Proc. Natl. Acad. Sci. USA
Vol. 74, No. 2, pp. 657–661, February 1977
Cell Biology

Dissociation of increases in levels of 3′:5′-cyclic AMP and 3′:5′-cyclic GMP from induction of ornithine decarboxylase by the tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate in mouse epidermis *in vivo*

(chemical carcinogenesis/polyamines/*dl*-isoproterenol)

R. A. MUFSON, E. G. ASTRUP, R. C. SIMSIMAN, AND R. K. BOUTWELL

McArdle Laboratory for Cancer Research, University of Wisconsin Medical Center, Madison, Wis. 53706

Communicated by Van R. Potter, December 2, 1976

ABSTRACT A single application of 17 nmol of 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) to mouse skin caused a marked (200- to 400-fold) induction of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxy-lyase) activity in mouse epidermal and epidermal-dermal preparations. No change in the basal level of 3′:5′-cyclic AMP occurred in epidermal-dermal preparations within 30 min of TPA application. Intraperitoneal injection of the β-agonist isoproterenol resulted in a dose-dependent accumulation of 3′:5′-cyclic AMP 10 min after injection, but caused no induction of ornithine decarboxylase. When isoproterenol was injected 10 min prior to an application of either 1.7 or 17 nmol of TPA, the magnitude of the ornithine decarboxylase induction was the same as induction with TPA alone.

Topical application of 17 nmol of TPA caused no increase in the level of 3′:5′-cyclic GMP present in the mouse epidermal-dermal preparations 2–20 min after application. Intraperitoneal injection of 1.75 μmol of dibutyryl 3′:5′-cyclic GMP caused a 6-fold increase in the level of cyclic GMP and/or butyryl derivatives of cyclic GMP in epidermal-dermal preparations within 5 min of injection, and the level remained elevated for at least 20–30 min. This dose of dibutyryl 3′:5′-cyclic GMP was incapable of inducing ornithine decarboxylase. Injection of dibutyryl 3′:5′-cyclic GMP 5 min before application of 1.7 nmol of TPA or 30 min before application of 17 nmol of TPA did not alter the magnitude of the ornithine decarboxylase induction produced by TPA alone. These results suggest that early increases in the total intracellular levels of either 3′:5′-cyclic AMP or 3′:5′-cyclic GMP are not part of the mechanism by which TPA induces ornithine decarboxylase in the epidermis.

One of the earliest and largest biochemical responses after the application of the hyperplasiogen and tumor promoter 12-*O*-tetradecanoyl phorbol-13-acetate (TPA) to mouse skin is the induction of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxy-lyase) (1). A 200- to 300-fold induction of ornithine decarboxylase occurs at about 4.5 hr after application of TPA, and the level returns to basal by 10–12 hr. It has been reported that a positive correlation exists between the ability of compounds to promote tumors in two-stage skin carcinogenesis and their ability to induce ornithine decarboxylase (2). Therefore, the induction of this enzyme may be an essential component of the tumor promotion process.

Changes in the levels of 3′:5′-cyclic AMP and 3′:5′-cyclic GMP have been implicated in the regulation of ornithine decarboxylase activity and the action of TPA in several systems. Rapid and transient increases in the levels of cyclic AMP precede the induction of ornithine decarboxylase in several tissues *in vivo* (3, 4) and in cell lines *in vitro* (5, 6). It has been proposed that an increase in cyclic AMP may be an essential component of the mechanism for ornithine decarboxylase induction (3, 4). Furthermore, rapid and transient elevations of cyclic GMP have been reported within minutes after *in vitro* exposure of epidermis and fibroblasts to TPA (7, 8). It has also been observed with lymphocytes in culture that exogenous addition of dibutyryl cyclic GMP may influence the level of ornithine decarboxylase induced by phytohemagglutinin (9).

The cyclic nucleotides have been suggested as second messengers for several classes of compounds acting at the cell membrane, and TPA is thought to have its initial action at the cell membrane (10). We have, therefore, attempted to determine whether an early alteration of cyclic nucleotide levels is a component of the induction of epidermal ornithine decarboxylase by TPA.

MATERIALS AND METHODS

Materials. $N^2,O^{2\prime}$-Dibutyryl cyclic GMP, the sodium salt of cyclic AMP, *dl*-isoproterenol·HCl, L-ornithine·HCl, bovine serum albumin, and calf thymus DNA (Type I) were all purchased from Sigma Chemical Comp. (St. Louis, Mo.). Cyclic [^{3}H]AMP (specific activity 37.7 Ci/mmol), cyclic [^{3}H]GMP (specific activity 9.92 Ci/mmol), and DL-[1-^{14}C]ornithine·HCl (specific activity 30–55 mCi/mmol) were obtained from New England Nuclear (Boston, Mass.). All ion exchange resins were purchased from Bio-Rad (Richmond, Calif.). TPA was purchased fom Consolidated Midland (Brewster, N.Y.). All other chemicals were of analytical or reagent grade.

Treatment of Animals. Female Charles River CD-1 mice (Wilmington, Mass.) were used at 7–9 weeks of age. The mice were housed in wire cages and were allowed Wayne Breeder Blox (Allied Mills, Chicago, Ill.) and water ad lib. Mice were allowed 12 hr of light and 12 hr of darkness on a cycle that corresponded to the natural day and night cycle. The dorsal skins of mice were clipped, and TPA was applied to the skin in acetone as described (1). All compounds used for injection were dissolved in 0.9% NaCl solution, and injected intraperitoneally (i.p.) in a 0.2-ml volume. Fresh solutions were made up every 10 min during the course of injection.

Determination of Ornithine Decarboxylase Activity. To obtain sufficient material, we killed groups of four mice by cervical traction and pooled their epidermis. The epidermis was separated from the dermis by the method of Raineri *et al.* (11). In determinations on epidermal-dermal preparations, skins were excised and the subcutaneous fat was thoroughly scraped off. Three epidermal-dermal preparations were homogenized in 5 ml of buffer. The enzyme activity was measured in supernatants, after centrifugation at 30,000 × *g* for 30 min, from

Abbreviations: cyclic AMP, 3′:5′-cyclic AMP; cyclic GMP, 3′:5′-cyclic GMP; TPA, 12-*O*-tetradecanoyl phorbol-13-acetate; i.p., intraperitoneal(ly).

Table 1. Effect of TPA on the basal level of cyclic AMP in frozen epidermal-dermal preparations

Topical application	pmol of cyclic AMP/ μg of DNA
Acetone control	0.22 ± 0.01
TPA 5 min	0.25 ± 0.02
10 min	0.22 ± 0.04
15 min	0.22 ± 0.04
30 min	0.20 ± 0.01

Acetone or 17 nmol of TPA was applied to the dorsal skin of mice. Mice were killed at the specified time, and the cyclic AMP and DNA content of frozen epidermal-dermal preparations were extracted and quantitated. Each value is the mean ±SEM of determinations on individual preparations from three to six different mice in a single experiment. Cyclic AMP and DNA determinations were performed in duplicate. Treatment with acetone did not change the control level of cyclic AMP during the time under investigation.

epidermal or epidermal-dermal homogenates prepared as published (1).

The activity of ornithine decarboxylase was assayed by measuring the release of $^{14}CO_2$ from DL-[1-^{14}C]ornithine as described by Russell and Snyder (12) with minor modifications (1). The substrate concentration most often used was nonsaturating (100 μM). To verify the results obtained in this way, we carried out some incubations at saturating levels (2 mM) simultaneously. Ornithine decarboxylase activity was expressed as nmol of CO_2 evolved in 30 min/mg of soluble protein. All enzyme assays were performed in triplicate, with a variation of ≤10%.

Determination of Cyclic AMP Levels. Animals were cervically dislocated, and the dorsal skin was immediately pressed against a block of solid CO_2 (−70°). Frozen epidermal-dermal preparations were flaked away from the lipid-rich plane and were immediately homogenized with a Polytron PT10 in cold 5% trichloroacetic acid. An aliquot of cyclic [^{3}H]AMP (10 × 10^3 dpm) was added to monitor recovery. The cyclic AMP was purified on columns of Dowex AG 50WX-4 H^+ (200–400 mesh) cation exchange resin from the ether-extracted acid-soluble fraction, and the column eluate was lyophilized. The residue was reconstituted in distilled water, and the cyclic AMP content was determined by the protein binding assay described by Brown et al. (13). The competing material extracted from the tissue was 96–99% destroyed after digestion with cyclic nucleotide phosphodiesterase for 60 min at 37°.

Determination of Cyclic GMP Levels. Epidermal-dermal preparations were harvested as described for cyclic AMP. Cyclic GMP was extracted into trichloroacetic acid exactly as described for cyclic AMP. An aliquot of cyclic [^{3}H]GMP (usually 5 × 10^3 dpm) was added to the extracts to quantitate recovery during purification. In experiments that were conducted with single epidermal-dermal preparations, the tracer cyclic GMP generally constituted 10% of the total cyclic GMP in the sample; in experiments in which preparations were pooled, the tracer represented 3% or less of the total cyclic GMP in the sample. The cyclic GMP was purified from the extracts on columns of Dowex AG 1-X8 (200–400 mesh) anion exchange resin in the formate form as described by Murad et al. (14). The Dowex AG 1-X8 eluates containing cyclic GMP were lyophilized, and the cyclic GMP content was quantitated by the radioimmunoassay procedure of Steiner et al. (15) using the antigen and antibodies supplied by Collaborative Research Inc. (Waltham, Mass.). In some determinations of the basal cyclic GMP levels in single epidermal-dermal preparations, cyclic GMP samples were succinylated (16) prior to assay to increase the sensitivity of the assay.

To be certain the competing material extracted from the tissue was actually cyclic GMP, an aliquot of the redissolved Dowex AG 1-X8 eluate was exposed to cyclic nucleotide phosphodiesterase for 60 min at 37°. It was found that 80–100% of the competing material disappeared after incubation with the cyclic nucleotide phosphodiesterase. Digestion of authentic cyclic GMP under the same conditions resulted in destruction of 88–100% of the competing material. The cyclic GMP measured in the unknown samples was directly proportional to the amount of reconstituted material added to the assay.

DNA and Protein Determinations. DNA was quantitated with the Burton diphenylamine procedure (17), with calf thymus DNA as standard. Protein was determined by the procedure of Lowry et al. (18), with bovine serum albumin as standard.

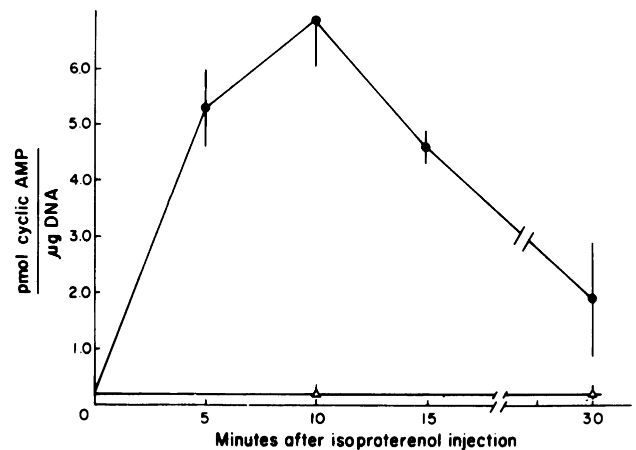

FIG. 1. Accumulation of cyclic AMP in epidermal-dermal preparations with time after injection of 300 nmol of dl-isoproterenol. Mice were injected i.p. with either 0.9% NaCl solution or isoproterenol and killed at the indicated times. Cyclic AMP levels after saline (△) or isoproterenol (●) injection are shown. Each point is the mean of duplicate cyclic AMP and DNA determinations from three different mice in a single experiment. Vertical lines represent the SEM.

RESULTS

Cyclic AMP and Ornithine Decarboxylase Induction. Bertsch and Marks (19) have shown that TPA has completely penetrated the skin and entered the blood within 30 min of topical application. Therefore, the effect of TPA on cyclic nucleotide levels was examined within 30 min of application. Cyclic AMP levels in epidermal-dermal preparations were found unchanged during this period of exposure to TPA (Table 1).

The effect of an increased intracellular level of cyclic AMP on the induction of ornithine decarboxylase was studied by i.p. injection of the pure β-agonist isoproterenol. Cyclic AMP accumulation in epidermal-dermal preparations was maximal at 10 min after i.p. injection of dl-isoproterenol (Fig. 1). Therefore, measurements of cyclic AMP level in response to isoproterenol were performed 10 min after injection. The curve in Fig. 2 shows that between 0 and 300 nmol of isoproterenol there is a dose-dependent accumulation of cyclic AMP, with a plateau appearing at doses higher than 300 nmol.

To determine whether elevations in cyclic AMP alone can induce ornithine decarboxylase, we injected 500 nmol of dl-isoproterenol i.p. into mice, and the mice were killed 1, 2, 3, or 4 hr later. At none of the times examined was the ornithine

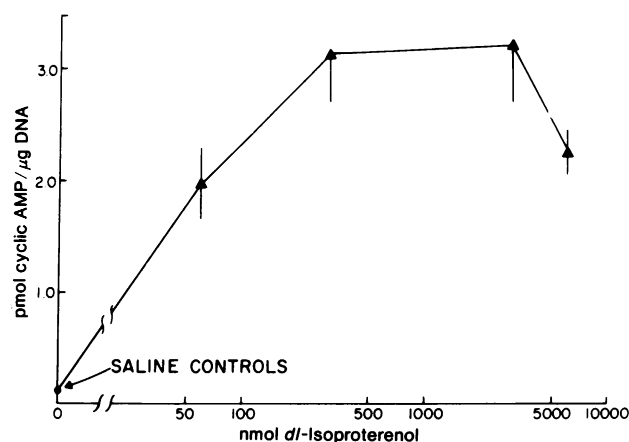

FIG. 2. Accumulation of cyclic AMP in epidermal-dermal preparations in response to increasing doses of *dl*-isoproterenol. Mice were injected i.p. with increasing doses of isoproterenol, and killed 10 min later. The cyclic AMP and DNA content were extracted and quantitated individually for each mouse. Each point is the mean of duplicate cyclic AMP and DNA determinations from six to nine different mice. The vertical lines represent the SEM.

decarboxylase level in epidermis from isoproterenol-injected mice above control levels. In contrast, a single application of 17 nmol of TPA to the skin induced a 400-fold increase in ornithine decarboxylase levels in epidermal cells 4 hr after application (Table 2). A comparable induction of ornithine decarboxylase by 17 nmol of TPA occurs in supernatants from epidermal-dermal preparations at 4 hr after application of TPA. Moreover, injection of isoproterenol, which resulted in a 10-fold accumulation of cyclic AMP in epidermal-dermal preparations, did not affect the induction of ornithine decarboxylase by TPA when the TPA was applied 10 min after isoproterenol injection (Table 2). Seven different doses of *dl*-isoproterenol of between 60 and 6000 nmol were also tested with no observable effect on the TPA-induced level of the enzyme. Of all the TPA doses previously tested (1), 17 nmol gave the maximal induction of ornithine decarboxylase. Therefore, to test the effect of increasing the cyclic AMP level on a less than maximal induction of the enzyme, we used one-tenth the dose of TPA. Ten minutes before the application of 1.7 nmol of TPA, 300 nmol of *dl*-isoproterenol were injected. No difference in enzyme induction

Table 2. Effect of *dl*-isoproterenol on induction of mouse epidermal ornithine decarboxylase

Treatment: Topical	Treatment: Injection i.p. (nmol)	Ornithine decarboxylase activity (nmol of CO_2 in 30 min/mg of protein)
Acetone	0.9% NaCl	≤0.02
TPA	0.9% NaCl	8.1 ± 0.5
TPA	*dl*-Isoproterenol (250)	6.7 ± 1.4
TPA	*dl*-Isoproterenol (500)	8.0 ± 0.3
Acetone	*dl*-Isoproterenol (500)	≤0.02

Groups of four mice were injected i.p. with saline or with two different doses of *dl*-isoproterenol 10 min prior to topical application of either acetone or 17 nmol of TPA to the dorsal skins. All mice were killed 4 hr after the topical application, and ornithine decarboxylase activity was determined. The substrate concentration used in these experiments was saturating (2 mM). Each of the enzyme activity levels shown is the mean ±SEM from three separate experiments.

Table 3. Effect of TPA on the basal level of cyclic GMP in frozen epidermal-dermal preparations

Topical application		fmol of cyclic GMP/ μg of DNA
(A)		
Acetone control		5.50 ± 0.66
TPA	2 min	5.87 ± 0.90
	5 min	6.78 ± 1.59
	10 min	6.69 ± 1.56
	20 min	6.30 ± 1.16
(B)		
Acetone	2 min	4.87 ± 1.7
TPA	2 min	3.70 ± 0.43
Acetone	5 min	3.70 ± 0.73
TPA	5 min	3.42 ± 0.44
Acetone	10 min	7.06 ± 0.60
TPA	10 min	4.17 ± 0.52
Acetone	20 min	3.38 ± 0.73
TPA	20 min	2.18 ± 0.17

Acetone or 17 nmol of TPA was applied to the backs of mice, and the mice were killed at specified times after application. In experiments in (A), preparations from each mouse were processed individually, and a complete time course was conducted in each experiment. In experiments in (B), preparations from three different animals were pooled, and each time point was examined independently. DNA and cyclic GMP were extracted and assayed in duplicate in all experiments. Each value in (A) is the mean ±SEM of individual determinations from nine to eighteen different mice from three separate experiments. The acetone level remained constant during the time under investigation and is thus presented as a control level in (A). Each value in (B) is the mean ±SEM of determinations from three to five separate experiments for each time point.

was found between control and isoproterenol-injected mice even with this much lower TPA dose.

Cyclic GMP and Ornithine Decarboxylase Induction. Two types of experiments were done to determine the effect of TPA on the basal level of cyclic GMP. The data in part A of Table 3 are generated from experiments in which an epidermal-dermal preparation from a single mouse was processed individually, and a complete time course was examined in each experiment. In the second type (part B, Table 3), epidermal-dermal preparations from three animals were pooled and each time point was examined separately. Basal level determinations were comparable for both types of experiments, and in none of the experiments was a significant increase in cyclic GMP detected.

To determine whether we could detect changes in the cyclic GMP level in our preparations, we determined cyclic GMP content after i.p. injection of dibutyryl cyclic GMP. The curve in Fig. 3 shows that 5 min after injection of 1.75 μmol of dibutyryl cyclic GMP there was a 6-fold increase in the amount of cyclic GMP and/or its butyrylated derivatives assayed in the epidermal-dermal preparations. Although the data in Fig. 3 are plotted as cyclic GMP against time, acylated derivatives of cyclic GMP are also immunoreactive in this assay (20). Thus, if any butyryl derivatives cochromatographed with cyclic GMP, their presence would also have been measured.

Intraperitoneal injections of dibutyryl cyclic GMP caused no induction of ornithine decarboxylase in epidermis when the enzyme activity was measured 4.5 hr after injection (Table 4). Topical application of TPA, as expected, caused a dramatic induction of ornithine decarboxylase when the enzyme activity

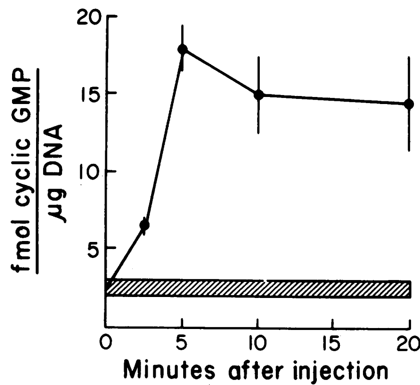

FIG. 3. Effect of dibutyryl cyclic GMP injection on the cyclic GMP content of epidermal-dermal preparations. Mice were injected i.p. with 1.75 μmol of dibutyryl cyclic GMP and killed at the specified times. Cyclic GMP and DNA were extracted and quantitated individually for each mouse. Each point is the mean of duplicate cyclic GMP and DNA determinations from three different mice; vertical lines represent the SEM. The stippled horizontal bar represents the mean ±SEM for the basal cyclic GMP content from six different animals injected with 0.9% NaCl solution and assayed individually. The experiment was performed twice with similar results.

was assayed 4 hr after application. Furthermore, the enzyme level obtained when dibutyryl cyclic GMP was injected 30 min before TPA application did not differ from the level obtained with TPA alone. We also tested the effect of dibutyryl cyclic GMP on the induction of the enzyme by one-tenth the dose of TPA. A 1.75-μmol dose of dibutyryl cyclic GMP was injected i.p. 5 min before the application of 1.7 nmol of TPA. Again there was no difference in the enzyme level between control mice and mice injected with dibutyryl cyclic GMP.

DISCUSSION

In the present study, we could find no evidence that a single topical application of TPA increases cyclic GMP levels in frozen epidermal-dermal preparations at early times after application. Voorhees *et al.* (7) have reported about a 2-fold increase in cyclic GMP levels within 1 min after addition of TPA to epidermal slices *in vitro*. The basal level of cyclic GMP in our preparations is of the same order as that reported by Voorhees *et al.*; however, the magnitude of the increase they report was

Table 4. Effect of dibutyryl cyclic GMP on induction of mouse epidermal ornithine decarboxylase

Treatment		Ornithine decarboxylase activity (nmol of CO_2 in 30 min/mg of protein)
Topical	Injection i.p.	
Acetone	0.9% NaCl	$\leq$0.02
Acetone	Dibutyryl cyclic GMP	$\leq$0.02
TPA	0.9% NaCl	3.3 ± 0.6
TPA	Dibutyryl cyclic GMP	3.2 ± 0.4

Groups of four mice were injected i.p. with either 0.9% NaCl solution or 1.75 μmol of dibutyryl cyclic GMP 30 min prior to application of either acetone or 17 nmol of TPA to the dorsal skin. All mice were killed 4 hr after the topical application, and the ornithine decarboxylase activity was determined. The substrate concentration used in these experiments was nonsaturating (100 μM). Each of the enzyme activity levels shown is the mean ±SEM from eight separate experiments.

within the range of physiological variation for the basal level found in this study (3.4–7 fmol/μg of DNA). We believe that the experiments with injected dibutyryl cyclic GMP suggest that significant increases in the level of cyclic GMP (or an immunoreactive derivative) in our preparations could be measured with the techniques used.

In neural tissue and heart cells, dibutyryl cyclic GMP has been used to mimic the effect of cholinergic agonists whose activity is associated with increases in intracellular cyclic GMP (21, 22). Mizoguchi *et al.* (9) have also reported that addition of dibutyryl cyclic GMP to lymphocytes in culture could increase the level of ornithine decarboxylase induced by phytohemagglutinin. In the present study dibutyryl cyclic GMP injection did not induce ornithine decarboxylase nor did it influence the induction mediated by TPA. Although it is tempting to suggest that the injection of this compound would mimic physiological increases in cyclic GMP, experiments with exogenous cyclic nucleotide derivatives are often difficult to interpret. In some systems the butyryl derivatives of cyclic GMP may interact differently from the free cyclic nucleotide with intracellular physiological substrates (23).

Examination of cyclic AMP levels at early times after TPA application also revealed no significant changes. Ten minutes after i.p. injection of *dl*-isoproterenol, however, a dose-dependent increase in the cyclic AMP level in epidermal-dermal preparations was observed. The agreement between the magnitude and kinetics of the accumulation curve observed in this study with that published for epidermis (24), as well as the localization of adenylate cyclase and its β receptor only in the epidermis by cytochemical and immunofluorescent techniques (25, 26), indicate that this increase is occurring in epidermal cells. The 10-fold increase in cyclic AMP accumulation after injection of 500 nmol of *dl*-isoproterenol was incapable of inducing ornithine decarboxylase. Moreover, when the level of cyclic AMP was raised in conjunction with the application of TPA, the resulting enzyme induction was the same as that produced after the application of TPA alone.

These results indicate that the induction of ornithine decarboxylase by TPA can be dissociated from early increases in the levels of either cyclic GMP or cyclic AMP. Richman *et al.* (27) have shown that in the thyroid gland the induction of ornithine decarboxylase can also be dissociated from increases in the level of cyclic AMP. Thus the regulation of ornithine decarboxylase by cyclic AMP may be a tissue-specific mechanism and not a general phenomenon, as has been previously suggested (3, 4). The role of cyclic nucleotides in the initiation of mitogenesis in general is controversial. Some workers have reported large increases in the cylic GMP content of lymphocytes after exposure to the mitogen phytohemagglutinin (28, 29), while other workers have been unable to reproduce these findings (30, 31). Most recently it has been demonstrated that mitogenic doses of phytohemagglutinin caused no changes in the levels of cyclic GMP or cyclic AMP in populations of adenoidal lymphocytes (32). In fact, very high concentrations of phytohemagglutinin were shown to increase the cyclic AMP content of these cells without producing mitogenesis (32). The results of our study indicate that the cyclic nucleotides are probably not second messengers for the growth-related increases in RNA and protein synthesis that occur in the epidermis after TPA application (33). The cyclic nucleotides could function, however, as regulatory molecules within the cell cycle, as has been suggested by Whitfield *et al.* (34).

This work was supported by Grants CA-07175 and CA-5002 from the National Cancer Institute. R.A.M. is the recipient of National Cancer Institute Postdoctoral Fellowship F22 CA 00586-01.

1. O'Brien, T. G., Simsiman, R. C. & Boutwell, R. K. (1975) *Cancer Res.* **35**, 1662–1670.
2. O'Brien, T. G., Simsiman, R. C. & Boutwell, R. K. (1975) *Cancer Res.* **35**, 2426–2433.
3. Byus, C. V. & Russell, D. H. (1974) *Life Sci.* **15**, 1991–1997.
4. Byus, C. V. & Russell, D. H. (1975) *Science* **187**, 650–652.
5. Bachrach, U. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 3087–3091.
6. Russell, D. H. & Stambrook, P. J. (1975) *Proc. Natl. Acad. Sci. USA* **72**, 1482–1486.
7. Voorhees, J. J., Colburn, N. H., Stawiski, M., Duell, E. A., Haddox, M. & Goldberg, N. D. (1974) in *Control of Proliferation of Animal Cells*, eds. Clarkson, B. & Baserga, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 635–648.
8. Estensen, R. D., Hadden, J. W., Hadden, E. M., Touraine, F., Touraine, J. L., Haddox, M. K. & Goldberg, N. D. (1974) in *Control of Proliferation of Animal Cells*, eds. Clarkson, B. & Baserga, R. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.), pp. 627–634.
9. Mizoguchi, Y., Otani, S., Matsui, I. & Morisawa, S. (1975) *Biochem. Biophys. Res. Commun.* **66**, 328–335.
10. Rohrschneider, L. R. & Boutwell, R. K. (1973) *Nature New Biol.* **243**, 212–213.
11. Raineri, R., Simsiman, R. C. & Boutwell, R. K. (1973) *Cancer Res.* **33**, 134–139.
12. Russell, D. H. & Snyder, S. H. (1968) *Proc. Natl. Acad. Sci. USA* **60**, 1420–1427.
13. Brown, B. L., Albano, J. M., Ekins, R. P. & Sgherzi, A. M. (1968) *Biochem. J.* **121**, 561–562.
14. Murad, F., Manganiello, V. & Vaughan, M. (1971) *Proc. Natl. Acad. Sci. USA* **68**, 736–739.
15. Steiner, A. L., Parker, C. W. & Kipnis, D. M. (1972) *J. Biol. Chem.* **247**, 1106–1113.
16. Frandsen, E. K. & Krishna, G. (1975) *Fed. Proc.* **34**, 231.
17. Burton, K. (1955) *Biochem. J.* **62**, 315–323.
18. Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275.
19. Bertsch, S. & Marks, F. (1974) *Cancer Res.* **34**, 3283–3288.
20. Cailla, H. L., Racine Weisbuch, M. S. & DeLaage, M. A. (1973) *Anal. Biochem.* **56**, 391–407.
21. Krause, E. G., Halle, W. & Wollenberger, A. (1972) in *Advances in Cyclic Nucleotide Research,* eds. Greengard, P. & Wollenberger, A. (Raven Press, New York), Vol. 1, pp. 301–305.
22. McAfee, D. A. & Greengard, P. (1972) *Science* **178**, 310–312.
23. Wells, J. N., Wu, Y. J., Baird, C. E. & Hardman, J. G. (1975) *Mol. Pharmacol.* **11**, 775–783.
24. Marks, F. & Grimm, W. (1972) *Nature New Biol.* **240**, 178–179.
25. Belman, S. & Troll, S. (1974) *Cancer Res.* **34**, 3446–3455.
26. Verma, A. K., Dixon, K. E., Froscio, M. & Murray, A. W. (1976) *J. Invest. Dermatol.* **66**, 239–241.
27. Richman, R., Park, S., Akbar, M., Yu, S. & Burke, G. (1975) *Endocrinology* **96**, 1403–1412.
28. Hadden, J. W., Hadden, E. M., Haddox, M. K. & Goldberg, N. D. (1972) *Proc. Natl. Acad. Sci. USA* **69**, 3024–3027.
29. Schumm, D. E., Morris, H. P. & Webb, T. E. (1974) *Eur. J. Cancer* **10**, 107–113.
30. Derubertis, F. R. & Zenser, T. (1976) *Biochim. Biophys. Acta* **428**, 91–103.
31. Wedner, J. H., Dankner, R. & Parker, C. W. (1975) *J. Immunol.* **115**, 1682–1687.
32. Weber, T. H. & Goldberg, M. L. (1976) *Exp. Cell Res.* **97**, 432–435.
33. Baird, W. M., Sedgwick, J. A. & Boutwell, R. K. (1971) *Cancer Res.* **31**, 1434–1439.
34. Whitfield, J. F., MacManus, J. P., Rixon, R. H., Boynton, A. L., Youdale, T. & Swierenga, S. (1976) *In Vitro* **12**, 1–18.