Calmodulin activation of adenylate cyclase in the mouse B16 melanoma

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1. Calmodulin antagonists inhibited hormone-stimulated cyclic AMP accumulation in both cultured cells and cell lysates of mouse B16 melanoma. 2. Particulate preparations of B16 melanoma contained 34–45% of total cell calmodulin, which could not be dissociated by extensive washing irrespective of the presence of EGTA in the buffer. The adenylate cyclase activity in such preparations was unaffected by the addition of exogenous calmodulin. 3. However, the rare-earth-metal ion La^{3+} , which can mimic or replace Ca^{2+} in many systems, produced an immediate inhibition of agonist-stimulated adenylate cyclase activity and preincubation of particulate preparations with La^{3+} followed by washing with La^{3+} -free buffer dissociated calmodulin (96% loss) from particulate preparations. 4. The loss of calmodulin from particulate preparations was associated with a decrease in agonist responsiveness (74%) and a marked change in the Ca^{2+} -sensitivity of the enzyme, low concentrations of calcium (approx. 10 nM) now failing to stimulate enzyme activity, high concentrations of calcium (≥ 100 nM) producing greater-than-normal inhibition of enzyme activity. 5. Direct activation of adenylate cyclase by the addition of pure calmodulin was now demonstrable in such calmodulin-depleted particulate preparations. Half-maximal stimulation of agonist-responsive adenylate cyclase occurred at 80 nM-calmodulin in the presence of 10 μM free Ca^{2+} . Maximal stimulation by calmodulin (at 300–600 nM) restored enzyme activity to $89 \pm 5\%$ (mean $\pm$ S.E.M., $n = 7$) of the activity in untreated, calmodulin-intact, preparations.

The adenylate cyclase enzyme of the mouse B16 melanoma responds to αMSH and related peptides (Mac Neil *et al.*, 1981) with increased production of cyclic AMP, the intracellular signal initiating an increase in tyrosinase activity and melanin synthesis (Pawelek *et al.*, 1973). Cyclic AMP phosphodiesterase rapidly terminates this signal by degrading cyclic AMP to inactive 5'-AMP, and we have recently reported that a large proportion (50%)

of this enzyme activity can be activated by Ca^{2+} + calmodulin (Walker *et al.*, 1984).

The intracellular Ca^{2+} -binding protein calmodulin mediates many of the intracellular actions of Ca^{2+} (Brostrom & Wolff, 1981) and can activate both the adenylate cyclase and phosphodiesterase enzymes in some tissues (Wang & Weisman, 1979). Although many hormone-responsive adenylate cyclase systems, including the B16 melanoma (De Graan *et al.*, 1981), are known to be Ca^{2+} -dependent (Bradham & Cheung, 1980), comparatively few have been shown to be regulated by calmodulin (Tomlinson *et al.*, 1984).

The present paper describes the inhibition of αMSH -stimulated cyclic AMP production in B16-melanoma cultured cells and cell lysates by calmodulin antagonists and the direct stimulation of agonist-responsive adenylate cyclase activity by calmodulin in calmodulin-depleted particulate preparations.

Abbreviations used: αMSH , melanocyte-stimulating hormone (α -peptide), melanotropin; PGE_1 , prostaglandin E_1 ; IBMX, isobutylmethylxanthine; MEM, minimal essential medium; phosphate-buffered saline (Dulbecco's), Na_2HPO_4 (1.15 g/l)/ KH_2PO_4 (0.2 g/l)/ NaCl (8 g/l)/ KCl (0.2 g/l)/ CaCl_2 (0.132 g/l)/ MgCl_2 (0.1 g/l), pH 7.2; W7, *N*-(6-aminohexyl)-5-chloro-1-naphthalenesulphonamide; PCP, prochlorperazine; PCPS, prochlorperazine sulphoxide.

Materials and methods

Chemicals

[α - 32 P]ATP (0.5–30 Ci/mmol), cyclic [8- 3 H]-AMP (20–30 Ci/mmol) and calmodulin radioimmunoassay kits were obtained from Amersham International, Amersham, Bucks., U.K. Cyclic AMP as the crystallized free acid, the sodium salt of ATP, phosphoenolpyruvate (tricyclohexylammonium salt), pyruvate kinase (EC 2.7.1.40; sp. activity approx. 200 units/mg) and calmodulin-deficient ox heart phosphodiesterase were obtained from Boehringer-Mannheim. King-cobra (*Ophiophagus hannah*) venom, Dowex 1 (X8; 200–400 mesh) anion-exchange resin, Coomassie Blue, prostaglandin E₁ (PGE₁), LaCl₃, bovine serum albumin (fraction V), isobutylmethylxanthine (IBMX) and theophylline were obtained from Sigma, Poole, Dorset, U.K.

Medium 199, MEM, phosphate-buffered saline (Dulbecco's), foetal-calf serum, glutamine, penicillin and streptomycin were all obtained from Gibco Europe Limited, Paisley, Renfrewshire, Scotland, U.K. Ham's F-10 medium was obtained from Flow Laboratories, Rickmansworth, Herts., U.K. All tissue-culture plastics were obtained from Sterilin, Teddington, Middx., U.K. Forskolin was obtained from Calbiochem-Boehringer Corp., La Jolla, CA, U.S.A.

Prochlorperazine and prochlorperazine sulphoxide were gifts from Smith, Kline and French Laboratories, Welwyn Garden City, Herts., U.K. *N*-(6-Aminoethyl)-5-chloro-1-naphthalene-sulphonamide (W7), used in initial studies was generously given by Dr. H. Hidaka, Mie University School of Medicine, Mie 514, Japan. α MSH was a gift from Dr. W. Rittel of Ciba-Geigy, Basel, Switzerland. All other chemicals were of analytical grade.

Preparation of calmodulin

Pig brain and eel electric-organ calmodulin were prepared by the method of Kakiuchi *et al.* (1981). Preparations were over 95% pure as determined by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970).

Culture of B16-melanoma cells

The mouse B16 melanoma was serially transplanted in C57BL mice. Melanotic cells were derived from the tumours and grown in 199 tissue-culture medium with Hank's 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 μ g/ml). Confluent cultures were used for the measurement of cyclic AMP accumulation in response to hormonal

stimulation and for the preparation of cell lysates, washed particulate preparations and cell cytosol.

Intact-cell cyclic AMP accumulation

In a comparison of three media, Ham's F-10, 199 and MEM, it was found that the greatest intracellular cyclic AMP response to hormone stimulation occurred with Ham's F-10 medium. Also, the cyclic AMP response to hormones decreased with successive passaging of the cells, the maximum response to α MSH on the second passage being only 50–60% of the response seen on the first passage. Accordingly, for studies of hormone-stimulated cyclic AMP accumulation, cells were grown to confluence in 24 (2 cm²) well plates using Ham's F-10 medium with 10% foetal-calf serum and penicillin (50 i.u./ml) and streptomycin (50 μ g/ml). At confluence, medium was removed and replaced with 0.5 ml of Ham's F-10 medium containing 0.1% bovine serum albumin and 800 μ M-IBMX for 10 min at 37°C. This medium was then removed and replaced with 0.5 ml of this same medium containing hormones and drugs as appropriate and incubated at 37°C for 15 min. Incubations were terminated by removal of medium and cultures were rinsed twice with phosphate-buffered saline and 1 ml of ethanol at 4°C added. Cells were detached by scraping (with a 1 ml plastic syringe plunger) and pelleted by spinning at 1000 *g* for 10 min. Cell pellets were washed with 1 ml of ethanol at 4°C and both ethanol washes combined and freeze-dried. For assay of intracellular cyclic AMP, 500 μ l of 50 mM-Tris/HCl (pH 7.4 at room temperature) containing 8 mM-theophylline, 6 mM-2-mercaptoethanol and 6 mM-tetrasodium EDTA was added to freeze-dried cell extracts and extracts assayed for cyclic AMP by using the protein-binding assay described by Brown *et al.* (1971). Cell pellets were retained for the determination of protein by the Lowry *et al.* (1951) method.

Lysate, particulate and cytosol preparations of B16 melanoma cells

Confluent cultures of B16-melanoma cells grown in 25 cm² Falcon flasks were washed free of culture medium with phosphate-buffered saline and drained, then 1 ml of 25 mM-Tris/HCl (pH 7.4 at 4°C) containing 250 mM-sucrose and EGTA as indicated was added per flask. Cells were scraped from the flask with a small metal spatula and lysed by homogenization with four strokes of a tight Dounce glass pestle. This lysate was used in the adenylate cyclase assay and also divided into particulate and supernatant fractions by centrifugation at 11950 *g* (*r*_{av}, 10.69 cm) using a Sorval RC-5B centrifuge for 10 min at 4°C. Particulate preparations were washed twice with the buffer

described and finally resuspended to the original volume of the lysate with either buffer or cell cytosol. Since we had previously shown that washed particulate preparations of B16 melanoma (Mac Neil *et al.*, 1981) are poorly responsive to hormones and other agonists in the absence of cell cytosol, experiments were done in both the presence and the absence of cytosol. Cytosol preparations were obtained by further centrifugation of supernatants at 70 000g for 60 min (r_{av} 6.52 cm) in a Sorvall OTD-65 centrifuge.

Measurement of adenylate cyclase activity

Adenylate cyclase activity was measured by the conversion of [α - 32 P]ATP into cyclic [32 P]AMP as described previously (Mac Neil *et al.*, 1980), and cyclic [32 P]AMP was isolated as described by Salomon *et al.* (1974). Assays were performed in triplicate and results were expressed as means $\pm$ S.E.M. of the triplicate determinations. Protein was determined by the method of Bradford (1976), with bovine serum albumin (fraction V) as standard.

Measurement of calmodulin activity

Calmodulin activity in particulate and supernatant preparations of B16 melanoma was determined as described previously (Mac Neil *et al.* 1984), with a calmodulin-dependent ox heart phosphodiesterase.

Ca²⁺ determination

Ca²⁺ present in homogenate, particulate and cell lysate preparations was determined by the method of Gitelman (1967).

Free-Ca²⁺ calculation

The dissociation constants of Caldwell (1970) for Ca²⁺, Mg²⁺ and EGTA at pH 7.4 were used to calculate free Ca²⁺ in the presence of EGTA.

Statistics

The difference between means was assessed for significance by Student's *t* test. Values of $P < 0.05$ were taken as statistically significant.

Results

Inhibitory effects of calmodulin antagonists on cultured-cell cyclic AMP accumulation and on cell-lysate adenylate cyclase activity

In both intact cells (IBMX present) and in broken-cell preparations the calmodulin antagonists PCP and W7 inhibited cyclic AMP production in response to a maximally stimulating concentration of α MSH (20 μ M) (Fig. 1). The drugs were more potent in cultured cells than in broken-cell preparations in this respect, 50% inhibition

occurring at 4 μ M-PCP and 22 μ M-W7 for intact cells but 45 μ M-PCP and 60 μ M-W7 for cell lysates. The PCP analogue PCPS, known to be a much less potent calmodulin antagonist than PCP, produced some inhibition of cyclic AMP accumulation in cells (40% at 100 μ M), but was ineffective in cell lysates up to 100 μ M.

Concentrations of PCP and W7 only 5-fold higher than those inhibiting α MSH-stimulated cyclic AMP accumulation in intact cells also caused some degree of cell detachment under the serum-free conditions, necessary for the measurement of cyclic AMP, used in these experiments. Further studies with broken-cell preparations showed that the inhibition produced by the calmodulin antagonists was increased by pre-incubation of particulate preparations with the drugs (up to a maximum at 10 min at 4°C), was apparently independent of the presence of Ca²⁺ and was more marked for α MSH- and PGE-stimulated enzyme activity than for basal, fluoride- or forskolin-stimulated enzyme activity. The drugs produced a similar degree of inhibition whether or not cytosol was present, as is shown in the example given in Table 1, where the inhibitory effect of PCP was compared in the presence and absence of cytosol.

Removal of endogenous calmodulin from particulate preparations with La³⁺

Adenylate cyclase activity in washed particulate preparations of B16 melanoma was not affected by the addition of pure calmodulin (from 10 ng to 100 μ g/ml). Such particulate preparations contained $0.82 \pm 0.06 \mu$ g of calmodulin activity per mg of particulate protein (mean $\pm$ S.E.M., $n = 6$). This amount represented 34–46% ($n = 3$) of the total cell calmodulin activity and was of a similar concentration to that found in a range of other cultured cells in this laboratory. Neither extensive washing with buffer nor incubation of membranes with buffer containing up to 20 mM-EGTA for 1 h at 37°C decreased this calmodulin concentration [$0.85 \pm 0.06 \mu$ g of calmodulin activity/mg of particulate protein (mean $\pm$ S.E.M., $n = 3$)]. However, following a report of the use of La³⁺ to remove membrane calmodulin in the ciliate protozoan *Paramecium* (Schultz & Klumpp, 1982), we found that up to 96% of the endogenous calmodulin could be removed from B16-melanoma particulate preparations using 15 min incubations with 200 μ M-LaCl₃ at 4°C, followed by two washes with buffer [$0.03 \pm 0.01 \mu$ g of calmodulin activity/mg of particulate protein (mean $\pm$ S.E.M., $n = 4$)]. Lower concentrations of LaCl₃ resulted in partial removal of calmodulin, as shown in Table 2. Loss of calmodulin from particulate preparations and the appearance of calmodulin in the washes was

Fig. 1. Inhibitory effect of calmodulin antagonists on α MSH-stimulated cyclic AMP accumulation in intact cells (a) and on α MSH-stimulated adenylate cyclase activity in cell lysates (b)

(a) Cells were incubated with IBMX and the drugs PCP (●), PCPS (○) and W7 (■) for 15 min at 37°C in the presence of a maximally stimulating concentration of α MSH (20 μ M) and in its absence (basal, unstimulated cyclic AMP accumulation). Results are expressed as a percentage of the control response to α MSH (basal values subtracted) in the absence of drugs. Values shown are means $\pm$ S.E.M. of quadruplicate cultures. Basal cyclic AMP accumulation, approximately one-tenth of that in the presence of α MSH, was inhibited 47% by 100 μ M-PCP, 30% by 100 μ M-W7 and unaffected by 100 μ M-PCPS. (b) Adenylate cyclase activity was measured in lysates of B16-melanoma cells containing 250 μ M-EGTA (otherwise as described in the Materials and methods section). Lysates were preincubated with the drugs PCP (●), PCPS (○) and W7 (■) for 10 min at 4°C before assay. Results are expressed as a percentage of the control response to α MSH (20 μ M) (basal enzyme activity subtracted) in the absence of drugs. Values shown are means $\pm$ S.E.M. for three experiments.

Table 1. Inhibitory effect of PCP (100 μ M) on B16-melanoma adenylate cyclase activity in the presence and absence of cytosol. Washed particulate preparations of B16-melanoma cultured cells and cell cytosol were prepared as described in the Materials and methods section. Particulate preparations were preincubated with cell cytosol (25 μ l per incubation; approx. 15 μ g of cytosolic protein) and buffer or PCP as appropriate for 10 min at 4°C before incubations were commenced by addition of ATP substrate. Results are means $\pm$ S.E.M. ($n = 3$) for an individual experiment, representative of results obtained on a number of occasions.

	Cytosol	Adenylate cyclase activity (nmol of cyclic AMP produced/10 min per mg of protein)		Inhibition produced by PCP (%)
		- PCP	+ PCP	
Basal	-	0.44 $\pm$ 0.02	0.36 $\pm$ 0.01	18.2
	+	0.78 $\pm$ 0.13	0.56 $\pm$ 0.10	28.2
PGE ₁ (1 μ M)	-	1.20 $\pm$ 0.02	0.84 $\pm$ 0.03	30.0
	+	2.89 $\pm$ 0.07	1.89 $\pm$ 0.17	34.6
α MSH (20 μ M)	-	1.06 $\pm$ 0.17	0.68 $\pm$ 0.14	35.8
	+	2.19 $\pm$ 0.05	0.86 $\pm$ 0.05	60.7
NaF (10 mM)	-	1.52 $\pm$ 0.09	1.04 $\pm$ 0.12	31.5
	+	2.00 $\pm$ 0.06	1.54 $\pm$ 0.04	23.0
Forskolin (10 μ M)	-	16.32 $\pm$ 0.23	6.08 $\pm$ 0.07	62.7
	+	22.50 $\pm$ 0.38	17.10 $\pm$ 0.16	24.0

confirmed by both assay of calmodulin activity and radioimmunoassay for calmodulin.

Effect of La³⁺ on adenylate cyclase assay

Addition of LaCl₃ to the assay mixture produced an immediate inhibition of (PGE₁ + forskolin)-stimulated enzyme activity. A 50% inhibition of the response to maximally stimulating concentrations of PGE₁ + forskolin (1 µg/ml + 10 µM) and NaF (10mM) in B16-cell lysates occurred at comparatively low concentrations of LaCl₃, namely 1.4 ± 0.7 µM (mean ± S.E.M., n = 6) and 1.7 ± 0.6 µM (mean ± S.E.M., n = 3) respectively. La³⁺ had much less effect on basal activity, approx. 50% inhibition occurring between 10 and 100 µM-LaCl₃. Similar results were obtained in washed particulate preparations. Despite the potency of La³⁺ against agonist-stimulated enzyme activity, the inhibition produced was incomplete: a maximum of 88.9 ± 4.7% (mean ± S.E.M., n = 6) for PGE₁ + forskolin, and 81.6 ± 5% (mean ± S.E.M., n = 5) for NaF. Addition of Ca²⁺ or calmodulin, alone or in combination, could not overcome this inhibition when La³⁺ was present in the assay.

Effect of La³⁺ pretreatment of particulate preparations on adenylate cyclase activity

Preincubation of particulate preparations with La³⁺ for 15 min at 4°C followed by two washes with assay buffer decreased endogenous calmodulin as already described, and was also associated with a decrease in both the agonist-responsiveness (Table 2) and the Ca²⁺-sensitivity of the adenylate cyclase (Fig. 2) as follows.

A progressive loss of agonist-responsiveness up to a maximum of 74 ± 10% loss (mean ± S.E.M., n = 3) occurred with increasing concentrations of LaCl₃ preincubation. Some agonist-responsiveness always remained, however, even in preparations that contained less than 4% of the original particulate calmodulin. Basal activity was less affected [32 ± 18% loss of activity (mean ± S.E.M., n = 3)] by La³⁺ treatment (Table 2).

Addition of cytosol failed to restore agonist responsiveness.

The effect of calmodulin depletion on the Ca²⁺-dependency of the adenylate cyclase enzyme is illustrated in Fig. 2, where the enzyme Ca²⁺-dependency of washed particulate preparations of B16 melanoma containing 100, 38, 23 and 3% of the original particulate calmodulin is compared. In the La³⁺-untreated preparations (100% original calmodulin) there is a clear biphasic response to Ca²⁺, optimal enzyme activity (in the presence of maximally stimulating concentrations of PGE₁ and forskolin) occurring at a calculated free-Ca²⁺ concentration between 1 and 10nM-Ca²⁺. On the basis of four such experiments we found this optimal calcium concentration to be 6.6 ± 1.4nM (mean ± S.E.M., n = 4). Ca²⁺ concentrations greater than 100nM were progressively inhibitory to enzyme activity. (Basal enzyme activity was similar to agonist-stimulated activity in its Ca²⁺-dependency, and neither was significantly altered by the inclusion of cytosol in the assay.) As Fig. 2 shows, loss of particulate calmodulin was associated with a loss of the biphasic response to Ca²⁺; the stimulation of enzyme activity seen at low Ca²⁺ disappeared, whereas the inhibition effect of Ca²⁺ on enzyme activity became more marked.

Ca²⁺ stimulation of adenylate cyclase activity in La³⁺-pretreated particulate preparations

In particulate preparations largely (90%) depleted of calmodulin by using La³⁺ pretreatment, we investigated the stimulation of the adenylate cyclase enzyme by calmodulin. Our results to date show that calmodulin stimulation of such enzyme preparations can be most consistently demonstrated if cytosol is present and at the higher (1 µM and above) concentrations of free Ca²⁺. In three experiments in which we examined calmodulin stimulation of enzyme activity in calmodulin-depleted preparations over a wide range of Ca²⁺ concentrations (0.1nM–100µM) we found little stimulation (0–31%) of enzyme activity by calmo-

Table 2. *Effect of La³⁺ pretreatment on adenylate cyclase activity*

Particulate preparations were incubated with LaCl₃ at the appropriate concentration for 15 min at 4°C, then washed twice with assay buffer. Enzyme activity in these preparations was then compared with that in preparations incubated and washed with assay buffer alone.

[LaCl ₃] (µM)	Decrease in particulate calmodulin activity (%)	Decrease in enzyme activity (%) [mean ± S.E.M. (n)]	
		Basal	PGE ₁ + forskolin
10	—	2 ± 8 (3)	18 ± 12 (3)
25	52 ± 10 (3)	5 ± 10 (3)	25 ± 17 (3)
100	87 ± 8 (4)	38 ± 14 (4)	69 ± 15 (4)
200	97 ± 2 (3)	32 ± 18 (3)	74 ± 10 (3)

Fig. 2. Effect of decreasing particulate calmodulin content on adenylate cyclase sensitivity to Ca^{2+} . Particulate preparations were prepared as described in the Materials and methods section and incubated with either buffer (●) or increasing concentrations [25 μM (■), 100 μM (○) or 200 μM (▲)] of LaCl_3 for 15 min at 4°C then washed twice with buffer. Enzyme activity was measured in the presence of a maximally stimulating concentration of PGE_1 (1 $\mu\text{g}/\text{ml}$) plus Forskolin (10 μM) at the different calculated free-calcium concentrations shown (obtained by using EGTA and calcium buffers).

Table 3. Characteristics of calmodulin activation of adenylate cyclase activity in La^{3+} -treated particulate preparations. Washed particulate preparations of B16-melanoma cultured cells and cell cytosol were prepared as described in the Materials and methods section. La^{3+} -treated particulate preparations were incubated with 100 μM - LaCl_3 for 15 min at 4°C, then washed twice with assay buffer. Control preparations were incubated with buffer alone. All preparations were resuspended in cell cytosol before assay. Calmodulin was added to La^{3+} -treated preparations for approx. 10 min at 4°C before assay. Results are means $\pm$ S.E.M. for triplicate determinations in individual experiments comparing for each preparation the enzyme activity pre- La^{3+} treatment with that post- La^{3+} treatment in the absence of calmodulin (significantly decreased in all cases at P 0.05 or less) and in the presence of a maximally stimulating concentration of calmodulin (usually 0.5–1.0 $\mu\text{g}/\text{incubation}$). All incubations were in the presence of maximally stimulating concentrations of PGE_1 (1 $\mu\text{g}/\text{ml}$) plus forskolin (10 μM) and 10 μM -free Ca^{2+} .

Expt. no.	Adenylate cyclase activity (nmol of cyclic AMP produced/10 min per mg of protein)			Calmodulin required for half-maximal activation (ng/incubation)
	Control	La^{3+} -treated	La^{3+} -treated + calmodulin	
1	2.01 $\pm$ 0.06	1.60 $\pm$ 0.09	2.09 $\pm$ 0.07	20
2	1.5 $\pm$ 0.03	0.57 $\pm$ 0.01	0.88 $\pm$ 0.01	16
3	1.41 $\pm$ 0.01	0.79 $\pm$ 0.15	1.35 $\pm$ 0.05	100
4	1.93 $\pm$ 0.01	0.66 $\pm$ 0.01	1.58 $\pm$ 0.02	70
5	0.70 $\pm$ 0.02	0.14 $\pm$ 0.02	0.59 $\pm$ 0.01	280
6	1.73 $\pm$ 0.06	0.97 $\pm$ 0.10	3.15 $\pm$ 0.21*	270
7	5.02 $\pm$ 0.15	1.70 $\pm$ 0.05	5.10 $\pm$ 0.45	200

* Stimulation of enzyme activity to levels greater than that of the control by addition of calmodulin was only seen in this one experiment.

dulin at low Ca^{2+} (0.1 nM), but clear stimulation (+70 to +156%) at concentrations of 1 μM and greater. Accordingly, for the purpose of determining whether the B16-melanoma adenylate cyclase is stimulated by calmodulin, we have concentrated on the calmodulin responsiveness of the enzyme in

the presence of cytosol at relatively high Ca^{2+} concentrations only (10 μM). Fig. 3 shows one of seven dose-response curves for calmodulin at 10 μM - Ca^{2+} . Results for all seven are summarized in Table 3. The concentration of calmodulin required for half-maximal activation of agonist-

Fig. 3. Stimulatory effect of calmodulin on B16-melanoma adenylate activity

Particulate preparations were prepared as described in the Materials and methods section and then incubated with $200\text{ }\mu\text{M}$ - LaCl_3 for 15 min at 4°C before washing twice with buffer and finally resuspending in cytosol. Adenylate cyclase activity was measured in the presence of either buffer (●) or a maximally stimulating concentration of PGE_1 ($1\text{ }\mu\text{g/ml}$) plus forskolin ($10\text{ }\mu\text{M}$) (○). Calmodulin was preincubated with particulate preparations for approx. 10 min at 4°C before commencement of assay. Values shown are means $\pm$ S.E.M. for a single representative experiment (for range of values, see Table 4.)

stimulated enzyme activity was $136 \pm 39\text{ ng}$ (80 nM) of calmodulin per incubation (mean $\pm$ S.E.M., $n = 7$), approx. 7 times as great as the amount of calmodulin calculated to be originally present in control untreated particulate preparations [incubation mixtures contained $22.8 \pm 9.2\text{ }\mu\text{g}$ of protein, of which $18.7 \pm 7.6\text{ ng}$ per incubation mixture was calmodulin (mean $\pm$ S.E.M., $n = 7$)]. Maximum stimulation of agonist-stimulated enzyme activity occurred between 0.5 and $1.0\text{ }\mu\text{g}$ of calmodulin per incubation mixture ($300\text{--}600\text{ nM}$) and this stimulation restored (4 out of 7) or partially restored (3 out of 7) enzyme activity in the calmodulin-depleted preparation to that seen in the calmodulin-intact control preparation. This stimulation ranged from $+30$ to $+321\%$ (mean $\pm$ S.E.M.; $148 \pm 37\%$, $n = 7$). Calmodulin had little effect on unstimulated enzyme activity until relatively high concen-

trations were used, i.e. greater than $1\text{ }\mu\text{g}$ per incubation mixture.

Discussion

Calmodulin activation of adenylate cyclase, originally described for brain (Brostrom *et al.*, 1975; Cheung *et al.*, 1975), has now been demonstrated in a variety of cell and tissue types, which include pancreatic islets, adrenal medulla, lung, kidney, testicular germ cells, intestine and smooth muscle (Tomlinson *et al.*, 1984).

The B16 melanoma contains calmodulin (Shar-rard *et al.*, 1983), where it activates a cyclic AMP phosphodiesterase (Walker *et al.*, 1984) and may be involved in the regulation of cell proliferation (Mac Neil *et al.*, 1984). However, there is no information on the possible calmodulin regulation of the B16-melanoma adenylate cyclase.

Initially the effects of drugs known to inhibit calmodulin activity were investigated for their action on the adenylate cyclase enzyme. Although it was possible to demonstrate inhibition of the enzyme by these agents, comparatively high concentrations of these were required in broken-cell preparations. Also, their inhibitory effects in such preparations did not appear to be Ca^{2+} -dependent, in contrast with their known requirement for Ca^{2+} in their action as calmodulin antagonists *in vitro* (Weiss *et al.*, 1980). Furthermore, the specificity of these agents as calmodulin antagonists is known to be poor (Vincenzi, 1982). For these reasons we decided to look for direct activation of the B16-melanoma adenylate cyclase by calmodulin.

Addition of calmodulin to membrane preparations did not affect adenylate cyclase activity, and it proved necessary to deplete membrane preparations of endogenous calmodulin. Although EDTA- or EGTA-containing buffers have been used successfully for this purpose in some tissues (e.g. brain; Brostrom *et al.*, 1977), in others they proved ineffective, e.g. guinea-pig sperm (Hyne & Garbers, 1979), rat pituitary GH_3 cells (Brostrom *et al.*, 1982) and in the present study. However, Schultz & Klumpp (1982) have shown that La^{3+} can deplete *Paramecium* ciliary membrane of calmodulin; this technique proved successful with the B16 melanoma and also in human thyroid membranes (T. Lakey, S. Mac Neil, S. Humphries, S. W. Walker, D. S. Munro & S. Tomlinson, unpublished work).

Addition of La^{3+} to the assay inhibited adenylate cyclase activity, as described in brain (Nathanson *et al.*, 1976) and also for *Paramecium* guanylate cyclase (Schultz & Klumpp, 1982). Some residual agonist-responsive activity always remained in the B16-melanoma adenylate cyclase, implying a part of the enzyme to be Ca^{2+} -

calmodulin-independent. Removal of endogenous calmodulin by La^{3+} was associated with a loss of the adenylate cyclase activation normally observed at submicromolar Ca^{2+} concentrations. This calmodulin requirement for submicromolar Ca^{2+} for adenylate cyclase activation has been reported for other cell types (Brostrom *et al.*, 1976, 1982; Ausiello & Hall, 1981; Ofulue & Nijjar, 1981; Piascik *et al.*, 1983).

Restoration of adenylate cyclase activity by addition of exogenous calmodulin to such calmodulin-depleted membranes was now demonstrable. The B16-melanoma adenylate cyclase was activated $148 \pm 37\%$ (mean $\pm$ S.E.M., $n = 7$), with maximal stimulation occurring at 300–600 nM, a concentration range comparable with that found by other authors [from 100 nM (Bodnaryk, 1983) to 6 μM (Brostrom *et al.*, 1982)]. This restoration of activity by added calmodulin occurred optimally at relatively high Ca^{2+} concentrations, in contrast with the situation with the native enzyme; the reasons for this discrepancy is not known, but has been observed by others (Piascik *et al.*, 1983).

These studies also suggest that the enhancement of agonist-stimulated adenylate cyclase activity mediated by (a) factor(s) in cytosol (Mac Neil *et al.*, 1980; Crawford *et al.*, 1980), is not due to calmodulin. Thus augmentation of agonist-stimulated activity occurred whether or not B16-melanoma membranes were calmodulin-depleted. This conclusion is in agreement with the work of Ofulue & Nijjar (1981) for rat lung.

In summary, the B16 melanoma contains adenylate cyclase that can be shown to be calmodulin-activated by the technique of depletion of endogenous membrane-bound calmodulin by La^{3+} washing. (This technique may prove valuable for the demonstration of calmodulin-dependent adenylate cyclase activity in other tissues also.) Therefore in the B16 melanoma, as in other tissues such as brain and pancreatic islets (Tomlinson *et al.*, 1984), calmodulin is involved in both the synthesis and degradation (Walker *et al.*, 1984) of cyclic AMP.

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