Calcium-Dependent Protein Modulator of Cyclic Nucleotide Phosphodiesterases from Mouse Epidermis

By ANDREW W. MURRAY and ANNE ROGERS

School of Biological Sciences, Flinders University of South Australia, Bedford Park, South Australia 5042, Australia

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1. A heat-stable modulator protein was partially purified from mouse epidermis. The protein stimulated modulator-depleted cyclic AMP phosphodiesterase from bovine brain in the presence of Ca^{2+} . 2. DEAE-cellulose chromatography of epidermal extracts demonstrated the presence of two main phosphodiesterase activities that hydrolysed both cyclic AMP and cyclic GMP. A minor peak was eluted between 0.1 and 0.3 M-sodium acetate and a major peak was eluted between 0.3 and 0.45 M-sodium acetate. 3. Cyclic AMP phosphodiesterase activity eluted at low salt concentrations was markedly activated by the epidermal modulator protein in the presence of Ca^{2+} . Storage of the enzyme led to a decrease in its sensitivity to the protein modulator. 4. Treatment of mouse skin with the tumour promoter 12-*O*-tetradecanoylphorbol 13-acetate, which leads to an increase in epidermal cyclic nucleotide phosphodiesterase activity, did not alter the amount of modulator present in soluble epidermal extracts. The tumour promoter decreased the amount of modulator extractable from particulate epidermal preparations with Triton X-100.

Considerable interest has developed in the role of cyclic AMP in the regulation of normal epidermal growth and in the development of disease states such as psoriasis and epidermal tumours (Marks, 1976; Murray *et al.*, 1976; Voorhees *et al.*, 1976). Consequently there is need for a detailed characterization of the individual components of the adenylate cyclase system in epidermis. The tissue has been shown to contain hormone-sensitive adenylate cyclase (Adachi *et al.*, 1977; Murray *et al.*, 1976), cyclic AMP-dependent protein kinases (Kumar *et al.*, 1971, 1973, 1976; Murray & Froscio, 1977) and cyclic nucleotide phosphodiesterases (Marks & Raab, 1974; Adachi *et al.*, 1976; Verma *et al.*, 1976).

Treatment of mouse skin with tumour-promoting agents leads to marked changes in the accumulation of cyclic AMP in response to β -adrenergic stimulation (Grimm & Marks, 1974; Murray *et al.*, 1976), to an increase in the activity of cyclic nucleotide phosphodiesterases (Verma *et al.*, 1976) and to changes in the basal concentrations of cyclic AMP in the epidermis (Grimm & Marks, 1974). The factors that interact with adenylate cyclase and cyclic AMP phosphodiesterase to regulate cyclic AMP concentrations are not understood, but of potential importance is the observation that both adenylate cyclase and phosphodiesterase are stimulated by a widely distributed Ca^{2+} -binding modulator protein (see Wells & Hardman, 1977, for references). The purpose of the present investigation was to determine

whether mouse epidermis contained a similar protein modulator and, if so, to determine its effect on epidermal cyclic nucleotide phosphodiesterase activity.

Materials and Methods

Unlabelled cyclic AMP and cyclic GMP, 5'-nucleotidase from *Crotalus adamanteus* venom (grade III) and adenosine deaminase from calf intestinal mucosa were obtained from Sigma, St. Louis, MO, U.S.A. Cyclic [8- ^3H]AMP (sp. radioactivity 21 Ci/mmol) and cyclic [8- ^3H]GMP (19 Ci/mmol) were obtained from The Radiochemical Centre, Amersham, Bucks., U.K. Triton X-100 was from Calbiochem, San Diego, CA, U.S.A., and 12-*O*-tetradecanoyl phorbol 13-acetate was from Cambrian Chemicals, Croydon, U.K.

Assays of phosphodiesterase activity

Cyclic nucleotide phosphodiesterase was assayed by both a spectrophotometric and a radioactive isotope method. The spectrophotometric assay of cyclic AMP phosphodiesterase was based on the conversion of AMP into inosine by the actions of 5'-nucleotidase and adenosine deaminase (Wallace & Harary, 1975). Assays were carried out in cuvettes with a 1 cm light-path and contained 125 μmol of Tris/HCl buffer, pH 7.4, 90 nmol of cyclic AMP, 5 nmol of MgCl_2 , 0.3 nmol of CaCl_2 , 7 μg of 5'-

nucleotidase, 0.8 μ g of adenosine deaminase and phosphodiesterase preparation in a final volume of 2.5 ml. Reaction mixtures were maintained at 37°C and the decrease in A_{260} was followed with a Zeiss PMQII spectrophotometer. A molar absorption coefficient of 8060 was used for the conversion of adenosine into inosine (Hrapchak & Rasmussen, 1972).

Alternatively, activities of cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase were measured by the procedure of Thompson & Appleman (1971) as modified by Bergstrand & Lindquist (1976). Reaction mixtures contained an appropriate concentration of either cyclic [3 H]AMP or cyclic [3 H]GMP (approx. 0.2 μ Ci), 15 nmol of CaCl_2 , 0.75 μ mol of AMP or GMP, 7.5 μ mol of Tris/HCl, pH 8, 0.15 μ mol of MgCl_2 and enzyme in a final volume of 150 μ l. Assays were carried out for 20 min at 37°C.

Assay of modulator activity

The ability of modulator protein to stimulate the activity of modulator-depleted cyclic AMP phosphodiesterase was tested by using the spectrophotometric assay described above, unless otherwise stated. Modulator-depleted phosphodiesterase was partially purified from bovine brain as described by Watterson *et al.* (1976a). Standard assays to detect the presence of modulator contained phosphodiesterase preparation (125 μ g of protein) and catalysed the hydrolysis of 14–15 nmol of cyclic AMP/min in the presence of saturating amounts of modulator. In the absence of added modulator, the phosphodiesterase activity was <0.08 nmol of cyclic AMP hydrolysed/min.

Preparation of phosphodiesterase modulator from mouse epidermis

The dermal layer of skin pieces was removed by scraping with a scalpel blade, and the epidermis homogenized in 0.1 M-sodium acetate/1 mM- β -mercaptoethanol/1 mM-EDTA (pH 7.2) in a Polytron homogenizer (PCU-2; Kinematica, Lucerne, Switzerland). The epidermis from 300 mice was processed in one batch (total volume of extraction buffer was 300 ml). The homogenate was strained through cheesecloth and centrifuged at 100 000g (30 min; 4°C). The supernatant was heated at 100°C for 5 min, clarified by centrifugation and dialysed overnight against 20 vol. of 10 mM-Tris/HCl/1 mM-EDTA/1 mM- β -mercaptoethanol (pH 7.5; buffer A) containing 0.2 M-NaCl. The sample was applied to a column of DEAE-Sephadex (25 cm $\times$ 2.5 cm) equilibrated with the same buffer and washed with buffer until the $A_{1\text{ cm}, 280}$ of the eluate was less than 0.1. The column was then eluted with buffer A containing 0.6 M-NaCl. Fractions (6 ml) were collected and tested for their ability to activate the modulator-depleted cyclic AMP phosphodiesterase prepared from bovine brain (see

above). The active fractions were pooled, dialysed against buffer A containing 0.2 M-NaCl, and re-applied to a DEAE-Sephadex column (20 cm $\times$ 1.5 cm) equilibrated with the same buffer. The column was eluted with a linear gradient of 0.2–0.6 M-NaCl in buffer A (total gradient volume 150 ml). Fractions (1.2 ml) were collected and assayed for modulator activity as described above. Active fractions were pooled, dialysed extensively against water, freeze-dried and the residue was redissolved in 3 ml of water. The total protein yield was 3.2 mg (total protein in the initial soluble epidermal extract was 400 mg).

Preparation of phosphodiesterase modulator from bovine brain

Modulator was partially purified from 200 g of bovine brain as described by Watterson *et al.* (1976b). The resultant material was further purified by DEAE-Sephadex chromatography as described by Watterson *et al.* (1976a).

Polyacrylamide-gel electrophoresis

Discontinuous polyacrylamide-gel electrophoresis of modulator preparations was carried out as described before (Watterson *et al.*, 1976a). Each sample was electrophoresed in duplicate gels. One gel was stained with Coomassie Brilliant Blue to localize protein and the other used to localize the modulator protein. The gels were cut into 0.5 cm sections and protein was eluted by crushing the gels into 1.0 ml (2 $\times$ 0.5 ml) of 50 mM-Tris/HCl, pH 7.4. The gel eluates were assayed for their ability to activate the modulator-depleted cyclic AMP phosphodiesterase from bovine brain by using the spectrophotometric assay described above.

Quantification of modulator in soluble and particulate preparations from epidermis

Epidermal homogenates (2–3 ml) were prepared from individual mice as described above for the isolation of the epidermal modulator, but in 50 mM-Tris/HCl, pH 7.4. A portion was removed for DNA determination (Murray & Verma, 1973) and the remainder centrifuged at 100 000g (20 min; 4°C). The supernatant was heated at 100°C for 3 min, clarified by centrifugation at 1600g (15 min) and assayed for modulator content as described below. The pellet was washed with 1 ml of 50 mM-Tris buffer and homogenized in 0.5 ml of 0.1% (v/v) Triton X-100. The homogenate was kept at 37°C for 5 min and centrifuged at 100 000g (20 min; 4°C). The supernatant was heated at 100°C for 3 min, clarified by centrifugation at 1600g (15 min) and assayed for modulator content.

Samples of the soluble and particulate extracts were tested for their ability to activate modulator-depleted brain phosphodiesterase by using either the radioactive isotope or spectrophotometric assay (see above). Standard curves were constructed in

which phosphodiesterase activity was plotted as a function of the amount of purified brain modulator (see above) added to the assay. One unit of modulator is defined as the amount resulting in a phosphodiesterase activation equivalent to $1\mu\text{g}$ of purified modulator. Triton X-100 was shown to inhibit phosphodiesterase activity. Consequently, to quantify modulator present in extracts of particulate fractions, standard curves were constructed in which assays contained an appropriate concentration of the detergent.

Other determinations

Protein was determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard and DNA by the method of Burton (1956).

Results

Isolation of epidermal Ca^{2+} -dependent modulator of cyclic nucleotide phosphodiesterases

Preliminary experiments demonstrated that heated extracts from mouse epidermis stimulated the hydrolysis of cyclic AMP catalysed by modulator-depleted phosphodiesterase from bovine brain.

The factor responsible for this activation was partially purified by DEAE-cellulose chromatography (see the Materials and Methods section).

Fig. 1. Polyacrylamide-gel electrophoresis of modulator protein preparations

Discontinuous gel electrophoresis was carried out as described in the Materials and Methods section. Gels were loaded with 13.4 and $12\mu\text{g}$ of protein from the partially purified modulator preparations of bovine brain (a) and mouse epidermis (b) respectively.

Activating material was eluted between 0.6 and 0.75M-sodium acetate. The phosphodiesterase modulator from a number of tissues is a low-molecular-weight, highly acidic protein with a rapid migration rate on discontinuous polyacrylamide gels (Watterson *et al.*, 1976a,b). The partially purified modulator from epidermis had an electrophoretic mobility similar to that of modulator protein purified from bovine brain (Fig. 1). The epidermal preparation was contaminated with at least one other major protein species.

As shown in Fig. 2, activation of brain cyclic AMP

Fig. 2. Effect of epidermal modulator and Ca^{2+} concentration on the activation of bovine brain cyclic AMP phosphodiesterase

Bovine brain cyclic AMP phosphodiesterase was assayed spectrophotometrically as described in the Materials and Methods section in the presence of $2.4\mu\text{g}$ of epidermal modulator and various concentrations of Ca^{2+} (a) or partially purified epidermal modulator (b). In (b) assays were carried out in the presence (●) or absence (▲) of $120\mu\text{M}$ - CaCl_2 .

phosphodiesterase by the epidermal modulator was dependent on the addition of Ca^{2+} to the assay mixtures. In assays containing $2.4\mu\text{g}$ of the modulator preparation, half-maximal activation was obtained at a CaCl_2 concentration of about $6.5\mu\text{M}$.

On the basis of these results, it is concluded that mouse epidermis contains a Ca^{2+} -dependent protein modulator of cyclic AMP phosphodiesterase with similar characteristics to the modulator present in other tissues (Wefts & Hardman, 1977).

Effect of the epidermal protein modulator on epidermal cyclic nucleotide phosphodiesterase activities

Previous studies have shown that extracts from mouse (Verma *et al.*, 1976) and pig epidermis (Adachi *et al.*, 1976) contain multiple phosphodiesterases that can be separated by chromatography on DEAE-cellulose.

As shown in Fig. 3, cell-free extracts of mouse

Fig. 3. Fractionation of epidermal cyclic nucleotide phosphodiesterases by DEAE-cellulose chromatography

A cell-free extract from epidermis (25ml ; 46mg of protein) was chromatographed on a column of DEAE-cellulose ($20\text{cm} \times 1.5\text{cm}$) as described by Verma *et al.* (1976). Fractions (6ml) were collected and $60\mu\text{l}$ samples assayed for cyclic GMP phosphodiesterase (a) and cyclic AMP phosphodiesterase (b) activity by using the radioactive isotopic assay described in the Materials and Methods section. Assays contained $1\mu\text{M}$ -cyclic nucleotide and were carried out in the absence (□) or presence (■) of partially purified mouse epidermal modulator protein ($2.4\mu\text{g}$ of protein). Samples ($150\mu\text{l}$) were also assayed for modulator protein (Δ) by using the spectrophotometric assay described in the Materials and Methods section.

epidermis contained two major peaks of both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activity (labelled peaks I and II), and a peak of modulator activity eluted at higher salt concentrations. The phosphodiesterase activities were modified to varying degrees by the addition of the epidermal modulator protein. Hydrolysis of cyclic AMP by enzyme in peak I was stimulated several-fold by the modulator (between 2- and 5.4-fold). The basal rate of hydrolysis of cyclic GMP by peak I in the absence of modulator was higher than that for cyclic AMP, but the activity was less affected by the modulator (activation between 1.5- and 1.7-fold). The hydrolysis of both cyclic AMP and cyclic GMP by peak II was relatively insensitive to the epidermal modulator.

Fractions 9 (peak I) and 15 (peak II) were re-assayed with both cyclic AMP and cyclic GMP in the presence and absence of the modulator protein and EGTA (Table 1). In each case stimulation by the modulator was essentially abolished by the inclusion of EGTA, suggesting a requirement for Ca^{2+} . These assays were carried out after storage of the column fractions at -20°C for 5 days. It was subsequently found that storage of the column fractions for longer periods of time led to an increase in the basal rates of cyclic AMP hydrolysis and to a decrease in the response to the protein modulator. Thus after a further period of storage at -20°C of 2 days, the basal rate of cyclic AMP hydrolysis by fraction 9 was increased from 0.15 (Table 1) to 0.25 pmol/min per assay and the activity in the presence of the protein modulator was decreased from 0.45 (Table 1) to 0.28 pmol/min per assay. Similarly, the basal rate of cyclic AMP hydrolysis catalysed by fraction 8 immediately after collection (see Fig. 3) was 0.08 pmol/min per assay and the rate in the presence of modulator was 0.42 pmol/min per assay (5.3-fold stimulation). After storage of fraction 8 for 24 days at -20°C the rates were 0.28 and 0.38 pmol/min per assay respectively (1.4-fold stimulation).

The loss of modulator sensitivity of peak-I cyclic AMP phosphodiesterase on storage was repeatedly observed. A decrease in the responsiveness of phosphodiesterase to the modulator protein has been previously reported to occur during enzyme purification (Ho *et al.*, 1977). As suggested by these authors, it is possible that these changes are a result of proteolytic attack during storage. It has been reported that limited proteolysis can lead to both phosphodiesterase activation and conversion of the enzyme into a modulator-insensitive form (Sakai *et al.*, 1977).

The elution profile of epidermal phosphodiesterases from DEAE-cellulose columns was not altered by the inclusion of 0.1mM -EGTA in the elution buffer. This experiment was necessary as it has been reported that, in the presence of Ca^{2+} , the Ca^{2+} -activatable phosphodiesterase is retarded on DEAE-

Table 1. *Effects of modulator and EGTA on epidermal cyclic nucleotide phosphodiesterases*

Samples (60 μ l) of fractions 9 and 15 from Fig. 3 were assayed for both cyclic AMP phosphodiesterase and cyclic GMP phosphodiesterase activities by using the radioactive isotope procedure described in the Materials and Methods section. Where indicated, assays contained partially purified epidermal modulator (2.4 μ g of protein) or 1.33 mM-EGTA. Assays were carried out in duplicate. Values in parentheses are the ratios of activity in the presence of the modulator to activity in the absence of modulator.

Substrate	Phosphodiesterase activity (pmol/min per assay)			
	Control	Control+modulator	Control+EGTA	Control+EGTA +modulator
Fraction 9				
1 μ M-Cyclic AMP	0.15	0.45 (3.0)	0.22	0.23 (1.05)
40 μ M-Cyclic AMP	5.55	11.38 (2.05)	5.98	6.66 (1.11)
1 μ M-Cyclic GMP	0.38	0.62 (1.63)	0.52	0.60 (1.15)
40 μ M-Cyclic GMP	19.28	20.31 (1.05)	18.80	21.72 (1.16)
Fraction 15				
1 μ M-Cyclic AMP	1.31	1.66 (1.27)	1.43	1.49 (1.04)
40 μ M-Cyclic AMP	34.46	46.31 (1.34)	40.62	36.77 (0.91)
1 μ M-Cyclic GMP	0.94	1.43 (1.52)	1.03	1.00 (0.97)
40 μ M-Cyclic GMP	35.85	51.85 (1.45)	41.38	41.23 (1.00)

cellulose columns and elutes in the same position as the Ca^{2+} -independent enzyme (Ho *et al.*, 1977).

Effect of 12-O-tetradecanoylphorbol 13-acetate treatment on the modulator content of mouse epidermal fractions

It has previously been observed that treatment of mouse skin with tumour promoters leads to a marked increase in epidermal cyclic nucleotide phosphodiesterase activity (Verma *et al.*, 1976). A series of experiments was carried out to determine whether treatment with 12-O-tetradecanoylphorbol 13-acetate, a powerful tumour promoter, also altered epidermal concentrations of the protein modulator.

Groups of five mice were treated with 12-O-tetradecanoyl phorbol 13-acetate (17 nmol in 0.2 ml of acetone) or with acetone (0.2 ml). After 16 h epidermal homogenates were prepared and modulator present in the soluble and particulate fractions was assayed as described in the Materials and Methods section.

Modulator contents of acetone- and promoter-treated mouse epidermis were 88 ± 11 and 80 ± 12 units/mg of DNA respectively in the soluble fractions ($P > 0.1$) and 18.9 ± 2.6 and 11.7 ± 1.2 units/mg of DNA respectively in the particulate fractions ($P < 0.05$). The promoter-induced decrease in modulator extractable from particulate preparations was confirmed in a separate experiment in which the extracts were assayed by the radioactive isotope method. In this experiment modulator contents of acetone- and promoter-treated epidermis were 47 ± 4 and 32 ± 2 units/mg of DNA ($P < 0.02$).

These values were calculated by reference to partially purified bovine brain modulator (see the Materials and Methods section). If it is assumed that

the brain and the epidermal modulators are equivalent, then an estimate can be made of the percentage contribution of the modulator to total epidermal protein. By using the ratios of DNA to protein in epidermis reported before (Murray & Froscio, 1977) it is calculated that modulator protein comprises approx. 1% of the soluble protein and 0.4% of the total protein of mouse epidermis. Similar estimates have been made for the modulator present in extracts of brain (Vanaman & Watterson, 1976) and cultured chicken-embryo fibroblasts (Watterson *et al.*, 1976b).

Discussion

The present studies demonstrate that epidermis can be added to the extensive list of tissues shown to contain both a modulator protein and a cyclic nucleotide phosphodiesterase sensitive to stimulation by the modulator. However, as is also the case in other tissues, the physiological role of the modulator is unclear. The protein is present in high concentrations in tissue extracts, and consequently the sensitive phosphodiesterase might be expected to be fully activated *in vivo*. In addition, the modulator has been shown to activate adenylate cyclase (Cheung *et al.*, 1975; Brostrom *et al.*, 1975) and skeletal-muscle actomyosin adenosine triphosphatase (Watterson *et al.*, 1976b) in the presence of Ca^{2+} . Consequently it is possible that the modulator may have other, as yet unknown, functions in cells.

No evidence was obtained that the amount of modulator protein present in soluble extracts from epidermal cells was altered 16 h after treatment with 12-O-tetradecanoylphorbol 13-acetate. At this time after promoter treatment epidermis has lower basal concentrations of cyclic AMP (Grimm & Marks,

1974) and enhanced cyclic nucleotide phosphodiesterase activities (Verma *et al.*, 1976). Thus it is unlikely that changes in cytoplasmic modulator concentration are associated with either of these two events. Treatment with 12-*O*-tetradecanoylphorbol 13-acetate did lead to a decrease in the amount of modulator extractable from a particulate epidermal fraction by detergent. It will be of interest to determine whether this decrease is associated with changes in adenylate cyclase activity or with changes in other membrane-associated activities. It has been reported previously that a decrease in the modulator content of rat striatal membranes decreases adenylate cyclase activation by dopamine (Gnegy *et al.*, 1976) and that an increase in striatal modulator is associated with catecholamine supersensitivity (Gnegy *et al.*, 1977). Consequently it is possible that a decrease in the modulator content of epidermal membranes could contribute to the loss of catecholamine sensitivity observed in promoter-treated skin (Grimm & Marks, 1974).

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