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CYCLIC 3′:5′-NUCLEOTIDE PHOSPHODIESTERASE DETERMINED IN VARIOUS HUMAN TISSUES BY DEAE-CELLULOSE CHROMATOGRAPHY

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Summary

Tissue extracts from human heart, lung, liver, kidney, skeletal muscle and cerebrum displayed at least 3 distinct cyclic 3′:5′-nucleotide phosphodieterase (EC 3.1.4.17) activity peaks (FI, FII, FIII) on DEAE-cellulose chromatography and various properties of these forms were compared in each tissue. FI eluted at about 0.08 M sodium acetate, hydrolyzed cyclic GMP more rapidly than it did cyclic AMP, and cyclic GMP hydrolysis by FI in most tissues was enhanced by a protein activator in the presence of $CaCl_2$. As only high concentrations of cyclic AMP inhibited cyclic GMP hydrolytic activity of FI, the enzyme probably has a low affinity for cyclic AMP. FII eluted at about 0.2 M sodium acetate, hydrolyzed both nucleotides at equal rates, and substrate affinities were relatively low. Cyclic GMP hydrolysis by FII was also stimulated by addition of a protein activator in the presence of $CaCl_2$ and cyclic AMP hydrolysis in this fraction was accelerated by a micromolar fraction of cyclic GMP. FIII eluted at about 0.35 M hydrolyzed cyclic AMP preferentially and was insensitive to protein activator. These two cyclic nucleotides act as mutual inhibitors of the hydrolysis in this fraction. Ratio of the cyclic GMP to cyclic AMP hydrolysis was in the order FI, FII, FIII. Four activity peaks were eluted from the cerebral extract and enzymes from this tissue exhibited much the same properties as observed in the other tissues examined herein.

Introduction

Multiple forms of cyclic nucleotide phosphodiesterase (EC 3.1.4.17) exist in mammalian tissue and isolation and characterization data have been reported in

considerable detail [1—12]. Russell et al. [13] found that rat liver contains at least three cyclic nucleotide phosphodiesterase fractions and these differ in substrate affinity, specificity and subcellular distribution. As the liver consists of heterogenous cells, it is not clear whether multiple forms of phosphodiesterase exist in tissues containing homogeneous cell populations. Three forms of cyclic nucleotide phosphodiesterase have also been demonstrated in human platelets [10] thus raising the possibility that a single cell may contain at least three forms of phosphodiesterase.

In recent experiments using DEAE-cellulose chromatography, we found evidence for the existence of at least three distinct phosphodiesterases in tissues from human heart, lung, liver, kidney and skeletal muscle. Various properties of these three forms were investigated and compared in each tissue.

Materials and Methods

Materials. Cyclic [^{3}H]adenosine 3′:5′-monophosphate (specific activity 33.2 Ci per mmol) and cyclic [^{3}H]guanosine 3′:5′-monophosphate (3.46 Ci per mmol) were purchased from the New England Nuclear Corp. Unlabeled cyclic AMP, cyclic GMP and snake venom (*Crotalus atrox*) were purchased from Sigma Chemical Co. Cation exchange resin (BioRad AG 50-X4, 200—400 mesh) was repeatedly washed with 0.5 M NaOH, 0.5 M HCl and deionized water to a final pH of 5.0. DEAE-cellulose (Whatman, DE 52) was equilibrated with the column buffer prior to use. All other chemicals were reagent grade and the best commercially available. Such were used without further purification.

Preparation of soluble phosphodiesterase from various tissues. Tissues were obtained within 4 h of autopsy from three Japanese patients. Included were tissues from a 54-year-old male who died of cardiac disorders, a 24-year-old female who had gastric cancer and a 62-year-old male with pneumonia. The excised tissues were washed in distilled water and stored at −70° C. Investigated were tissues from the kidney (cortex), heart (ventricle), lung (both lobes), liver (a part of right lobe), skeletal muscle (M. psoas major) and cerebrum (frontal cortex). All tissues were homogenized at 4° C in 3 vols. of 50 mM Tris/acetate buffer (pH 6.0) containing 3.75 mM 2-mercaptoethanol. The homogenate was centrifuged at 105 000 × g for 60 min. The clear supernatant fluid served as the source of soluble phosphodiesterase.

Phosphodiesterase assay. The two-step assay for enzymatic activity was similar to the previously described [14]: 5′-[^{3}H]AMP or 5′-[^{3}H]GMP formed by the phosphodiesterase was converted to [^{3}H]adenosinde or [^{3}H]guanosine by the action of nucleotidase and the product isolated by cation exchange resin was counted in a liquid scintillation counter. An appropriate dilution of enzyme was incubated in 50 mM HCl, pH 8.0, 5 mM $MgCl_2$, 50 μg of bovine serum albumin, 0.4 μM cyclic [^{3}H]AMP or 0.4 μM cyclic [^{3}H]GMP, in a total volume of 0.5 ml. When higher concentrations of substrate were required, indicated amounts of unlabeled cyclic nucleotide were included. After 20 min incubation at 30° C, the reaction was terminated by boiling for 5 min. Next 0.05 ml of snake venom (1 mg/ml H_2O) was added and a further incubation was carried out for 10 min at 30° C. Then 1.0 ml of water was added and the mixture applied to a small ion exchange resin column (AG 50-X4, 0.8 × 2 cm column).

Adenosine or guanosine was eluted with 1.5 ml of 3 M NH_4OH after washing the column with 15 ml of distilled water. In routine assays, less than 20% of the substrate was converted into the product. Recovery of adenosine or guanosine from the column was 95%.

Chromatography. DEAE-cellulose chromatography was performed by the method of Hidaka and Asano [10] in columns (1.5 × 20 cm) with bed volumes of 35 ml. The buffer was 50 mM Tris/acetate, pH 6.0, containing 3.75 mM 2-mercaptoethanol. The enzyme preparation was applied to the column, followed by elution with several column volumes of buffer. The initial wash contained no detectable phosphodiesterase activity. A linear gradient of 300 ml from 0 to 1 M sodium acetate was then applied with a flow rate of 0.5 ml/min. Fractions were pooled and usually stored at 4°C in the presence of 0.1 mg/ml of bovine serum albumin. At this temperature 50% of the activity remained after 3 weeks. The amount of activator capable of doubling the enzyme activity in the standard system was defined as 10 units. In most experiments, the effect of protein activator was estimated by comparing the activity in the presence of Ca^{2+} (20 μM) and protein activator (40 units) with that in the presence of activator and EGTA (200 μM). Activator protein was partially purified from bovine brain by heat treatment, ammonium sulfate fractionation and DEAE-cellulose chromatography according to the method of Cheung [15].

Results

DEAE-cellulose column chromatography

Human tissues homogenized with 3 volumes of 50 mM Tris/acetate buffer pH 6.0 containing 3.75 mM 2-mercaptoethanol revealed substantial amounts of the phosphodiesterase as recovered from the washed particulate fractions of cardiac muscle (60—70%), lung 25—40%), liver (35—45%), kidney (25—50%) and skeletal muscle (40—80%). The supernatants of these tissues contained 18—80% of the total activities. The 105 000 × *g* supernatant fractions contained 23, 70, 60, 40 and 18%, or 40, 40, 60, 75 and 55%, respectively, of the total homogenate cyclic AMP or cyclic GMP activities present in cardiac muscle, lung, liver, kidney and skeletal muscle. Cerebral tissue contained the highest activity of both nucleotides per gram of wet tissue while the lowest activity was noted in liver tissue. An extract (105 000 × *g* supernatant) of each tissue was subjected to DEAE-cellulose chromatography.

Fig. 1 is a typical profile seen in kidney and cerebral tissues. Each tissue examined contained three separable phosphodiesterases and such were designated FI, FII and FIII according to the order of elution from the column. FI eluted at about 0.08 M sodium acetate, FII at about 0.2 M and FIII at about 0.35 M. Recovery of cyclic AMP (30—40%) and GMP (50—60%) hydrolytic activities was fairly constant in all tissues and the ratio is shown in Table I. FI enzyme hydrolyzed cyclic GMP preferentially, FII hydrolyzed both nucleotides at equal rates, while FIII hydrolyzed cyclic AMP. When the activity was measured at a higher concentration of substrate (100 μM), FI (except liver and kidney) and FII (most tissues) showed a slightly higher hydrolytic activity for cyclic GMP and FIII catalyzed the hydrolysis of cyclic AMP faster than that of cyclic GMP. FI appears to be a relatively specific cyclic GMP phosphodiester-

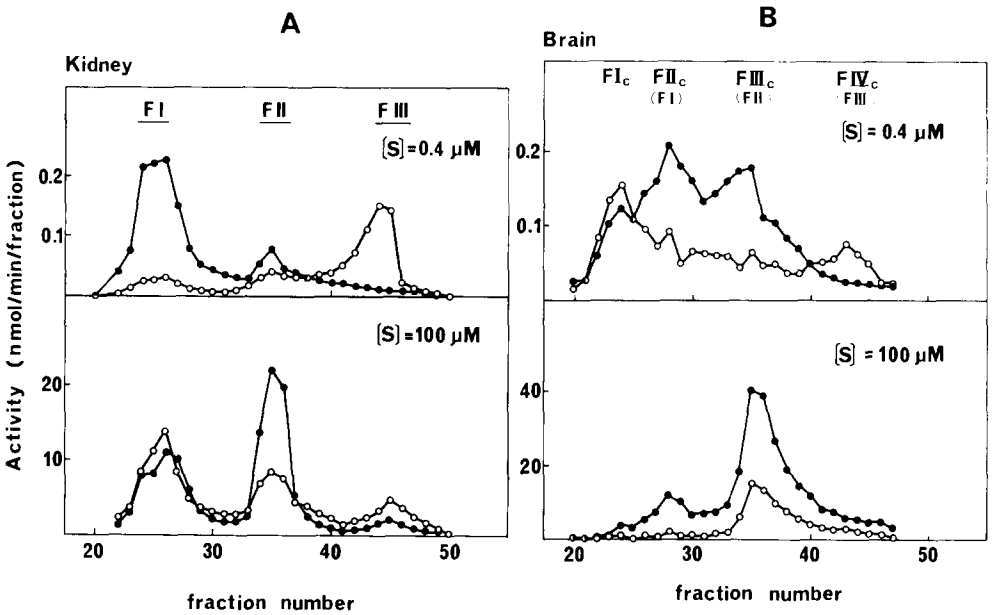


Fig. 1. DEAE-cellulose column elution profile of 105 000 × *g* supernatant of human kidney and cerebral tissues. A linear gradient from 0 to 1 M sodium acetate was applied from Fraction 20 to 75. Aliquots 0.2 ml for high substrate (100 μM) or 0.01 ml for low substrate level (0.4 μM) were assayed directly.

ase, FII a cyclic nucleotide phosphodiesterase and FIII a relatively specific cyclic AMP phosphodiesterase.

Kinetic analysis was carried out for FI using cyclic GMP as substrate, for FII using cyclic GMP and cyclic AMP as substrates and for FIII using cyclic AMP. Data obtained from Lineweaver-Burk plots of these three forms in each tissue are summarized in Table II.

FI, a relatively specific cyclic GMP phosphodiesterase, showed a normal kinetics in liver and kidney but such were abnormal in other tissues. K_m values of FI in these five tissues ranged from 0.65 to 3.0 μM suggesting that FI has a higher affinity for cyclic GMP. However, FI of heart, lung and muscle showed two K_m values of 10, 3.1 and 20 μM respectively (Table II). Kinetic analysis of

TABLE I

RATIO OF CYCLIC GMP TO CYCLIC AMP HYDROLYSIS BY DEAE-CELLULOSE RESOLVED ENZYMES (FI, FII, FIII) FROM HUMAN TISSUES

Tissue	Cyclic GMP hydrolysis/cyclic AMP hydrolysis					
	FI, substrate concn.:		FII, substrate concn.:		FIII, substrate concn.:	
	0.4 μM	100 μM	0.4 μM	100 μM	0.4 μM	100 μM
Heart	8.0	2.4	1.2	1.7	0.45	0.45
Lung	3.4	1.6	1.2	1.3	0.079	0.25
Liver	7.9	0.75	0.71	1.3	0.25	0.26
Kidney	8.9	0.85	1.9	2.6	0.10	0.33
Skeletal muscle	4.6	2.7	2.3	2.1	0.14	0.45

TABLE II

APPARENT K_m VALUES OF DEAE-CELLULOSE RESOLVED CYCLIC NUCLEOTIDE PHOSPHODIESTERASES IN VARIOUS HUMAN TISSUES

Tissue	K_m values (μM)			
	FI, substrate:	FII, substrate		FIII, substrate:
	Cyclic GMP	Cyclic GMP	Cyclic AMP **	Cyclic AMP
Heart	2.0 10	15	—	0.30
Lung	0.93 3.1	29 *	15	1.0
Liver	3.0	27 *	90	0.80
Kidney	0.65	10	44	0.75
Skeletal muscle	1.5 20	24 *	50	0.44

* Positively cooperative at low substrate concentration.

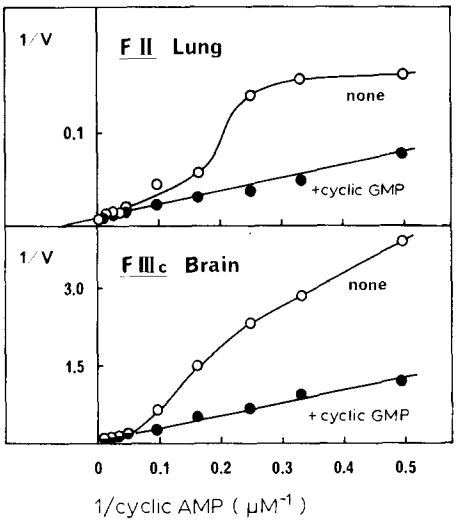
** K_m values were determined in the presence of 5 μM cyclic GMP.

FI for cyclic AMP hydrolysis was not performed because the activity was low at a low concentration of the substrate. FIII in all tissues except muscle revealed a normal kinetics for cyclic AMP hydrolysis with K_m values ranging from 0.3 to 1.0 μM. This enzyme in muscle showed abnormal kinetics for cyclic AMP hydrolysis and the K_m was 0.44 and 1.5 μM. Cyclic GMP hydrolysis by FIII was lower than its cyclic AMP hydrolytic activity and K_m values of FIII enzymes for cyclic GMP were not studied further.

FII showed a relatively lower substrate affinity for nucleotides (Table II and Fig. 2). FII in tissues except heart and kidney showed abnormal kinetics for cyclic GMP and K_m values ranged from 10 to 29 μM (Table II). Lineweaver-Burk plots for cyclic AMP hydrolysis by FII were not linear, particular when the substrate concentrations were between 2 and 10 μM of cyclic AMP (Fig. 2). However, with addition of 5 μM of cyclic GMP the kinetics for cyclic AMP hydrolysis showed normal values (Fig. 2) and these K_m values in various tissues are shown in Table II.

Fig. 3 shows that the cyclic AMP hydrolysis by FII was stimulated by addition of micromolar amounts of cyclic GMP and maximum stimulation was observed at approximately 5 μM of cyclic GMP. This stimulation was also dependent on the substrate (cyclic AMP) concentration, and 2—10 μM proved to be adequate amounts for the stimulation (Fig. 2). The effect of cyclic GMP on cyclic AMP hydrolysis by FII of heart tissue could not be investigated as the activity was too low.

Cyclic AMP hydrolysis by other forms (FI and FIII) was inhibited by the addition of cyclic GMP. Cyclic GMP hydrolytic activity of the three forms was also inhibited by cyclic AMP. When 0.05 μM to 40 μM of cyclic GMP was used as a substrate either in the presence or absence of 0.1 to 200 μM of cyclic AMP, Ki values of FI, FII and FIII for cyclic AMP were obtained by Dixon plits [16].



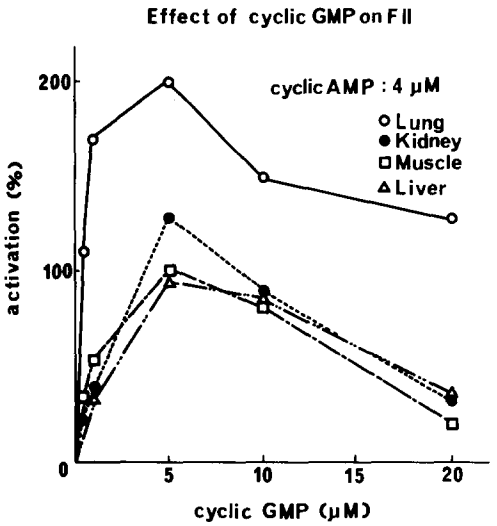


Fig. 2. Kinetic analysis of cyclic AMP hydrolysis by FII of lung and cerebral tissues in the presence or absence of 5 μM of cyclic GMP. Substrate concentrations of reaction mixture were from 2 μM to 1.0 mM.

Fig. 3. Effect of cyclic GMP on cyclic AMP hydrolysis by FII of various human tissues. Cyclic AMP hydrolytic activity in the absence of cyclic GMP was taken as a standard. Concentration of cyclic GMP described in the figure indicates the averaged amounts of cyclic GMP throughout the incubation; (Initial concentration of cyclic GMP + the concentration at the end of incubation)/2. Cyclic GMP concentration at the end of the incubation was determined as follows; various concentration of cyclic [^{3}H]GMP were incubated with enzyme in the presence of 4 μM of non-radioactive cyclic AMP in the same condition and the hydrolysis of cyclic [^{3}H]GMP was determined.

These values were as follows (μM): 80, 0.3 (heart FI and FIII), 16, 0.8 (lung FII and FIII), 56, 35, 0.95 (liver FI, FII and FIII), 70, 25 (kidney FI and FIII) 8, 15, 10 (muscle FI, FII and FIII). The optimal concentration of Mg^{2+} for cyclic nucleotides hydrolytic activities in the three forms was from 0.1 to 10 mM. Mg^{2+} was required for demonstration of activity with all three forms.

Effect of protein activator on the three forms

When phosphodiesterase activities of the three forms were compared in the presence of 20 μM $CaCl_2$ and saturated amounts of partially purified bovine brain protein activator (40 units) or in the presence of 200 μM of EGTA and the protein activator, Ca^{2+}-dependent stimulation of FI (except liver) and FII enzyme activities by a protein activator was obsevered (Table III). FIII was not significantly stimulated by bovine brain protein activator in the presence of 200 μM $CaCl_2$. Cyclic GMP rather than AMP hydrolytic activity was more markedly stimulated in all tissues except lung, in which case the reverse was noted.

Cyclic nucleotide phosphodiesterase in cerebral tissue

The 105 000 $\times g$ supernatant fraction contained 50 and 30% respectively of the total homogenate cyclic AMP and cyclic GMP hydrolytic activities. When this supernatant was applied to DEAE-cellulose column, four forms of cyclic nucleotides hydrolytic activities were observed and were designated as FIc,

TABLE III

EFFECT OF A PROTEIN ACTIVATOR ON DEAE-CELLULOSE RESOLVED ENZYMES (FI, FII, FIII) FROM HUMAN TISSUES

$CaCl_2$: 20 μM; EGTA: 200 μM. Protein activator from bovine brain (40 units) was added with maximum stimulation.

	Activity with $CaCl_2$/activity with EGTA					
	FI		FII		FIII	
	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP	Cyclic AMP	Cyclic GMP
Heart	2.3	3.5	1.2	2.0	1.0	1.1
Lung	1.8	1.3	1.5	2.5	1.0	1.2
Liver	1.0	1.0	1.9	2.7	1.0	1.3
Kidney	1.0	2.3	1.3	1.8	1.0	1.0
Skeletal muscle	2.3	3.8	1.0	2.6	1.0	—

FIIc, FIIIc and FIVc (Fig. 1B). Properties of FIIc, FIIIc and FIVc such as substrate specificity and the effect of a protein activator or cyclic GMP (Figs. 2 and 4 and Table IV) resembled, to some extent, those of FI, FII and FIII in other tissues. Namely, FIIc hydrolyzed cyclic GMP faster than cyclic AMP and was stimulated by a protein activator in the presence of Ca^{2+} (Table IV). FIIIc was also stimulated by a protein activator in the presence of Ca^{2+} and this stimulation was observed at a substrate concentration below 10 μM. FIIc and FIIIc

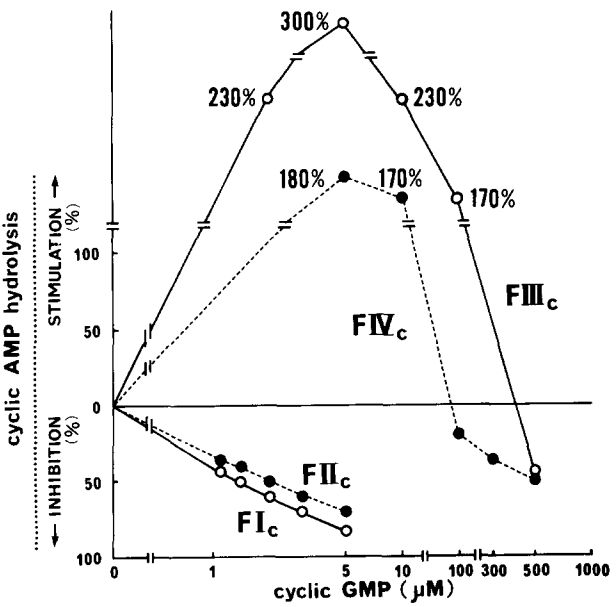


Fig. 4. Effect of cyclic GMP on cyclic AMP hydrolysis by DEAE-cellulose resolved human cerebral enzymes (FIc, FIIc, FIIIc and FIVc seen in Fig. 1B). Cyclic AMP hydrolytic activity in the absence of cyclic GMP was taken as a standard. Substrate concentration (cyclic AMP) was 2 μM and cyclic GMP concentration described in the figure indicated the inital concentration of cyclic GMP. Degradation of the added cyclic GMP in this condition was below 20%.

TABLE IV

EFFECT OF VARIOUS SUBSTANCES ON HUMAN CEREBRAL PHOSPHODIESTERASE

40 units (saturated amounts) of purified bovine cortex activator protein were added and the substrate concentration used in this experiment was 0.4 μM. Ca (20 μM), EGTA (200 μM) and cyclic GMP 5 μM) were added to reaction mixture.

Addition	DEAE-cellulose resolved enzymes			
	FIc	FIIc	FIIIc	FIVc
Cyclic AMP hydrolysis				
None	100	100	100	100
Activator + Ca	115	436	183	102
Activator + EGTA	96	78	64	100
Cyclic GMP hydrolysis				
None	100	100	100	— *
Activator + Ca	121	463	329	— *
Activator + EGTA	111	46	66	— *

* Cyclic GMP hydrolytic activity of FIVc was extremely low and not measured.

TABLE V

KINETIC PARAMETERS OF DEAE-CELLULOSE-RESOLVED CYCLIC NUCLEOTIDE PHOSPHODIESTERASES OF HUMAN CEREBRAL TISSUE

K_m values of FIIIc for both nucleotides hydrolysis were not be obtained.

Enzyme	K_m values (μM) for substrate:	
	Cyclic AMP	Cyclic GMP
FIc	0.65, 16.6	—
FIIc	0.27, 16.6	0.11, 1.25
FIVc	0.55	—

only were stimulated by a protein activator. Similar relations were observed among FI, FII and FIII in other tissues. Cyclic AMP hydrolytic activity of FIIIc was stimulated by micromolar cyclic GMP (Fig. 4). The kinetics of cyclic AMP hydrolysis by FIIIc were at first nonlinear but in the presence of 5 μM cyclic GMP became linear (Fig. 2). These properties of FIIIc were similar to those of FII in other tissues. FIVc hydrolyzed cyclic AMP faster than did cyclic GMP and application of a protein activator had no effect. The kinetic analyses of FIc, FIIc and FIVc are summarized in Table V. The kinetics of FIIIc were nonlinear for both nucleotides. FIc hydrolyzing cyclic AMP preferentially was not stimulated by a protein activator and the cyclic AMP hydrolytic activity was inhibited by cyclic GMP. These properties of FIc and FIVc are similar to those seen with FIII.

Discussion

FI, FII and FIII phosphodiesterases were found herein to be similar to the three forms noted in platelet phosphodiesterase already reported by the authors [10]. These activities in particulate fractions of platelet tissue and the rela-

tionship between soluble and insoluble particulate enzymes were however, not investigated in that study. Pichard and Cheung [17] recently reported interconvertible changes of sephadex column resolved phosphodiesterases of human platelets and rat brain and this phenomenon in rat platelet phosphodiesterase was also reported by our group [18]. The three activity peaks as determined herein by DEAE-cellulose chromatography of human tissue were not interconvertible. FI, FII and FIII were rechromatographed individually after isolation and such were eluted in exactly the same fraction as the original with recovery of 15—30% (cyclic AMP hydrolytic activity) and 20—40% (cyclic GMP activity). The protein activator from all tissues eluted mainly at the fractions under Nos. 45—50, however a small amount of the fraction was also evident in fractions under Nos. 33—44. FII contained a small amount of the activator and the activity was inhibited by 20—40% as measured after addition of 200 μM of EGTA. Sensitivity of cerebral FIIc to stimulation by the protein activator was not so stable and storage of the preparation at 0° C for about one week reduced the sensitivity to about half. Stimulation of FII cyclic AMP hydrolysis by a micromolar concentration of cyclic GMP was apparent in all tissues studied herein. Thus cyclic GMP is not only a potent stimulator but also an effective inhibitor of AMP hydrolysis. George et al. [19] and Gardner and Allen [20] found an increase in tissue levels of cyclic GMP with application of acetylcholine to be associated with a decrease in levels of cyclic AMP in tissue. These workers reported tissue levels of cyclic GMP to be 0.2—0.4 μM and we found in our present work that these same concentrations of cyclic GMP had a significant stimulatory effect on hydrolysis of cyclic AMP (Fig. 3). Such effects may be of physiological significance. The FII enzyme in most tissues showed a relatively low affinity for both nucleotides, however, the cyclic AMP hydrolytic activities at a 1 μM concentration were stimulated to a greater extent than when over 10 μM of micromolar amounts of cyclic AMP was applied (Fig. 2). As cyclic AMP hydrolytic activity appears to be regulated mainly by cyclic GMP in various human tissues and cyclic GMP hydrolytic activity is controlled by a protein activator, the three substances are probably intricately involved in regulation of related metabolism.

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References

1 Brooker, G., Thomas, L. and Appleman, M.M. (1968) Biochemistry 7, 4177—4181
2 Thompson, W.J. and Appleman, M.M. (1971) Biochemistry 10, 311—316
3 Thompson, W.J. and Appleman, M.M. (1971) J. Biol. Chem. 246, 3145—3150
4 Jard, S. and Bernard, M. (1970) Biochem. Biophys. Res. Commun. 41, 781—788
5 Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1970) J. Biol. Chem. 245, 5649—5655
6 Beavo, J.A., Hardman, J.G. and Sutherland, E.W. (1971) J. Biol. Chem. 246, 3841—3846
7 Huang, Y.C. and Kemp, R.G. (1971) Biochemistry 10, 2278—2283
8 Amer, M.S. and McKinney, G.R. (1972) J. Pharmacol. Exp. Ther. 183, 535—548
9 Russell, T.R., Thompson, W.J., Schneider, F.W. and Appleman, M.M. (1972) Proc. Natl. Acad. Sci. U.S. 69, 1791—1795
10 Hidaka, H. and Asano, T. (1976) Biochim. Biophys. Acta. 429, 485—497

11 Adachi, K., Levine, V., Halprin, K., Iizuka, H. and Yoshikawa, K. (1976) Biochim. Biophys. Acta. 429, 498—507
12 Bergetrand, H. and Lundguist, B. (1976) Biochemistry 15, 1727—1735
13 Russell, T.R., Terasaki, W.L. and Appleman, M.M. (1973) J. Biol. Chem. 248, 1334—1340
14 Hidaka, H. and Shibuya, M. (1974) Biochem. Med. 10, 301—311
15 Cheung, W.Y. (1970) J. Biol. Chem. 246, 2859—2869
16 Dixon, M. (1953) Biochem. J. 55, 170—176
17 Pichard, A.L. and Cheung, W.Y. (1976) J. Biol. Chem. 251, 5726—5737
18 Hidaka, H. and Asano, T. (1976) J. Biol. Chem. 251, 7508—7516
19 George, W.J., Ignarro, L.J., Paddock, R.J., White, L. and Kadowitz, P.J. (1975) J. Cyclic Nucleotide Res. 1, 339—347
20 Gardner, R.M. and Allen, D.O. (1976) J. Pharm. Exp. Ther. 198, 412—419