Identification and characterization of a Ca^{2+} –calmodulin-sensitive cyclic nucleotide phosphodiesterase in a human lymphoblastoid cell line

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This study examines the pattern and regulatory properties of cyclic nucleotide phosphodiesterases in a human lymphoblastoid B-cell line (RPMI 8392) established from a patient with acute lymphocytic leukaemia. In this cell line, phosphodiesterase activity measured at $0.25\ \mu\text{M}$ -cyclic AMP is approx. 7-fold greater than that in isolated human peripheral-blood lymphocytes, and 16% of the phosphodiesterase activity in RPMI 8392 cells is associated with particulate fractions. Phosphodiesterase activity in crude fractions of this cell line is reproducibly stimulated by about 60–80% by Ca^{2+} –calmodulin. In the presence of 20 nM-calmodulin, half-maximal stimulation occurs at $0.7\ \mu\text{M}$ - Ca^{2+} . The cytosolic phosphodiesterase activity of RPMI 8392 cells is separated into two forms by DEAE-Sephacel chromatography. The first form is eluted at approx. 0.2 M-sodium acetate, catalyses the hydrolysis of both cyclic AMP and cyclic GMP, and is stimulated 3-fold by Ca^{2+} –calmodulin. This form exhibits non-linear kinetics for cyclic AMP in the absence of calmodulin, with extrapolated K_m values of 0.8 and $4\ \mu\text{M}$, and non-linear kinetics in the presence of calmodulin, with extrapolated K_m values of 0.5 and $1\ \mu\text{M}$. The $V_{\max}$ values are increased approx. 3-fold by calmodulin. The second form is eluted at approx. 0.6 M-sodium acetate, is specific for cyclic AMP, and insensitive to stimulation by Ca^{2+} –calmodulin. The Ca^{2+} –calmodulin-sensitive phosphodiesterase from the DEAE-Sephacel column can be adsorbed to a calmodulin–Sepharose affinity column and eluted with EGTA. This enzymic activity can also be immunoprecipitated by a monoclonal antibody directed against a calmodulin–bovine heart phosphodiesterase complex. This study documents the existence of Ca^{2+} –calmodulin-sensitive phosphodiesterase in a cultured lymphoblastoid cell line derived from a leukaemic patient.

INTRODUCTION

In many mammalian tissues, 3',5'-cyclic nucleotide phosphodiesterases (EC 3.1.4.17) have been shown to consist of multiple forms, differing in physical, kinetic and regulatory properties (Wells & Hardman, 1977; Strada & Thompson, 1978; Appleman *et al.*, 1982; Beavo *et al.*, 1982). These forms can often be classified as one of three general types: a Ca^{2+} –calmodulin-sensitive form, a form with a regulatory site for cyclic GMP, which stimulates the hydrolysis of cyclic AMP, and a form with no apparent regulators, which exhibits a high affinity and relative specificity for cyclic AMP (Appleman *et al.*, 1982). Not all tissues contain all three of these types of phosphodiesterase. With lymphocytes in particular, the presence of a Ca^{2+} –calmodulin-sensitive form of the enzyme has never been described. Hait & Weiss (1977, 1979) examined normal and leukaemic mouse splenic lymphocytes for the presence of this enzyme activity. Although the presence of calmodulin in these lymphocytes was shown by the Ca^{2+} -dependent stimulation of a calmodulin-sensitive rat brain phosphodiesterase by these cell supernatants, both the normal and leukaemic lymphoid cells nevertheless appeared to lack a Ca^{2+} –calmodulin-sensitive phosphodiesterase, since phosphodiesterase activity in these cells was unaffected by EGTA,

Ca^{2+} , or Ca^{2+} plus calmodulin. Human monocytes were also reported to be devoid of Ca^{2+} –calmodulin-dependent phosphodiesterase (Thompson *et al.*, 1980). Similarly, HPBL isolated from defibrinated blood have been reported to contain only high-affinity cyclic AMP phosphodiesterase activity, and this enzyme activity was insensitive to stimulation by Ca^{2+} in crude supernatants (Thompson *et al.*, 1976). This was confirmed during a subsequent study of the properties of phosphodiesterase in isolated HPBL (Epstein & Hachisu, 1984). It was shown by anion-exchange chromatography, isoelectric focusing and glycerol-density-gradient centrifugation that HPBL contain only a single form of high-affinity Ca^{2+} –calmodulin-insensitive phosphodiesterase activity.

There is some controversy surrounding the nature of phosphodiesterases in isolated HPBL, in that some investigators have reported the presence of both high- and low-affinity cyclic AMP phosphodiesterase activity in HPBL isolated from heparinized blood (Lagarde & Colobert, 1972; Monahan *et al.*, 1975; Wedner *et al.*, 1979; reviewed by Epstein & Hachisu, 1984). However, in those studies, the possible regulation by calmodulin of these phosphodiesterase activities was never examined. In the present study, we report on the pattern and properties of cyclic nucleotide phosphodiesterases in the cultured human B-cell lymphoblastoid cell line, RPMI

Abbreviations used: HPBL, human peripheral-blood lymphocytes; Tos-Lys- CH_2Cl , tosyl-lysylchloromethane.

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8392, established from a patient with acute lymphocytic leukaemia. This cultured human leukaemic cell line contains Ca^{2+} -calmodulin-sensitive phosphodiesterase, which appears to be absent from normal HPBL when analysed under the same conditions. A preliminary account of this work has been published previously (Epstein & Hachisu, 1983).

MATERIALS AND METHODS

Growth and maintenance of the lymphoblastoid cell line RPMI 8392

The cell line RPMI 8392 was established from a patient with acute lymphocytic leukaemia by Dr. Jun Minowada of the Roswell Park Memorial Institute, Buffalo, NY, U.S.A. These cells were obtained for this study through the courtesy of Dr. Stanley Cohen, of the Department of Pathology, University of Connecticut Health Center. These cells were maintained in suspension in Falcon flasks at 37 °C in a humidified air/ CO_2 (19:1) environment by continuous passage (three times weekly, 50–75% of the cells and medium were replaced by fresh medium) in RPMI 1640 medium supplemented with 10% heat-inactivated fetal-calf serum, 100 units of penicillin/ml, 100 μg of streptomycin/ml and 2 mM-L-glutamine. These cells possess Epstein-Barr virus and surface immunoglobulins (Schook *et al.*, 1981). For these studies, the cells were grown to a density of approx. 10^6 /ml before harvesting.

Isolation of HPBL

HPBL were isolated from leucocyte-rich fractions of cells in Fenwol Anticoagulant Citrate (Fenwol Labs, Deerfield, IL, U.S.A.), obtained from the American Red Cross, Farmington, CT, U.S.A., by leukapheresis of normal donors. The lymphocytes were purified to > 98% purity by incubation with tetracarboxylon, separation by Ficoll-Hypaque density-gradient centrifugation, and lysis of remaining erythrocytes by incubation with hypo-osmotic Tris/ NH_4Cl buffer, as described previously (Epstein *et al.*, 1977; Epstein & Hachisu, 1984).

Preparation of cell extracts

For preparation of cell extracts, both HPBL and RPMI 8392 cells were resuspended in 40 mM-Tris/HCl/50 μM -Tos-Lys- CH_2Cl (pH 8.0) at 2×10^7 cells/ml and sonicated for 3×10 s at 70 W in a Heat Systems/Ultrasonics Sonifier Cell Disruptor model W185D. For preparation of supernatants, sonicated cell preparations were centrifuged at 100 000 g for 60 min.

Assay of cyclic nucleotide phosphodiesterase activity

This was done by the two-step radioisotope procedure of Thompson *et al.* (1979), by using Dowex 1-X2 columns to separate reaction products from unchanged substrate. Reaction mixtures (0.4 ml) contained 40 mM-Tris/HCl (pH 8.0), 10 mM- MgCl_2 , 2.5 mM-2-mercaptoethanol, 1×10^5 c.p.m. of either cyclic [^3H]AMP (0.25 μM) or cyclic [^3H]GMP (0.25 μM), 50 μg of bovine serum albumin, and enzyme to yield $\leq 20\%$ hydrolysis. For calmodulin-activated enzyme activity, reactions were supplemented with 0.2 mM- CaCl_2 and 15–20 nM-calmodulin, purified from bovine brain as described previously (Epstein *et al.*, 1982).

Determination of calmodulin contents

This was done in heat-treated 100 000 g supernatants of HPBL and RPMI 8392 cells by comparing their ability to stimulate a calmodulin-sensitive rat brain phosphodiesterase with that of purified bovine brain calmodulin, as described previously (Epstein *et al.*, 1987).

Materials

Cyclic [$2,8\text{-}^3\text{H}$]AMP (sp. radioactivity 38.1 Ci/mmol) and cyclic [$5,8\text{-}^3\text{H}$]GMP (sp. radioactivity 36.9 Ci/mmol) were purchased from New England Nuclear, Boston, MA, U.S.A., purified by anion-exchange chromatography on Dowex 1-X2 (200–400 mesh) and stored at -20 °C in acidic 50% (v/v) ethanol. Ficoll, DEAE-Sephacel and CNBr-activated Sepharose 4B were from Pharmacia, Piscataway, NJ, U.S.A.; Hypaque (50%, w/v) was from Winthrop Laboratories, New York, NY, U.S.A.; Dowex 1-X2 (200–400 mesh) was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; phosphate-buffered saline (Dulbecco's formula), Hanks balanced salt solution (modified) and minimal essential medium (Eagle, modified), RPMI 1640, fetal-calf serum and penicillin/streptomycin were from Flow Laboratories, McLean, VA, U.S.A.; Tos-Lys- CH_2Cl was from Sigma Chemical Co., St. Louis, MO, U.S.A. Protein A-positive *Staphylococcus aureus* cells (Cowan 1 strain, heat-killed and fixed in formalin) were obtained from Boehringer Mannheim, Indianapolis, IN, U.S.A., and washed twice by centrifugation and resuspension in 40 mM-Tris/HCl (pH 8.0)/2 mM- MgCl_2 before use. Other purchased chemicals were of commercial analytical-grade quality.

RESULTS

Subcellular distribution and Ca^{2+} -sensitivity of cyclic AMP phosphodiesterase

The cyclic AMP phosphodiesterase activity in RPMI 8392 cells was determined in homogenates of sonically disrupted cells, and in the supernatants and pellets after high-speed centrifugation of the homogenates and washing of the pellets. Table 1 shows that approx. 80% of the activity was recovered in the supernatant, and approx. 16% of the activity was retained in the pellet, after trapped supernatant activity was removed by washing the pellet. The Ca^{2+} -sensitivities of these fractions were examined by assaying in the presence of EGTA (1 mM), CaCl_2 (0.2 mM), and CaCl_2 (0.2 mM)+calmodulin (15 nM) (Table 1). In both the homogenates and supernatants, EGTA decreased the activity by 10%, Ca^{2+} increased the activity by 54%, and Ca^{2+} +calmodulin increased the activity by 77%. In the pellets, EGTA decreased the activity by 6%, Ca^{2+} increased the activity by 13%, and Ca^{2+} +calmodulin increased the activity by 60%. The activity determined in the presence of Ca^{2+} and calmodulin is as much as 2-fold greater than that determined in the presence of EGTA, for homogenates and supernatants, and 71% greater in pellets.

The concentration of Ca^{2+} required to activate cyclic AMP phosphodiesterase in RPMI 8392 cells was examined by measuring Ca^{2+} dose-responses in the presence and absence of added calmodulin. By using a Ca^{2+} -EGTA buffer system (Rodan *et al.*, 1980), in the absence of added calmodulin, the free Ca^{2+} concentration required to produce half-maximal stimulation of a

Table 1. Ca^{2+} -calmodulin-sensitivity of cyclic AMP phosphodiesterase activity in RPMI 8392 cells

Homogenates were prepared by sonicating cells in 40 mM-Tris/HCl/50 μM -Tos-Lys- CH_2Cl (pH 8.0) as described in the Materials and methods section. For preparation of supernatant, homogenates were centrifuged at 100000 g for 60 min. The resulting pellet from the centrifugation was washed twice by resuspension in a volume of Tris/Tos-Lys- CH_2Cl homogenization buffer equal to that for the initial homogenization, and re-centrifugation at 100000 g for 60 min. Phosphodiesterase activity recovered in the supernatant was $80.2 \pm 9.6\%$ of the total; activity recovered in the first wash of the pellet was $7.2 \pm 0.5\%$ and in the second wash of the pellet it was $2.6 \pm 0.2\%$; activity remaining in the final washed pellet was $16.0 \pm 7.8\%$. Phosphodiesterase activity was assayed at 0.25 μM -cyclic AMP as described in the Materials and methods section, with various additions as indicated below. Results represent means $\pm$ S.E.M. for three separate preparations of cells assayed in duplicate.

Assay conditions	Cyclic AMP phosphodiesterase activity					
	Homogenate		Supernatant		Pellet	
	(pmol/min per 10^6 cells)	(% of control)	(pmol/min per 10^6 cells)	(% of control)	(pmol/min per 10^6 cells)	(% of control)
Control (no additions)	4.79 ± 0.70	100	3.84 ± 0.46	100	0.767 ± 0.375	100
+ EGTA (1 mM)	4.25 ± 0.55	89	3.45 ± 0.47	90	0.718 ± 0.369	94
+ CaCl_2 (0.2 mM)	7.36 ± 0.27	154	5.91 ± 0.08	154	0.870 ± 0.441	113
+ Calmodulin (15 nM) + CaCl_2 (0.2 mM)	8.49 ± 0.45	177	6.78 ± 0.36	177	1.23 ± 0.51	160

Table 2. Effect of mixing HPBL and RPMI 8392-cell extracts on calmodulin stimulation of phosphodiesterase

Phosphodiesterase activity was determined as the rate of hydrolysis of cyclic AMP by using either 7.75 μg of HPBL supernatant or 3.84 μg of RPMI 8392-cell peak-I phosphodiesterase isolated by DEAE-Sephacel chromatography as described in the legend to Fig. 1, or a mixture of both of these enzyme sources. Activity was measured at 0.25 μM -cyclic AMP as described in the Materials and methods section, with the inclusion of either 1 mM-EGTA or 0.2 mM- CaCl_2 + 15 nM purified bovine brain calmodulin (CaM) as indicated. Results represent means of duplicate determinations.

Cell extracts and assay conditions	Cyclic AMP phosphodiesterase activity (pmol/min)
HPBL (+ EGTA)	0.107
HPBL (+ Ca^{2+} + CaM)	0.106
RPMI 8392 (+ EGTA)	0.335
RPMI 8392 (+ Ca^{2+} + CaM)	0.655
HPBL + RPMI 8392 (+ EGTA)	0.417
HPBL + RPMI 8392 (+ Ca^{2+} + CaM)	0.756

supernatant of RPMI 8392 cells was determined to be 1.3 μM ; under these assay conditions, the amount of endogenous calmodulin present, added along with the cell supernatant, was estimated to be about 2 nM, on the basis of the measured content of calmodulin in RPMI 8392 cells (see below). When 20 nM purified bovine brain calmodulin was added to the assays, the Ca^{2+} concentration required for half-maximal stimulation was decreased to 0.7 μM .

Comparison of HPBL and RPMI 8392 phosphodiesterase activities

When the supernatant cyclic AMP phosphodiesterase activity in RPMI 8392 cells was compared with that in

isolated HPBL, it was found to be approx. 6.9-fold higher. The average activity of isolated HPBL assayed at 0.25 μM -cyclic AMP was 0.558 ± 0.043 pmol/min per 10^6 cells (mean $\pm$ S.E.M.; $n = 8$), whereas RPMI 8392 cytosol had an average activity of 3.84 ± 0.46 pmol/min per 10^6 cells (mean $\pm$ S.E.M., $n = 3$). Moreover, in contrast with RPMI 8392 cells, which displayed significant Ca^{2+} -calmodulin-sensitivity, this activity of isolated HPBL was not significantly altered when assayed in the presence of EGTA, Ca^{2+} , or Ca^{2+} + calmodulin.

The lack of stimulation of HPBL phosphodiesterase activity by Ca^{2+} -calmodulin could result from the action of proteinases, not inhibited by Tos-Lys- CH_2Cl , which render the phosphodiesterase unable to be stimulated by calmodulin, or by the actions of other diffusible factors which might inhibit the calmodulin stimulation. We tested these possibilities by examining the calmodulin stimulation of RPMI 8392-cell phosphodiesterase mixed with HPBL supernatant. If diffusible factors account for the lack of calmodulin stimulation of HPBL phosphodiesterase, then they should also attenuate the calmodulin stimulation of RPMI 8392 phosphodiesterase. As shown in Table 2, this was not the case. Ca^{2+} -calmodulin added to HPBL phosphodiesterase activity alone had no effect; added to RPMI 8392-cell phosphodiesterase alone, Ca^{2+} -calmodulin stimulated the activity by 2-fold. When RPMI 8392-cell phosphodiesterase was mixed with crude supernatant HPBL phosphodiesterase, the two activities were nearly completely additive, and no attenuation of the calmodulin stimulation of the RPMI 8392-cell phosphodiesterase activity was observed. These results suggest that the lack of calmodulin stimulation of HPBL phosphodiesterase is probably not due to proteinase activity or other diffusible factors.

Characterization of the cytosolic cyclic nucleotide phosphodiesterases in RPMI 8392 cells

The cytosolic phosphodiesterase activity in RPMI 8392 cells was characterized by DEAE-Sephacel anion-exchange chromatography. As shown in Fig. 1, enzyme

Fig. 1. DEAE-Sephacel chromatography of phosphodiesterases in RPMI 8392-cell cytosol

A 100 000 *g* supernatant from 7×10^8 RPMI 8392 cells was applied to a 1.6 cm $\times$ 2 cm column of DEAE-Sephacel equilibrated in 5 mM-Mes (pH 6.5)/30% (v/v) ethylene glycol/1 mM-dithiothreitol/5% (w/v) glucose/10 mM-sodium acetate/1 mM-EGTA/10 mM-NaF/20 μ M-Tos-Lys-CH₂Cl. The column was washed with 30 ml of the same buffer, and enzyme activity was eluted with a 40 ml linear gradient of sodium acetate (NaAc; 0.01–1.0 M), in the same buffer. The flow rate was 0.5 ml/min, and 1 ml fractions were collected. Fractions were then analysed for protein and sodium acetate content (a), and for phosphodiesterase activity at 0.25 μ M-cyclic AMP (b) and 0.25 μ M-cyclic GMP (c) in the absence (●) or presence (○) of 0.2 mM-Ca²⁺/15 nM-calmodulin. Recovery of activities from the column, given as basal and calmodulin-stimulated activities respectively, were 53% and 47% measured at 0.25 μ M-cyclic AMP, and 64% and 68% measured at 0.25 μ M-cyclic GMP.

activity was eluted in two separate peaks; the first peak, eluted at approx. 0.2 M-sodium acetate, was sensitive to stimulation by Ca²⁺-calmodulin and hydrolysed both cyclic AMP and cyclic GMP substrates with approximately equal efficiency at 0.25 μ M. The second peak, eluted at approx. 0.6 M-sodium acetate, was insensitive to stimulation by Ca²⁺-calmodulin, and appeared relatively specific for cyclic AMP as substrate. This is in sharp contrast with the profile obtained with isolated HPBL, which exhibits a single peak of cyclic nucleotide phosphodiesterase activity on DEAE-Sephacel anion-exchange chromatography (Takemoto *et al.*, 1978; Epstein & Hachisu, 1984). The enzyme from HPBL behaves similarly to the second peak obtained from RPMI 8392 cells, in that it is eluted at approx. 0.6 M-sodium acetate, is unaffected by Ca²⁺-calmodulin, and is relatively specific for cyclic AMP (Takemoto *et al.*, 1978; Epstein & Hachisu, 1984).

After DEAE-Sephacel chromatography, the Ca²⁺-calmodulin stimulation of the cytosolic cyclic AMP

Fig. 2. Hofstee analysis of RPMI 8392-cell Ca²⁺-calmodulin-sensitive phosphodiesterase isolated by DEAE-Sephacel chromatography

Fractions 13–18 from the DEAE-Sephacel column elution shown in Fig. 1 were pooled and analysed kinetically in the presence (○) or absence (●) of 20 nM-calmodulin, with 0.25–100 μ M-cyclic AMP (*s* is given as μ M-cyclic AMP). Kinetic constants were determined from the extrapolated linear portions of the curves, and are as follows. + Calmodulin: $K_{m_1} = 0.53 \mu$ M; $V_{max_1} = 180$ pmol/min per ml; $K_{m_2} = 1.0 \mu$ M; $V_{max_2} = 222$ pmol/min per ml. No calmodulin: $K_{m_1} = 0.83 \mu$ M; $V_{max_1} = 39$ pmol/min per ml; $K_{m_2} = 4.0 \mu$ M; $V_{max_2} = 79$ pmol/min per ml.

phosphodiesterase in RPMI 8392 cells is somewhat greater, approx. 3-fold for both cyclic AMP and cyclic GMP substrates (Fig. 1). Kinetic analysis of the Ca²⁺-calmodulin-sensitive phosphodiesterase form isolated by DEAE-Sephacel chromatography, when plotted according to Hofstee (1952), displays a non-linear kinetic profile. The extrapolated K_m values are 0.8 and 4 μ M. In the presence of Ca²⁺-calmodulin, the kinetic profile was still non-linear, with extrapolated K_m values of 0.5 and 1 μ M, and the low and high V_{max} values were both increased approx. 3-fold (Fig. 2).

Demonstration of a direct interaction of calmodulin with cyclic AMP phosphodiesterase in RPMI 8392 cells

In order to confirm that the cytosolic Ca²⁺-sensitive phosphodiesterase in RPMI 8392 cells can directly interact with calmodulin in a Ca²⁺-dependent manner, a portion of the enzyme activity obtained from the DEAE-Sephacel column was passed over a calmodulin-Sepharose affinity column in the presence of Ca²⁺; 100% of the activity was retained on the column, and 76% of the activity was recovered after elution with EGTA (Fig. 3). This demonstrates that the enzyme can interact directly with calmodulin in a Ca²⁺-dependent manner.

Immunoprecipitation of RPMI 8392-cell Ca²⁺-calmodulin-sensitive phosphodiesterase

Immunoprecipitation studies provide further evidence of an interaction between calmodulin and RPMI 8392-cell phosphodiesterase. Using a monoclonal anti-

Fig. 3. Calmodulin-Sepharose chromatography of RPMI 8392-cell phosphodiesterase

Calmodulin-Sepharose was prepared by coupling calmodulin to CNBr-activated Sepharose 4B by the procedure of Klee & Krinks (1978). A portion (0.5 ml) of pooled fractions 13–18 from the DEAE-Sephacel column shown in Fig. 1 was diluted 8-fold into 40 mM-Tris/HCl (pH 8.0)/0.2 mM- CaCl_2 /50 mM-NaCl/3 mM- MgCl_2 /0.1 mM-dithiothreitol and applied to a 1.6 cm $\times$ 2.0 cm column of calmodulin-Sepharose. The column was washed with 20 ml of the same buffer containing 0.2 M-NaCl, and then eluted with the same buffer containing 50 mM-NaCl and 2 mM-EGTA in place of CaCl_2 . The flow rate was 0.5 ml/min, and 2 ml fractions were collected. Phosphodiesterase activity was determined in the absence (●) or presence (○) of 2 mM- Ca^{2+} /15 nM-calmodulin (CaM) at 0.25 μM -cyclic AMP.

body directed against a Ca^{2+} -calmodulin-bovine heart phosphodiesterase complex, we were able to precipitate 75% of the soluble RPMI calmodulin-sensitive phosphodiesterase in the presence of heat-killed *Staphylococcus aureus* bacteria, Ca^{2+} , and calmodulin (Table 3). In the presence of EGTA, however, only 6% of the phosphodiesterase was precipitated. Further studies showed that this 6% activity precipitated in the presence of EGTA

was due to non-specific adsorption of the phosphodiesterase to the *S. aureus* bacteria. For comparison, a calmodulin-sensitive rat brain phosphodiesterase was also tested, and under the same conditions only 60% of the rat brain phosphodiesterase was immunoprecipitated. Hence, the antibody appears to be even more reactive against the calmodulin-human leukaemia-cell phosphodiesterase complex than against the calmodulin-rat brain phosphodiesterase complex. In both cases, the antibody recognized only the Ca^{2+} -calmodulin-phosphodiesterase complex and was inactive when Ca^{2+} and calmodulin were not present.

Comparison of calmodulin contents in normal lymphocytes and RPMI 8392 cells

Since our data clearly indicated the presence of calmodulin-sensitive phosphodiesterase activity in RPMI 8392 cells, and the apparent absence of this activity in HPBL, we measured the amounts of calmodulin in HPBL and RPMI 8392 cells. The calmodulin content determined in HPBL was 11.8 ± 3.7 pmol/ 10^7 cells ($\pm$ S.E.M., $n = 5$), and that in RPMI 8392 cells was 31.9 ± 7.5 pmol/ 10^7 cells ($\pm$ S.E.M., $n = 7$), which is significantly greater ($P < 0.025$) by about 3-fold than that in HPBL.

DISCUSSION

Our data clearly document the presence of Ca^{2+} -calmodulin-sensitive phosphodiesterase activity in the human cultured lymphoblastoid cell line RPMI 8392. When analysed similarly, normal isolated HPBL appear to lack Ca^{2+} -calmodulin-sensitive phosphodiesterase activity. Although proteolysis and possibly other factors can render calmodulin-sensitive phosphodiesterases refractory to stimulation by calmodulin, the experiments in which we mixed crude HPBL supernatant with RPMI 8392 phosphodiesterase and found no attenuation of the calmodulin stimulation (Table 2) argue against these possibilities as explanations for the lack of calmodulin stimulation of HPBL phosphodiesterase. Nevertheless, the presence of calmodulin-sensitive phosphodiesterase

Table 3. Immunoprecipitation of RPMI 8392-cell Ca^{2+} -calmodulin-sensitive phosphodiesterase

Protein (22.5 μg) obtained from freeze-drying of the ascites fluid of a mouse injected with an ACC-1 monoclonal-antibody-producing hybridoma was incubated on ice with 0.1 ml of a 10% suspension of heat-killed formalin-treated *S. aureus* Cowan 1 strain bacteria for 60 min in 40 mM-Tris/HCl (pH 8.0)/2 mM- MgCl_2 . The *S. aureus* cells with bound ACC-1 were then centrifuged at 5000 g for 1 min and resuspended in 500 μl of a dilution of either RPMI 8392 or rat brain phosphodiesterase in 40 mM-Tris/HCl (pH 8.0) containing 0.5 mg of bovine serum albumin/ml and either 0.5 mM- CaCl_2 /120 nM-calmodulin or 0.1 mM-EGTA. The samples were then incubated for 2 h on ice, and supernatants and pellets were obtained by centrifugation at 5000 g for 1 min. The pellet was washed once with buffer containing either Ca^{2+} -calmodulin or EGTA, to remove any trapped supernatant. Samples of the supernatants and washed pellets were then assayed for phosphodiesterase activity at 1 μM -cyclic AMP. The source of RPMI 8392 phosphodiesterase was the Ca^{2+} -calmodulin-sensitive peak obtained from DEAE-Sephacel chromatography as shown in Fig. 1. The rat brain phosphodiesterase used was also isolated by DEAE-Sephacel chromatography as described previously (Volpi *et al.*, 1981). Results represent means of duplicate determinations.

Phosphodiesterase	Precipitation conditions	Phosphodiesterase activity (%)	
		Supernatant	Pellet
RPMI 8392	ACC-1 Ca^{2+} /calmodulin	25	75
	ACC-1 EGTA	94	6
Rat brain	ACC-1 Ca^{2+} /calmodulin	40	60
	ACC-1 EGTA	97	3

activity in HPBL cannot be ruled out completely, since conditions may yet be found which will enable the detection of this enzyme in HPBL. This was seen with human neutrophils, for example, where one study (Smolen & Geosits, 1984) reported the complete absence of any Ca^{2+} -calmodulin-dependent phosphodiesterase activity in these cells, whereas another study (Engerson *et al.*, 1986), using different homogenization and assay conditions, reported the detection of this activity.

Our characterization of the cytosolic calmodulin-sensitive phosphodiesterase shows it to have a high affinity for the hydrolysis of cyclic AMP. In that regard it is similar to a high-affinity calmodulin-sensitive phosphodiesterase which we have detected in developing chick heart (Andrenyak & Epstein, 1982; Epstein *et al.*, 1987), and which has been found in several other tissues as well (Smoake *et al.*, 1981; Purvis *et al.*, 1981; Vandermeers *et al.*, 1983; Geremia *et al.*, 1984). Although monoclonal antibody directed against a Ca^{2+} -calmodulin-bovine heart phosphodiesterase complex immunoprecipitates the RPMI 8392-cell phosphodiesterase activity, this does not necessarily mean that high- and low-affinity phosphodiesterases have similar immunological determinants, because the ACC-1 antibody may be directed largely against calmodulin (Charbonneau *et al.*, 1986). Nevertheless, the observations that most of the RPMI 8392-cell enzyme can be precipitated by this antibody only in the presence of Ca^{2+} -calmodulin and that it can be adsorbed to and eluted from a calmodulin-Sepharose column strongly suggest that this enzyme can interact directly with calmodulin. Since this enzyme has a high affinity for cyclic AMP, it can conceivably function to regulate the contents of cyclic AMP in these cells, in a Ca^{2+} -dependent manner.

In addition to the clear detection of Ca^{2+} -calmodulin-sensitive phosphodiesterase activity in RPMI 8392 cells as compared with HPBL, in which this activity was not detectable, the RPMI 8392 cells contain 3 times as much calmodulin as did HPBL. Takemoto & Jilka (1983) also reported that the cultured human leukaemic cell lines, MOLT-4 and IM-9, have calmodulin contents approx. 10 times that of HPBL. In many other types of cells it has also been shown that transformed cells have higher calmodulin contents than do their normal counterparts (Watterson *et al.*, 1976; LaPorte *et al.*, 1979; Chafouleas *et al.*, 1981; Wei & Hickie, 1981), and many of these investigators suggested that the increased content of calmodulin may contribute to the state of abnormal proliferation of these transformed cells. This suggestion has also been made on the basis of observations that (1) calmodulin increases 2-fold at the late G_1 /early S phase of the growth cycle of CHO-K₁ cells (Chafouleas *et al.*, 1982), (2) that calmodulin antagonists inhibit cell growth at the G_1 /S-phase boundary (Chafouleas *et al.*, 1982; Hidaka *et al.*, 1981), and (3) that a positive correlation exists between calmodulin content and the growth rate of different hepatomas (Wei *et al.*, 1982). Some investigators have reported that DNA synthesis can actually be initiated by the exogenous addition of calmodulin to hepatoma and spleen cells (Boynton & Whitfield, 1981; Gosnell & Mathews, 1983). We have been unable to promote the initiation of DNA synthesis by the exogenous addition of calmodulin to isolated HPBL; however, upon micro-insertion of calmodulin into HPBL by a poly(ethylene glycol)-mediated erythrocyte ghost fusion procedure, we observe a small enhancement of the

lectin-induced mitogenic response (P. M. Epstein & I. Damon, unpublished work).

Numerous studies have shown that transformed cells have a much lower Ca^{2+} requirement for growth than normal cells (reviewed by Durham & Walton, 1982). Connor *et al.* (1983) examined two rat kidney cell lines transformed with temperature-sensitive viruses for their calmodulin content and ability to grow in low Ca^{2+} . In contrast with most cells, which increase their calmodulin during transformation, one of these cell lines did not alter its calmodulin content when grown at the permissive temperature for transformation, even though at this temperature it was able to grow in low- Ca^{2+} medium. On further analysis, they found that, although calmodulin itself was not altered in this cell line, there were alterations in the proportions of calmodulin-acceptor proteins between the permissive and non-permissive temperatures, and they suggested that the alteration in the calmodulin-acceptor proteins may be responsible for a modification of the response of the cell to a regulatory Ca^{2+} signal. This may be true for RPMI 8392 cells as well, where both the increased calmodulin content and the appearance of calmodulin-sensitive phosphodiesterase in these cells may contribute to their rapid proliferation associated with their transformed state.

We have observed an induction of phosphodiesterase activity after mitogenic activation of HPBL and have shown that pharmacological antagonism of this induced phosphodiesterase activity inhibits lymphocyte proliferation (Epstein *et al.*, 1980; Epstein & Hachisu, 1984). Additionally, we have found that blast cells isolated from patients with acute myelogenous leukaemia contain a form of phosphodiesterase not found in normal lymphocytes or monocytes (Onali *et al.*, 1985). If the calmodulin-sensitive phosphodiesterase that we have identified in RPMI 8392 cells represents a transformation-specific calmodulin-dependent enzyme, not present in normal HPBL, then it is possible that specific antagonists directed against this enzyme may modulate the growth of these leukaemic cells.

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