Thymidine Kinase of Rat Liver

ACTIVATION BY PHOSPHOLIPASE C AND SUBCELLULAR DISTRIBUTION IN THE LIVER

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1. The thymidine kinase activity of rat liver is greatly enhanced on addition of phospholipase C to the assay mixture. 2. Most of the thymidine kinase activity of the liver is recovered in the mitochondrial and in the impure 'nuclear' fractions. No activity was detected in purified nuclei prepared in high-density sucrose. 3. A substantial thymidine kinase activity could be detected, with the aid of phospholipase C, in all rat tissues examined.

The activity of thymidine kinase (ATP–thymidine 5'-phosphotransferase, EC 2.7.1.21) has been reported to be very scarce in preparations of adult rat liver and to be higher in preparations from foetal (Bresnick, Thompson, Morris & Liebelt, 1964; Klemperer & Haynes, 1968) and regenerating liver (see Bucher, 1963) and in hepatomas (see Morris, 1965). Shiosaka, Omura, Okuda & Fujii (1970) observed that the filtrate from cultures of *Clostridium perfringens* contains a protein factor 'activating' *in vitro* thymidine kinase of normal liver preparations, whose activity was enhanced to reach that of regenerating liver; the filtrate did not affect the enzymic activity of preparations from regenerating liver or from Yoshida sarcoma.

We report in this paper that a commercial preparation of phospholipase C activates thymidine kinase of the liver and other organs of the rat. The subcellular distribution of thymidine kinase activity in rat liver has also been studied.

EXPERIMENTAL

Chemicals. [2-¹⁴C]Thymidine (58 mCi/mmol) was obtained from The Radiochemical Centre, Amersham, Bucks., U.K.; thymidine was from Calbiochem, Los Angeles, Calif., U.S.A.; ATP from Boehringer and Soehne G.m.b.H., Mannheim, Germany; α -glycerophosphate, phospholipase C (type I, from *C. perfringens*, lot 0898-1920 or 90C-0900), phospholipase D (type I, from cabbage), *Crotalus adamanteus* venom (as a source of phospholipase A), trypsin (type XI), chymotrypsin (type II), protease (type VII, subtilisin), collagenase (type I), hyaluronidase (type IV), deoxyribonuclease I, ribonuclease-A (type IA), and neuraminidase (type V) were from Sigma Chemical Co., St Louis, Mo., U.S.A.; lysozyme and bovine serum albumin were from Armour Laboratories, Armour and Co. Ltd., London E.C.1, U.K.; sodium deoxycholate was from Sigma Chemical Co.;

sodium lauryl sulphate was from Mann Research Laboratories Inc., New York, N.Y., U.S.A.; other detergents were a kind gift from Dr G. Barbanti-Brodano. All other reagents were of analytical grade.

Animals. Male rats of the Wistar–Glaxo strain, 4–5 months old and weighing 250–300 g were used. Partial hepatectomies were performed by the method of Higgins & Anderson (1931).

Culture filtrate of *C. perfringens*. A 78 h culture of *C. perfringens* type A (strain A.T.C.C. 13124) in fluid thioglycollate medium [Difco: Pharmacopeia of the U.S., XIV Revision (1950) p. 758] was filtered through a EKSII Seitz filter, under 0.5 atm of N₂ pressure, and stored frozen at –80°C until used. Sterile culture medium, filtered and stored as indicated above, was used as a control.

Tissue extracts and subcellular fractions. Liver extracts were prepared initially as described by Shiosaka *et al.* (1970) (homogenate 2:25, w/v, in 1 mM tris–maleate buffer, pH 6.5, centrifuged at 8000g for 15 min). For fractionations and subsequently for all experiments, the liver and other tissues were homogenized in 0.25 M-sucrose (1 g of tissue with 9 ml of sucrose). Blood was collected with heparin, and the plasma and cells were separated by centrifugation. The following fractions were prepared from liver homogenate by centrifugation at the indicated speed and time: 'nuclear' (10 min at 700g); mitochondrial (15 min at 10000g); microsomal and supernatant (1 h at 105000g). All fractions were resuspended in 0.25 M-sucrose by gentle homogenization, and their volumes were adjusted to the original volume of the homogenate from which the fractions were prepared. Purified nuclei were prepared as described by Chauveau, Moulé & Rouiller (1956) from liver homogenate in 2.2 M-sucrose containing 10 mM-magnesium acetate, and were resuspended in 0.25 M-sucrose–1 mM-MgCl₂.

Determination of thymidine kinase activity. Initial experiments were performed as described by Shiosaka *et al.* (1970). For the reasons given in the Results section, the assay conditions were subsequently modified as follows: the amount of tissue extract was decreased, the pH of the reaction mixture was raised from 6.5 to 8.0,

tris-HCl buffer was used instead of tris-maleate buffer, and the length of incubation was decreased from 30 to 20 min. The reaction was stopped by immersing the tubes in boiling water for 3 min, and the tubes were centrifuged at 1000g for 10 min. Samples (50 μ l) of the clear supernatant were put on discs of DEAE-cellulose paper (Whatman DE-81) which were washed as described by Bresnick & Karjala (1964), dried and transferred to counting vials with 10 ml of scintillation [0.01% 1,4-bis-(5-phenyloxazol-2-yl)benzene and 0.4% 2,5-diphenyloxazole in toluene] fluid. The radioactivity was determined in a Nuclear-Chicago Mark I scintillation spectrometer with an efficiency of approx. 80%. Values obtained with zero-time blanks were subtracted. Protein was determined by the method of Lowry, Rosebrough, Farr & Randall (1951), with bovine serum albumin as a standard.

RESULTS

Effect of C. perfringens culture filtrate and of phospholipase C on thymidine kinase activity. The stimulation of thymidine kinase by the culture filtrate of *C. perfringens* was confirmed (Table 1). Since phospholipase C is one of the main toxic products of *C. perfringens* the effect of its addition was tested on thymidine kinase, which indeed was markedly stimulated (Table 1). In the presence of the culture filtrate or of phospholipase C the thymidine kinase activity of normal and regenerating liver preparations became similar, as reported by Shiosaka *et al.* (1970), although in our experiments the unstimulated enzyme activity of regenerating liver was not as high as that observed by these investigators, which could explain why it was further stimulated by the experimental treatment. A less-marked activation was obtained with phospholipase D, whereas the addition of *Crotalus adamanteus* venom (as a crude source of phospholipase A) was practically ineffective; various other

enzymes tested were completely ineffective (Table 2).

The effect of various amounts of phospholipase C on thymidine kinase is shown in Fig. 1. The stimulation was proportional to the amount of

Table 2. *Effect of various phospholipases and other enzymes on the activity of rat liver thymidine kinase*

Experimental conditions were as described in Fig. 2, with 0.025 ml of liver homogenate (1:40, w/v, in 0.25M-sucrose).

Expt. no.	Additions	Thymidine kinase activity (pmol/20 min per mg of liver)
1	None	59
	Phospholipase C (200 μ g)	5010
	Phospholipase D (500 μ g)	1849
	Phospholipase A (500 μ g)	406
	(from <i>Crotalus adamanteus</i> venom)	
2	None	99
	Phospholipase C (200 μ g)	1613
	Trypsin (100 μ g)	18
	Chymotrypsin (100 μ g)	27
	Protease (subtilisin)	0
3	None	136
	Phospholipase C (200 μ g)	4346
	Collagenase (100 μ g)	117
	Hyaluronidase (100 μ g)	126
	Deoxyribonuclease (100 μ g)	99
	Lysozyme (100 μ g)	136
4	None	54
	Phospholipase C (200 μ g)	5414
	Ribonuclease (100 μ g)	77
5	None	28
	Phospholipase C (200 μ g)	5342
	Neuraminidase (100 μ g)	18

Table 1. *Effect of culture filtrate of C. perfringens and of phospholipase C on thymidine kinase activity of normal and regenerating rat liver*

Regenerating liver was taken 30h after partial hepatectomy. The reaction mixture contained, in a final volume of 0.5 ml: 100 μ mol of tris-maleate buffer, pH 6.5; 2.5 μ mol of $MgCl_2$; 3 μ mol of α -glycerophosphate; 2.5 μ mol of ATP; 25 μ mol of thymidine, containing 0.05 μ Ci of [2- 14 C]thymidine; 0.1 ml of liver extract prepared as described by Shiosaka *et al.* (1970) and, when present, 0.1 ml of culture filtrate of *C. perfringens* or of phospholipase C solution. Incubation was at 37°C for 30 min. Values obtained from zero-time blanks were subtracted. The culture filtrate of *C. perfringens* and the preparation of phospholipase C did not show any thymidine kinase activity.

Liver ...	Thymidine kinase activity			
	Normal		Regenerating	
	(pmol/30 min per mg of liver)	(pmol/30 min per mg of protein)	(pmol/30 min per mg of liver)	(pmol/30 min per mg of protein)
Additions				
None	23	223	57	650
Sterile culture medium	16	156	56	630
<i>C. perfringens</i> culture filtrate	208	1977	237	2638
Phospholipase C (0.5 mg)	602	5738	406	4517

Fig. 1. Effect of various amounts of phospholipase C on rat liver thymidine kinase activity. Experimental conditions were as described in Table 1.

Fig. 2. Activity of liver thymidine kinase at various pH values. Experimental conditions were as described in Table 1, except for the buffer, the amount of liver used [0.025 ml of a 1:20 (w/v) homogenate in 0.25 M-sucrose] and the length of incubation (20 min). Phospholipase C (200 µg) was included in the reaction mixture. ●, Tris-maleate buffer; ○, tris-HCl buffer.

phospholipase added up to 75 µg/0.5 ml; it was maximum with 200 µg and decreased progressively with higher amounts.

Subcellular distribution of thymidine kinase. The observation that thymidine kinase was present, although in a 'masked' condition, in normal liver extract, and that it could be detected by the aid of phospholipase C, suggested that the distribution of the enzyme in subcellular fractions should be studied. The first experiments, in which the assays were performed as described by Shiosaka *et al.* (1970) showed that most of the enzyme activity was

present in the 'nuclear' (highly impure) and mitochondrial fractions. However, the sum of the activity recovered in all subcellular fractions was much higher (160%) than the activity present in the unfractionated homogenate. This led us to re-investigate the method of assay, which was modified as described in the Experimental section to ensure better linearity of the reaction with respect to the amount of enzyme added and to the time of incubation. Moreover, it was observed that the pH giving optimum activity was 8.0, which is consistent with the results of Bresnick & Thompson (1965) and of Klemperer & Haynes (1968) (Fig. 2).

The assays with the subcellular fractions, repeated under these modified conditions, showed (Table 3) that most of the thymidine kinase activity was present in the 'nuclear' and mitochondrial fraction. In all fractions, as well as in the whole homogenate, the activity could be detected only on addition of phospholipase C. Since the 'nuclear' fraction was highly impure, and contaminated at least by unbroken cells, thymidine kinase activity was determined with purified nuclei, prepared by centrifugation in high-density sucrose. This preparation did not show any enzyme activity, even after addition of phospholipase C (Table 3).

To ascertain whether the stimulation of thymidine kinase activity by phospholipase C was due to solubilization of the enzyme from subcellular particles, mitochondria were preincubated with phospholipase C and centrifuged, and thymidine kinase was assayed in the sedimented mitochondria as well as in the preincubation medium; the activity was recovered entirely in the mitochondria. Disruption of mitochondria by sonication did not unmask thymidine kinase activity. However, in preparations treated in this way a higher stimulation of thymidine kinase was observed on addition of phospholipase C (Table 4). A similar effect (i.e. no unmasking, but potentiation of stimulation by phospholipase C) was observed on freezing or on addition of non-ionic detergents to assay mixtures with whole liver homogenate. The addition of ionic detergents abolished or decreased the effect of phospholipase C (Table 4).

Thymidine kinase activity in organs other than liver. A considerable activity of thymidine kinase was detected, in the presence of phospholipase, in all tissues examined (Table 5). The activity assayed in the absence of phospholipase C was always very scarce or nil.

DISCUSSION

Our results confirm the observation of Shiosaka *et al.* (1970) on the activation of thymidine kinase by the culture filtrate of *C. perfringens* and demonstrate that the same effect can be obtained with

Table 3. *Distribution of thymidine kinase activity in rat liver subcellular fractions*

Experimental conditions were as described in Fig. 2, with tris-HCl buffer, pH 8.0; 0.025 ml of homogenate (1:40, w/v, in 0.25M-sucrose) or of 'nuclear' or mitochondrial fractions and 0.050 ml of 'microsomes' or supernatant (all 1:20, w/v, in sucrose) were used in Expt. 1; 0.025 ml of homogenate (as above) and 0.050 ml of purified nuclei were used in Expt. 2. Results in Expt. 1 are mean results from two experiments.

Phospholipase C ...		Thymidine kinase activity			
		Absent	Present		
			(pmol/20 min per mg of liver)	(%)	(pmol/20 min per mg of protein)
Expt. no.					
1	Whole homogenate	67	5447	100	23 030
	'Nuclei'	38	1967	36.1	51 557
	Mitochondria	64	2373	43.5	56 708
	Microsomes	0	1074	19.7	28 439
	Supernatant	0	372	6.8	4 014
	Recovery			106.1	
2	Whole homogenate		5268		27 154
	Purified nuclei		120		488

Table 4. *Effect of sonic disruption or freezing and of detergents on the activity of thymidine kinase*

Experimental conditions were as described in Fig. 2, with 0.1 ml of mitochondrial suspension (Expt. 1) or with 0.025 ml of liver homogenate (1:40, w/v, in 0.25M-sucrose) in other experiments. Sonic disruption was for 2 min at peak intensity with a MSE ultrasonic disintegrator. Detergents were added, to a final concentration of 0.25%.

Phospholipase C ...		Thymidine kinase activity (pmol/20 min per mg of liver)			
		Absent		Present	
Expt. no.	Treatment or addition				
1	None		9		1110
	Sonic disruption		0		1417
2	None		136		4346
	Storage at -20°C for 24 h		54		5414
3*, 4†	None	99	62	4170	5679
	Triton X-100	82	18	6360	7787
	Tween-80	54	82	6379	7638
	Nonidet P-40	54	0	6075	7466
	TN-101	54	72	5968	7101
	Sodium lauryl sulphate	82	0	82	0
	Sodium deoxycholate	35	0	1147	1186

* Left columns.

† Right columns.

commercial preparations of phospholipase C. Commercial preparations of phospholipase C are not pure, and therefore we are not sure that the thymidine kinase-activating factor is identical with phospholipase C. If this factor is different from the enzyme, it must be present also in the preparation of phospholipase D (from cabbage) which can also activate thymidine kinase, although with a lower efficiency than phospholipase C. Phospholipase C does not act by solubilizing the enzyme: the possibility should be considered that phospholipase

C acts by damaging the subcellular structures containing thymidine kinase, thus facilitating a better access of substrates to the enzyme. This possibility seems unlikely, since other drastic disruptive treatments such as sonication do not activate thymidine kinase. Further, after this work had been completed, Toide, Okuda, Shiosaka & Fujii (1970) reported that even a purified preparation of thymidine kinase is activated by the factor purified from the culture filtrate of *C. perfringens*, and this eliminates the modification of subcellular

Table 5. *Thymidine kinase activity of various rat tissues*

Experimental conditions were as described in Fig. 2, with 0.025 ml of tissue homogenate (1:40, w/v, in 0.25 M-sucrose), undiluted blood plasma, or blood cells (diluted 1:3 with water).

Phospho- lipase C ...	Thymidine kinase activity		
	Absent	Present	
	(pmol/20 min per mg of tissue)	(pmol/20 min per mg of tissue)	(pmol/20 min per mg of protein)
Spleen	98	11094	95641
Kidney	30	7795	40600
Heart	14	7069	57006
Skeletal muscle (psoas)	0	6971	49093
Lung	8	6776	80666
Testis	8	5197	64960
Liver	37	4238	16556
Small intestine	0	4059	78061
Brain	59	3744	29715
Blood plasma	0	22	383
Blood cells	0	737	3449

structures as the mechanism of the activation of thymidine kinase.

Information on the intracellular distribution of thymidine kinase in normal liver is scarce, although soluble supernatant has been used as source of the enzyme in most studies on thymidine kinase. In regenerating rat liver, the highest activity was recovered from the soluble supernatant fraction (Baugnet-Mahieu, Goutier & Semal, 1968). The present study of the intracellular distribution of thymidine kinase showed that the mitochondrial fraction is the richest in the enzyme. The activity recovered in the impure 'nuclear' fraction is at least in part due to contaminating mitochondria and unbroken cells, and to residual blood. No enzyme activity could be detected in purified nuclei: however, the possibility cannot be excluded that the enzyme leaks out from nuclei during the isolation in high-density sucrose, as has been demonstrated for other enzymes.

The activity of thymidine kinase has been considered to be negligible in resting tissues, and to increase in growing tissues, such as foetal and regenerating liver and in tumours. These results will have to be re-interpreted, since the results of Shiosaka *et al.* (1970) and ours demonstrate that the enzyme is present, although in a 'masked' or inactive condition, even in typically non-proliferating tissues such as muscle and brain. Thus the possibility should be considered that during growth the enzyme may be simply activated or released

from subcellular particles, as postulated by Brent, Butler & Crathorn (1965), and it remains to be seen whether this activation is due to a mechanism similar to that involving phospholipase C.

Moreover, we believe that the treatment with phospholipase C could represent a useful means of investigation of the source of the high thymidine kinase activity induced in various cell-culture systems by different cytolytic and oncogenic DNA viruses. In this regard, unequivocal experimental evidence is still lacking, particularly for oncogenic viruses, to ascertain whether the increased enzymic activity is dependent on (a) the synthesis of a virus-coded enzyme, or (b) the de-repression or unmasking of a host-cell-coded enzyme (Kit & Dubbs, 1969; Basilico, Matsuya & Green, 1969; Hatanaka, Twiddy & Gilden, 1969).

Note added in proof. After this paper had been accepted, we observed no direct relationship between the thymidine kinase-activating capacity of various batches of commercial preparations of phospholipase C and their phospholipase activity assayed on lecithin.

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