

Effect of Tumor Promoters on the Activity of Cyclic Adenosine 3':5'-Monophosphate-dependent and -independent Protein Kinases from Mouse Epidermis¹

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SUMMARY

Cyclic adenosine 3':5'-monophosphate (cyclic AMP)-dependent and -independent protein kinases were detected and partially characterized in soluble extracts from mouse epidermis. Cyclic AMP-dependent histone kinase activity was separated from cyclic AMP-independent casein kinase activity by DEAE-Sephadex chromatography. The application of the tumor promoters croton oil or 12-*O*-tetradecanoyl-phorbol-13-acetate to mouse skin caused a rapid increase in the soluble protein extractable from the epidermis resulting in a decrease in the specific activity of both classes of protein kinase when expressed on a protein basis. No change in the activities of either the cyclic AMP-dependent or -independent enzymes was observed when expressed relative to the DNA content.

INTRODUCTION

A number of recent publications have described changes in epidermal cyclic nucleotide metabolism following the treatment of mouse skin with carcinogens or tumor promoters (4, 16, 17, 23-25). Alterations are observed both in the hormone responsiveness of epidermal adenylate cyclase and in the basal levels of cyclic AMP.² Interpretation of such data requires information on possible changes in cyclic AMP-stimulated protein kinases (22). It has previously been shown that the epidermis from a number of animal species contains cyclic AMP-dependent protein kinase activity (7-10, 13). As part of a continuing program to characterize the molecular changes occurring during epidermal carcinogenesis, the effect of tumor promoters on the activity of epidermal protein kinases has been studied and the results are presented in this paper.

MATERIALS AND METHODS

Materials. Female Swiss albino mice were used at approximately 3 months of age.

Whole calf thymus histone (Type 2A) and croton oil were

obtained from the Sigma Chemical Co., St. Louis, Mo., and TPA was from Cambrian Chemicals, Croydon, England. DL-[1-¹⁴C]Ornithine (specific activity, 61 mCi/mole) and [8-³H]cyclic AMP (specific activity, 27 Ci/mole) were obtained from the Radiochemical Centre, Amersham, England. [γ -³²P]ATP was prepared as described by Glynn and Chappell (3).

The heat-stable inhibitor of cyclic AMP-dependent protein kinases was prepared from rabbit muscle as described before (26).

Treatment of Mice. Animals were kept in a room with an artificial rhythm of dark and light periods (dark from 4 a.m. to 4 p.m.). The back skin of the animals was shaved, and the animals used for experimentation were that which did not show a regrowth of hair over 7 days. Shaved areas of individual mice were treated with croton oil [0.2 ml of a 0.5% (v/v) solution in acetone] or with TPA (17 nmoles in 0.2 ml acetone); control animals were treated with 0.2 ml acetone.

Preparation of Epidermal Extracts. Soluble extracts for assay of protein kinase activity were prepared as described before (24) but in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.45) containing 5 mM 2-mercaptoethanol. Extracts for the assay of ornithine decarboxylase activity were prepared in 50 mM sodium phosphate (pH 7.2)-0.1 mM pyridoxal phosphate-0.1 mM EDTA.

Experiments were also carried out to determine the effect of TPA on the soluble protein and DNA content of defined areas of skin. Skin was pinned on a cork board (epidermis down), and a circle of tissue was cut with a punch (17.5 mm in diameter; 2.4 sq cm). The epidermis was separated from the dermis by brief heat treatment (12). Epidermis so obtained from 2 mice was homogenized in 1.5 ml of the above-described buffer. An aliquot (1 ml) was removed to cold 10% (w/v) trichloroacetic acid (1 ml) for the determination of DNA (17); the remainder was centrifuged at 100,000 \times *g* (20 min) to give a soluble extract.

Protein was measured by the method of Lowry *et al.* (11).

Assay of Protein Kinase Activities. Assays contained 5 μ moles *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid buffer (pH 7.45), 1 μ mole of MgCl₂, 4.7 to 5.1 nmoles [γ -³²P]ATP (specific activity greater than 0.06 mCi/ μ mole), 0.1 mg histone or 0.33 mg casein, and epidermal extract (5 to 25 μ g protein) in a final volume of 100 μ l. Where appropriate, assays included 0.2 nmole cyclic AMP and/or 25 μ g of rabbit muscle protein kinase inhibitor. Assays were carried out for 15 min at 37°. Reactions were terminated and incorporation of ³²P into protein was measured as described before (14).

¹ This work was supported by the Australian Research Grants Committee and the Australian Tobacco Research Foundation.

² The abbreviations used are: cyclic AMP, cyclic adenosine 3':5'-monophosphate; cyclic GMP, cyclic guanosine 3':5'-monophosphate; TPA, 12-*O*-tetradecanoyl-phorbol-13-acetate.

Received October 27, 1976; accepted February 4, 1977.

Assay of Ornithine Decarboxylase. Assays were carried out as described by O'Brien *et al.* (19) but contained 0.2 μCi of labeled ornithine. Incubations were continued for 30 min at 37°.

RESULTS

General Characteristics of Epidermal Protein Kinases.

Although most of the studies to be reported used crude cell-free extracts from epidermis (see below), preliminary experiments were carried out to characterize the kinases present. Fractionation of extracts by DEAE-Sephadex chromatography (Chart 1) resulted in the elution of 2 peaks of activity which catalyzed the phosphorylation of histone. These 2 peaks could be resolved more distinctly when assays of each fraction were carried out (data not shown). The histone kinase activities were followed by a more diffuse peak of casein kinase activity. Both histone kinase activities were strongly inhibited by the inclusion of the rabbit muscle protein kinase inhibitor in assays (greater than 80% inhibition); casein kinase activity was not inhibited by this inhibitor. Consequently, it is concluded that histone phosphorylation is catalyzed predominantly by cyclic AMP-dependent enzymes, and that of casein, by cyclic AMP-independent enzymes. The protein kinase pattern present in epidermal extracts closely resembled that reported in soluble extracts from human lymphocytes (6).

A series of experiments established that the activities of these separate histone and casein kinase enzymes could be adequately distinguished in assays with crude epidermal extracts. Thus the phosphorylation of histone by crude extracts was stimulated by cyclic AMP and inhibited by the heat-stable inhibitor from rabbit muscle (to about 16% of the activity measured in the presence of 2×10^{-6} M cyclic AMP). Routinely, assays were carried out with histone both in the presence of cyclic AMP and in the presence of the

heat-stable inhibitor; the difference between the 2 values is referred to as cyclic AMP-dependent protein kinase activity. Separate experiments established that the concentration of protein kinase inhibitor used resulted in maximum inhibition of histone phosphorylation. However, the phosphorylation of casein was insensitive to both cyclic AMP (2×10^{-6} M) and to the protein kinase inhibitor, suggesting that assays with this substrate measured the separate cyclic AMP-independent kinase activity identified in Chart 1. The specificity was not complete, however, as a residual kinase activity with histone remained in the presence of an excess of the protein kinase inhibitor.

Consequently, it is felt that assays of crude extracts carried out as described with casein and histone primarily detect different molecular species of protein kinase.

Protein kinase activity in crude epidermal extracts measured with either casein or histone as a substrate was linear with increasing concentrations of protein (up to 30 μg /assay) and with time of incubation (up to 30 min). Routinely, assays were carried out for 15 min and contained less than 25 μg of protein. The phosphorylated product formed when either casein or histone was used as a substrate was alkali labile (73 and 75%, respectively; 0.1 M NaOH; 100°; 20 min) and stable in acid (90 and 100%, respectively; 0.1 M HCl; 100°; 20 min).

Effect of Tumor Promoters on Epidermal Protein Kinase Activity. As shown in Table 1, croton oil induced a decrease in the specific activity of both cyclic AMP-independent (casein) and cyclic AMP-dependent kinase activity in epidermal extracts. Significant decreases ($p < 0.05$ at 2 and 4 hr; $p < 0.001$ at other time intervals) were maintained for at least 48 hr. A significant depression in cyclic AMP-dependent activity ($p < 0.02$) was observed 2 hr after application of croton oil, although there was no significant effect on casein kinase activity at this time ($p > 0.1$). Maximum depression of both specific activities was observed 4 hr after treatment.

As discussed before (2), the decrease in protein kinase-

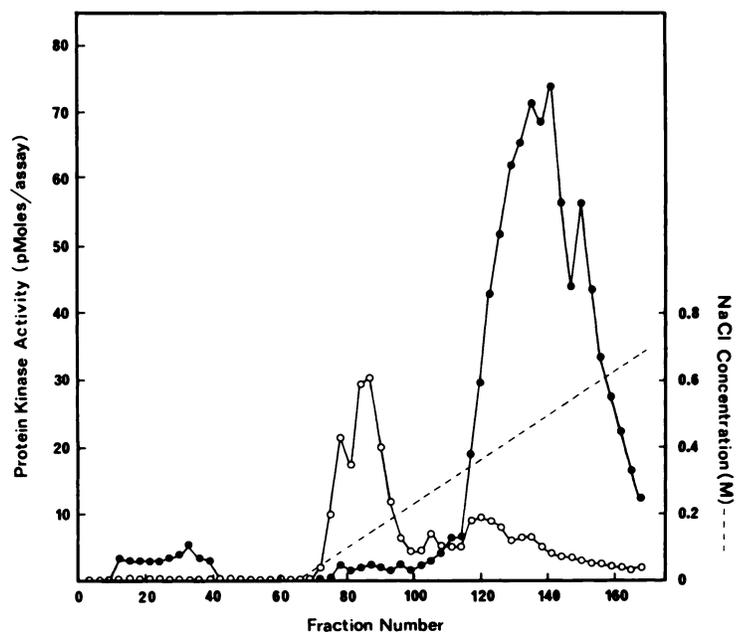


Chart 1. DEAE-Sephadex chromatography of epidermal extracts. Extract (6.8 mg protein) was fractionated on a column (1.7 x 9 cm) as described before (6). Fractions (1.3 ml) were collected and every 3rd fraction was assayed for protein kinase activity as described in "Materials and Methods" with histone (○) or casein (●) as a substrate. Assays with histone contained 2×10^{-6} M cyclic AMP.

specific activity could have a number of explanations, one of which is an increase in total soluble protein without a corresponding increase in protein kinase synthesis. An increase in the incorporation of tritiated leucine into soluble protein has been observed 2 hr after the application of tumor promoters (5). As shown in Table 2, the decrease in protein kinase-specific activity following TPA or croton oil treatment was solely due to an increase in soluble protein; no decrease was observed when enzyme activities were expressed on a DNA basis. In separate experiments (Table 3), it was confirmed that TPA induced a marked increase in the soluble protein extractable from a fixed area of epidermis, with no change in the DNA content.

In view of the lack of effect of both TPA and croton oil on protein kinase activity, the effects of these materials on

ornithine decarboxylase activity were determined. The activity of this enzyme has been reported to increase rapidly in epidermal extracts following promoter treatment (18-20). In these experiments, extracts were prepared as described in "Materials and Methods," and, within each treatment group, extracts were prepared from the pooled scrapings from 6 animals; enzyme assays were carried out in triplicate.

The activity of the epidermal cyclic AMP-dependent kinase was reduced from 182 (acetone control) to 36.5 pmoles/min/mg protein, 6 hr after croton oil treatment. In the same extracts, casein kinase activities were 482 and 85.4 pmoles/min/mg protein, respectively, and ornithine decarboxylase activities were 4.1 and 25.9 pmoles/min/mg protein, respectively.

Table 1
Effect of croton oil on protein kinase activities in mouse epidermal extracts

Extracts were prepared and assayed for cyclic AMP-dependent histone kinase and casein kinase activity as described in "Materials and Methods."

Treatment and activity measured	Protein kinase activity (pmoles/min/mg protein)					
	0 hr	6 hr	15 hr	24 hr	30 hr	48 hr
Experiment 1						
Acetone						
Cyclic AMP-dependent	180 ± 14 ^a	192 ± 17				179 ± 16
Casein	539 ± 36	561 ± 28				594 ± 70
Croton oil						
Cyclic AMP-dependent		80 ± 17	132 ± 16	114 ± 9	103 ± 8	99 ± 7
Casein		229 ± 46	359 ± 60	389 ± 58	401 ± 47	358 ± 39
	2 hr	4 hr	6 hr			
Experiment 2						
Acetone						
Cyclic AMP-dependent	586 ± 48					
Casein	830 ± 26					
Croton oil						
Cyclic AMP-dependent	381 ± 55	168 ± 30	195 ± 27			
Casein	637 ± 116	280 ± 57	364 ± 48			

^a Each value represents the mean ± S.E. of determinations carried out on 6 separate preparations.

Table 2

Effect of TPA and croton oil on epidermal protein kinase activities expressed relative to soluble protein or to total DNA

Extracts for enzymic assay were prepared and assayed for cyclic AMP-dependent histone kinase and casein kinase activity as described in "Materials and Methods." In Experiment 1, extracts were prepared 4 hr after the application of 17 nmoles of TPA; in Experiment 2, extracts were prepared 27 hr after the application of 0.2 ml 0.5% croton oil in acetone.

Treatment	Protein kinase activity			
	Cyclic AMP-dependent		Casein	
	Protein basis ^a	DNA basis ^b	Protein basis ^a	DNA basis ^b
Experiment 1				
Acetone	322 ± 32 ^c	4.75 ± 0.40	429 ± 27	6.33 ± 0.28
TPA	109 ± 5	5.33 ± 0.77	174 ± 21	8.03 ± 0.73
<i>p</i>	<0.001	>0.1	<0.001	<0.1
Experiment 2				
Acetone	128 ± 15	2.77 ± 0.53	255 ± 7	5.33 ± 0.43
Croton oil	32 ± 5	1.91 ± 0.26	95 ± 15	5.51 ± 0.37
<i>p</i>	<0.001	>0.1	<0.001	>0.1

^a pmoles phosphate transferred per min per mg protein.

^b pmoles phosphate transferred per min per μg DNA.

^c Each value represents the mean ± S.E. of determinations carried out on 6 separate preparations.

Table 3

Effect of TPA on soluble protein and total protein and DNA content of mouse epidermis

DNA, soluble, and total protein measurements were carried out as described in "Materials and Methods" 5 hr after the application of acetone or TPA (20 nmoles) to mouse skin.

Treatment	DNA/sq cm (μg)	Soluble protein/sq cm (mg)	Total protein/sq cm (mg)
Acetone	17.4 ± 2.3 ^a	0.13 ± 0.03	0.46 ± 0.08
TPA	18.4 ± 1.6	0.41 ± 0.04	0.87 ± 0.07
p	>0.1	<0.001	<0.01

^a Each value represents the mean ± S.E. of 6 separate determinations.

In a separate experiment, the cyclic AMP-dependent kinase was reduced from 127 to 30.7 pmoles/min/mg protein 6 hr after TPA treatment. The respective casein kinase activities in the same extracts were 290 and 92 pmoles/min/mg protein, and those of ornithine decarboxylase were 0.15 and 9.35 pmoles/min/mg protein.

DISCUSSION

A number of components of epidermal cyclic AMP metabolism have been shown to alter following exposure to tumor promoters. These include basal levels of cyclic AMP (4, 23), activities of cyclic nucleotide phosphodiesterases (25), the responsiveness of adenylate cyclase to β-adrenergic stimulation (4, 16, 25), and the accumulation of cyclic AMP during ischemia (17). The present experiments were designed to determine whether promoter treatment altered the specific activity of epidermal protein kinases.

Soluble epidermal extracts contained both cyclic AMP-dependent and -independent protein kinases, but neither activity was modified by treatment with tumor promoters. As expected (18-20), the application of TPA resulted in an early rise in ornithine decarboxylase activity. In view of the negative effect of TPA application on kinase activity, it was thought essential to establish this point under the experimental conditions used in the present study. Although a significant decrease in protein kinase specific activity was induced by both TPA and croton oil when soluble protein was used as a base line, no decrease was observed when activities were expressed relative to DNA content. This result presumably implies that the average protein kinase activity per cell remains constant following exposure to tumor promoters. Consequently, if changes in protein phosphorylation patterns accompany tumor promotion (21), they are most likely to be triggered by alterations in cyclic AMP levels leading to changes in the activity of pre-existing enzymes. However, it is not established that cyclic nucleotides are the only important regulators of epidermal protein kinase activity. For example, it has recently been shown that polyamines inhibit epidermal cyclic AMP-dependent protein kinases and stimulate cyclic AMP-independent enzymes (15). Tumor promoters lead to a rapid increase in epidermal ornithine decarboxylase activity and to an associated accumulation of polyamines (18-20).

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