ACTIVATION OF RAT LIVER CHOLINE KINASE BY POLYAMINES

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1. Introduction

Several lines of evidence have indicated that polyamines, such as spermidine and spermine, are related to increased cellular proliferation in animal tissues. Striking increases in the levels of the enzymes of polyamine biosynthesis, and enhanced cellular levels of these compounds have been observed during periods of rapid tissue growth [1,2]. It has been observed that polyamine accumulation is accompanied by an increase in the rate of RNA synthesis as well as of protein synthesis [2–5]. On the other hand, phospholipids have been demonstrated to play a crucial role in biological membranes [6]. Therefore, it is very likely that active phospholipid synthesis is required for rapid cellular proliferation, and that polyamines might stimulate phospholipid synthesis. In the present communication, we present evidence which indicates that polyamines activate choline kinase, the initial enzyme in lecithin biosynthesis (reaction 1) [7,8]. The results of the present study suggest that polyamines are involved in the control of membrane lipid biosynthesis.

$$\text{choline} + \text{ATP} \longrightarrow \text{phosphorylcholine} + \text{ADP} \quad (1)$$

2. Materials and methods

Choline kinase was assayed as follows. The reaction mixture contained 40 μmol of glycine–NaOH, 2 μmol of $MgSO_4$, 2 μmol of ATP, 0.25 μmol of [*methyl*-^{14}C] choline (the Radiochemical Center, Amersham, England), and the enzyme in a total volume of 1.0 ml. The final pH of the reaction mixture was 9.6. After a 20 min incubation period at 37°C, the reaction was terminated by placing the tube in a boiling water bath for 2 min. A 0.5 ml aliquot was applied to a 0.77 ml column of Dowex 50W-X8, 100–200 mesh, H^+ form. After the column was washed with 2.0 ml of water, phosphorylcholine was eluted with 5.0 ml of 0.5 N HCl. Radioactivity was determined with 1 ml aliquot in 7.5 ml of toluene–Triton X-100 scintillant [9] with a Packard liquid scintillation spectrometer (Downers Grove, USA).

Choline kinase was purified 55-fold from the 100 000 × *g* supernatant fraction of rat liver as follows. Male Wistar rats (approximately 200 g of body weight) were generously provided by Dr M. Igarashi, Department of Obstetrics and Gynecology, Gunma University School of Medicine. The liver was homogenized in 3 vol of 0.25 M sucrose. The homogenate was centrifuged at 100 000 × *g* for 60 min. The pH of the supernatant solution was adjusted to 5.0 by the addition of 1 N acetic acid, and the resulting precipitate was removed by centrifugation. To the clear supernatant solution was added solid ammonium sulfate to 30% saturation. The fraction sedimenting between 30 and 45% saturation was dissolved in 0.05 M Tris–HCl, pH 7.6, and dialyzed against 0.04 M acetate buffer, pH 5.6. The enzyme was adsorbed to calcium phosphate gel at a gel/protein ratio of 0.5 (protein concentration, 5 mg/ml), and eluted from the gel with 0.2 M potassium phosphate, pH 7.6. The eluate (18 ml) was passed through a sephadex G-200 column (4.5 × 55 cm) equilibrated with 0.05 M Tris–HCl, pH 7.6. The fractions with highest specific activity were combined and applied to a DEAE–cellulose column (2.6 × 37 cm) equilibrated with 0.05 M Tris–HCl, pH 7.6. The column was washed with 200 ml of 0.1 M NaCl in 0.05 M Tris–HCl, pH 7.6, and then

eluted with a 500 ml linear gradient from 0.1 M to 0.4 M NaCl containing 0.05 M Tris–HCl, pH 7.6. The fractions with highest specific activity were combined and used as enzyme. All procedures were performed at 0–4°C.

Cadaverine, putrescine, spermidine and spermine were purchased from Nakarai chemicals (Kyoto, Japan).

3. Results

The choline kinase reaction proceeded linearly with time for at least 40 min under the conditions employed in the present investigation. Maximal activity was obtained over a pH range from 9.2 to 9.6 in the presence of polyamines which were found to activate the enzyme (see below). The activities at pH 8.5 and 10.5 were 80% of that at the pH optimum. Therefore, in the present study, incubation for enzyme assay was carried out for 20 min at pH 9.6 unless otherwise stated.

Table 1 shows the effects of Mg^{2+} and spermine on choline kinase. When spermine was omitted from the reaction mixture, very low activity was observed with 2 mM Mg^{2+}. Although increase in the Mg^{2+} concentration to 10 mM resulted in some increase in activity, the value attained was much lower than that in the presence of both spermine and Mg^{2+}. Likewise, when Mg^{2+} was omitted, the enzyme activity was very low as reported by Wittenberg and Kornberg [7]. In a separate experiment spermine was shown to protect

Table 1
Requirement of choline kinase for spermine and Mg^{2+}

Additions	Activity
	nmol/min/mg
None	0
2 mM Mg^{2+}	8.3
10 mM Mg^{2+}	15.3
2 mM Spermine	3.1
10 mM Spermine	0
2 mM Mg^{2+} + 2 mM spermine	76.6

Reaction mixture contained 40 μmol of glycine–NaOH, 2 μmol of ATP, 0.25 μmol of [*methyl*-^{14}C]choline, 20 μg of partially purified choline kinase and the additions as indicated. The final pH was 9.6. Other conditions were as described in 'Materials and methods'.

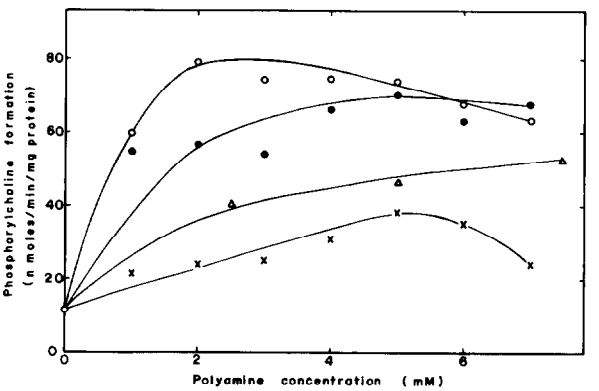

Fig.1. Effects of polyamines on the formation of phosphorylcholine. Choline kinase was assayed in the presence of increasing concentrations of spermine (○), spermidine (●), cadaverine (△), or putrescine (x) as described in 'Materials and methods'.

the enzyme against inactivation. However, the dependence of the enzyme on spermine cannot be ascribed to the stabilization of the enzyme; the reaction proceeded linearly with time both in the presence and absence of spermine, and virtually no enzyme inactivation occurred during assay even in the absence of spermine under the present conditions. Therefore, it is evident that choline kinase requires spermine as well as Mg^{2+} for its maximal activity. Figure 1 illustrates the effects of increasing concentrations of various polyamines and diamines on enzyme activity. Polyamines were more effective than diamines. Spermine was the most efficient activator. Under the experimental conditions employed, the addition of spermine, spermidine and putrescine increased enzyme activity by 6.8-, 6.1- and 3.3-fold, respectively. When cadaverine was used, a higher concentration of the diamine was required for maximal activation; a 5.2-fold increase in activity was attained at a concentration of 15 mM.

The product formed by the polyamine-activated reaction was examined. Choline kinase was incubated with labeled choline and Mg^{2+}-ATP in the presence and absence of spermine. The radioactive material derived from labeled choline was examined by paper chromatography with a solvent system consisting of 2 N NH_4OH/acetone/methanol (3:2:5, by vol). As shown in fig.2, the reaction product was identified as phosphorylcholine. As in other experiments

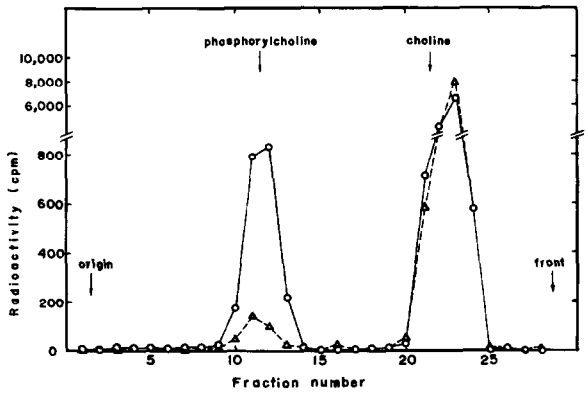

Fig.2. Paper chromatography of the product by the polyamine-activated choline kinase reaction. The reaction mixture was incubated as described in 'Materials and methods' in the presence (○) and absence of 2 mM spermine (△). Twenty micrograms of enzyme was used. A 0.5 ml aliquot was applied to Whatman No. 3 MM paper and developed in 2 N NH_4OH/acetone/methanol (3:2:5, by vol). The entire chromatogram was cut into 1 cm strips and counted for radioactivity in 5 ml of toluene scintillator liquid containing 0.4% 2,5-diphenyloxazole and 0.01% 1,4-bis-2-(5-phenyloxazolyl)-benzene. Choline chromatographed in an adjacent lane was located by spraying the paper with 1% $K_4Fe(CN)_6$ solution and then with 0.5% cobalt sulfate solution. Phosphorylcholine was visualized by the method of Bandurski and Axelrod [10].

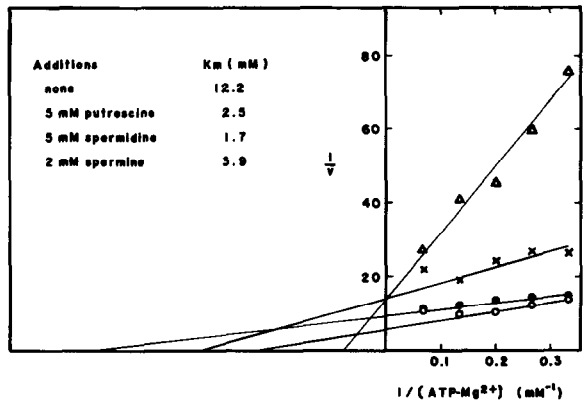

Fig.3. Double reciprocal plots of initial velocity of phosphorylcholine formation. Choline kinase was assayed as described in 'Materials and methods' with the following modifications. The concentrations of ATP-Mg^{2+}, polyamines and diamines were as indicated, and the final pH was 9.2 instead of 9.6. Initial velocity (v) is expressed as pmol phosphorylcholine formed per min per mg protein. Symbols: (- -△- -) in the absence of polyamine; (- -x- -) 5 mM putrescine; (- -●- -) 5 mM spermidine; (- -○- -) 2 mM spermine.

described above, it can be seen that the addition of spermine markedly enhanced the formation of phosphorylcholine.

Kinetic experiments were carried out to investigate the effects of amines on the K_m for substrate and the V_{max} exhibited by the enzyme. The Lineweaver-Burk representation gave a linear relationship for ATP except at high concentrations. The apparent K_m for ATP in the absence of polyamines was 12.2 mM. This value decreased to 3.9, 1.7 and 2.5 mM in the presence of 2 mM spermine, 5 mM spermidine and 5 mM putrescine, respectively. The V_{max} was affected by the amines to a lesser extent. On the other hand, these amines did not change the K_m values of the enzyme for choline significantly.

4. Discussion

The pathway leading to phospholipid synthesis has been well formulated [8]. However, little is known about the enzymatic mechanism by which the rate of phospholipid synthesis is controlled. The present study shows that choline kinase, the first enzyme in lecithin biosynthesis, is stimulated markedly by polyamines and, to a lesser extent, by diamines. Although polyamines are known to replace Mg^{2+} in several Mg^{2+}-requiring reactions, polyamines did not substitute for Mg^{2+} in the choline kinase reaction. The present data indicates that both polyamines and Mg^{2+} are required for the enzyme. Kinetic experiments revealed that in the presence of polyamines the K_m value of the enzyme for ATP is notably decreased. These data are compatible with the view that polyamines are involved in the control of phospholipid biosynthesis. In connection with the present results, noteworthy is the recent finding by Lockwood and East [11] on insulin-like actions of polyamines on lipid metabolism. They demonstrated that polyamines facilitate glucose transport and inhibit lipolysis in isolated rat adipose cells.

Acknowledgement

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References

[1] Morris, D. R. and Fillingame, R. H. (1974) Ann. Rev. Biochem. 43, 303–325.
[2] Tabor, H. and Tabor, C. W. (1972) Adv. Enzymol. 36, 203–268.
[3] Dykstra, W. G. Jr. and Herbst, E. J. (1965) Science 149, 428–429.
[4] Caldarera, C. M., Barbiroli, B. and Moruzzi, G. (1965) Biochem. J. 97, 84–88.
[5] Kay, J. E. and Lindsay, V. J. (1973) Exptl. Cell Res. 77, 428–436.
[6] Singer, S. J. and Nicolson, G. L. (1972) Science 175, 720–731.
[7] Wittenberg, J. and Kornberg, A. (1953) J. Biol. Chem. 202, 431–444.
[8] Kennedy, E. P. (1961) Fed. Proc. 20, 934–940.
[9] Patterson, M. S. and Greene, R. C. (1965) Anal. Chem. 37, 854–857.
[10] Bandurski, R. S. and Axelrod, B. (1951) J. Biol. Chem. 193, 405–423.
[11] Lockwood, D. H. and East, L. E. (1974) J. Biol. Chem. 249, 7717–7722.