Ca^{2+} -independent cyclic GMP phosphodiesterases from rat liver and HTC hepatoma cells

Gordon J. STREWLER,* Mary Ann DANIELLO,† Vincent C. MANGANIELLO† and Martha VAUGHAN†

*University of California Service, Veterans Administration Medical Center, San Francisco, CA 94121, and
†Laboratory of Cellular Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Bethesda, MD 20205, U.S.A.

(Received 14 March 1983/Accepted 8 April 1983)

We have separated and characterized a Ca^{2+} - and calmodulin-insensitive cyclic nucleotide phosphodiesterase from rat liver supernatant as well as an analogous enzyme from HTC hepatoma cells. Chromatography of rat liver supernatant on DEAE-cellulose in the presence and subsequently in the absence of 0.1 mM- CaCl_2 , resulted in the separation of two distinct phosphodiesterase activities, both of which preferentially hydrolysed cyclic GMP rather than cyclic AMP. One enzyme, E-Ib, was activated in the presence of Ca^{2+} and calmodulin, and the other, E-Ia, was not. The E-Ia enzyme, which did not bind to calmodulin–Sephadex, had M_r 325 000 and displayed anomalous kinetic behaviour [K_m (cyclic GMP) 1.2 μM ; K_m (cyclic AMP) 15.4 μM]. The E-Ib enzyme, which bound to calmodulin–Sephadex in the presence of Ca^{2+} , had M_r 150 000 and exhibited Michaelis–Menten kinetics for hydrolysis of cyclic GMP [K_m (basal) 6.5 μM ; K_m (activated) 12.0 μM]. E-Ia activity was diminished by incubation with α -chymotrypsin and was unaffected by the action of a rat kidney lysosomal proteinase. Partial hydrolysis of E-Ib enzyme by α -chymotrypsin or the kidney proteinase resulted in irreversible activation of the enzyme. The E-I enzyme isolated from HTC hepatoma cells was similar to the rat liver E-Ia enzyme in many respects. Its apparent M_r was 325 000. Its activity was unaffected by calmodulin in the presence of Ca^{2+} or by incubation with the kidney proteinase, and was decreased by digestion with α -chymotrypsin. Unlike the liver E-Ia enzyme, however, the hepatoma enzyme exhibited normal kinetic behaviour, with K_m (cyclic GMP) 3.2 μM . Although HTC cells contain two other phosphodiesterases analogous to those in rat liver and a calmodulin-like activator of phosphodiesterase, no calmodulin-sensitive phosphodiesterase was detected.

At least three forms of soluble cyclic nucleotide phosphodiesterase (EC 3.1.4.17) have been found in many mammalian tissues. One type (E-I) has a higher affinity for cyclic GMP than for cyclic AMP, and can be activated by Ca^{2+} in the presence of the calcium-regulatory protein calmodulin or by limited proteolysis (Wells & Hardman, 1977). A second type (E-II) has similar affinities for cyclic AMP and for cyclic GMP, and displays positively co-operative kinetics; hydrolysis of cyclic AMP by this phosphodiesterase is stimulated by cyclic GMP (Beavo *et al.*, 1971; Moss *et al.*, 1977; Martins *et al.*, 1982). A third type of phosphodiesterase (E-III) has a higher affinity for cyclic AMP than for cyclic GMP

and is also activated by limited proteolysis (Wells & Hardman, 1977). Previous work in our laboratory showed that when cyclic GMP phosphodiesterases from rat liver supernatant were separated by chromatography on DEAE-cellulose the first phosphodiesterase activity to be eluted, although incompletely separated from the calmodulin-stimulated activity, was insensitive to Ca^{2+} plus calmodulin (Moss *et al.*, 1978). We now describe the separation of the Ca^{2+} -independent cyclic GMP phosphodiesterase (E-Ia) from the enzyme that is stimulated by Ca^{2+} plus calmodulin (hereinafter termed E-Ib) and from the E-II phosphodiesterase. Some characteristics of the three phosphodiesterases are com-

pared. An enzyme very similar to rat liver E-Ia enzyme was also found in cultured HTC hepatoma cells.

Experimental

Preparation of supernatants

Male Osborne-Mendel rats were decapitated and liver was rapidly homogenized (Dounce homogenizer, five strokes) at 4°C in 20 mM-potassium phosphate buffer, pH 7.0, containing 0.25 M-sucrose, 0.1 M-CaCl₂, 1 mM-NaN₃ and 10 µM-leupeptin (4 ml/g). In some experiments the buffer also contained 10 µM-pepstatin A and 10 µM-phenylmethanesulphonyl fluoride. Confluent HTC cells were scraped from dishes and homogenized with 30 strokes of a Dounce homogenizer in 50 mM-Tris/HCl buffer, pH 8.0, containing 0.3 M-sucrose, 0.1 mM-EGTA, 1 mM-MgCl₂ and 10 µM-leupeptin. Supernatants of liver and HTC homogenates were prepared by centrifugation at 100 000 g for 60 min.

Assay of phosphodiesterase activity

For most experiments phosphodiesterase activity was assayed at 30°C in a final volume of 0.3 ml

containing 0.5 µM-cyclic [³H]AMP or 0.5 µM-cyclic [³H]GMP, 8 mM-MgCl₂ and 83 mM-Tris/HCl buffer, pH 8.0 (for assay of liver phosphodiesterase), or 50 mM-Bes {2-[bis-(2-hydroxyethyl)amino]ethanesulphonic acid}/NaOH buffer, pH 7.0 (for assay of HTC phosphodiesterase), as described previously (Ross *et al.*, 1977). In other experiments assays were performed in the presence of the indicated substrate concentration or other additions.

Cell culture

Hepatoma cells, which were kindly provided by Dr. E. B. Thompson (National Cancer Institute, Bethesda, MD, U.S.A.), were grown in Eagle's basal medium supplemented with Earle's salts and 10% (v/v) foetal-calf serum as described by Ross *et al.* (1977), and phosphodiesterase activity was measured in supernatant fractions obtained from homogenates of confluent monolayers grown in 100 mm or 250 mm dishes.

Materials

Calmodulin was purified from bovine brain by a modification of the method of Watterson *et al.*

Fig. 1. Chromatography of rat liver supernatant on DEAE-cellulose in the presence of CaCl₂. Fresh rat liver (30 g) was homogenized in 120 ml of 20 mM-potassium phosphate buffer, pH 7.0, containing 0.25 M-sucrose, 0.1 mM-CaCl₂, 1.0 mM-NaN₃ and 10 µM concentrations of leupeptin, pepstatin A and phenylmethanesulphonyl fluoride. The homogenate was centrifuged at 100 000 g for 60 min, and the supernatant (120 ml) was applied to a column (2.5 cm × 16.5 cm) of DE-52 DEAE-cellulose equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-CaCl₂, 1.0 mM-NaN₃ and the proteinase inhibitors. These proteinase inhibitors were included in all remaining column buffers. Phosphodiesterase activity was eluted with a gradient of 500 ml of 20 mM-potassium phosphate buffer, pH 7.0, and 500 ml of 200 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-CaCl₂. Cyclic GMP phosphodiesterase activity was assayed with: ●, 0.5 µM-cyclic [³H]GMP plus 0.5 mM-EGTA; O, 0.5 µM-cyclic [³H]GMP plus 0.5 mM-CaCl₂ and 0.16 µg of calmodulin. Cyclic AMP phosphodiesterase activity was assayed with: ▲, 0.5 µM-cyclic [³H]AMP; △, 0.5 µM-cyclic [³H]AMP plus 0.5 µM-cyclic GMP. Recovery of cyclic GMP phosphodiesterase activity (assayed with EGTA) was 54%.

(1976) and coupled to CNBr-activated Sepharose (Pharmacia) as described by Kincaid & Vaughan (1979). Kidney phosphodiesterase activator was purified from rat kidney cortex as described by Strewler & Manganiello (1979). Chymotrypsin, pepstatin and phenylmethanesulphonyl fluoride were purchased from Sigma Chemical Co., DEAE-cellulose (DE-52) was from Whatman, Ultrogel AcA-34 from LKB, and DEAE-Bio-Gel from Bio-Rad Laboratories. Tissue-culture media were prepared by the Media Production Unit, National Institutes of Health, and foetal calf serum was from GIBCO.

Results

Separation of phosphodiesterase E-Ia and E-Ib enzymes

Ho *et al.* (1977) showed that addition of Ca^{2+} and calmodulin to crude preparations of a soluble calmodulin-sensitive phosphodiesterase from bovine heart altered the chromatographic behaviour of the enzyme, presumably because of a Ca^{2+} -dependent association of the enzyme with calmodulin, a highly acidic protein. As shown in Fig. 1, chromatography of rat liver supernatant under conditions previously used (Moss *et al.*, 1978), except that buffers contained 0.1 mM- CaCl_2 instead of EDTA and EGTA, clearly separated E-Ia phosphodiesterase

activity (fractions 20–35), unaffected by Ca^{2+} and calmodulin, from calmodulin-sensitive E-Ib activity (fractions 50–60) and from cyclic GMP-stimulated E-II activity (fractions 50–80). After addition of excess EGTA, the pooled fractions (50–60) that contained E-Ib activity were subjected to DEAE-cellulose chromatography as before, except that the column was equilibrated and eluted with buffer containing EGTA (Fig. 2). Under these conditions, phosphodiesterase activity sensitive to Ca^{2+} and calmodulin (E-Ib) was eluted earlier than in Fig. 1, in fractions corresponding approximately to those containing E-Ia activity on the previous column (Fig. 1) and to its own position when the initial chromatographic separation was performed with buffers containing EGTA (results not shown). The elution of E-II activity was unaffected by the presence of Ca^{2+} or EGTA (Figs. 1 and 2).

E-Ia and E-Ib enzymes, separated by chromatography on DEAE-cellulose in CaCl_2 , were subjected to chromatography on calmodulin-Sepharose as described by Kincaid & Vaughan (1979). Only 7% of E-Ia activity was retained, whereas 87% of E-Ib enzyme activity was retained and 77% was eluted with EGTA.

Phosphodiesterase activity in HTC hepatoma cells

Chromatography of HTC-cell supernatants on DEAE-Bio-Gel partially separated three forms of

Fig. 2. Chromatography of the E-Ib + E-II pool from Fig. 1 on DEAE-cellulose in the presence of EGTA. Fractions 52–60 from Fig. 1, which contained E-Ib and E-II activity, were pooled, diluted to 500 ml with distilled water containing 0.1 mM-EGTA and applied to the same DE-52 DEAE-cellulose column equilibrated with 20 mM-potassium phosphate buffer, pH 7.0, containing 0.1 mM-EGTA. Enzymic activity was eluted as described in Fig. 1, except that buffers contained 0.1 mM-EGTA instead of 0.1 mM- CaCl_2 . Recovery of cyclic GMP phosphodiesterase activity was 74%. Symbols are as described in Fig. 1.

phosphodiesterase, which are analogous to E-I, E-II and E-III enzymes from rat liver as previously reported (Ross *et al.*, 1977). Ca^{2+} alone or calmodulin or both did not increase phosphodiesterase activity assayed with cyclic AMP (results not shown) or cyclic GMP (Fig. 3). As shown in Fig. 3,

Fig. 3. Assay of calmodulin-sensitive phosphodiesterase activity, calmodulin-like activity and calmodulin inhibitors in HTC-cell supernatant

Confluent HTC cells were homogenized in 50 mM-Tris/HCl buffer, pH 8.0, containing 0.3 M-sucrose, 0.1 mM-EGTA, 1 mM-MgCl₂ and 10 μM -leupeptin with 30 strokes of a Dounce homogenizer. The homogenate was centrifuged at 100 000 *g* for 60 min, and the supernatant, containing approx. 40 mg of protein in 70 ml, was applied to a column (1.2 cm $\times$ 20 cm) of DEAE-Bio-Gel equilibrated with 50 mM-Tris/HCl buffer, pH 8.0, containing 50 mM-NaCl, 0.1 mM-EGTA, 1 mM-MgCl₂ and 10 μM -leupeptin. The column was washed with 100 ml of this buffer and eluted with 400 ml of a linear gradient of 50–200 mM-NaCl in Tris buffer without leupeptin. (b) Samples (150 μl) were assayed with 0.5 μM -cyclic [³H]GMP alone (●) or in the presence (○) of 0.18 μg of bovine brain calmodulin and 3.0 mM-CaCl₂. Activity in the presence of 3.0 mM-CaCl₂ alone was identical with that in the absence or in the presence of CaCl₂ and calmodulin. Recovery of cyclic GMP phosphodiesterase was 57%. (a) Heated (5 min, 95°C) samples (150 μl) were assayed in the presence of a partially purified calmodulin-sensitive phosphodiesterase from bovine brain with 0.5 μM -cyclic [³H]GMP in the absence (▲) or in the presence (△) of 3 mM-CaCl₂ alone or with 3 mM-CaCl₂ plus 0.003 μg of calmodulin (■). Activity of the brain phosphodiesterase (pmol of cyclic GMP/10 min) was 13.9 alone, 10.7 in the presence of CaCl₂ and 35.2 in the presence of CaCl₂ and calmodulin.

incubation of heated (90°C, 5 min) fractions with Ca^{2+} plus a calmodulin-deficient phosphodiesterase from bovine brain indicated that fractions 50–60 contained a heat-stable Ca^{2+} -dependent activator of phosphodiesterase. Incubation of heated fractions (Fig. 3) or unheated fractions (results not shown) with Ca^{2+} plus a submaximally effective concentration of calmodulin and calmodulin-deficient phosphodiesterase revealed no evidence of heat-stable or heat-labile inhibitors of phosphodiesterase activation similar to those from brain (Sharma *et al.*, 1978). Thus E-I enzyme from HTC cells appears to be analogous to E-Ia enzyme rather than E-Ib enzyme, and, although HTC cells appear to contain a calmodulin-like activator of phosphodiesterase, no calmodulin-sensitive phosphodiesterase (E-Ib) was detected.

Relative molecular masses

On gel filtration, the predominant species of phosphodiesterase E-I from HTC cells and E-Ia enzyme from rat liver both displayed apparent M_r 325 000, although the recovery of activity from the HTC-cell enzyme was poor (21%). E-Ib and E-II enzymes from rat liver had apparent M_r 150 000 and 430 000 respectively. Determination of relative molecular mass by gel filtration assumes that the enzymes have axial ratios similar to those of the proteins used for calibrations. This assumption is unverified for the cyclic GMP phosphodiesterases, and Goren & Rosen (1972) reported that relative molecular masses of phosphodiesterases obtained by gel filtration did not agree with determinations by other methods. E-Ia enzyme, separated from a mixture of E-Ia and E-Ib enzymes [prepared as described previously (Moss *et al.*, 1978)] by chromatography on calmodulin-Sepharose in the presence of CaCl₂, exhibited the same M_r (325 000) as did E-Ia enzyme prepared by DEAE-cellulose chromatography in the presence of CaCl₂ (results not shown).

Enzyme kinetics

E-Ia enzyme from rat liver exhibited anomalous kinetics with an apparent low K_m for cyclic GMP of $1.2 \pm 0.6 \mu\text{M}$ and a low V_{max} of 257 ± 124 pmol/min per mg of protein (mean $\pm$ s.d., $n=5$). The high K_m for cyclic GMP was $27 \pm 17 \mu\text{M}$ and the high V_{max} was 0.76 ± 0.36 nmol/min per mg of protein (Fig. 4b). The Hill coefficient was 0.54. For cyclic AMP the low K_m was $15.4 \pm 2.1 \mu\text{M}$ and the low V_{max} was 328 ± 35 pmol/min per mg of protein; the high K_m was $302 \pm 148 \mu\text{M}$ and the high V_{max} was 5.0 ± 2.1 nmol/min per mg of protein ($n=4$, detailed results not shown). E-I enzyme from HTC cells exhibited normal kinetics with an apparent K_m for cyclic GMP of $3.8 \pm 1.0 \mu\text{M}$ and V_{max} of 184 ± 43 pmol/min per mg of protein ($n=3$) (Fig. 4a). E-Ib enzyme from rat liver displayed normal

Fig. 4. Hydrolysis of cyclic GMP by phosphodiesterases from rat liver and HTC cells

Assays were performed under standard conditions (pH 8.0) with the indicated substrate concentrations in the presence of 0.5 mM-EGTA with 40 μ g of liver E-Ia enzyme (b) or 5.7 μ g of HTC E-I enzyme (a). Activity is expressed as pmol of cyclic GMP/10 min. Each point represents the mean of duplicate determinations. For E-Ia enzyme the pseudo-linear portion of the curve (from 0.04 μ M- to 2 μ M-cyclic GMP) was extrapolated to estimate the apparent 'low' K_m .

Fig. 5. Hydrolysis of cyclic GMP by E-Ib phosphodiesterase

Assays were performed under standard conditions with the indicated substrate concentrations in the presence of 0.5 mM-EGTA (●) or 2.5 mM- CaCl_2 and 0.16 μ g of calmodulin (○) with 2 μ g of liver E-Ib enzyme. Activity is expressed as pmol of cyclic GMP/10 min. Each point represents the mean of duplicate determinations.

kinetics at cyclic GMP concentrations up to 100 μ M in the basal state [apparent K_m for cyclic GMP $6.5 \pm 1.5 \mu$ M (mean $\pm$ half range, $n = 2$)] and in the presence of Ca^{2+} plus calmodulin [apparent K_m for cyclic GMP $12.0 \pm 1.0 \mu$ M (mean $\pm$ half range, $n = 2$)] (Fig. 5). At low substrate concentrations both E-Ia and E-Ib enzymes preferentially hydrolysed cyclic GMP, whereas at much higher substrate concentrations (0.1–1.2 mM) hydrolysis of cyclic AMP was greater than (E-Ia) enzyme or similar (E-Ib enzyme) to that of cyclic GMP. High concentrations of cyclic AMP inhibited hydrolysis of cyclic GMP by E-Ib enzyme to a greater extent than that by E-Ia enzyme (results not shown).

Effect of partial proteolysis

Incubation with chymotrypsin increased the activity of E-Ib (Fig. 6a) but resulted in inactivation of E-Ia enzyme from rat liver and of E-I enzyme from HTC cells (Figs. 6a and 6b). After proteolytic activation, E-Ib enzyme was unresponsive to Ca^{2+}

plus calmodulin (Fig. 6a), as previously noted (Moss *et al.*, 1978). When E-Ib enzyme from liver was subjected to chromatography on Ultrogel AcA-34 after limited proteolysis by chymotrypsin, the predominant species eluted had an apparent M_r of 30000 (results not shown). E-Ib enzyme from liver was also activated irreversibly by incubation with a lysosomal proteinase purified from rat kidney, as previously reported (Strewler & Manganiello, 1979). E-Ia enzyme from liver and E-I enzyme from HTC cells were unaffected by the kidney proteinase, but E-III enzyme obtained from HTC cells was activated (results not shown).

Discussion

Rat liver has long been known to contain two soluble cyclic GMP phosphodiesterases (Table 1). One, E-Ib enzyme, is characterized by its activation by Ca^{2+} and calmodulin (Wells & Hardman, 1977); the second, E-II enzyme, is distinguished by anomalous kinetic properties (cyclic AMP hydrolysis is stimulated by cyclic GMP) (Beavo *et al.*, 1971; Moss *et al.*, 1977; Martins *et al.*, 1982). As reported in the present paper, we have now separated and characterized a third cyclic GMP phosphodiesterase (E-Ia) in rat liver supernatant. This enzyme is unaffected by Ca^{2+} plus calmodulin, does not bind to a calmodulin–Sepharose column,

Fig. 6. Effect of chymotrypsin on liver and HTC-cell E-I phosphodiesterases

(a) E-Ia and E-Ib fractions from the same liver supernatant were prepared by chromatography on DE-52 DEAE-cellulose and calmodulin–Sephacrose as described in Fig. 1. Samples of E-Ia enzyme (16 μg) and E-Ib enzyme (4 μg) were incubated with the indicated concentrations of chymotrypsin (final volume 0.1 ml) for 5 min at 30°C. Trypsin inhibitor (125 μg in 25 μl) was added, and the samples were assayed for phosphodiesterase activity with 0.5 μM -cyclic [^3H]GMP. Δ , E-Ia enzyme assayed in the presence of 0.5 mM-EGTA; $\bullet$, E-Ib enzyme assayed in the presence of EGTA; $\circ$, E-Ib enzyme assayed in the presence of 0.5 mM- CaCl_2 and 0.16 μg of calmodulin. (b) Samples (90 μg of protein) of E-I enzyme from rat liver ($\bullet$) [prepared by chromatography on DE-52 DEAE-cellulose as described by Moss *et al.* (1978)], or samples (6 μg of protein) of E-I enzyme from HTC cells (Δ) after chromatography on DEAE-Bio-Gel (analogous to fractions 30–36 in Fig. 3) were incubated at 30°C for 5 min with the indicated concentration of chymotrypsin before addition of 125 μg of trypsin inhibitor, and were assayed for phosphodiesterase activity with 0.5 μM -cyclic [^3H]GMP.

Table 1. Properties of cyclic GMP phosphodiesterases

Property	E-I enzyme (HTC cells)	E-Ia enzyme (rat liver)	E-Ib enzyme (rat liver)	E-II enzyme (rat liver)
Effect of Ca^{2+} + calmodulin	None	None	Activation	None
Affinity for calmodulin–Sephacrose	None	None	High	None
Kinetics	Linear	Non-linear	Linear	Non-linear*
Apparent K_m (cyclic GMP)	2–4 μM	1–2 μM	5–8 μM	30 μM^*
Apparent K_m (cyclic AMP)	—	12–16 μM	40 μM	100 μM^*
Effect of chymotrypsin	Inactivation	Inactivation	Activation	Inactivation*
Effect of kidney proteinase	None	None	Activation	None
M_r	325 000	325 000	150 000	430 000
M_r after chymotryptic activation	—	—	30 000	—

* Moss *et al.* (1977).

and exhibits anomalous kinetics, with a low K_m for cyclic GMP and a high K_m for cyclic AMP (Table 1). Its presence has hitherto been unnoticed, because, with buffers containing chelators, it is eluted from DEAE-cellulose at approximately the same ionic strength as is the E-Ib enzyme. It can be separated from E-Ib enzyme by chromatography in

the presence of Ca^{2+} , which alters the elution of E-Ib enzyme, presumably because of its Ca^{2+} -dependent association with endogenous calmodulin (Ho *et al.*, 1977), or by chromatography on calmodulin–Sephacrose. Since limited proteolysis converts E-Ib enzyme into a form that is insensitive to Ca^{2+} plus calmodulin, the possibility that E-Ia enzyme is an

altered form of E-Ib enzyme was considered. Isolation of E-Ia enzyme was performed in the presence of leupeptin, which inhibits proteolytic activation of a phosphodiesterase in rat liver homogenates (Strewler & Manganiello, 1979). Moreover, the apparent M_r of E-Ia enzyme was significantly greater than that of E-Ib enzyme, which was converted into a still smaller form by limited proteolysis with chymotrypsin. Thus it is unlikely that E-Ia enzyme arose from limited proteolysis of E-Ib enzyme.

The E-I enzyme from HTC cells was previously found to have a high affinity for cyclic GMP and to be decreased in HTC cells incubated with dexamethasone, but it was not further characterized (Ross *et al.*, 1977). As shown in the present paper, E-I enzyme from HTC cells resembles liver E-Ia enzyme in its failure to respond to Ca^{2+} and calmodulin, its elution from DEAE-cellulose, its M_r value, its inactivation by chymotrypsin and its resistance to the kidney proteinase. Unlike rat liver and other rat tissues (Kakiuchi *et al.*, 1975), HTC cells apparently do not contain a phosphodiesterase that is sensitive to Ca^{2+} and calmodulin. We considered the possibility that E-I enzyme is an anomalous or altered form of the calmodulin-sensitive enzyme. The presence of calmodulin in fractions containing E-I phosphodiesterase activity could result in unresponsiveness to exogenous calmodulin. Calmodulin is not demonstrable in fractions with E-I activity, although HTC-cell supernatants do contain a heat-stable Ca^{2+} -dependent phosphodiesterase activator that resembles calmodulin. No inhibitor of calmodulin-dependent phosphodiesterase was detected in fractions with E-I phosphodiesterase activity. It is unlikely that proteinases present in the homogenate converted E-I enzyme into a calmodulin-insensitive form during isolation, since inhibitors of proteolysis that block activation of phosphodiesterase in HTC-cell homogenates (G. J. Strewler, unpublished work) were present, and E-III enzyme in the same preparations remained sensitive to activation by a kidney proteinase. Whether the absence of the Ca^{2+} -dependent phosphodiesterase from the hepatoma cells is related to the transformed state or whether, perhaps, hepatocytes also lack a Ca^{2+} -dependent phosphodiesterase is at present unknown.

A cyclic GMP phosphodiesterase that is unresponsive to Ca^{2+} or Ca^{2+} plus calmodulin is reported to occur in human (Bergstrand & Lundquist, 1976) and guinea-pig lung (Davis & Kuo, 1977). The enzyme from guinea-pig lung, which exhibited normal kinetics, seemed more specific for cyclic GMP and had a lower apparent M_r (estimated by chromatography on Sephadex G-200) than the E-Ia enzyme from liver (Davis & Kuo, 1977). Rabbit lung contains a phosphodiesterase that responds to Ca^{2+} but not to calmodulin; this enzyme appears to

be associated with tightly bound calmodulin, which is not removed by chromatography in the presence of EGTA (Sharma & Wirch, 1979). Nasu *et al.* (1978) have reported the separation of Ca^{2+} - and calmodulin-dependent and -independent cyclic GMP phosphodiesterases from pig heart. The Ca^{2+} -independent enzyme had M_r 400 000 by gel filtration, had optimum pH 7.5 and exhibited anomalous kinetics, with an apparent K_m for cyclic GMP of $0.4 \mu\text{M}$. It was less susceptible to inhibition by cyclic AMP than was the Ca^{2+} - and calmodulin-dependent enzyme (Nasu *et al.*, 1978). These properties closely resemble those of E-Ia enzyme from rat liver (Table 1), although the effects of limited proteolysis on the enzyme were not investigated, nor was its chromatographic behaviour on calmodulin-Sepharose ascertained. Taken together, the data from the present study and those of Nasu *et al.* (1978) are consistent with the view that cyclic GMP phosphodiesterases similar to liver E-Ia enzyme and unresponsive to Ca^{2+} plus calmodulin may be widely distributed. As is the case with previously known phosphodiesterases, the specific role of this enzyme in the control of cellular cyclic nucleotide concentrations remains to be determined.

We thank Dr. R. Kincaid for calmodulin-Sepharose, Mrs. F. Lieberman and Dr. S. Jaffe for assistance in the laboratory, and Mrs. D. Marie Sherwood and Ms. Sally K. Danekas for secretarial help.

References

- Beavo, J. A., Hardman, J. G. & Sutherland, E. W. (1971) *J. Biol. Chem.* **246**, 3841–3846
- Bergstrand, H. & Lundquist, B. (1976) *Biochemistry* **15**, 1727–1735
- Davis, C. W. & Kuo, J. F. (1977) *J. Biol. Chem.* **252**, 4078–4084
- Goren, E. N. & Rosen, O. M. (1972) *Arch. Biochem. Biophys.* **153**, 384–397
- Ho, H. C., Wirch, E., Stevens, F. C. & Wang, J. H. (1977) *J. Biol. Chem.* **252**, 43–50
- Kakiuchi, S., Yamazaki, R., Teshima, Y., Uenishi, K. & Miyamoto, E. (1975) *Biochem. J.* **146**, 109–120
- Kincaid, R. A. & Vaughan, M. (1979) *Proc. Natl. Acad. Sci. U.S.A.* **76**, 4903–4907
- Martins, T. J., Mumby, M. C. & Beavo, J. A. (1982) *J. Biol. Chem.* **257**, 1973–1979
- Moss, J., Manganiello, V. C. & Vaughan, M. (1977) *J. Biol. Chem.* **252**, 5211–5215
- Moss, J., Manganiello, V. C. & Vaughan, M. (1978) *Biochim. Biophys. Acta* **541**, 279–287
- Nasu, S., Shioji, K., Ueda, I., Tanigawa, Y. & Shimoyama, M. (1978) *J. Biochem. (Tokyo)* **83**, 1449–1458
- Ross, P. S., Manganiello, V. C. & Vaughan, M. (1977) *J. Biol. Chem.* **252**, 1448–1452
- Sharma, R. K. & Wirch, E. (1979) *Biochem. Biophys. Res. Commun.* **91**, 338–344

Sharma, R. K., Wirch, E. & Wang, J. H. (1978) *J. Biol. Chem.* **253**, 3575–3580

Strewler, G. J. & Manganiello, V. C. (1979) *J. Biol. Chem.* **254**, 11891–11898

Watterson, D. M., Harrelson, W. G., Jr., Keller, P. M., Sharief, F. & Vanaman, T. C. (1976) *J. Biol. Chem.* **251**, 4501–4513

Wells, J. N. & Hardman, J. G. (1977) *Adv. Cyclic Nucleotide Res.* **8**, 119–143