Calmodulin activation of cyclic AMP phosphodiesterase in the B16 mouse melanoma

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(Received 15 December 1983/Accepted 2 February 1984)

Mouse B16 melanoma extracts of both cultured cells and tumour tissue contain cyclic AMP phosphodiesterase activity, with 95% present in the soluble fraction. Although activation of the enzyme by added calmodulin did not occur, it was found that endogenous calmodulin was present at a level sufficient to activate fully the enzyme. The ability of Ca-calmodulin to stimulate cyclic AMP phosphodiesterase in this tissue was shown by the inhibitory effect of *N*-(6-aminohexyl)-5-chloronaphthalene-sulphonamide (W7), a known calmodulin antagonist; by the activation of the enzyme with exogenous calmodulin observed in supernatants depleted of endogenous calmodulin by passage over fluphenazine-Sepharose 6B in the presence of Ca^{2+} ; by the Ca-dependent binding of the enzyme to calmodulin-agarose and its activation by Ca-calmodulin after elution from the column with EGTA-containing buffer. It was calculated that about 50% of the total cyclic AMP phosphodiesterase activity was calmodulin-activated in this tissue.

The B16 mouse melanoma responds to α MSH and related peptides with a rise in intracellular cyclic AMP (Mac Neil *et al.*, 1981). This signal is believed to result in an increase in tyrosinase activity and melanin synthesis (Pawelek *et al.*, 1973). However, the rise in cyclic AMP is transitory since cyclic AMP phosphodiesterase rapidly degrades the second messenger to 5'-AMP.

It has recently been shown that the action of α MSH is dependent upon Ca^{2+} , both for hormone-receptor binding and intracellular action (De Graan *et al.*, 1981). Both the adenylate cyclase which synthesizes cyclic AMP from ATP, and the cyclic AMP phosphodiesterase, have been shown to be activated by Ca-calmodulin in a number of tissues (Wang & Weissman, 1979); calmodulin is an intracellular Ca-binding protein which mediates many of the intracellular actions of calcium (Brostrom & Wolff, 1981).

This paper describes the Ca-calmodulin-dependent activation of cyclic AMP phosphodiesterase in calmodulin-depleted supernatant of mouse B16 melanoma homogenate and the calcium-dependent binding of the calmodulin-activated cyclic

AMP phosphodiesterase to calmodulin-agarose.

Materials and methods

Materials

Cyclic [8- ^3H]AMP (20–30 Ci/mmol) and *N*-propionyl-[^3H]calmodulin (31 Ci/mmol) were obtained from Amersham International, Amersham, Bucks., U.K. Cyclic AMP, as the crystallized free acid, was obtained from Boehringer Mannheim, London. Snake venom (*Ophiophagus hannah*), Dowex 1-X8 (200–400 mesh) anion exchange resin, bovine heart 3',5'-cyclic nucleotide activator-deficient phosphodiesterase, dithiothreitol and Mes (4-morpholine-ethanesulphonic acid) were from Sigma. Medium 199, phosphate-buffered saline, foetal calf serum, glutamine, penicillin and streptomycin were from Gibco-Europe, Paisley, U.K. All tissue culture plates were obtained from Sterilin, Teddington, Middx., U.K. Epoxy-activated Sepharose 6B was from Pharmacia, Hounslow, Middx., U.K. Calmodulin-agarose (Affi-gel calmodulin) was from Bio-Rad Laboratories, St. Albans, Herts., U.K.

Fluphenazine hydrochloride was a gift from E. R. Squibb, Reeds Lane, Moreton, Wirral, Mersey-

Abbreviations used: α MSH, α -melanotropin; PMSF, phenylmethanesulphonyl fluoride.

side, U.K. Trifluoperazine hydrochloride was a gift from Smith, Kline and French, Welwyn Garden City, Herts., U.K. The *N*-(6-amino-hexyl)-5-chloro-1-naphthalenesulphonamide (W7) was generously given by Dr. Hidaka, Mie University School of Medicine, Mie 514, Japan.

All other chemicals were of analytical grade.

Preparation of calmodulin

Pig brain calmodulin was prepared according to the method of Kakiuchi *et al.* (1981). The extraction and two-dimensional gel electrophoresis have been reported in brief elsewhere (Sharrard *et al.*, 1983).

The fluphenazine-Sepharose 6B, as used in the purification of calmodulin and the calmodulin depletion of supernatant preparations, was prepared by the method of Kakiuchi *et al.* (1981).

Culture of B16 melanoma cells

The B16 mouse melanoma was serially transplanted in C57BL mice. Melanotic cells were derived from the tumours and grown in 199 tissue culture medium with Hanks' salts and 20 mM-Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid] buffer supplemented with 20% (v/v) foetal calf serum and containing penicillin (50 i.u./ml) and streptomycin (50 µg/ml). Confluent cultures were used to prepare cell lysates, washed particulate preparations and supernatant preparations.

Homogenate, lysate, particulate and supernatant preparations

B16 melanoma tumours were homogenized with approx. 10 vol. of 40 mM-Tris/HCl (pH 7.0)/4 mM-2-mercaptoethanol with four strokes of a loose Dounce glass pestle homogenizer. After filtering through two layers of surgical gauze, part of this homogenate was used immediately in the assay of phosphodiesterase activity. Supernatant and washed particulate preparations were prepared from the remaining homogenate by centrifugation of homogenate at 11950g (r_{av} , 10.69 cm), using a Sorvall RC-5B for 10 min at 4°C. Supernatant from this stage was used immediately in the assay of phosphodiesterase activity. Particulate preparations were washed twice with the buffer described and each time centrifuged at 11950g as described and finally resuspended to the original volume of the homogenate before use in the assay of phosphodiesterase activity. For cultured cells derived from B16 tumours, Falcon flasks of 25 cm² area were washed free of culture medium with phosphate-buffered saline and drained, and then 1 ml of 40 mM-Tris/HCl (pH 7.0 at 37°C)/4 mM-2-mercaptoethanol was added and cells were scraped from the flask with a small metal spatula. Cells

were lysed by homogenization using four strokes of a tight Dounce glass pestle homogenizer. After filtering through two layers of surgical gauze, part of this lysate was used immediately in the assay of phosphodiesterase activity. Particulate and supernatant fractions were prepared from this cell lysate as described for the tumour homogenate.

Removal of calmodulin from B16 melanoma supernatant preparations

Fluphenazine-Sepharose 6B was used in the removal of calmodulin from preparations of B16 tumour and cultured cell supernatant as follows. Preparations were diluted 1:1 with 50 mM-Tris/HCl (pH 7.5 at room temperature) containing 5 mM-CaCl₂, 0.5 M-NaCl, 50 mg of PMSF/l and 50 µl of pepstatin A/l, and 1 ml was applied to a 6 cm × 1 cm column containing approx. 3 ml of fluphenazine-Sepharose 6B equilibrated with this buffer. The column was eluted with this buffer and 1 ml fractions were collected and kept at 4°C. Of each fraction, 100 µl was taken for protein assay using the method of Bradford (1976) which gives an immediate colour reaction. Fractions containing protein (by eye) were pooled and used immediately in the assay for cyclic AMP phosphodiesterase. Protein values were later measured at 595 nm.

Phosphodiesterase assay and measurement of calmodulin using calmodulin-dependent phosphodiesterase

Phosphodiesterase activity was measured by the method of Thompson *et al.* (1979) in which cyclic [³H]AMP substrate is converted to 5'-[³H]AMP by the phosphodiesterase and then to [³H]adenosine by snake venom 5'-nucleotidase. Phosphodiesterase incubations of final volume 400 µl contained 40 mM-Tris/HCl (pH 7.0 at 37°C), 4 mM-2-mercaptoethanol, 5 mM-MgCl₂, cyclic [³H]AMP (2 × 10⁵ c.p.m./incubation, approx. 0.1 µCi), cyclic AMP at 10 µM as detailed in the Results section, 100 µM-CaCl₂, and calmodulin or calmodulin antagonist as appropriate.

Calmodulin activity in supernatant preparations of B16 melanoma was determined by diluting the sample in question 1:1 with 40 mM-Tris/HCl (pH 7.0 at room temperature) containing 100 µM-CaCl₂, 50 mg of PMSF/l and 50 µl of Pepstatin A/l. A portion of this was heated at 95°C for 6 min, centrifuged (high speed, Micro Centaur bench centrifuge, 2 min) and the supernatant retained and stored at -20°C until calmodulin estimation.

A calmodulin-deficient phosphodiesterase (Sigma) was used as previously described (MacNeil *et al.*, 1982) which gave up to 4-fold activation with pure calmodulin, with half-maximal activation requiring 7 ng of calmodulin/incubation

(1.04×10^{-9} M) in the presence of a supra-maximal concentration of calcium, 25 μ M.

Binding of cyclic AMP phosphodiesterase to calmodulin-agarose

The validation of calmodulin-agarose affinity chromatography for the isolation of calmodulin-dependent phosphodiesterase was confirmed in preliminary experiments with beef heart phosphodiesterase. The binding of beef heart cyclic AMP phosphodiesterase was carried out by using a 1.0–1.5 ml column of calmodulin-agarose. Approx. 200 units of the enzyme were made up in 600 μ l of 50 mM-Tris/HCl (pH 7.5)/5 mM-CaCl₂ (buffer B); 100 μ l was removed for assay and the remainder was applied to the column, which had been pre-equilibrated with buffer B. Fractions (1 ml) were collected while the column was eluted with 10 column volumes of buffer B. The elution buffer was then changed to buffer B+0.2 M-NaCl for a further 10 column volumes. Finally, elution of enzyme was achieved with 50 mM-Tris/HCl (pH 7.5)/2 mM-EGTA, collecting 1 ml fractions into 1 ml of 5 mM-Mes (pH 6.5)/30% (v/v) ethylene glycol/1 mM-dithiothreitol/5% (w/v) glucose/50 mM-NaF/50 mg of PMSF/l buffer (phosphodiesterase storage buffer). Of each fraction, 100 μ l was tested for cyclic AMP phosphodiesterase activity as described above, after adjusting the free Ca²⁺ concentration to approx. 10 μ M in each case (using the dissociation constants for Ca²⁺ and EGTA given by Caldwell, 1970) and assaying with and without a maximally stimulating amount of calmodulin (1.5×10^{-8} M final concentration). At this calmodulin concentration it is known that the Sigma enzyme requires 5–10 μ M-Ca²⁺ for full activation.

The same procedure was also carried out with supernatants prepared from cultured cells as described above. Before application to the column, 2.5 ml of supernatant (protein content approx. 0.5 mg/ml) was diluted with an equal volume of buffer B.

Results

In homogenates of B16 tumour and lysates of B16 cultured cells at least 95% of the total cyclic AMP hydrolysis, with 10 μ M substrate, occurred in the 12000g supernatant fraction.

In supernatant preparations of both tumours and cultured cells the cyclic AMP phosphodiesterase activity was unaffected by the addition of exogenous calmodulin (up to 1.5×10^{-8} M). However, the calmodulin antagonist W7 at 5×10^{-5} M produced a significant reduction in cyclic AMP phosphodiesterase activity (Table 1).

Assay of calmodulin activity, using a heated

Table 1. Effect of calmodulin and of the calmodulin antagonist W7 on cyclic AMP hydrolysis in untreated and calmodulin-depleted preparations of B16 tumour supernatant and B16 cultured cell supernatant

System	Expt.	[Cyclic AMP] (μ M)	Treatment	Cyclic AMP hydrolysis (nmol/mg of protein)			Calmodulin-dependent activity (%)
				No additions	+ Calmodulin	+ W7	
Tumour supernatant	1	10	None	5.50 $\pm$ 0.75	6.24 $\pm$ 0.39	2.43 $\pm$ 0.37*	61.0
	2	100	Calmodulin depletion	1.85 $\pm$ 0.27	4.74 $\pm$ 0.75*	1.80 $\pm$ 0.10	62.0
Cultured cell supernatant	3	10	None	85.0 $\pm$ 4.30	93.2 $\pm$ 2.90	64.0 $\pm$ 10.80	31.3
	4	100	Calmodulin depletion	60.9 $\pm$ 0.10	95.4 $\pm$ 2.80*	62.6 $\pm$ 2.10	34.3
			None	3.94 $\pm$ 0.05	5.65 $\pm$ 0.44*†	3.0 $\pm$ 0.08*	46.9
			Calmodulin depletion	3.06 $\pm$ 0.05	5.69 $\pm$ 0.50*	3.15 $\pm$ 0.10	44.6
			None	78.7 $\pm$ 4.20	86.2 $\pm$ 2.70	54.4 $\pm$ 5.80*	36.9
			Calmodulin depletion	83.4 $\pm$ 0.16	108.9 $\pm$ 2.30*	85.9 $\pm$ 0.50*	21.1

* Denotes values differing from control incubations by $P < 0.05$.
† Activation by calmodulin of untreated supernatant was significant only in this one experiment and was not observed on seven other occasions.

supernatant preparation to destroy any endogenous cyclic nucleotide phosphodiesterase activity, showed supernatants to contain 0.69 ± 0.07 (mean $\pm$ S.E.M., $n = 3$) μ g of calmodulin activity per mg of soluble protein.

In order to demonstrate the direct activation of cyclic AMP phosphodiesterase by calmodulin, supernatant preparations were depleted of endogenous calmodulin. This was achieved by passage of supernatant over fluphenazine-Sepharose 6B in the presence of calcium. Using this technique 75–85% of the original protein was found in the first 12 (1ml) fractions eluted from the column. The efficiency of calmodulin removal (by using [3 H]calmodulin) with this affinity column was $94 \pm 2\%$ (mean $\pm$ S.E.M., $n = 3$) for [3 H]calmodulin in buffer but $72\% \pm 8\%$ (mean $\pm$ S.E.M., $n = 4$) for [3 H]calmodulin added to B16 melanoma supernatant.

Fig. 1. Detection of calmodulin-dependent cyclic AMP phosphodiesterase using calmodulin-agarose

B16 melanoma cultured cell supernatant in buffer containing a final concentration of 2.5 mM-CaCl₂ (full details in the Materials and methods section) was applied to calmodulin-agarose, equilibrated in 50 mM-Tris/HCl (pH 7.5)/5 mM-CaCl₂, at the point indicated by the arrow. Fractions (1 ml) were collected into 1 ml of phosphodiesterase storage buffer. At the point indicated by the second arrow, buffer containing 5 mM-CaCl₂ + 0.2 M-NaCl was used to elute the column until no further protein was detectable (approx. fraction 24), followed by a further 20 ml buffer wash (results not shown). At the point indicated by the third arrow, buffer containing 2 mM-EGTA was used to elute calcium-dependent protein from the column (1.3% of total protein in this experiment). The protein content of fractions is indicated by ●---●. Phosphodiesterase activity in fractions was measured in the absence (●---●) and presence (○—○) of a maximally stimulating concentration of calmodulin (200 ng/ml). Results show mean $\pm$ S.E.M. of triplicate determinations.

Boiling of supernatant increased the efficiency of [3 H]calmodulin removal by the column, e.g. from 70% efficiency before boiling to 80% after boiling.

When such calmodulin-depleted preparations were assayed for cyclic AMP phosphodiesterase activity with and without calmodulin (1.5×10^{-8} M) a significant effect of added calmodulin could now be seen (Table 1). The enzyme activity of supernatant preparations depleted of calmodulin in this way was not significantly affected by 5×10^{-5} M-W7, in the absence of calmodulin (Table 1).

For the four preparations shown in Table 1 which were divided and part of each subjected to calmodulin depletion, good agreement was found for the percentage of the enzyme calculated to be calmodulin-dependent, whether this figure was based on the untreated or the calmodulin-depleted preparation. Based on the difference in activity between the maximally stimulating amount of calmodulin (1.5×10^{-8} M) and maximally inhibitory amount of W7 (5×10^{-5} M) the percentage of total enzyme activity which was calmodulin-activated ranged from 21% (seen with 100 μ M-cyclic AMP) to 62% (observed with 10 μ M-cyclic AMP). The average activity (based on eight such experiments) calculated to be calmodulin-dependent was $49 \pm 4.6\%$ (mean $\pm$ S.E.M., $n = 8$).

Fig. 2. Calmodulin activation of B16 cyclic AMP phosphodiesterase eluted from calmodulin-agarose

Calmodulin-responsive cyclic AMP phosphodiesterase in pooled fractions eluted from calmodulin-agarose by EGTA-containing buffer was activated over 3-fold by added calmodulin. Half-maximal activation occurred at 10^{-8} M-calmodulin.

Subsequent study of this enzyme activity made use of a calmodulin-agarose affinity column. The bovine heart cyclic AMP phosphodiesterase enzyme, which is known to be calmodulin-activated, was tested for its ability to bind to the column in the presence of calcium. A peak of enzyme activity which could be further activated 3–4-fold by exogenous calmodulin could be eluted from the column with EGTA-containing buffer.

Using supernatant derived from B16 cells grown in culture (2.5 ml of a preparation containing approx. 0.5 mg of protein/ml), it was possible to demonstrate calcium-dependent binding of cyclic AMP phosphodiesterase activity present in B16 cytosol to the calmodulin-agarose gel. Only 1–2% of the supernatant protein exhibited calcium-dependent binding to the gel and it was only in these EGTA-eluted fractions that calmodulin-responsive cyclic AMP phosphodiesterase activity was detected (Fig. 1). Enzyme activity in these fractions was stimulated 3–4-fold by the addition of calmodulin, half-maximal activation occurring at 10^{-8} M-calmodulin (Fig. 2).

Discussion

Calmodulin is present in the mouse B16 melanoma (Sharrard *et al.*, 1983). It is known that α MSH elevates cyclic AMP in the B16 melanoma (Mac Neil *et al.*, 1981), so that one possible function of calmodulin in the B16 melanoma is to modulate the cyclic AMP signal. Thus, cyclic nucleotide phosphodiesterase is activated by calmodulin in a number of tissues, as summarized by Lin & Cheung (1980). We have therefore looked for cyclic AMP phosphodiesterase activity in the B16 melanoma and investigated its possible stimulation by Ca-calmodulin.

In agreement with other work, we found that cyclic AMP phosphodiesterase activity was predominantly in the supernatant fraction (Smoake *et al.*, 1974). Initial attempts to enhance further the activity of the enzyme by added pig brain calmodulin were unsuccessful. In view of the high degree of amino acid sequence conservation in calmodulins from different mammalian species, it seemed very unlikely that the pig brain calmodulin would be ineffective in the mouse system. The possibility of an unusual tumour-specific calmodulin was not suggested by our own studies on the B16 melanoma (Sharrard *et al.*, 1983). The most likely explanation is that the endogenous calmodulin content of the B16 supernatant preparation was already maximally activating for the phosphodiesterase; this was confirmed by assay of calmodulin activity in supernatant.

By using the calmodulin antagonist W7 it was possible to achieve significant inhibition of cyclic

AMP phosphodiesterase activity. However, such agents have been criticized for their lack of specificity against calmodulin. We therefore used two approaches to demonstrate the direct activation of B16 cyclic AMP phosphodiesterase by exogenous pig brain calmodulin.

The first approach was to deplete B16 melanoma supernatant of endogenous calmodulin by passage over fluphenazine-Sepharose 6B in the presence of calcium. Levels of depletion of more than 70% were achieved with good protein recovery, making this technique applicable to other soluble, calmodulin-activated enzymes. Although ion-exchange (DEAE-cellulose) and phenyl-Sepharose affinity chromatography produced adequate calmodulin removal, protein recovery was inferior by these techniques (results not shown). In this way, calmodulin-depleted supernatant now showed significant enhancement of cyclic AMP phosphodiesterase activity by added calmodulin. In the absence of exogenous calmodulin, W7 had little effect on enzyme activity, consistent with most of the endogenous calmodulin having been removed. This finding also argues for specificity of the action of W7 against the cyclic AMP phosphodiesterase in this system. Based on these observations it was calculated that approx. 50% of the total B16 cyclic AMP phosphodiesterase activity was calmodulin-sensitive.

The second approach was to separate the enzyme from endogenous calmodulin by calmodulin-agarose affinity chromatography. Preliminary studies with beef heart cyclic AMP phosphodiesterase showed that the enzyme bound to the gel in a calcium-dependent fashion.

Studies with B16 supernatant showed that this, too, contained cyclic AMP phosphodiesterase activity which would bind to the column in the presence of calcium and could be eluted by using EGTA-containing buffer. Moreover, the EGTA-eluted fractions contained enzyme activity which was further stimulated by added calmodulin. Although some phosphodiesterase activity failed to bind to the column in the presence of calcium, this enzyme activity was not activated by added calmodulin.

Although we have not in this study determined the biochemical characteristics of this enzyme, it is clear from these investigations that the mouse B16 melanoma contains a calmodulin-activated cyclic AMP phosphodiesterase. The problem of demonstrating calmodulin activation in unfractionated cytosol reflects the high endogenous levels of this protein. Calmodulin depletion by fluphenazine-Sepharose 6B affinity chromatography or separation of the enzyme from endogenous calmodulin by use of calmodulin-agarose are two simple ways to overcome this problem. Such methods should be

applicable to other soluble enzymes. The same principle of separation of enzyme from endogenous calmodulin may also be important in a study of calmodulin activation of membrane-bound enzymes such as the adenylate cyclase.

We are grateful to the MRC and the Yorkshire Cancer Research Campaign for financial support. S. T. is a Wellcome Trust Senior Lecturer.

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