Effects of 3-Isobutyl-1-methylxanthine and Cyclic Nucleotides on 12-O-Tetradecanoylphorbol-13-acetate-induced Ornithine Decarboxylase Activity in Mouse Epidermis in Vivo

Jean-Pierre Perchellet and R. K. Boutwell

ABSTRACT

A single topical application of 8.5 nmol of 12-O-tetradecanoylphorbol-13-acetate (TPA) to mouse skin induced rapid and transient increases in the levels of both guanosine cyclic 3':5'-monophosphate and adenosine cyclic 3':5'-monophosphate, followed by the stimulation of ornithine decarboxylase (ODC, L-ornithine carboxy-lyase, EC 4.1.1.17) activity. Peak syntheses of guanosine cyclic 3':5'-monophosphate and adenosine cyclic 3':5'-monophosphate were achieved within 10 and 60 min, respectively, while ODC activity was maximally stimulated between 4.5 and 6 hr. The increased levels were dose dependent and correlated with the tumor-promoting abilities of diverse compounds. However, the magnitudes of the cyclic nucleotide and ODC responses to TPA were quite different (3- to 7- and 70-fold, respectively).

A single topical application of 10 μmol of 3-isobutyl-1-methylxanthine (IBMX), which was able to raise the levels of cyclic nucleotides almost as much as did TPA, produced only a 9-fold increase in ODC activity. IBMX, as well as other phosphodiesterase inhibitors, enhanced the magnitude and the duration of the increases in cyclic nucleotide levels and ODC activity produced by TPA. Maximum stimulation of TPA-induced ODC activity was achieved when IBMX was applied within the 30-min time interval preceding TPA treatment. As a result of the IBMX pretreatment, the same or higher levels of cyclic nucleotides and ODC activity could be induced with about 10 times less TPA or with weak and moderate tumor promoters, as compared with the increased levels attributable to TPA alone. However, in the presence of IBMX, a discrepancy was still observed between the magnitude of the increased cyclic nucleotide response to TPA (6- to 14-fold) and the 150-fold induction of ODC activity produced by TPA.

In addition, single or combined topical treatments with guanosine cyclic 3':5'-monophosphate and adenosine cyclic 3':5'-monophosphate were unable to mimic the effect of TPA on ODC activity and even depressed significantly basal and TPA-induced ODC activities in the presence of IBMX. Therefore, the findings presented in this and an accompanying paper suggest that, in mouse epidermis in vivo, if TPA-induced cyclic nucleotides participate in the molecular mechanism by which ODC activity is induced, their controlled degradation may be equally important.

INTRODUCTION

In the 2-step model system for the production of skin tumors in mice, repeated topical applications of the potent tumor promoter TPA2 initiated skin led to the formation of skin papillomas and carcinomas (7). The molecular basis for the action of TPA in mouse epidermis is not understood. Most of the early biochemical responses observed following TPA treatment (reviewed in Ref. 7), particularly the stimulation of polyamine synthesis (6, 37, 38), have been proposed to be necessary components of the promotion process. However, little is known about the biochemical mechanism by which signals resulting from TPA contact with the plasma membrane could be transmitted into the interior of the epidermal cell to induce gene expression.

In other systems, an increasing body of literature indicates that ODC activity may be regulated via stimulation of a cyclic nucleotide-dependent process (reviewed in Ref. 47). The presence of active adenylate cyclase, guanylate cyclase, phosphodiesterases, and protein kinases has been shown in the epidermis (26, 32, 53, 59). The possibility that some of the effects of TPA might be mediated by phosphorylation of cellular components through a cyclic nucleotide-dependent process is quite attractive. Although conflicting data have been reported concerning early changes in cyclic nucleotide levels after TPA treatment (reviewed in Ref. 41), regulation of the enzymes controlling the metabolism of the cyclic nucleotides by TPA has been described (32, 34, 45, 53, 57). Recently, investigations in this laboratory demonstrated that TPA did stimulate rapidly the production of cyclic GMP and cyclic AMP in vitro, using suspensions of freshly isolated epidermal cells (41). In the same system, time- and dose-dependent relations were observed between TPA-increased cyclic nucleotide levels and ODC activity. Moreover, the phosphodiesterase inhibitor IBMX and the adenylate cyclase stimulator cholera toxin that raised cyclic AMP levels also induced ODC activity and further enhanced the stimulation of ODC activity produced by TPA (41).

In the present study, the magnitude, the duration, and the specificity of the increases in cyclic nucleotide levels and ODC activity were determined in mouse epidermis in vivo at early stages following topical treatment of mouse skin with IBMX, cyclic nucleotides, and/or agents of varying tumor-promoting abilities. New evidence is provided that alterations in the mag-

1 This material was presented in part at the Annual Meeting of the American Association for Cancer Research, San Diego, Calif., May 1980 (42). This investigation was sponsored by Grants CA-07175 and CA-22484 from the NIH. This is Paper 1 in a series exploring the role of cyclic nucleotides in the mechanism of action of TPA. Ref. 43 is the following paper in this series.

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2 The abbreviations used are: TPA, 12-O-tetradecanoylphorbol-13-acetate; ODC, ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17); cyclic GMP, guanosine cyclic 3':5'-monophosphate; cyclic AMP, adenosine cyclic 3':5'-monophosphate; IBMX, 3-isobutyl-1-methylxanthine; PDD, phorbol-12,13-didecanoate; PDB, phorbol-12,13-dibenoate; PDA, phorbol-12,13-diacetate; DMBA, 7,12-dimethylbenz[a]anthracene.
plitude and the duration of the early and transient elevation of cyclic GMP and cyclic AMP observed after TPA may modulate the effect of the tumor promoter on ODC activity.

MATERIALS AND METHODS

Chemicals. TPA, mezerein, PDD, PDB, PDA, and phorbol were all purchased from Peter Borchert, Chemical Carcinogenesis (Eden Prairie, Minn.), and DMBA was from Eastman Organic Chemicals (Rochester, N. Y.). Cyclic AMP, cyclic GMP, aminophylline, caffeine, papaverine, theophylline, crystalline bovine serum albumin, and L-ornithine hydrochloride were obtained from Sigma Chemical Co. (St. Louis, Mo.), and IBMX was from Aldrich Chemical Co. (Milwaukee, Wis.). Cyclic \[^{3}H\]GMP (39.9 Ci/mmol), cyclic \[^{14}C\]AMP (40 mCi/mmol), and dl-\[^{14}C\]ornithine hydrochloride (52.8 mCi/mmol) were purchased from New England Nuclear (Boston, Mass.). Ion-exchange resins were obtained from Bio-Rad (Rockville Center, N. Y.). Calf thymus DNA was from Calbiochem-Behring Corp. (La Jolla, Calif.). All other chemicals were reagent grade and were obtained commercially.

Treatment of Mice. Female Charles River CD-1 mice (Wilmington, Mass.), housed in screen-bottomed stainless steel cages in light- and temperature-controlled rooms with food and water ad libitum, were used for experimentation when 7 to 9 weeks old. The dorsal skin of the mice was shaved at least 2 days before treatment, and only those mice showing no hair regrowth were used for experimentation. The solutions of mezerein, phorbol, the 4 phorbol diesters, and DMBA were prepared in acetone and delivered to the shaved backs of individual mice in a volume of 0.2 ml. The phosphodiesterase inhibitors used for these experiments, dissolved in 96% acetone-4% water, were applied to mouse skin in 0.2-ml volumes, with the exception of the dose of 5 \(\mu\)mol of papaverine which was given in 2 consecutive applications of 0.2 ml, each with 2.5 \(\mu\)mol. The doses of 0.25 \(\mu\)mol of cyclic AMP or 0.16 \(\mu\)mol of cyclic GMP were delivered topically to mouse skin in 2 consecutive volumes of 0.2 ml of 96% acetone-4% water, each containing 0.125 or 0.08 \(\mu\)mol, respectively. Multiple treatments were administered at twice-weekly intervals; the mice were given the phosphodiesterase inhibitors and/or the cyclic nucleotides immediately before and to the same area of skin as the application of TPA or the other agents of varying tumor-promoting abilities. In every experiment, all mice received the same volume of solvent.

Determination of Cyclic GMP and Cyclic AMP Levels. At the designated time after treatment, the mice were killed by cervical dislocation and immediately frozen flat on solid CO\(_2\). After immersion for 15 sec in liquid N\(_2\), the epidermis were scraped off with a surgical blade, and the scrapings from one mouse were immediately homogenized in 5 ml of ice-cold 5% trichloroacetic acid with a Polytron PT 10 homogenizer for 3 x 15 sec at Setting 6. Trace amounts of cyclic \[^{3}H\]GMP and cyclic \[^{14}C\]AMP (each with 5000 dpm) were added to the samples to correct for the recoveries of the 2 cyclic nucleotides, and the homogenates were processed as described previously (41). Briefly, cyclic AMP and cyclic GMP were purified from the ether-extracted acid-soluble samples on 8 x 0.5-cm Dowex AG 1-X8 columns (200 to 400 mesh) in the formate form. After the above column was washed with 10 ml of 0.1 n formic acid, complete separation of the 2 cyclic nucleotides was achieved by sequential elution with 7 ml of 2 n formic acid and 7 ml of 4 n formic acid (33), as assessed by counting aliquots of the 2 fractions for \(^{3}H\) and \(^{14}C\) dpm of the tracers. Recovery of each cyclic nucleotide was at least 75%. After lyophilization, the cyclic AMP and cyclic GMP-containing samples were reconstituted in 1 ml of 50 mM sodium acetate buffer, pH 6.2. The cyclic AMP (50) and succinylated cyclic GMP (16) contents were determined by radioimmunoassay, using the reagents, antigens, and antibodies supplied by Collaborative Research, Inc. (Waltham, Mass.). Under these conditions, the assays of cyclic AMP and cyclic GMP were highly sensitive and specific; cross-reactivity of even closely related nucleotides was minimal.

Determination of ODC Activity. At the appropriate time after treatment, the mice were killed by cervical dislocation and epidermis from individual mice was separated by a brief heat treatment (57° for 30 sec) as described previously (27). The epidermal preparations from 2 mice were pooled in 2 ml of 25 mM Tris-HCl buffer, pH 7.6, containing 4 mM dithiothreitol, 1 mM EDTA, and 0.2 mM pyridoxal 5'-phosphate, homogenized with a Polytron PT 10 homogenizer for 10 sec at Setting 6, and centrifuged at 30,000 \(\times\) g for 30 min to give a soluble supernatant (38). ODC activity was determined in 0.2-ml aliquots of the clear supernatants by measuring the release of \(^{14}C\)CO\(_2\) from dl-\[^{1-14}C\]ornithine hydrochloride as described previously (54). The substrate concentration routinely used was 0.4 mM L-ornithine hydrochloride containing 0.5 \(\mu\)Ci of dl-\[^{1-14}C\]ornithine hydrochloride. The assays were carried out in duplicate, and all values were corrected against no enzyme or boiled enzyme blanks; ODC activity was expressed as nmol of CO\(_2\) released in 60 min per mg of protein.

DNA and Protein Determinations. The acid-insoluble pellet obtained after cyclic nucleotide extraction was washed twice with 2 ml of ice-cold 0.5 n perchloric acid and once with 2 ml of 100% ethanol, and the DNA was hydrolyzed in 2 ml of 0.5 n perchloric acid for 10 min at 90°. DNA content in the clear supernatant was determined by the diphenylamine procedure of Burton (6) with calf thymus DNA as the standard. The protein concentration of the epidermal extracts was assayed with Bio-Rad dye reagent, using crystalline bovine serum albumin as the standard.

RESULTS

Effects of IBMX on the Time Courses for the Increases in Cyclic Nucleotide Levels and ODC Activity by TPA. A single topical application of 8.5 nmol of TPA to mouse skin induced rapid increases (3- to 7-fold) in the levels of epidermal cyclic GMP and cyclic AMP relative to DNA content within the 120-min time interval studied. As shown in Chart 1, the level of cyclic GMP increased within 2 min of TPA treatment, reaching a peak at 10 min and declining to the basal level by 60 min. In contrast, the cyclic AMP level was increased 10 min after TPA treatment and continued to rise until 60 min before declining. Acetone treatment did not induce any significant change in the basal levels of either cyclic nucleotide over the course of the experiment. As reported previously (6, 37, 38), epidermal ODC activity was maximally induced (70-fold) between 4 and 6 hr following TPA treatment and returned to basal levels beyond a 12-hr period (Chart 2).

Treatment of the same area of skin with 10 \(\mu\)mol of IBMX elicited a slow but steady accumulation of cyclic GMP and cyclic AMP (Chart 1). The effect of IBMX seemed to fade beyond 60 min. In contrast with TPA, the 3- to 5-fold increases in cyclic nucleotide levels produced by IBMX were accompanied by only a 9-fold enhancement of basal ODC activity at 5 hr (Chart 2).

Topical application of IBMX immediately before TPA enhanced (about 2-fold) the magnitude of both the cyclic nucleotide and the ODC responses to TPA (Charts 1 and 2, respectively) without altering the general time courses observed previously. However, as a result of the IBMX pretreatment, the peak levels of cyclic GMP, cyclic AMP, and ODC activity were broadened so that the same or higher levels of cyclic nucleotides, compared with the response to TPA alone, were maintained for about 90 min longer, and the ODC activity exceeded that induced by TPA alone through the period from 2.5 to 9 hr.

Effects of the Dose and Time of Treatment with IBMX on the Induction of ODC Activity by TPA. As shown in Table 1,
stimulations of basal and TPA-induced ODC activities by topical applications of IBMX were dose dependent. The induction of ODC activity became apparent with a dose of 0.5 µmol of IBMX and was stimulated maximally at 10 to 20 µmol of IBMX. The effects of IBMX and TPA were clearly more than additive.

The stimulation of TPA-induced ODC activity was dependent on the time of IBMX application. Maximal stimulation occurred when IBMX was given concurrently with TPA or 30 min before TPA application. Effectiveness was rapidly lost at treatment times further from the time of application of TPA (Chart 3), so that the ability of IBMX to potentiate the effect of TPA was reduced by more than 50% when it was applied to mouse skin 2 hr before or after the treatment with TPA. This is in accord with the observation that IBMX becomes ineffective in preventing the degradation of epidermal cyclic nucleotides beyond 60 min of its application to the skin. However, addition of TPA 4 to 6 hr after IBMX treatment, at a time when basal ODC activity was maximally stimulated by the IBMX pretreatment (Chart 2), resulted in a slight but significant increase of the ODC response to TPA.

**Effects of IBMX on the Dose-dependent Stimulations of Cyclic Nucleotide Levels and ODC Activity by TPA.** In order to determine the levels of both cyclic nucleotides in the same epidermal extracts, 20 min was selected as the best time interval following TPA treatment to measure nearly peak levels of cyclic GMP and cyclic AMP in the presence and absence of IBMX. As shown in Chart 4, both cyclic GMP and cyclic AMP levels, as well as ODC activity, were stimulated in the same dose-dependent manner by TPA. The lowest dose of TPA eliciting a significant increase in both cyclic nucleotide levels and ODC activity was 0.07 nmol; maximal levels occurred with 5.66 and 17 nmol of TPA.

The effect of each dose of TPA tested was enhanced after IBMX treatment (Chart 4). Therefore, as a result of the IBMX treatment, a dose of TPA about 10 times lower than that routinely used in a single treatment was sufficient to induce

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**Table 1**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC activity (nmol CO₂ in 60 min/mg protein)</th>
<th>+ acetone</th>
<th>+ TPA (0.5 nmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetone</td>
<td>0.115 ± 0.019#</td>
<td>4.432 ± 0.310</td>
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</tr>
<tr>
<td>IBMX (0.1 µmol)</td>
<td>0.125 ± 0.018b</td>
<td>4.601 ± 0.320#</td>
<td></td>
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<tr>
<td>IBMX (0.5 µmol)</td>
<td>0.164 ± 0.022d</td>
<td>5.067 ± 0.356#</td>
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<td>IBMX (1 µmol)</td>
<td>0.198 ± 0.037</td>
<td>5.664 ± 0.452</td>
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<tr>
<td>IBMX (2.5 µmol)</td>
<td>0.261 ± 0.050</td>
<td>7.357 ± 0.685</td>
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</tr>
<tr>
<td>IBMX (5 µmol)</td>
<td>0.392 ± 0.060</td>
<td>9.312 ± 0.708</td>
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<td>IBMX (10 µmol)</td>
<td>0.467 ± 0.068</td>
<td>13.865 ± 1.040</td>
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<td>IBMX (15 µmol)</td>
<td>0.487 ± 0.090</td>
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<tr>
<td>IBMX (20 µmol)</td>
<td>0.494 ± 0.086</td>
<td>14.710 ± 1.180</td>
<td></td>
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*a* Mean ± S.D. 
*b* Results not significant versus acetone plus acetone. 
*c* Results not significant versus acetone plus TPA. 
*d* p < 0.005, significantly greater versus acetone plus TPA. 
*e* p < 0.01, significantly greater versus acetone plus TPA.
Effects of Multiple Applications of IBMX and TPA on Induction of Epidermal ODC Activity. Development of skin tumors requires repeated applications of a tumor promoter to initiated skin. Furthermore, it has been shown previously that repeated applications of TPA led to much greater increases in induction of ODC activity than those observed after the first treatment (38, 56). Therefore, it is of interest to determine if the phosphodiesterase inhibitor IBMX is able to further enhance the greater induction of ODC activity produced by repeated TPA treatments. The levels of ODC induction were determined following multiple applications of IBMX plus TPA to mouse skin initiated with DMBA. The results shown in Chart 5 indicate that the degree of stimulation of ODC activity by TPA was increased about 2-fold after the second TPA treatment and plateaued at about 2.5-fold between the sixth and tenth applications of TPA, as compared with the response to the first TPA treatment. IBMX, which has been shown to enhance the induction of ODC activity produced by a single application of TPA, was able to further increase the greater ODC responses elicited by each successive promoter treatment.

Effects of IBMX on the Cyclic Nucleotide and ODC Responses to Compounds of Varying Tumor-promoting Abilities. Within a series of agents of varying tumor-promoting potencies, the magnitude of the increases in cyclic nucleotide levels and ODC activity correlated with their promoting abilities (3, 37) (Chart 6). Phorbol and the nonpromoting phorbol ester, PDA, caused no detectable increase in the levels of either cyclic GMP, cyclic AMP, or ODC activity, whereas PDB, PDD, or TPA treatments increased the cyclic nucleotide levels by about 2-, 3-, and 5-fold, respectively, and induced ODC activity by about 10-, 20-, and 50-fold, respectively. Mezerein, which is not a phorbol derivative, is much less active than TPA to promote tumor formation but is very effective in Stage II promotion (30, 49). However, mezerein caused increases in cyclic nucleotide levels and ODC activity comparable with those induced by TPA. A single topical application of 10 μmol of IBMX enhanced by about 2-fold the stimulation of cyclic nucleotide levels and ODC activity produced by each of the above compounds (Chart 6). Moreover, after IBMX pretreatment, the levels of cyclic nucleotides and ODC activity produced by the weak or moderate tumor promoters were boosted to values corresponding to the effects of the strong promoting agents in the absence of IBMX. For instance, the moderate tumor promoter PDD was able, in the presence of IBMX, to increase the levels of cyclic GMP, cyclic AMP, and ODC activity as much as the powerful TPA did alone.

Effects of Other Phosphodiesterase Inhibitors. Not only...
IBMX but a variety of other phosphodiesterase inhibitors as well were able to increase basal and TPA-stimulated cyclic nucleotide levels and ODC activities (Chart 7). Their effectiveness as stimulators of the cyclic nucleotide and ODC responses to TPA was as follows. Papaverine and IBMX appeared to be the most effective, followed by caffeine and aminophylline, with theophylline having a much lesser effect. Although papaverine- and IBMX-increased cyclic nucleotide levels were comparable to those produced by TPA, the ODC inductions that resulted from papaverine and IBMX treatments were 7 to 9 times smaller than the ODC response to TPA. In the presence of the above compounds, the degree of stimulation of ODC activity by TPA was also much greater than that of the cyclic nucleotides.

Effects of Cyclic Nucleotides on Basal and TPA-induced ODC Activities. If the TPA-increased levels of cyclic nucleotides play some role in the regulation of ODC (41), then elevations in epidermal cyclic GMP and cyclic AMP produced in vivo by phosphodiesterase inhibitors and/or exogenously added cyclic nucleotides should mimic the enzymic induction caused by the tumor promoter. As shown in Table 2, topical applications of cyclic GMP or cyclic AMP to mouse skin were totally ineffective in raising, and even slightly depressed, basal and TPA-stimulated ODC activities; additive inhibitory effects were observed when the 2 cyclic nucleotides were applied together. No change in ODC activity was observed when the cyclic nucleotides were applied 4 hr prior to the acetone, TPA, or IBMX plus TPA treatments (data not shown), suggesting that the enzyme inhibition by cyclic GMP and cyclic AMP was not caused by a local toxic effect. Since applications of large amounts of exogenous cyclic nucleotides could trigger the activation of epidermal cyclic nucleotide phosphodiesterases, combined treatments including IBMX, cyclic GMP, and cyclic AMP were applied. In the presence of IBMX, cyclic GMP and cyclic AMP treatments were again unable to reproduce the activity of TPA and very significantly reduced the enhancement of basal and TPA-induced ODC activities caused by IBMX alone. Moreover, incubation of isolated epidermal cells (41) with 1 μM to 1 mM cyclic GMP, cyclic AMP, or their dibutyryl derivatives resulted in a concentration-dependent inhibition of basal and TPA-induced ODC activities, whether or not 0.5 mM IBMX was present in the incubation medium (data not shown). A dye exclusion test performed at the end of each incubation period revealed no change in cell viability, and normal ODC induction by TPA was restored following replacement of the cells in cyclic nucleotide-free medium. The same doses of sodium butyrate did not elicit any modification of basal and TPA-induced ODC activities.

DISCUSSION

Recent evidence of sequential oscillations in the levels of both cyclic GMP and cyclic AMP induced by TPA (22, 41, 46) but not by nonpromoting phorbol esters (46) has been reported in vitro. In mouse epidermis (36, 58) and various cell systems (15, 51), it is noteworthy that cyclic GMP levels are increased...
within minutes of TPA treatment. However, the effect of TPA on cyclic AMP is more controversial (4, 21, 31, 36, 53, 55). Other reports concerning the impaired balance of cyclic nucleotides at 6 (44), 12 (19), and 36 (5) hr after single or multiple (17) treatments with TPA, when TPA-induced phenomena have already been maximally stimulated, reflect the high ratio of cyclic GMP to cyclic AMP characteristic of benign and malignant proliferative skin diseases (59) but do not preclude the involvement of cyclic nucleotides at earlier stages of the mechanism of action of TPA. In accord with some of the previous observations reported in the literature (22, 46) and with our recent findings in vitro using isolated epidermal cells (41), this paper extends to mouse epidermis in vivo in that

TPA has been demonstrated to induce the activity of the enzymes controlling the metabolism of cyclic nucleotides, including adenylate cyclase (45), guanylate cyclase (45, 57), and phosphodiesterases (32, 53). Therefore, the early responses in cyclic nucleotide levels observed in our system and elsewhere suggest that TPA may interact primarily at the plasma membrane level, allowing a sequential stimulation of the synthesis and subsequent degradation of cyclic GMP and cyclic AMP, resulting in the early and transient accumulation of cyclic nucleotides. At physiological concentrations of substrate, the rate of cyclic GMP hydrolysis by cytoplasmic phosphodiesterase is usually greater than that of cyclic AMP (26). Therefore, after TPA treatment, newly synthesized cyclic GMP may simply be degraded much faster than is cyclic AMP, resulting in a cyclic AMP level that is still increasing while cyclic GMP is declining. Epidermal phosphodiesterase activity, which may be induced as early as 10 min after TPA treatment as suggested by our data, has been demonstrated to reach higher activities later (32, 53), thus allowing the degradation of the accumulated cyclic AMP.

It is difficult to pinpoint the reasons why, in an earlier work, no significant change in cyclic nucleotide levels could be detected after TPA treatment using epidermal-dermal preparations (29). The inability to measure increased level of cyclic nucleotides does not mean that they have not occurred. The use of a potent phosphodiesterase inhibitor (IBMX) that enhanced the magnitude and the duration of the cyclic nucleotide response to TPA was particularly helpful in our studies in vitro (41) and in vivo, since epidermal cyclic GMP and cyclic AMP seem to be rapidly synthesized and rapidly turned over after TPA treatment. However, we did not determine the possible changes of cyclic nucleotide levels in the extracellular compartment that could result from cyclic nucleotide excretion from the cells. In our experiments, the levels of epidermal cyclic GMP and cyclic AMP, expressed per µg of DNA, are in the fmol and pmol ranges, respectively. Since 2 × 10^6 isolated epidermal cells contain about 10 µg of DNA, the levels of cyclic nucleotides measured in vivo appear to be about 5 times lower than those assayed in vitro (41). Furthermore, in vitro, the changes in cyclic nucleotide levels are determined in a rather purified epidermal preparation containing a high ratio of active and TPA-responsive isolated basal cells (41), whereas in vivo the variations of cyclic nucleotide levels are determined in scrapings of epidermis with probably a lower ratio of basal cells due to the presence of all other more differentiated and keratinized cell types. In addition, we were obliged to compromise for an experimental time interval of 20 min after TPA treatment, giving near-maximal levels for both cyclic GMP and

Table 2

<table>
<thead>
<tr>
<th>Treatment</th>
<th>ODC activity (nmol CO₂ in 60 min/mg protein)</th>
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</thead>
<tbody>
<tr>
<td>+ acetone</td>
<td>+ TPA (8.5 nmol)</td>
</tr>
<tr>
<td>Acetone</td>
<td>0.134 ± 0.018 ± a 5.187 ± 0.306</td>
</tr>
<tr>
<td>IBMX</td>
<td>0.336 ± 0.045 ± b 9.873 ± 0.630</td>
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<tr>
<td>Cyclic AMP</td>
<td>0.098 ± 0.014 ± c 4.513 ± 0.275</td>
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<td>Cyclic GMP</td>
<td>0.125 ± 0.015 ± d 4.938 ± 0.281</td>
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<tr>
<td>IBMX + cyclic AMP</td>
<td>0.231 ± 0.028 ± f 7.787 ± 0.610</td>
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<tr>
<td>IBMX + cyclic GMP</td>
<td>0.267 ± 0.032 ± g 8.455 ± 0.575</td>
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<tr>
<td>IBMX + cyclic AMP + cyclic GMP</td>
<td>0.208 ± 0.036 ± h 6.712 ± 0.507</td>
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a Mean ± S.D.
b Results not significant versus acetone plus acetone.
c Results not significant versus acetone plus TPA.
d p < 0.01, significantly smaller versus IBMX plus acetone.
cyclic AMP (Chart 1). Therefore, it is obvious that the values reported in Charts 4, 6, and 7 represent only 70 to 80% of the phenomenon studied, as compared with the values of cyclic GMP and cyclic AMP that could have been obtained at 10 and 60 min, respectively.

The exact role of this early and transient increase in cyclic GMP and cyclic AMP levels in the mechanism of action of the tumor promoters remains to be determined. ODC activity has been induced in vivo and in vitro by raising the extracellular concentration of cyclic nucleotides (reviewed in Ref. 41) or by elevating intracellular cyclic nucleotides with the use of compounds known to affect the activity of the enzymes controlling the metabolism of the cyclic nucleotides such as phosphodiesterase inhibitors (1, 9, 10, 12, 23, 41, 48), cholera toxin (35, 41), prostaglandins (1, 23, 40), adrenergic agents (1, 52), and growth factors and diverse hormones (reviewed in Ref. 24).

Furthermore, sequential increases in cyclic nucleotide levels, cyclic nucleotide-dependent protein kinase, ODC, and RNA polymerase I activities have been described (2, 14, 25, 28, 39, 47). In quiescent cultures of Chinese hamster ovary cells, the major action of cyclic AMP during the initial 2 hr of the induction of ODC activity by serum is thought to be the activation of type I cyclic AMP-dependent protein kinase which then may phosphorylate nuclear proteins, which in turn enhance the transcriptional process leading to the induction of ODC (13). Based on results obtained in vitro, a recent report suggested that cyclic nucleotides might play a mediatory role in the regulation of epidermal ODC activity by TPA (41). In the present study, however, although peak levels of cyclic GMP and cyclic AMP precede the increase in ODC activity when TPA is topically applied to mouse skin, the degree of stimulation of the cyclic nucleotides and that of ODC activity are not proportional.

To enhance basal and TPA-increased cyclic nucleotide levels in mouse epidermis in vivo, single or multiple topical treatments with cholera toxin, prostaglandins, isoproterenol, or epinephrine are not feasible for their cost, for their possible side effects, and because the reciprocal effects of β-adrenergic agonists and TPA on β-adrenergic receptors (18) and normal adrenergic responsiveness of the epidermis (21, 31, 36, 55) have not been clarified. In contrast, IBMX and related phosphodiesterase inhibitors have consistently been reported to increase ODC activity (1, 9, 10, 12, 23, 41, 48) and to enhance its induction by TPA (41). Topical applications of phosphodiesterase inhibitors dissolved in acetone have been demonstrated to reach the target epidermal cells (60). We chose this mode of application not only because it is consistent with the way TPA is administered to the skin but also because this is certainly a more effective way than by systemic routes to act directly without delay or systemic toxicity on a large number of epidermal target cells. As shown in our results, IBMX or other phosphodiesterase inhibitors alone raised epidermal cyclic GMP and cyclic AMP to levels corresponding to the effect of TPA but failed to reproduce the induction of ODC activity by TPA. Since the ability of IBMX to inhibit, or at least slow down, the degradation of cyclic nucleotides appeared to last for about 1 hr, this compound was applied immediately before TPA treatment in order to alter as much as possible the effects of TPA. The magnitude of the cyclic nucleotide response in the presence of IBMX was also dependent upon the dose of TPA and correlated with the promoting abilities of diverse tumor promoters. Much higher levels of cyclic GMP and cyclic AMP were maintained for a longer period of time after IBMX plus TPA treatment, suggesting that IBMX, by inhibiting or slowing down temporarily the degradation of newly synthesized cyclic GMP and cyclic AMP, increased the effectiveness of the tumor promoters as stimulators of the production of cyclic nucleotides. In accord with our previous findings in vitro (41), IBMX pretreatment also enhanced the magnitude of the ODC response to the diverse tumor promoters tested in vivo. In the presence of IBMX, only one-tenth the quantity of TPA was required to induce both peak levels of cyclic nucleotides and maximally induced ODC activity as compared with the effects of TPA alone. However, even in the presence of the phosphodiesterase inhibitors, TPA and the other tumor promoters tested still caused disproportionate increases in cyclic GMP and cyclic AMP compared to the magnitudes of their induction of ODC activity.

In the experiment depicted in Table 1, treatments with 10 μmol of IBMX or 8.5 nmol of TPA raised ODC activity from a basal value of 0.115 nmol CO₂ in 60 min per mg protein to stimulated levels of 0.467 and 4.432 nmol CO₂, respectively. However, when these compounds were added together, they did not raise the ODC activity to a value of only 4.899 nmol CO₂ but to a more than additive activity of 13.865 nmol CO₂. Therefore, the synergistic effects of IBMX and TPA on ODC induction cannot be explained solely on the basis of increased cyclic nucleotide levels. Our data indicate that TPA seems to have an effect distinct from the cyclic nucleotides which dramatically induces ODC. Separate routes for the induction of ODC activity by aminophylline and triiodothyronine involving cyclic AMP-mediated events or direct nuclear interaction have also been suggested (11). Whether the enhancement of basal and TPA-induced ODC activities by phosphodiesterase inhibitors is entirely due to an elevation of cyclic nucleotides is difficult to determine since methylxanthines do have other biochemical effects.

Based on the facts that several discrepancies were observed between the degree of tumor promoter-increased cyclic nucleotide levels and that of ODC induction in vivo and that phosphodiesterase inhibitor treatments raised cyclic nucleotide levels as much as did TPA without eliciting a comparable induction of ODC activity, it would be tempting to conclude that the early and transient elevation of epidermal cyclic GMP and cyclic AMP by TPA is unlikely to mediate the mechanism by which TPA induces ODC in mouse skin. However, the better correlations observed between the production of cyclic nucleotides and induction of ODC activity following incubation of isolated epidermal cells with TPA, IBMX, and cholera toxin have led us to a rather opposite conclusion in a previous report (41). Our various studies illustrate the difficulties inherent in correlating quantitative changes in cyclic nucleotide levels with subsequent ODC induction in cells stimulated by a variety of agents. It has been emphasized that a stimulation of cyclic AMP-dependent protein kinase need not be preceded by a proportionately large increase in cyclic AMP (47). Therefore, only the study of the activation of cyclic AMP-dependent protein kinase, which is a more reliable index or a cyclic AMP-mediated event (47), would provide definitive answers concerning cyclic nucleotide involvement in ODC induction by TPA. However, the other findings of the present and accompanying paper (43) provide new and substantial evidence that, under certain conditions, there is a dissociation of in-
increases in levels of cyclic nucleotides from induction of ODC activity and skin tumors by the tumor promoter (29). Topical applications of cyclic GMP and cyclic AMP in solution in acetone were unable, in the presence or absence of IBMX, to successfully mimic the stimulatory effect of TPA on ODC. The additional inhibition of basal and TPA-induced ODC activities observed after cyclic GMP plus cyclic AMP treatment indicates that the exogenously added cyclic nucleotides do enter into the epidermal cells and suggests that both cyclic GMP and cyclic AMP may actually decrease polyamine biosynthesis. The negative effect of exogenously added cyclic nucleotides on ODC activity is apparently not due to increased cyclic nucleotide phosphodiesterase activities, since this inhibition persists in the presence of IBMX. Cyclic AMP was slightly more effective than cyclic GMP in decreasing basal and TPA-stimulated ODC activities, but cyclic AMP was applied at a dose of 0.25 μmol whereas only 0.16 μmol of cyclic GMP was administered to the mice. Furthermore, at physiological levels, cyclic AMP is usually 10 times more concentrated in the epidermis than is cyclic GMP, an observation compatible with the fact that the 2 cyclic nucleotides have different physiological importance. However, the levels of epidermal cyclic nucleotides have not been measured following topical applications of the above doses of cyclic AMP and cyclic GMP to the skin. Therefore, one should not discard the possibility that excessively high concentrations of exogenously added cyclic GMP may interact nonspecifically with components of the cyclic AMP-dependent system (20), resulting in an effect on ODC activity similar to that obtained with cyclic AMP. Despite the better correlations between increases in cyclic nucleotide levels and induction of ODC activity described in vitro (41), a dose-dependent inhibition of basal and TPA-induced ODC activities was reproduced with isolated epidermal cells during incubation with diverse concentrations of cyclic nucleotides or their dibutyryl derivatives, in both the presence and the absence of IBMX. Finally, the results presented in the accompanying paper (43) indicate that both IBMX and cyclic nucleotide treatments inhibit epidermal ODC activity in situ and decrease dramatically TPA-induced polyamine accumulation, macromolecule synthesis, and formation of skin in situ and decrease dramatically TPA-induced polyamine activity (41), a dose-dependent inhibition of basal with cyclic AMP. Despite the better correlations between in vivo and in vitro (41), a dose-dependent inhibition of basal and TPA-stimulated ODC activity. Biochem. J., 62: 315-323, 1968.


Effects of 3-Isobutyl-1-methylxanthine and Cyclic Nucleotides on 12-O-Tetradecanoylphorbol-13-acetate-induced Ornithine Decarboxylase Activity in Mouse Epidermis in Vivo

Jean-Pierre Perchellet and R. K. Boutwell


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