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Increased Activity of Low-K_m Cyclic Adenosine 3′:5′-Monophosphate Phosphodiesterase in Plasma Membranes of Morris Hepatoma 5123tc (h)[1]

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SUMMARY

The total cyclic adenosine 3′:5′-monophosphate (cAMP) phosphodiesterase activities as well as the activities of the low- and high-K_m enzyme forms were investigated in homogenates, 100,000 × *g* supernatants, and plasma membrane fractions of rat liver and Morris hepatoma 5123tc (h); the responsiveness of hepatoma and liver plasma membrane (low-K_m) phosphodiesterases to imidazole (40 mM) and theophylline (5 mM) were also compared at cAMP concentrations of 1 and 7.5 μM.

The total cAMP phosphodiesterase activities of tumor homogenates and 100,000 × *g* supernatant fractions were found to be less than one-half those of liver; kinetic studies of homogenates indicated that this finding was largely due to a substantial reduction (53%) in activity of the hepatoma high-K_m enzyme.

In contrast, low-K_m cAMP phosphodiesterase activities for tumor homogenate and plasma membrane fractions were significantly (50%) higher than liver; this was particularly evident when cAMP concentrations were between 0.5 and 2 μM. Since these concentrations are in the range of basal physiological levels of cAMP in hepatocytes, the present results suggest that the reduced levels of cAMP, previously observed in hepatoma 5123tc (h), are primarily due to an increased rate of cAMP metabolism by low-K_m cAMP phosphodiesterase in plasma membranes of the tumor.

Imidazole increased the activity of the low-K_m cAMP phosphodiesterase of liver plasma membranes by 22 (1 μM cAMP) and 38% (7.5 μM cAMP); tumor activity was enhanced 35 and 50%, respectively, at 1 and 7.5 μM cAMP. Theophylline inhibited the plasma membrane phosphodiesterase activity of liver 79 and 53% at cAMP concentrations of 1 and 7.5 μM, respectively; hepatoma activity was inhibited 82 (1 μM cAMP) and 62% (7.5 μM cAMP).

INTRODUCTION

Morris hepatoma 5123tc (h) was recently shown to have basal cAMP[2] levels that are considerably lower than those of liver (8). In subsequent studies (7) it was found that the basal adenylate cyclase activities in the plasma membranes of this tumor were similar to those of liver suggesting that the reduced cAMP levels in this hepatoma were not due to a decrease in the basal rate of cAMP formation. Since tissue levels of this nucleotide also depend on the rate of its metabolism by cAMP phosphodiesterase, it is of particular interest in this study to determine whether the rate of cAMP metabolism has been altered in hepatoma 5123tc (h).

It is well documented that cAMP phosphodiesterase exists as 2 main forms in various tissues, including liver and hepatoma (1, 4, 5, 14–17, 19). The "high-K_m" (low-affinity) form is believed to be present primarily in the soluble fraction of the cell (although some low-affinity sites appear to be associated with the particulate fraction as well); the "low-K_m" (high-affinity) form is mainly associated with particulate fractions such as plasma membrane. Some work has been carried out on hepatoma cAMP phosphodiesterases in various supernatant fractions, *i.e.*, 100,000 × *g* (4), 78,000 × *g* (14), and 18,000 × *g* (17); in each case significant alterations in the activity of this enzyme complex were found to occur in the neoplastic tissue. There are, however, no known reports on the activity of this enzyme in hepatoma plasma membranes; similarly, little is currently known about the relative responsiveness of hepatoma and liver plasma membrane cAMP phosphodiesterases to the stimulant, imidazole, or to the inhibitor, theophylline.

In this study, the total cAMP phosphodiesterase activities of hepatoma and liver homogenates and 100,000 × *g* supernatants were determined (2) in the presence of 1 mM cAMP; the activities of the low- and high-K_m phosphodiesterases were also measured (19) for homogenate and plasma membrane fractions. In addition, the responsiveness of liver and hepatoma plasma membranes to imidazole and theophylline was investigated.

MATERIALS AND METHODS

Animals and Tumor. Male Buffalo rats (200 to 400 g) were obtained from Simonsen Laboratories, Gilroy, Calif. Morris hepatoma 5123tc (h), an intermediate-growth-rate tumor, was grown s.c. and bilaterally in the inguinal region of the animal as described previously (8).

Reagents. Unlabeled cAMP, 5′-nucleotidase (*Crotalus adamanteus* venom), imidazole, and theophylline (as aminophylline) were purchased from Sigma Chemical Co., St. Louis, Mo. The [8-^{3}H]cAMP (30 Ci/mmole) was obtained from Amersham-Searle Corp., Don Mills, Ontario, Can-

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[2] The abbreviation used is: cAMP, cyclic adenosine 3′:5′-monophosphate.

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ada; both the unlabeled and labeled cAMP were further purified by chromatography on Bio-Rad AG 50W-X4 columns. This resin and the one used for the phosphodiesterase assays (AG1-X2, 200 to 400 mesh) were obtained from Bio-Rad Laboratories, Richmond, Calif.; the latter was routinely washed with 0.5 N sodium hydroxide, 0.5 N HCl, and deionized water to a final pH of 5.0.

Preparation of Tissue Fractions. For the preparation of homogenate and supernatant fractions, rats were decapitated and exsanguinated; 2 g of liver or hepatoma tissue were quickly excised at 0–4°, taking special care with tumor to exclude necrotic, hemorrhagic, and connective tissue. The tissue was homogenized in 18 ml of cold 0.25 M sucrose, 1 mM potassium phosphate (pH 7.2) and 0.1 mM EDTA, using a glass Potter-Elvehjem homogenizer. One-half of the homogenate was saved for assays while the other one-half was centrifuged at 100,000 × *g* for 60 min, and the supernatant was removed (excluding the lipid layer) and saved for the assay. Plasma membrane preparations were prepared as described previously (7), suspended in 1 mM $KHCO_3$ (40 μg protein per ml), and stored in liquid nitrogen until required.

Phosphodiesterase Assays. The total cAMP phosphodiesterase activities were determined for homogenates and 100,000 × *g* supernatants by measuring the release of P_i from cAMP in a reaction coupled with 5′-nucleotidase (2, 14). The assay mixture (total volume, 1 ml) contained the following: 50 mM Tris-HCl (pH 7.4); 5 mM $MgCl_2$; 1 mM cAMP; tissue fraction, *i.e.*, 1.2 to 1.7 mg protein for homogenates or 0.6 to 0.9 mg for supernatants. This mixture was incubated at 37° for 15 min (for supernatant) or 20 min (for homogenate), and the reaction was terminated by placing the tubes in a boiling water bath for 3 min. The samples were reequilibrated to 37°, 5′-nucleotidase (1 unit) was added, and the mixture was incubated for 15 min. This reaction was terminated by the addition of 0.1 ml of trichloroacetic acid (55%, w/v) and the released P_i was measured (3). Blanks were prepared by placing the tissue fractions in a boiling water bath for 3 min prior to adding cAMP and were subsequently treated the same as the samples.

To differentiate between low-K_m and high-K_m phosphodiesterase activity, the "2-stage" assay method of Appleman's group was used (16, 19). The reaction mixture in these experiments (0.5 ml total volume) had the same concentrations of Tris-HCl and $MgCl_2$ as in the above method; however, the cAMP concentrations varied from 0.5 to 1,500 μM. In addition, labeled cAMP (^3H in position 8; 200,000 cpm) was also added. Tissue protein concentrations varied from 40 to 90 μg for plasma membrane assays to 400 to 800 μg for homogenate assays. In some plasma membrane assays, with cAMP concentrations of 1 and 7.5 μM, theophylline (5 mM) or imidazole (40 mM) were added to the reaction mixture. The samples were incubated at 37° for 10 min (when determining low-K_m activity) or 20 min (when measuring the high-K_m activity), and the reaction was terminated by heating in a boiling water bath for 3 min. After equilibration to 37°, 5′-nucleotidase (1 unit) was added, and the samples were incubated for 10 min. A 1-ml slurry of Bio-Rad AG 1-X2 resin in water (1:2, w/v) was added to each sample, mixed thoroughly, and centrifuged at 1800 × *g* for 15 min; 0.5 ml of the supernatant was added to 12 ml of Aquasol (New England Nuclear, Montreal, Canada) and assayed in a Beckman LS100 liquid scintillation counter. Blanks were prepared as in the above method except that one set contained resin and the other did not. Total hydrolysis of the substrate was usually low (*i.e.*, less than 20%) since the sample protein concentration was adjusted to keep hydrolysis at a minimum. The specific activity of the enzyme was expressed as nmoles of cAMP hydrolyzed per min per mg protein.

Protein was determined using the method of Lowry *et al.* (13).

RESULTS

From Table 1 it can be seen that the total cAMP phosphodiesterase activities of hepatoma homogenate and 100,000 × *g* supernatant fractions are substantially lower (*i.e.*, 53% lower) than those of liver.

Hofstee plots (9) of the homogenate cAMP phosphodiesterase activities (Chart 1) indicate that liver and hepatoma contain low-K_m phosphodiesterase activity in the substrate concentration range of 0.5 to 30 μM; the activity of the hepatoma enzyme is, however, consistently higher than that of liver. Chart 1 also shows that liver and hepatoma have high-K_m phosphodiesterase activity at cAMP concentrations of 30 to 1000 μM; the hepatoma high-K_m phosphodiesterase appears, however, to have different kinetic properties as well as net activities that are substantially lower than liver; the latter finding probably accounts for the reduced total cAMP phosphodiesterase activity in hepatoma mentioned above. In these homogenate preparations, the low-K_m activity represents 14% of the total cAMP phosphodiesterase activity in liver and 27% in tumor.

Double-reciprocal plots (12) of high-K_m enzyme activities in homogenates (Chart 2) indicate that the apparent K_m for the liver enzyme is about 300 μM and its V_{max} is 4.0 nmoles/min/mg protein; in contrast, hepatoma appears to have 2 types of high-K_m activities, *i.e.*, between cAMP concentrations of 30 to 200 μM the enzyme has an apparent K_m of 50 μM and V_{max} of 1.3 nmoles/min/mg protein. In the 400 to 1000 μM range the enzyme has an apparent K_m of approximately 600 μM and a V_{max} of 3.0 nmoles/min/mg protein.

Kinetic analysis of the low-K_m cAMP phosphodiesterase activities in homogenates (Charts 1 and 3*A*) shows that this form of liver enzyme has an apparent K_m of 16.6 μM and V_{max} of 0.5 nmole/min/mg protein while the hepatoma enzyme has an apparent K_m of 12.5 μM and V_{max} of 0.7 nmole/min/mg protein.

With corresponding studies on liver and hepatoma plasma membranes (Chart 3*B*), it is evident that the activity of the hepatoma low-K_m enzyme is substantially higher than that of liver, particularly at cAMP concentrations of 0.5 to 2 μM. By drawing best-fitting straight lines through the points in this range, the resulting apparent K_m of liver is 3.0

Table 1

Comparison of total cAMP phosphodiesterase activities[a] for homogenate and 100,000 × g supernatant fractions from normal liver and Morris hepatoma 5123tc (h)

	cAMP hydrolyzed (nmoles/min/mg protein)
Liver	
Homogenate	4.96 ± 0.88[b] (4)[c]
Supernatant	11.63 ± 1.21 (4)
Hepatoma	
Homogenate	2.35 ± 0.33 (4)
Supernatant	5.50 ± 0.76 (4)

[a] Initial cAMP concentration, 1 mM.
[b] Mean ± S.E.
[c] Numbers in parentheses, number of experiments.

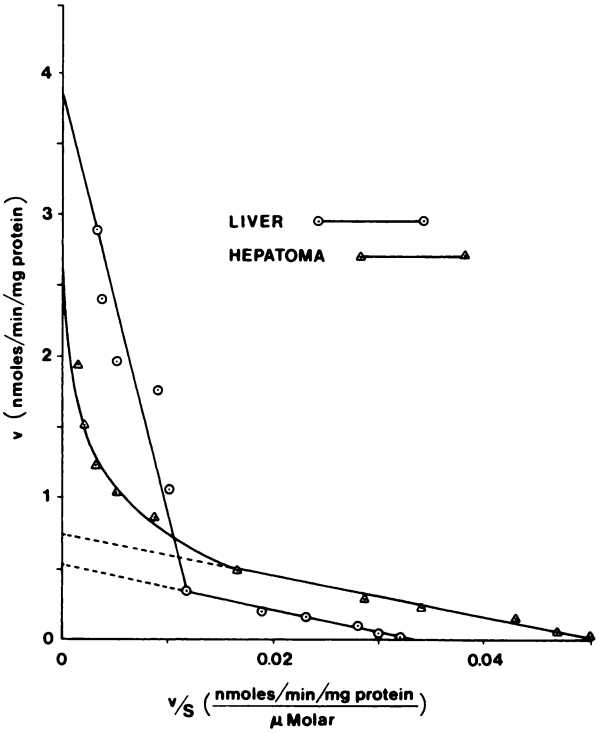

Chart 1. Hofstee plots (9) of the cAMP phosphodiesterase activities of liver and Morris hepatoma 5123tc (h) homogenate fractions. Points, means of 4 separate experiments. *v*, initial velocities expressed as nmoles of cAMP hydrolyzed per min per mg protein; *S*, cAMP concentrations ranging from 0.5 to 1000 μM.

μM while that of hepatoma is 1.9 μM; the V_{max} values are 0.5 nmole/min/mg protein for both preparations.

Table 2 summarizes the effects of imidazole (40 mM) and theophylline (5mM) on liver and hepatoma plasma membrane cAMP phosphodiesterase activities. When the cAMP concentration is 1 μM, imidazole increases the activities (relative to the corresponding control values) 22% in liver and 35% in hepatoma preparations; theophylline inhibits liver activity by 79% and tumor activity by 82%. At 7.5 μM cAMP, imidazole enhances the activities 38 and 50% for liver and hepatoma, respectively; theophylline decreases the liver activity by 53% and hepatoma by 62%.

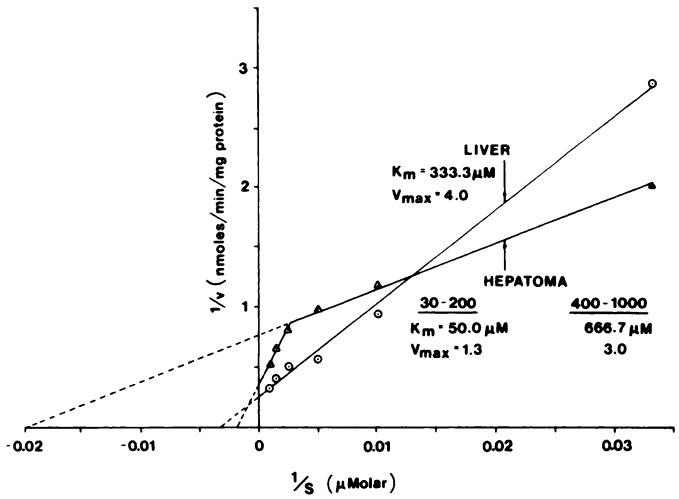

Chart 2. Lineweaver and Burk plots (12) of the high-K_m cAMP phosphodiesterase activities of liver and Morris hepatoma 5123tc (h) homogenate fractions. The apparent K_m and V_{max} values were estimated by extending best-fitting straight lines through appropriate points. *Points*, means of 4 separate experiments. Range of cAMP concentrations (*S*), 30 to 1000 μM.

In view of the current lack of agreement on the mechanism(s) involved in the action of insulin on liver (6, 10, 11) and the report that this hormone activates liver membrane cAMP phosphodiesterase (10), the effect of various concentrations of insulin (0.2 to 100 nM) on liver and hepatoma plasma membrane preparations was also studied. However, the results obtained (not included) were inconclusive as insulin occasionally stimulated this enzyme, but this response was inconsistent and did not follow a dose-response curve; hence, this aspect requires further investigation.

DISCUSSION

A few studies have been carried out on the cAMP phosphodiesterases of transplantable hepatomas; there are, however, no known reports on this enzyme complex in hepatoma plasma membranes.

Weber's group (4, 20) has recently studied the phosphodiesterase activities in 100,000 × *g* supernatant fractions of rapidly growing (3924A) and slowly growing (47C) hepatomas; these activities were compared to those of normal resting liver and of normal proliferating liver (*i.e.*, livers of 5-day rats and of regenerating livers of hepatectomized animals). These workers found that 2 cAMP phosphodiesterases were present in these tissues, one having a low apparent K_m of 2 to 3 μM and the other having a high apparent K_m of 100 to 600 μM; the V_{max} of the low-K_m enzyme in liver represented 4% of the total activity, while in tumor it constituted 25% of the total activity. Of particular interest are the findings that the low-K_m hepatoma enzyme activities increased markedly relative to normal liver (*i.e.*, by 185% in the slowly growing and by 265% in the rapidly growing hepatomas), whereas the activity of the high-K_m enzyme was decreased substantially (*i.e.*, by

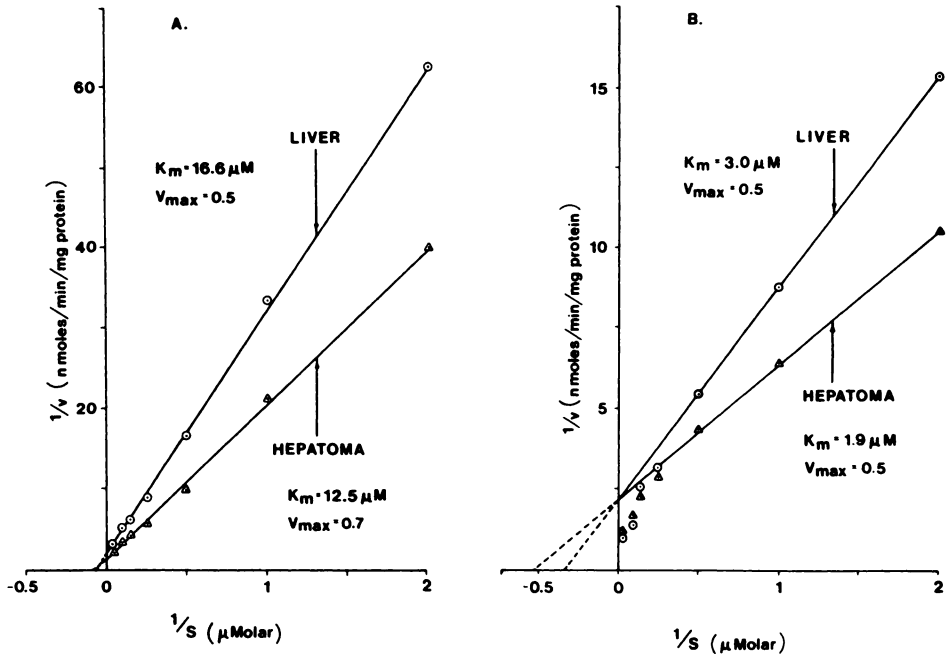

Chart 3. Lineweaver and Burk plots of the low-K_m cAMP phosphodiesterase activities of liver and Morris hepatoma 5123tc (h). *A*, mean homogenate data for 4 experiments; *B*, mean plasma membrane data for 2 separate experiments. cAMP concentration (*S*) range, 0.5 to 30 μM.

Table 2

The effects of imidazole and theophylline on the low-K_m cAMP phosphodiesterase activities of normal liver and Morris hepatoma 5123tc (h) plasma membranes

	cAMP concentration (μM)	Control		Imidazole (40 mM)		Theophylline (5 mM)	
		Enzyme activity (nmole/min/mg protein)	% of appropriate control	Enzyme activity (nmole/min/mg protein)	% of appropriate control	Enzyme activity (nmole/min/mg protein)	% of appropriate control
Liver plasma membranes	1	0.073 ± 0.004[a]	100	0.089 ± 0.005	122[b]	0.015 ± 0.004	21[b]
	7.5	0.213 ± 0.019	100	0.294 ± 0.001	138[b]	0.100 ± 0.015	47[b]
Hepatoma plasma membranes	1	0.130 ± 0.008	178[b]	0.175 ± 0.013	135[c]	0.023 ± 0.003	18[c]
	7.5	0.308 ± 0.001	145[b]	0.462 ± 0.013	150[c]	0.116 ± 0.002	38[c]

[a] Mean ± S.E. of 3 experiments.
[b] With respect to liver controls.
[c] With respect to hepatoma controls.

42% in tumor 47C and by 87% in hepatoma 3924A); however, these differences were not found with either regenerating liver or newborn liver. On the basis of these results Weber postulated that an "isozyme shift" occurs in hepatoma (*i.e.*, shift from the high-K_m to low-K_m enzyme form) which appears to be related to the neoplastic process rather than to simply a change in cell proliferation. Rhoads *et al.* (14) investigated the cAMP phosphodiesterase activities in the 78,000 × *g* supernatant fractions of various Morris hepatomas (not including 5123tc) and found the presence of 1 low-K_m enzyme (apparent K_m, 2.5 to 7.6 μM) and 1 high-K_m form (apparent K_m, 39 to 54 μM). The total phosphodiesterase activity (at 1 mM cAMP) decreased in all hepatomas by 40% or more. Schröder and Plagemann (17) studied 18,000 × *g* supernatants of cultured Novikoff rat hepatoma cells (subline N1S1-67). This tissue was found to contain 1 low-K_m cAMP phosphodiesterase with an apparent K_m of 120 μM; the specific activity of this enzyme (0.8 mM cAMP) was only about 5 to 10% that of rat liver extracts.

Our findings that hepatoma 5123tc (h) has increased low-K_m and reduced high-K_m cAMP phosphodiesterase activities are in general agreement with those reported above; however, they differ in some respects. For example, we found that low-K_m form in liver homogenate represented 14% of the total activity rather than 6% reported by Weber's group. This difference is probably due to the fact that our studies utilized whole homogenates rather than 100,000 × *g* supernatants; *i.e.*, it is possible that not all of the low-K_m enzyme (*e.g.*, that present in larger plasma membrane fragments) may have been recovered in the supernatants. In contrast, our hepatoma findings on this aspect agree more closely with those of Weber. This is probably explained by the fact that hepatoma plasma membranes tend to fragment more readily into less dense particles than do liver membranes; hence, recovery of the tumor low-K_m form in the supernatants would be greater than liver. Our finding of 2 high-K_m enzyme activities in hepatoma 5123tc (h) also differs from the above studies; it is not clear whether this difference is due to the use of

homogenate (rather than supernatant) fractions or whether an actual difference exists between the high-K_m enzyme forms in these hepatomas.

The present findings indicate that plasma membranes of hepatoma 5123tc (h) contain the low-K_m cAMP phosphodiesterase and that the activity of this enzyme is substantially higher (*i.e.*, 50% higher) than that of liver at physiological concentrations of cAMP (18); furthermore, the kinetic studies suggest that the substrate affinity of the tumor membrane enzyme is greater than that of liver. On this basis it is concluded that the reduced basal cAMP levels observed previously in this hepatoma (8) are mainly due to an increased rate of cAMP metabolism by the low-K_m phosphodiesterase in plasma membranes; the apparently enhanced affinity for cAMP may contribute to this increase in metabolism efficiency.

Rhoads *et al.* (14) found that the imidazole responsiveness of their hepatomas were generally less than that of liver. Our studies, in contrast, suggest that the hepatoma low-K_m cAMP phosphodiesterase is more responsive to imidazole than is liver. This difference may be related to the fact that our studies were carried out on plasma membrane preparations rather than 78,000 $\times$ *g* supernatants.

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