorylation was similar under both in vivo and in vitro conditions. Because a nuclear preparation was used for the in vitro studies, it is likely that the observed phosphorylation occurred in the nucleus in both cases.

Dose Dependency. The tumor promotion dose-response curve of 12-O-tetradecanoyl-phorbol-13-acetate increases sharply between 0.105 and 10.5 µg. The effects of 10.5, 1.05, and 0.105 µg of the phorbol ester on histone phosphorylation were studied 24 hr after administration (Table 4). These doses have been described by Baird and Boutwell (2) as very effective, good, and very weak promoting doses, respectively. The largest dose of 12-O-tetradecanoyl-phorbol-13-acetate caused an increase in histone phosphorylation of 406% of control for the slower migrating histones and 468% of control for the faster migrating histones. Administration of 1.05 µg of the phorbol ester (good promoter) resulted in substantially lower histone phosphorylation of both histone fractions. The smallest dose of 12-O-tetradecanoyl-phorbol-13-acetate resulted in no increased phosphorylation of the slower migrating histones and a relatively small (but repeatable) increase of 151% of control in the phosphorylation of the faster migrating histones.

Effect of Cycloheximide. It has been demonstrated that cycloheximide greatly inhibits the incorporation of leucine-$^3$H into mouse skin protein (19) and the incorporation of lysine-$^3$H into mouse epidermal histones (manuscript in preparation). Table 5 illustrates the effect of cycloheximide on the 12-O-tetradecanoyl-phorbol-13-acetate-induced phosphorylation of epidermal histones. The increased phosphorylation

<table>
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<tr>
<th>Compound</th>
<th>Histone (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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</tr>
<tr>
<td>None</td>
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</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>54,390</td>
</tr>
<tr>
<td>Phorbol</td>
<td>7,511</td>
</tr>
</tbody>
</table>

Table 3
In vitro phosphorylation of mouse epidermal histone

Groups of 10 mice were treated with either 0.1 ml of acetone or 0.017 µmole of 12-O-tetradecanoyl-phorbol-13-acetate or phorbol dissolved in acetone and killed 24 hr after treatment. Nuclei were isolated as described in the "In Vitro Experiments" and incubated with ATP-γ-$^{32}$P.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Histone (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1707</td>
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<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>5870</td>
</tr>
<tr>
<td>Phorbol</td>
<td>2676</td>
</tr>
</tbody>
</table>

Table 4
Dose-response effect of 1 application of 0.00017, 0.0017, or 0.017 µmole of 12-O-tetradecanoyl-phorbol-13-acetate on the phosphorylation of mouse epidermal histones

Groups of 5 mice were treated with either 0.1 ml of acetone or phorbol ester dissolved in acetone and killed 3 days after treatment. Each mouse was given an i.p. injection of 500 µCi of H$_2$PO$_4$$^{-32}$P 1 hr before killing.

<table>
<thead>
<tr>
<th>12-O-Tetradecanoyl-phorbol-13-acetate (µg)</th>
<th>Histone (dpm/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Slow band</td>
</tr>
<tr>
<td></td>
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</tr>
<tr>
<td>0.105</td>
<td>4,870</td>
</tr>
<tr>
<td>1.05</td>
<td>8,645</td>
</tr>
<tr>
<td>10.5</td>
<td>20,190</td>
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liver. It has been postulated that this phenomenon occurs in the nucleus after the synthesis of the histones is completed, and it has been proposed that the phosphorylation affects DNA-histone interaction causing an increase in template activity (13).

In whole mouse skin, following 1 topical application of croton oil, there is a rapid increase in appropriate radioactive precursor incorporation into RNA, protein, and DNA with peak incorporation times occurring at 6, 12, and 18 hr, respectively (9). Similar observations were made in mouse epidermal tissue after treatment with phorbol esters (3, 4).

In these studies we have shown that there is a correlation between the effect of phorbol esters on histone phosphorylation, macromolecular biosynthesis, and skin tumor promotion. Stimulation of histone phosphorylation has been associated with template activation, and these observations provide further support for the relevance of gene activation to the phenomenon of tumor promotion in mouse skin.

In mice treated with 1 application of 10.5 μg of 12-O-tetradecanoyl-phorbol-13-acetate (0.017 μmole), a peak in the phosphorylation of the electrophoretically slower migrating epidermal histones occurred at 3 days after the administration of the phorbol ester. Maximum phosphorylation of the electrophoretically faster migrating histones was observed 1 day after treatment. These 2 peaks coincide to a reasonable degree with the times of maximum DNA synthesis of 18 hr and 2 days found by Baird et al. (4). In addition, a minor peak in histone phosphorylation was observed 2 hr after treatment with 12-O-tetradecanoyl-phorbol-13-acetate. This occurs prior to the time of maximum RNA synthesis which is found 6 hr after 12-O-tetradecanoyl-phorbol-13-acetate administration (4). The relationship of the early peak in histone phosphorylation with stimulated RNA synthesis is strengthened by the observation that significant increases in the synthesis of specific species of RNA occurred as early as 30 min after treatment with 12-O-tetradecanoyl-phorbol-13-acetate (3).

There is no direct way now available for establishing the relevance of histone phosphorylation to the tumor promotion process. However, correlation was found between structure, promoting activity, and degree of phosphorylation. Relevance of phosphorylation induced by the highly effective promoter, 12-O-tetradecanoyl-phorbol-13-acetate is further strengthened by the parallelism between dose dependency for tumor formation and phosphorylation.

Topical administration of 1 mg of cycloheximide 1 hr before treatment with 12-O-tetradecanoyl-phorbol-13-acetate prevents a 4-fold stimulation of incorporation of lysine-3H into epidermal histones and causes a 55% inhibition of incorporation into histones of the control mice (manuscript in preparation). In the present studies, we have found that cycloheximide also prevents the 12-O-tetradecanoyl-phorbol-13-acetate-induced stimulation of the phosphorylation of the histones in both electrophoretic bands. Cycloheximide did not, however, significantly affect the phosphorylation of the slower migrating histones (which comprise 80 to 90% of the total histone) of the control mice. One explanation for these observations may be the possibility that the phorbol ester-stimulated increase in histone phosphorylation takes place during histone synthesis. However, because the slower migrating histones of the control group are unaffected by cycloheximide and because cycloheximide greatly inhibits tritiated precursor incorporation into epidermal histones, it is likely that histone phosphorylation in the absence of phorbol ester treatment does not occur during histone synthesis. Furthermore, the stimulation of histone phosphorylation observed by incubating nuclei (with ATPγ32P) isolated from 12-O-tetradecanoylphorbol-13-acetate-treated mice is consistent with the possibility that stimulation of histone phosphorylation observed in vivo occurs in the nucleus. Although the nature of the nuclear preparation does not discount the involvement of cytoplasmic contamination, no evidence has been previously presented demonstrating the phosphorylation of histones during histone synthesis. One alternate possibility

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**Table 5**
The effect of cycloheximide on the 12-O-tetradecanoyl-phorbol-13-acetate stimulation of mouse epidermal histone phosphorylation

Groups of 5 mice were treated with either 0.1 ml of acetone or 0.017 μmole of 12-O-tetradecanoyl-phorbol-13-acetate dissolved in acetone and killed at various times after treatment. One hr before, 0.1 ml of acetone or 1 mg of cycloheximide in acetone was applied topically. Each mouse was given an i.p. injection of 500 μCi of H3PO4·32P 1 hr before killing.

<table>
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<th>Compound</th>
<th>Cycloheximide</th>
<th>Histone (dpm/mg)</th>
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<td></td>
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<td>–</td>
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<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>–</td>
<td>10,190</td>
</tr>
<tr>
<td>None</td>
<td>+</td>
<td>3,122</td>
</tr>
<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>+</td>
<td>1,740</td>
</tr>
</tbody>
</table>

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**Table 6**
The effect of 1 application of 0.017 μmole of 12-O-tetradecanoylphorbol-13-acetate, phorbol-12,13-dibenzoate, or phorbol on the uptake of 32P into the mouse epidermis

Groups of 4 mice were treated with either 0.1 ml of acetone or phorbol ester dissolved in acetone and killed at various times after treatment. Epidermal tissue was obtained by treatment at 55°as described in “Materials and Methods.” The tissue from each group was extracted by homogenizing with 0.25 N HCl. Phosphorus determinations were performed by the methods of Chen et al. (8).

<table>
<thead>
<tr>
<th>Compound</th>
<th>t (hr)</th>
<th>Phosphorus (dpm/mg ± av. deviation)</th>
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<td>4</td>
<td>65.9 ± 14.9</td>
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<td>151 ± 6.8</td>
</tr>
<tr>
<td>None</td>
<td>8</td>
<td>76.9 ± 17.8</td>
</tr>
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<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
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<td>157 ± 31.2</td>
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<tr>
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<td>24</td>
<td>45.8 ± 4.7</td>
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<tr>
<td>12-O-Tetradecanoyl-phorbol-13-acetate</td>
<td>24</td>
<td>121 ± 10.5</td>
</tr>
<tr>
<td>Phorbol-12,13-dibenzoate</td>
<td>24</td>
<td>77.7 ± 14.5</td>
</tr>
<tr>
<td>Phorbol</td>
<td>24</td>
<td>48.2 ± 0.5</td>
</tr>
</tbody>
</table>
may be that the prevention of the 12-O-tetradecanoyl-phorbol-13-acetate stimulation of histone phosphorylation by cycloheximide is due to inhibition of new protein kinase synthesis.

A correlation was observed between the tumor-promoting activities of phorbol, phorbol-12,13-dibenzzoate, and 12-O-tetradecanoyl-phorbol-13-acetate and the abilities of these compounds to cause an increase in the uptake of $^{32}$P into epidermal tissue. This increase may be a reflection upon the ability of these compounds to interact with membranes, a likely site of action for promoting agents (18). The increase in $^{32}$P uptake may also be responsible for the stimulation of histone phosphorylation that occurs following phorbol ester administration. However, the increase in the uptake of $^{32}$P occurs at 4 and 8 hr after treatment; these are periods when no histone phosphorylation is observed. Therefore, if the increase in the uptake of $^{32}$P is involved, it is probably not the sole factor governing the stimulation of histone phosphorylation.

The present studies show that phosphorylation of histones occurs in mouse epidermis under both in vivo and in vitro conditions. At least 2 time-dependent peaks in histone phosphorylation were observed in vivo following 12-O-tetradecanoyl-phorbol-13-acetate treatment, and these peaks may be related to the peaks in RNA and DNA synthesis in mouse epidermis observed by Baird et al. (3, 4). Furthermore, the degree of stimulation in histone phosphorylation was related to the level of the tumor-promoting activity of the phorbol derivative and to the dose of the phorbol ester. It appears that histone phosphorylation may be an essential component of the promotion stage of skin tumor formation and that a role for gene activation in the process is indicated.

ACKNOWLEDGMENTS

We wish to thank Dr. Thomas Slaga for his helpful suggestions.

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Stimulation of the Phosphorylation of Mouse Epidermal Histones by Tumor-promoting Agents

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