Biochimica et Biophysica Acta, 522 (1978) 477—490

BBA 68328

INCREASED ACTIVITY OF CYCLIC AMP PHOSPHODIESTERASE FROM FROZEN-THAWED RAT LIVER

A ROLE OF LYSOSOMAL PROTEASE IN ENZYME ACTIVATION

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(Received April 13th, 1977)

Summary

The activity of cyclic AMP phosphodiesterase (3′:5′-cyclic-nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) in 105 000 × *g* supernatant fraction from frozen-thawed rat liver was 2.5 times higher than the corresponding preparation from fresh liver. This increased activity of frozen liver enzyme was accompanied by a decreased sensitivity of the enzyme to known activators such as α-tocopheryl phosphate and trypsin. Neither membrane-bound cyclic AMP phosphodiesterase, nor supernatant cyclic GMP phosphodiesterase increased in frozen liver preparation. It is unlikely that the activator protein of phosphodiesterase participated in the observed change of enzyme activity. Among rat tissues so far tested, the increased level of cyclic AMP phosphodiesterase was noted only in tissues rich in lysosome content. In the recombination experiment where phosphodiesterase from fresh liver was incubated with lysosomal fraction, stimulation of the enzyme activity was observed with a concomitant loss of sensitivity to above-mentioned activators. Since the stimulation by lysosomal fraction was effectively inhibited by cathepsin B1 inhibitors, leupeptin and antipain, it was deduced cathepsin-B1 (EC 3.4.12.3) type protease(s) was the main causative of activating the cyclic AMP phosphodiesterase. The freezing-thawing process of rat liver made the lysosomal membrane more permeable, and hence lysosomal proteases were released into soluble fraction during phosphodiester-

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Abbreviations: Cyclic AMP, adenosine 3′,5′-monophosphate; cyclic GMP, guanosine 3′,5′-monophosphate; TLCK, N^{α}-tosyl-L-lysyl-chloromethyl ketone; leupeptin, acetyl- and propionyl-L-leucyl-L-leucyl-L-arginine; antipin, *S*-1-carboxy-2-phenylethyl-carbamoyl-L-arginyl-L-varyl-L-arginine; pepstatin, isovaleryl-valyl-valyl-4-amino-3-hydroxy-6-methylheptanoyl-alanine; EGTA, ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

ase preparation. These results provide a warning not to use frozen liver for phosphodiesterase preparation, otherwise altered properties of the enzyme will be seen.

Introduction

Our previous papers showed that cyclic AMP phosphodiesterase (3′:5′-cyclic nucleotide 5′-nucleotidohydrolase, EC 3.1.4.17) of the supernatant fractions from rat brain [1] and from rat liver [2] were stimulated by α-tocopheryl phosphate, a vitamin E derivative. In the course of these experiments, we had consistently noted that cyclic AMP phosphodiesterase with markedly increased activity was obtained from frozen-thawed rat liver as compared to fresh liver preparation. Furthermore, this increased activity of frozen liver preparation was accompanied by a diminished sensitivity of the enzyme to α-tocopheryl phosphate. These findings aroused questions as to (a) whether the increased enzyme activity is extractable from other frozen tissues, (b) what type of multiple forms of liver phosphodiesterases [3—9] is stimulated in frozen-thawed preparation and, (c) by what mechanism the phosphodiesterase was activated during freezing and thawing procedure. In this communication, we shall report that the increased activity of cyclic AMP phosphodiesterase with frozen liver preparation is a result of modifications by protease(s) released from liver lysosomes. We shall discuss the roles of activator protein [10—17] as well as of inhibitor protein [18,19] with regard to the observed stimulation of phosphodiesterase by frozen liver.

Materials and Methods

Materials. Cyclic [G-^{3}H]AMP (38 Ci/mmol) and [G-^{3}H]GMP (2.1 Ci/mmol) were purchased from New England Nuclear. Unlabeled cyclic AMP, cyclic GMP, snake venom (*Crotalus atrox*), trypsin and soybean trypsin inhibitor were purchased from Sigma Chemical Co. TLCK was obtained from Cyclo Chemical Co., Los Angeles. Leupeptin, antipain and pepstatin were from Institute of Microbial Chemistry, Tokyo. α-Tocopheryl phosphate (sodium salt) was obtained from Eisai Pharmaceutical Co. Tokyo. DEAE-cellulose, DE-52 microgranular (Whatmann Biochem.), Dowex 1-X8, 200—400 mesh (Bio-Rad Laboratories), DEAE-Sephadex A-50 (Pharmacia) were purchased as indicated. All other chemicals were of analytical grade quality.

Preparation of the supernatant fraction from fresh rat liver. Unless otherwise indicated, all operations were carried out at 4°C. Male Wistar rats weighing 150—200 g were killed by decapitation and liver was excised. The tissue was minced with scissors and was homogenized in five volumes (w/v) of 0.32 M sucrose/40 mM Tris · HCl buffer (pH 8.0) in a glass homogenizer with five strokes of a Teflon pestle. The liver homogenate was then centrifuged at 105 000 × *g* for 60 min. The supernatant was stored in small aliquots at −20°C until used.

Preparation of frozen-thawed liver supernatant. Rat liver was taken out as described above. The liver lobes in glass vials were quickly frozen in a solid CO_2/ethanol bath and were kept in a freezer (−20°C) for 72 h prior to use. They

were allowed to thaw at 37°C for 3—5 min and then were minced with scissors. These liver pieces were homogenized and centrifuged to obtain the frozen liver supernatant fraction according to the same method as employed for the fresh liver preparation. The supernatant fraction thus obtained from frozen liver were stored —20°C up to 3 weeks without appreciable change of phosphodiesterase activity.

Preparation of membrane-bound phosphodiesterase. Membrane-bound phosphodiesterase was prepared by discontinuous sucrose gradient [4,20]. Rat liver was homogenized with a Teflon-glass homogenizer in five volumes (w/v) of 0.04 M Tris · HCl buffer (pH 8.0) containing 0.32 M sucrose. A density gradient was made 1.7 ml each of 1.2 M sucrose (bottom), 0.8 M sucrose (middle) and 1.7 ml of liver homogenate containing 0.32 M sucrose (top) in centrifuge tubes of 5.5 ml capacity. The tubes were centrifuged at 92 000 × *g* for 90 min using a swinging bucket rotor. The cytoplasmic membrane fraction [4,20] at 1.2–0.8 M interface was collected through a syringe needle, and was used as preparation of membrane-bound phosphodiesterase.

Phosphodiesterase assay. The enzyme activity was assayed by Boudreau-Drummond's modification [21] of the method of Thompson and Appleman [22]. The reaction mixture (final volume, 0.2 ml) contained 10 mM $MgCl_2$, 40 mM Tris · HCl (pH 8.0), 25 μg of bovine serum albumin, 1 μM cyclic [^{3}H]-AMP or cyclic [^{3}H]GMP (90 000 cpm), and an appropriate amount of the enzyme, and was incubated at 37°C for 5 min. The reaction was terminated by boiling for 45 s, and then 150 μg of snake venom were added. The mixture was incubated at 37°C for another 15 min. No more than 15% of the substrate was generally hydrolyzed during the enzyme assay. Enzyme activity is expressed as pmol of cyclic nucleotide hydrolyzed per min per mg protein or per ml of enzyme fraction.

Preparation of a lysosome fraction of rat liver. A lysosome fraction was prepared from rat liver according to the method of Sawant et al. [23]. This isolation method was based on both differential centrifugation and density gradient centrifugation techniques. The F III pellet fraction [23] which was suspended in a small amount of 70 mM acetate buffer (pH 6.5) was frozen in solid CO_2/ethanol) and thawed (in ice/water) five times to disrupt the lysosomal membrane. The lysed lysosome was centrifuged at 105 000 × *g* for 60 min and the clear supernatant fraction was obtained. The supernatant was used as "lysosomal fraction" in our current studies.

DEAE-cellulose chromatography. A similar system to that reported by Terasaki and Appleman [8] was employed for DEAE-cellulose chromatography. 2—4-ml aliquots of 105 000 × *g* liver supernatant were applied to a column (bed volume, 20 ml) equilibrated with 90 mM acetate buffer (pH 6.5). Fractions were developed with a linear 200 ml gradient from 70 mM to 1 M acetate buffer (pH 6.5). Fractions of 7 g eluent were collected, and aliquots (25—50 μl) of the fraction were examined for phosphodiesterase activity. Although the pH of phosphodiesterase reaction mixture (pH 8.0, Tris · HCl buffer) dropped to 7.9—7.6 by addition of the aliquots, the pH decrease did not cause an appreciable change in phosphodiesterase activity.

Estimation of activator protein. Standard activator protein or protein modulator [10—17] was purified from bovine brain by the procedure of Kato et al.

[24]. The homogeneity and physical properties of thus purified activator protein were described elsewhere [24]. The activator protein-sensitive phosphodiesterase that was required to estimate the content of endogenous activator protein in rat liver was prepared as described previously [1]. In brief, the 105 000 × *g* supernatant of rat brain homogenate was fractionated by DEAE-cellulose chromatography. In the presence of activator protein and Ca^{2+}, the P2 fraction of activator-sensitive phosphodiesterase was stimulated eight times over the basal (non-stimulated) activity [1]. Since the activator protein was heat stable, the liver supernatant was first boiled at 100°C for 60 s to denature the phosphodiesterase, and then the boiled supernatant was used for the estimation of endogenous activator protein.

Gel filtration. Gel filtration of liver supernatant fraction was carried out on a Sephadex column (2 × 30 cm) with a bed volume of 100 ml. The gel was equilibrated and eluted with 70 mM acetate buffer (pH 6.5) which was the identical buffer employed in DEAE-cellulose chromatography. As a routine, 2 ml of liver supernatant were applied to the column and 1.8-g fractions were collected. The void volume (V_0) was calibrated using Blue Dextran-2000.

Results

Cyclic nucleotide phosphodiesterase activities from fresh or frozen liver preparations

As indicated in Table I, the 105 000 × *g* supernatant fraction from frozen rat liver contained 2.3 times higher activity of cyclic AMP phosphodiesterase than the supernatant of fresh liver preparation. This increased activity of cyclic AMP phosphodiesterase was accompanied by the abolishment of the stimulatory effects of known phosphodiesterase activators such as trypsin and α-tocopheryl phosphate. Alternatively, these phosphodiesterase activators were effective only on the supernatant from fresh liver preparation. In contrast to cyclic AMP phosphodiesterase activity, there was no significant difference in cyclic GMP phosphodiesterase activity between fresh and frozen liver preparations (Table I). Virtually no difference was noted in the membrane-bound cyclic AMP phosphodiesterase activity from preparation of either fresh or frozen liver. To test whether increased cyclic AMP phosphodiesterase activity from frozen liver was due to increased amount of activator protein in the supernatant [10–17], the contents of endogenous activator protein from both preparations were estimated using purified activator protein as standard. As seen in Table I, almost identical amounts of activator protein were present in fresh and frozen liver supernatants. Unlike phosphodiesterase from the brain [1], we were unable to induce cyclic AMP phosphodiesterase activation, regardless of enzyme preparation, by addition of purified activator protein. Therefore, it is unlikely that activator protein participated in the observed activation of cyclic AMP phosphodiesterase. Since there was little difference in the protein content between fresh and frozen liver preparation (see legend of Table I), we concluded from Table I that cyclic AMP phosphodiesterase itself was activated being accompanied by a concomitant loss of trypsin and α-tocopheryl phosphate activation.

Kinetic analysis of cyclic AMP phosphodiesterases from the supernatant fractions of fresh and frozen liver gave an equal apparent K_m value of 3.1 μM,

TABLE I

CYCLIC NUCLEOTIDE PHOSPHODIESTERASE ACTIVITIES AND ACTIVATOR PROTEIN CONTENTS IN FRESH AND FROZEN LIVER PREPARATIONS

The 105 000 × *g* supernatant fraction and the membrane-bound fraction from fresh and frozen liver were prepared as described in Materials and Methods. Substrate (cyclic AMP or cyclic GMP) concentration was 1 μM. The supernatant fraction was assayed for cyclic AMP phophodiesterase activity with or without various activators; a, trypsin, 0.5 μg/tube; b, α-tocopheryl phosphate, 0.25 mM; c, activator protein, 0.4 μg/tube in the presence of 0.5 mM $CaCl_2$. These activators, where indicated, were directly added to assay tubes 5 min before the assay was started. Values are means ± S.D. of triplicate experiments. Percent relative to each basal activity is given in parentheses. Activator protein contents were determined as described in Materials and Methods. Protein, determined by the method of Lowry et al. [44], was 57.5 and 55.5 mg/g wet liver in the supernatant fractions from fresh and frozen liver, respectively.

Sources	Cyclic AMP phosphodiesterase					Cyclic GMP phosphodiesterase	Activator protein content (μg/g wet liver)
	105 000 × *g* supernatant (pmol/min per mg protein)				Membrane bound (pmol/min per 10 μl)	105 000 × *g* supernatant (pmol/min per mg protein)	
	None (basal)	+ Trypsin	+ α-Tocopheryl phosphate	+ Activator protein	None (basal)		
Fresh liver	139 ± 15 (100)	377 ± 34 (271)	384 ± 27 (276)	126 ± 11 (90.5)	98.4 ± 13.5	186 ± 16	480 ± 22
Frozen liver	323 ± 28 (100)	300 ± 12 (92.9)	326 ± 48 (101)	316 ± 17 (97.9)	104.0 ± 8.6	189 ± 19	442 ± 57

when substrate concentration was between 1 and 50 μM. On the other hand, V of frozen liver preparation was 8.4 nmol/min per mg protein, which was 2.4 times higher than the corresponding preparation of fresh liver.

Effects of tissue freezing

As a means of characterizing the activation, several rat tissues were tested to see whether preparations from frozen tissues contained more cyclic AMP phosphodiesterase activity than fresh tissue preparation. Table II shows the increased phosphodiesterase activity in the supernatant from frozen spleen and kidney, but not from the brain. It was also proved with spleen and kidney that the increment in the activity was accompanied by the insensitivity to trypsin or α-tocopheryl phosphate activation. Since liver, spleen and kidney are reported to be rich in lysosome content, whereas the brain contains few [25,26], and since trypsin activation was noted only by the supernatant of fresh tissues (liver, spleen and kidney), we assumed that the observed enzyme activation of frozen liver was a result of attack by lysosomal protease(s) released by freezing-thawing procedure on the latent form of cyclic AMP phosphodiesterase.

To prepare the supernatant enzyme from frozen liver, we routinely stored

TABLE II

EFFECTS OF TRYPSIN AND α-TOCOPHERYL PHOSPHATE ON CYCLIC AMP PHOSPHODIESTERASES PREPARED FROM FRESH OR FROZEN TISSUES

Rat tissues were homogenized in five volumes of 0.32 M sucrose/40 mM Tris buffer (pH 8.0). The 105 000 $\times g$ supernatant was used for phosphodiesterase assay. To prepare phosphodiesterase from frozen tissues, rat tissues were kept at —20°C for 72 h prior to homogenization. Cyclic AMP concentration was 1 μM. Values shown are mean ± S.D. of duplicate determinations. Percent relative to each basal activity is given.

Phosphodiesterase preparation	Additions	Cyclic AMP phosphodiesterase activity	
		pmol/min per mg protein	Relative (%)
Kidney, fresh	None (basal)	338 ± 10	100
	+ Trypsin (0.5 μg/tube)	676 ± 29	199
	+ α-Tocopheryl phosphate (0.25 mM)	801 ± 16	237
Kidney, frozen	None (basal)	470 ± 8	100
	+ Trypsin (0.5 μg/tube)	372 ± 24	79.2
	+ α-Tocopheryl phosphate (0.25 mM)	412 ± 29	87.6
Spleen, fresh	None (basal)	215 ± 17	100
	+ Trypsin (0.5 μg/tube)	302 ± 16	140
	+ α-Tocopheryl phosphate (0.25 mM)	353 ± 9	164
Spleen, frozen	None (basal)	257 ± 11	100
	+ Trypsin (0.5 μg/tube)	219 ± 5	85.4
	+ α-Tocopheryl phosphate (0.25 mM)	231 ± 19	89.8
Brain, fresh	None (basal)	806 ± 48	100
	+ Trypsin (0.5 μg/tube)	2840 ± 220	353
	+ α-Tocopheryl phosphate (0.25 mM)	3040 ± 273	378
Brain, frozen	None (basal)	856 ± 137	100
	+ Trypsin (0.5 μg/tube)	2740 ± 246	320
	+ α-Tocopheryl phosphate (0.25 mM)	2760 ± 192	323

the tissue at −20°C for 72 h prior to homogenization. It was also a useful procedure to obtain increased enzyme activity by five times repeated freezing of intact liver lobes in solid CO_2/ethanol, followed by thawing in an ice-and-water slurry. In other experiment (data not shown), a supernatant fraction was prepared from the liver which was kept at 4°C from 3 to 24 h. This procedure did not cause the activity change of cyclic AMP phosphodiesterase. It was noted that storing the 105 000 × *g* supernatant fraction, once it has been separated from particulates, did not cause an appreciable change in the basal phosphodiesterase activity as well as enzyme sensitivity to trypsin and α-tocopheryl phosphate, whether fresh or frozen liver was used as source, even after the enzyme had been kept at −20°C up to 3 weeks (data not shown). These results led us to suspect that a necessary process for the enzyme activation involved protease leakage from liver lysosomes.

Effects of lysosomal fraction on cyclic AMP phosphodiesterase activity

To obtain the direct evidence that lysosomal proteases are participating in activation step of phosphodiesterase, a lysosomal fraction was isolated from rat liver as described in Materials and Methods. When a liver supernatant fraction was preincubated with the lysed lysosomal fraction, only the fresh liver phosphodiesterase was stimulated by lysosomal fraction and no stimulation occurred with the frozen liver enzyme (Table III). Since lysosomes contain a series of cathepsin group proteases, several classes of protease inhibitors were tested to

TABLE III

EFFECTS OF LYSOSOMAL FRACTION ON THE ACTIVITY OF CYCLIC AMP PHOSPHODIESTERASE

Rat liver 105 000 × *g* supernatant fraction from either fresh or frozen liver was obtained as described in Materials and Methods. The lysosomal fraction was prepared from rat liver according to the method of Sawant et al. [23]. The F-III fraction [23] was subjected five times to a freezing and thawing cyclic in 70 mM acetate buffer, pH 6.5, and then centrifuged at 105 000 × *g* for 60 min to obtain "lysosomal fraction". Phosphodiesterases and the "lysosomal fraction" (50 μg/tube) were preincubated at 15°C in 70 mM acetate buffer, pH 6.5, containing 5 mM 2-mercaptoethanol and bovine serum albumin (50 μg/tube) for 15 min in a final volume of 150 μl. Various protease inhibitors, where indicated, were added prior to the addition of the "lysosomal fraction". Cyclic AMP concentration was 1 μM. Values are means of duplicate experiments. Percent relative to each basal activity is given. STI, soybean trypsin inhibitor.

Phosphodiesterase preparation	Additions	Cyclic AMP phosphodiesterase activity	
		pmol/min per mg protein	Relative (%)
Fresh liver	None (basal)	154	100
	+ Lysosomal fraction	376	244
	+ Lysosomal fraction + leupeptin (5 μM)	149	97.0
	+ Lysosomal fraction + antipain (5 μM)	151	98.2
	+ Lysosomal fraction + TLCK (5 μM)	157	102
	+ Lysosomal fraction + STI (2 μg/tube)	270	175
	+ Lysosomal fraction + pepstatin (5 μM)	276	179
Frozen liver	None (basal)	235	100
	+ Lysosomal fraction	229	97.5

see whether inhibitors preferentially stunt the observed activation of phosphodiesterase. As seen in Table III, complete inhibition of lysosome-induced activation was exhibited by leupeptin and antipain, the known cathepsin B1 inhibitor [27,28], while pepstatin, a recognized antagonist of cathepsin D [29,30] and soybean trypsin inhibitor were found to be moderately effective inhibitors, giving approx. 50% depression of protease-induced phosphodiesterase activation. A substantial inhibitory effect was also noted in TLCK, which is known to suppress the activity of trypsin-like protease as well as these of sulfhydryl-active sites [31,32]. These data strongly suggest that a cathepsin B1-type protease is the main lysosomal enzyme which induces the phosphodiesterase activation.

DEAE-cellulose column chromatography

In the next series of experiment, chromatographic profiles of supernatant phosphodiesterase from fresh and frozen liver were compared using DEAE-cellulose columns. In case of fresh liver supernatant, DEAE-cellulose chromatography yielded three active peaks of cyclic nucleotide phosphodiesterases (F-1, F-2 and F-3 in Fig. 1A). However, the supernatant fraction prepared from frozen rat liver gave four active peaks designated as F-1, F-2, F-3 and F-4. Despite the different elution pattern of cyclic AMP phosphodiesterase, there was a good similarity between fresh and frozen liver in the elution profile of cyclic GMP phosphodiesterase activity. This observation is predictable from the results of Table I, in which no appreciable difference in cyclic GMP phosphodiesterase activity was noted between fresh and frozen liver preparations. The recovery of cyclic AMP phosphodiesterase activity from all the DEAE-cellulose fraction was 38% for fresh liver and 42% for frozen liver. The recovery of cyclic GMP phosphodiesterase activity was 54% for fresh liver and 52% for frozen liver.

Effects of phosphodiesterase activators on DEAE-cellulose fractions

Experiments were conducted to test the effects of various phosphodiesterase activators [1,2] on the different types of cyclic AMP phosphodiesterases separated by DEAE-cellulose chromatography (Table IV). The F-3 from fresh liver was demonstrated to be preferentially stimulated by trypsin as well as α-tocopheryl phosphate, whereas, the same fraction from frozen liver was not activated. Moreover these chemicals were even inhibitory on F-2 and F-4 from frozen liver. These findings support the concept that cyclic AMP phosphodiesterase from frozen liver was already activated by proteolytic process. Thus, no further activation was attained by trypsin treatment in the case of frozen liver preparation. None of the DEAE-cellulose fractions was stimulated by addition of activator protein (Table IV).

Gel filtration of liver supernatant

The elution patterns of cyclic AMP phosphodiesterases were compared between fresh and frozen liver preparations using Sephadex G-200 column. The results from typical experiments are shown in Fig. 2. The top diagram (A) shows the elution pattern obtained with freshly prepared 105 000 $\times g$ supernatant fraction; the bottom diagram (B) was obtained with frozen liver superna-

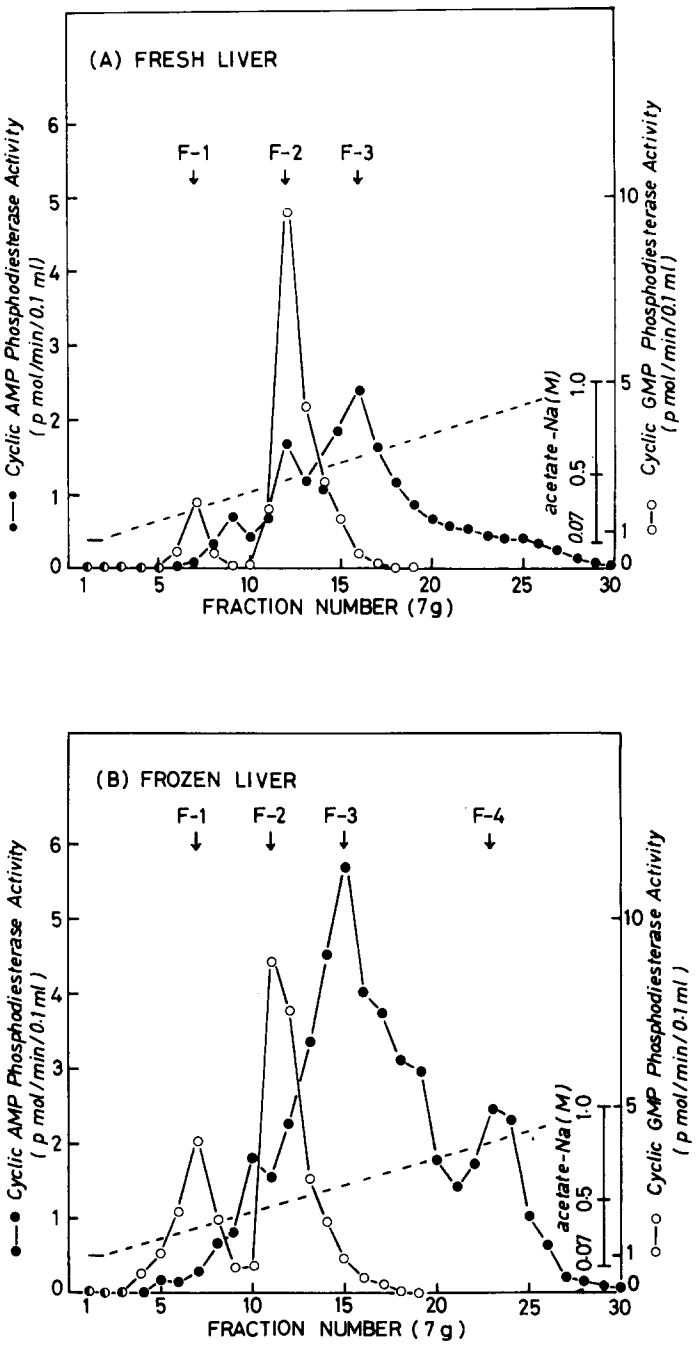

Fig. 1. DEAE-cellulose column chromatography of fresh (A) and frozen (B) liver preparations. Rat liver 105 000 × *g* supernatant fraction from either fresh (A) or frozen (B) liver was prepared as described in Materials and Methods. The supernatant was applied to the DEAE-cellulose column equilibrated with 70 mM acetate buffer (pH 6.5). The column was developed with a linear 200 ml gradient from 0.07 to 1 M acetate buffer (pH 6.5). Fraction of 7 g was collected. Phosphodiesterase activity was assayed using 1 μM cyclic AMP (●———●) or 1 μM cyclic GMP (○———○) as substrate. The major peaks of cyclic nucleotide phosphodiesterase activity were designated to F-1, F-2, F-3 (and F-4) in order of elution.

TABLE IV

EFFECTS OF PHOSPHODIESTERASE ACTIVATORS ON DEAE-CELLULOSE FRACTION

Experimental conditions were described in Materials and Methods. The identification of the DEAE-cellulose fraction is given in Figs. 1A and 1B. DEAE-cellulose fractions from either fresh or frozen liver were assayed for cyclic AMP phosphodiesterase activity in the absence (basal) and presence of various activators. The concentration of cyclic AMP was 1 μM. Values are means of duplicate assay. Percent relative to each basal activity is given in parentheses.

Additions	Cyclic AMP phosphodiesterase activity (pmol/min per 100 μl fraction)				
	Fresh liver DEAE-cellulose fraction		Frozen liver DEAE-cellulose fraction		
	F-2	F-3	F-2	F-3	F-4
None (basal)	2.06 (100)	2.46 (100)	0.99 (100)	5.25 (100)	3.09 (100)
+ Trypsin, 0.5 μg/tube	1.57 (76.2)	7.55 (307)	0.95 (96.4)	5.46 (104)	2.02 (65.5)
+ α-Tocopheryl phosphate, 0.25 mM	2.43 (118)	7.30 (297)	0.76 (78.3)	5.41 (103)	2.69 (86.9)
+ Activator protein, 0.5 μg/tube	2.10 (102)	2.41 (98.0)	1.08 (109)	5.67 (108)	3.00 (97.2)

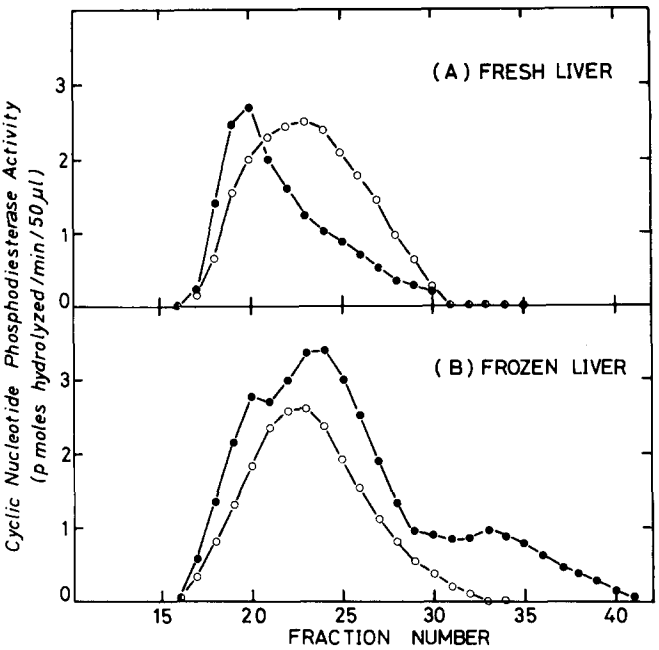

Fig. 2. Sephadex G-200 gel filtration of the 105 000 × *g* supernatant fraction from either fresh (A) or frozen (B) rat liver. The supernatant fraction (2 ml) was chromatographed on a Sephadex G-200 column (100 ml). Fractions of 1.8 g were collected and assayed for phosphodiesterase activity using 1 μM cyclic AMP (●———●) or 1 μM cyclic GMP (○———○). Details are described in Materials and Methods.

tant. In case of fresh liver the main peak of cyclic AMP phosphodiesterase activity appeared immediately behind the void volume with a V_e/V_0 value of 1.11. On the other hand the principal cyclic AMP phosphodiesterase activity of frozen liver moved into fraction No. 24, which gave the V_e/V_0 value of 1.33. Furthermore, an additional small peak of the enzyme activity appeared in fraction No. 33 (V_e/V_0 = 1.83). These results suggest that cathepsin effects on phosphodiesterase of frozen liver preparation consisted of production of a smaller form of the enzyme with a concomitant increase of enzyme activity. The main peak of cyclic AMP phosphodiesterase from fresh liver was activated three times over the basal activity in the presence of trypsin, or α-tocopheryl phosphate (data not shown). However, there was no stimulatory effect of these agents upon the cyclic AMP phosphodiesterase activity from frozen liver fractions. As expected from the results of DEAE-cellulose chromatography, the elution pattern of cyclic GMP phosphodiesterase activity did not differ significantly between fresh and frozen liver supernatant (Fig. 2).

Discussion

We postulate that freezing and thawing of rat liver makes lysosomal membranes "leaky", and consequently a large amount of lysosomal protease(s) is released into the soluble fraction during phosphodiesterase preparation. As a result, the cyclic AMP phosphodiesterase is activated by the released protease(s). Judging from Table III that leupeptin and antipain effectively inhibited the

protease-induced activation of phosphodiesterase, it was concluded that cathepsin B1 was the main protease participating in phosphodiesterase activation. So far as we are aware, this is the first example in which the cyclic AMP phosphodiesterase was activated by endogenous protease.

To prepare the supernatant fraction from frozen liver, we routinely kept the liver at −20°C for 72 h; a procedure that was found to be useful in obtaining acid proteases from monkey liver lysosomes [25]. Earlier studies showed that kinetic changes of phosphodiesterase were seen when homogenates of rat liver [5] and lymphocytes [33] or a kidney enzyme preparation [34] were either stored at 4°C or frozen and thawed. It is conceivable that lysosomal proteases modified the nature of phosphodiesterase, since, in addition to storing the preparations at low temperature, these authors sonicated them for the purpose of releasing the particulate phosphodiesterase. Presumably lysosomal membranes could be disrupted by sonication.

It wash shown by Cheung [10] that trypsin treatment of brain phosphodiesterase caused the enzyme activation. Desensitization of phosphodiesterase to EGTA inhibition was also reported after trypsin treatment [35]. Activation of enzymes by limited proteolysis were reported in several other enzymes including phosphorylase phosphatase [36], protein kinase [37] and fructose-1,6-diphosphatase [38]. Common to these enzymes, inactive form of an enzyme existed as enzyme-inhibitor protein complex. Recently it was shown a protein inhibitor of phosphodiesterase was prepared from retina [18] and brain [19]. In both cases, the inhibitor was susceptible to trypsin. These results suggest that cyclic AMP phosphodiesterase of rat liver may exist in the inactive form as an enzyme-inhibitor complex. A possible reason why the membrane-bound enzyme was not activated by freezing and thawing (Table I), is that lysosomal protease(s) was not accessible to the membrane-integrated enzyme.

Among the multiple types of phosphodiesterase, the enzyme sensitive to trypsin activation was also stimulated by α-tocopheryl phosphate [1]. This observation was also confirmed in our present experiments. The activation of phosphodiesterase by α-tocopheryl phosphate is likely to be due to a dissociating effect of inhibitor from the latent enzyme-inhibitor complex. We have questioned the origin of the multiple forms of the enzyme from liver. As evident in DEAE-cellulose studies (Fig. 1), a more complicated elution pattern of cyclic AMP phosphodiesterase activity was observed with frozen and thawed then fresh liver preparations. It is reasonable to suggest that the multiple forms of phosphodiesterase may be artifacts generated, at least in part, by partial proteolysis of the phosphodiesterase during preparation.

Regarding the extent of activation attained by activator protein [10—17], there is a considerable variation among the sources of phosphodiesterase. In the present study, neither crude nor partially purified liver enzyme was stimulated by activator protein. It was recently observed [39] that chick liver cyclic AMP phosphodiesterase was also refractory to the activator protein, while the enzyme from early stage embryo was sensitive to the activator protein. Kakiuchi et al. [7] found that cyclic GMP hydrolysis was more enhanced by activator protein than the cyclic AMP hydrolysis by rat liver preparation. A considerable amount of activator protein was found in liver (Table I). Thus the protein appears not to function as an activator of cyclic AMP phosphodiesterase. Possi-

bly the protein serves as an activator of adenylate cyclase system [40—43]. Alternatively, the activator may function as an modulator of cyclic GMP hydrolysis [7].

The present work shows that no increased activity of cyclic GMP phosphodiesterase was obtained in the frozen liver preparation. Unlike cyclic AMP phosphodiesterase, cyclic GMP phosphodiesterase was unaffected by, or resistant to, the lysosomal proteases. It may be deduced that the enzyme capable of hydrollyzing cyclic GMP is different entity from the enzyme specific for cyclic AMP hydrolysis.

Acknowledgements

We are indebted to Dr. S. Kakiuchi for his helpful discussion, to Misses H. Yamanaka and T. Okano for their technical assistance.

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