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Does Phospholipase C Stimulate Thymide Kinase Activity of Rat Liver Extracts Prepared after Partial Hepatectomy? (38644)

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Stirpe and La Placa (1) reported that addition of phospholipase C, E.C. 3.1.4.3, to extracts of rat liver greatly enhanced the thymidine kinase, E.C. 1.7.1.75, activity of these extracts. More recently, Salser and Balis (2) reported that the thymidine kinase activity of epithelial cells isolated from the intestinal mucosa of humans or rats also was markedly stimulated by the addition of phospholipase C to assay mixtures.

Thymidine can be enzymatically phosphorylated to TMP either by thymidine kinase or by nucleoside phosphotransferase, E.C. 1.7.1.77, (3). In the first reaction the phosphate donor is a nucleoside triphosphate, usually ATP; in the second reaction the phosphate donor is a nucleoside monophosphate, usually AMP. Thymidine kinase activity is very low in adult rat liver, but increases markedly following partial hepatectomy (4–8).

Shiosaka *et al.* (9) observed that addition of a culture filtrate of *Clostridium perfringens* to an extract of adult rat liver homogenate markedly stimulated apparent thymidine kinase activity of these extracts. Subsequent studies (10, 11) revealed that thymidine phosphorylation was not mediated by thymidine kinase, but rather was mediated by two enzyme reactions: (a) ATPase activity (either ATP phosphohydrolase, E.C. 3.6.1.5, or ATP pyrophosphohydrolase, E.C. 3.6.1.8) which was contributed by liver extracts, catalyzed the conversion of ATP to AMP; and (b), nucleoside phosphotransferase activity, which was contributed by *Cl. perfringens* filtrates, catalyzed the conversion of thymidine to TMP using AMP as the phosphate donor.

In view of the foregoing considerations, it seemed possible that phospholipase C, like filtrates of *Cl. perfringens* (11), might contain nucleoside phosphotransferase activity. Since Shiosaka *et al.* (9) observed that *Cl. perfringens* filtrates did not stimulate thymidine kinase activity of regenerating rat liver, we studied effects of phospholipase C on thymidine phosphorylation in liver extracts at various time-intervals following partial hepatectomy; this permitted evaluation of phospholipase C in liver extracts containing both low and high levels of thymidine kinase activity.

Materials and Methods. Female Holtzman rats were housed in a constant temperature (30°) room having an 0800–2000 light cycle. Rats were partially hepatectomized (12) either at 0900 (24 hr and 48 hr rats) or at 2100 (12 hr and 36 hr rats). They were fed Teklad Mouse and Rat Diet.

Livers were homogenized in 0.25 *M* surose and homogenates were centrifuged 15 min at 10,000 *g*. The supernatant was the source of the "liver extract." Phospholipase C isolated from *Cl. perfringens* was purchased from General Biochemicals, Chagrin Falls, Ohio (now purchased through Grand Island Biologicals Co., Grand Island, NY).

Reaction mixtures, similar in composition to those used by Stirpe and La Placa (1), contained: Tris–HCl buffer, 200 m*M*, pH 8.0; $MgCl_2$, 5 m*M*; thymidine, 50 μM containing 0.05 μCi (2-^{14}C) thymidine; and α-glycerophosphate, 6 m*M*. Total volume was 0.5 ml. When present in reaction mixtures, ATP, AMP and NaF were added at 5 m*M*, 5 m*M* and 20 m*M*, respectively, while liver extract and phospholipase C were added in amounts equivalent to 2 mg and 0.5 mg of protein/ml of reaction mixture, respectively. Incubation was for 10 min at 37°.

Reactions were stopped by dispersing 0.05 ml aliquots of reaction mixture on 2 cm × 2.2 cm rectangles of DEAE-cellulose and dropping them into 20 ml of 1 m*M* ammonium formate (13). Papers were washed (13) and counted in a phosphor solution (14) using either a Mark I Nuclear Chicago or a Beckman LS-245 liquid scintil-

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TABLE I. THE EFFECT OF ATP, AMP, NaF OR PHOSPHOLIPASE C UPON THYMIDINE PHOSPHORYLATION BY EXTRACTS FROM REGENERATING LIVER.[a]

Composition of reaction mixtures[a]	Amount of TMP formed μmoles/g of extract protein — Hours after partial hepatectomy: 0	12	24	36	48
I ATP	0.1 ± 0.05[b]	0.1 ± 0.05	1.9 ± 0.7	3.7 ± 0.5	2.5 ± 0.3
II ATP + NaF	0.1 ± 0.03	0.1 ± 0.02	2.7 ± 0.8	4.2 ± 0.6	2.7 ± 0.3
III ATP + PLC	4.5 ± 0.6	6.3 ± 1.3	5.8 ± 0.9	5.9 ± 0.9	5.3 ± 0.6
IV ATP + PLC + NaF	0.2 ± 0.01	0.1 ± 0.01	1.0 ± 0.3	2.1 ± 0.2	1.2 ± 0.09
V AMP	0.01 ± 0.01	0.2 ± 0.1	0.02 ± 0.01	0.03 ± 0.01	0.06 ± 0.04
VI AMP + PLC	5.6 ± 0.2	5.8 ± 0.3	5.4 ± 0.4	6.4 ± 0.3	7.0 ± 0.3
VII AMP + PLC + NaF	3.3 ± 0.3	2.8 ± 0.7	3.6 ± 0.8	4.1 ± 0.4	3.3 ± 0.3

[a] Abbreviations are: ATP and AMP, adenosine triphosphate and monophosphate, respectively; NaF, sodium fluoride; PLC, phospholipase C. The concentration of reactants was: ATP and AMP, 5 m*M* each; NaF, 20 m*M*; liver extract and phospholipase C, 2 mg and 0.5 mg of protein/ml of reaction mixture, respectively. For other details see Materials and Methods.

[b] Means ± standard errors for four to eight extracts from rats at each time-interval.

lation system; quenching was corrected by channels ratio (15). The protein content of liver extracts was estimated by the procedure of Lowry *et al.* (16) using crystalline bovine serum albumin as a standard. TMP formation was expressed relative to the protein content of liver extracts.

Results and Discussion. The influence of phospholipase C upon thymidine phosphorylation at various time-intervals after partial hepatectomy is shown in Table I. When ATP was added to liver extract (Reaction Mixture I), appreciable TMP formation occurred only in extracts made from 24 hr, 36 hr or 48 hr regenerating liver. Addition of NaF (Reaction Mixture II) exerted no deleterious effects. Both the timing of appreciable TMP formation relative to partial hepatectomy and the failure of Naf to inhibit TMP formation are consistent with previously published characteristics of thymidine kinase (4–8, 18, 19), and support a conclusion that thymidine phosphorylation in Reaction Mixtures I and II was catalyzed by thymidine kinase.

When phospholipase C and ATP were present (Reaction Mixture III), thymidine was phosphorylated irrespective of the time after partial hepatectomy. Upon the further addition of NaF (Reaction Mixture IV), appreciable TMP formation occurred only at 24 hr, 36 hr and 48 hr following operation. These data are consistent with a conclusion that TMP formation in Reaction Mixture III was mediated principally by ATPase and nucleoside phosphotransferase; with thymidine kinase probably contributing to phosphorylation at 24 hr, 36 hr and 48 hr. The addition of NaF (Reaction Mixture IV) caused inhibition of ATPase activity (2) and only thymidine kinase-mediated phosphorylation at 24 hr, 36 hr and 48 hr could proceed. As shown in Fig. 1, under conditions for optimal thymidine kinase activity, ATPase activity of liver extract was especially sensitive to NaF inhibition.

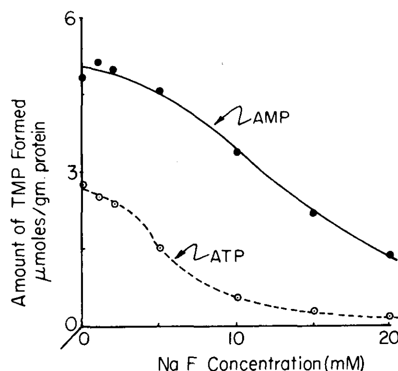

FIG. 1. The effect of NaF upon TMP formation from ATP or AMP catalyzed by enzymes in normal liver extract and phospholipase C preparations. Both nucleotides were present at 5 m*M*. Other conditions are described in Materials and Methods.

Liver extract contained no demonstrable nucleoside phosphotransferase activity (Reaction Mixture V) under conditions optimal for thymidine kinase activity; but, upon addition of phospholipase C (Reaction Mixture VI), thymidine was rapidly phosphorylated. Nucleoside phosphotransferase activity of phospholipase C also was sensitive to NaF. This is shown in Reaction Mixture VII and in Fig. 1. We also observed that TMP was formed from AMP by phospholipase C in the absence of liver extract; e.g., in the absence of liver extract, 16.8 $\pm$ 0.2 nmoles TMP/ml of reaction mixture or 33.4 $\pm$ 0.7 μmoles TMP/g of phospholipase C were formed.

The data in Table I support a conclusion that thymidine phosphorylation in liver extracts containing ATP and phospholipase C is catalyzed by thymidine kinase, which is contributed by liver extract, and by nucleoside phosphotransferase, which is a contaminant in the phospholipase C preparation. AMP, the substrate for nucleoside phosphotransferase, is formed from ATP by action of a NaF-sensitive ATPase which also is contributed by liver extract.

Stirpe and La Placa (1) reported that commercial preparations of phospholipase C activated rat liver thymidine kinase. Direct proof would require showing that transfer of the gamma phosphate of ATP occurred at faster rates in the presence of phospholipase C. In Table I, thymidine kinase activity was not inhibited by NaF (Reaction Mixture II), but ATPase activity was severely inhibited by NaF, thus appreciably slowing transfer of the alpha phosphate of AMP to thymidine by nucleoside phosphotransferase (Reaction Mixture IV). When thymidine kinase activity at 24 hr, 36 hr and 48 hr in Reaction Mixture II was compared to the same activity in Reaction Mixture IV, it was clear that the presence of phospholipase C in reaction mixtures exerted no activating influence upon thymidine kinase; in fact, at two time-intervals, i.e., 36 hr and 48 hr, thymidine kinase activity in phospholipase C-containing reaction mixtures was significantly lower. Therefore, these data do not support the contention voiced previously, i.e., that phospholipase C activated rat liver thymidine kinase (1). It seems likely to us that the "... remarkable stimulation of kinase activity ..." observed with extracts of mammalian intestines (2) involved TMP formation mediated by ATPase and nucleoside phosphotransferase activity.

Summary. Our purpose was to determine whether phospholipase C stimulated thymidine kinase activity of regenerating rat liver. We determined effects of phospholipase C upon TMP formation by rat liver extracts prepared at 0, 12, 24, 36 and 48 hr following partial hepatectomy. Data were obtained which supported these conclusions: (a) Commercial preparations of phospholipase C contained nucleoside phosphotransferase activity; (b) phospholipase C exerted no appreciable stimulatory influence upon thymidine kinase activity of regenerating rat liver; and (c), apparent stimulation of thymidine kinase was associated with linked activities of two enzymes, viz., liver extract-ATPase activity and nucleoside phosphotransferase activity.

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