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PHOSPHOLIPID-INDUCED ACTIVATION OF TYROSINE HYDROXYLASE FROM RAT BRAIN STRIATAL SYNAPTOSOMES*

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Abstract—Lysolecithin and phosphatidylserine stimulate rat striatal tyrosine hydroxylase, partially purified from the crude synaptosomal fraction. The stimulatory effect is associated with a 3- to 4-fold decrease in K_m for 6-methyl-tetrahydropterin or tetrahydrobiopterin without an alteration in the K_m for L-tyrosine or in the V_{max}. In addition, the K_i for dopamine inhibition is increased approximately 3-fold. Centrifugation of the enzyme on linear sucrose gradients gave a sedimentation coefficient ($S_{20,w}$) of 8.6, either in the absence or presence of lysolecithin, indicating that no significant changes in the molecular weight are caused by the phospholipid. The enzyme obtained after high speed centrifugation of whole striatal tissue was activated by a combination of lysolecithin plus a cyclic AMP–ATP mixture to a greater extent than that obtained by either activating condition alone. The data presented suggest a potential regulatory function of phospholipids in the control of striatal neurotransmitter synthesis.

Tyrosine hydroxylase [L-tyrosine, tetrahydropteridine, oxygen oxidoreductase (3-hydroxylating), EC 1.14.16.2] catalyzes the first and rate-limiting step in the biosynthesis of catecholamines [1]. The early finding that dihydroxyphenylalanine (dopa),§ norepinephrine and dopamine are competitive inhibitors of the enzyme with respect to the pteridine cofactor [1–3] led to the postulation of intraneuronal feedback regulation by catecholamines [4].

Kuczenski and Mandell [5] showed that tyrosine hydroxylase exists in rat striatum as a soluble and a particulate enzyme form, exhibiting a significantly different affinity for $DMPH_4$ and sensitivity to dopamine inhibition. They also found that the kinetic properties of the soluble enzyme from hypothalamus [6] and striatum [5] became similar to those of the particulate enzyme after addition of the polyanion heparin.

Treatment of soluble tyrosine hydroxylase from brain and adrenal medulla with trypsin or chymotrypsin reduces the molecular weight and increases the catalytic activity associated with a decreased K_m for substrate and cofactor [7–9]. Recently it was shown that tyrosine hydroxylase is stimulated by the addition of EGTA [10] or cyclic AMP [11, 12] to rat brain homogenates. In addition, activation of bovine caudate tyrosine hydroxylase by phosphatidylserine has also recently been reported [13]. These studies indicate that modifications in enzyme environment and/or structure can alter the kinetic properties of tyrosine hydroxylase and suggest possible mechanisms of alterations *in vivo* in enzyme activity.

This report describes the effects of phospholipids on tyrosine hydroxylase from rat brain striatal synaptosomes and also describes the interaction of phospholipids with the cyclic AMP–ATP-dependent activation.

MATERIALS AND METHODS

Trizma base, cyclic AMP, ATP, lysolecithin (from egg yolk), lysophosphatidylethanolamine, brain lipid extract (Folch-Fraction VI), catalase (bovine liver), alcohol dehydrogenase (yeast), EGTA, hemoglobin (horse) and bovine serum albumin were obtained from Sigma. Phosphatidylserine and sucrose (gradient grade) were from Schwartz-Mann; L[^{14}C-U]tyrosine (sp. act. 500 mCi/m-mole) from Amersham-Searle; NAD, NADH and NADPH from Boehringer–Mannheim Co.; $DMPH_4$ from Aldrich; 6-MPH_4 and DTT from CalBiochem; dopamine and biopterin from Regis Chemical Co.; 2-mercaptoethanol from Eastman Organic Chemicals; and dinitroindophenol from Fisher Scientific Co.

Preparation of enzymes. Male Sprague–Dawley rats (200–300 g) were decapitated, striata dissected from the brain and homogenized in cold 0.32 M sucrose, and the crude synaptosomal fraction (P_2) was prepared as described [14]. Most of the tyrosine hydroxylase activity in this fraction has been shown to be associated with synaptosomes after sucrose density gradient centrifugation [15]. The P_2 fraction was lysed by homogenization in 5 mM Tris–HCl, pH 7.4 (10 vol/g of original weight). The lysate was centrifuged for 30 min at 80,000 *g* at 4°. Saturated $(NH_4)_2SO_4$ solution, pH adjusted to 7.0, was added to the supernatant to give a 30% $(NH_4)_2SO_4$ saturation. The solution was stirred for 20 min and centrifuged for 30 min at 50,000 *g*, and the supernatant

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§ Abbreviations used in the text: dopa, dihydroxyphenylalanine; $DMPH_4$, 6,7-dimethyl-5,6,7,8-tetrahydropterin; cyclic AMP, adenosine-3′,5′-cyclic monosphoric acid; 6-MPH_4, 6-methyl-5,6,7,8-tetrahydropterin; DTT, dithiothreitol; and BH_4, tetrahydropterin.

brought to 60% saturation by further addition of saturated $(NH_4)_2SO_4$ solution. The solution was stirred for 20 min, centrifuged for 30 min at 50,000 *g* and the pellet dissolved in a small volume of 5 mM Tris-HCl, pH 7.4, and dialysed overnight against a large excess of the same buffer.

Membrane-bound tyrosine hydroxylase from striatal P_2 fraction was prepared according to Kuczenski and Mandell [5].

Sheep liver dihydropteridine reductase was purified by the method of Craine *et al.* [16] through the second ammonium sulfate step.

Enzyme assays. Tyrosine hydroxylase was assayed using a modification of the method by Coyle [15]. The assay mixture contained sodium acetate (pH 6.0) 0.1 M, NADPH 0.5 mM, catalase 2000 units, sheep liver pteridine reductase, 0.07 to 0.08 mg protein, L[^{14}C-U]tyrosine and pteridines at the concentrations indicated in Results, usually in a vol of 0.2 ml. In some experiments, the pteridine-regenerating system was replaced by 40 mM mercaptoethanol.

Alcohol dehydrogenase was assayed as described by Vallee and Hoch [17], catalase by the method of Beers and Sizer [18], and hemoglobin was read at 540 nm in a Cary spectrophotometer. Protein was determined by the method of Lowry *et al.* [19] using bovine serum albumin as a standard.

Density gradient centrifugation. Centrifugation of tyrosine hydroxylase on linear 5–20% sucrose gradients was performed according to Martin and Ames [20]. Gradients were prepared in 0.01 M Tris–HCl, pH 7.4, or 0.05 M potassium-phosphate buffer, pH 6.5, containing 0.1 M KCl and 2 mM mercaptoethanol or 0.2 mM DTT.

Centrifugation was carried out in a Spinco ultracentrifuge (model L2-65B) at 4° for 14–18 or 9 hr, using either a Spinco SW 40 or SW 56 rotor. With gradient volumes of 13 or 4.2 ml, fractions of 0.52 or 0.1 ml, respectively, were collected by puncturing the bottom of the tubes. Sedimentation coefficients ($S_{20,w}$) of the marker-proteins used for molecular weight determinations were: hemoglobin (horse) 4.09, alcohol dehydrogenase 7.6 and catalase 11.15 [21].

Other methods. Biopterin was reduced to tetrahydrobiopterin by the method of Lloyd and Weiner [22]. The degree of reduction (83–100 per cent) to the tetrahydro compound was estimated by dinitroindophenol titration [23]. Ascorbate was used to standardize the oxidizing dye [24]. Kinetic constants were determined using a computer program by Cleland [25]. Significance of the results was determined using Student's *t*-test [26]. A P value of less than 0.05 was considered significant.

RESULTS

Addition of from 5–40 μg lysolecithin caused a significant increase in tyrosine hydroxylase activity using subsaturating concentrations of either 6-MPH_4 or $DMPH_4$ as cofactor (Fig. 1). Assuming equal substitution of the phosphatidyl moiety of lysolecithin with stearyl and palmityl acids, a maximal stimulatory concentration of about 0.4 mM can be calculated.

Various lipids exhibit differences in the degree to which they stimulate tyrosine hydroxylase, indicating some degree of structural specificity for lipid-induced

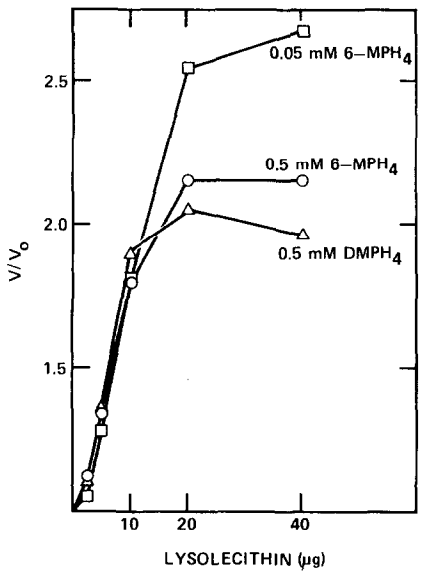

Fig. 1. Stimulation of rat striatal tyrosine hydroxylase by lysolecithin. Partially purified tyrosine hydroxylase from rat striatal P_2 fraction was incubated at various concentrations of lysolecithin and subsaturating pteridine cofactor concentrations. The complete assay mixture contained in a volume of 0.2 ml: sodium acetate, pH 6.0, 0.1 M; L[^{14}C-U]tyrosine (0.25 μCi), 0.02 mM; catalase, 2000 units, 6-MPH_4 or $DMPH_4$, 0.5 or 0.05 mM; and tyrosine hydroxylase preparation (0.005 mg protein). The pteridines were dissolved in mercaptoethanol to give a final concentration of 40 mM mercaptoethanol. Lysolecithin was added at the start of the reaction. Control units (nmoles dopa/mg of protein/hr) were 11.2 (0.05 mM 6-MPH_4), 45 (0.5 mM 6-MPH_4) and 7.0 (0.5 mM $DMPH_4$).

V = velocity in the presence of phospholipid
V_0 velocity in the absence of phospholipid

Each point represents the average of duplicate determinations.

enzyme activation. An increase of approximately 3-fold in enzyme activity was obtained with 200 μg of crude lipid extract (bovine brain, Folch-Fraction VI), 40 μg lysolecithin or 10 μg phosphatidylserine, whereas lysophosphatidylethanolamine was only slightly stimulatory (Fig. 2). Cardiolipin was found to be inhibitory (data not shown). Half maximal stimulation was obtained with 4 μg phosphatidyl-L-serine, 10 μg lysolecithin and 28 μg Folch-Fraction VI.

In agreement with earlier work using $DMPH_4$ as cofactor [5], we find that the particular enzyme has a significantly lower K_m for 6-MPH_4 than does the soluble form (Table 1). In addition, treatment of the soluble enzyme with lysolecithin significantly lowers the K_m for 6-MPH_4 to a value similar to that found for the particulate enzyme form (Table 1). The ability of phospholipids to alter the kinetic properties of tyrosine hydroxylase could be further demonstrated by the ability of phosphatidyl-L-serine to increase the apparent K_i [27] of the soluble enzyme for dopamine approximately 3-fold (Table 2).

The K_m of the soluble enzyme for what may be the natural tyrosine hydroxylase cofactor, tetrahydrobiopterin [22], is also significantly lowered by lyso-

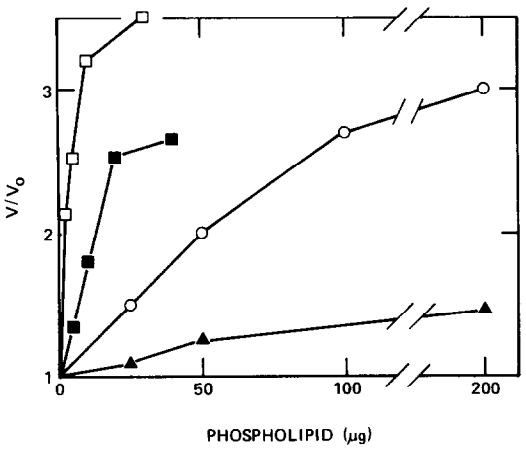

Fig. 2. Effect of various phospholipids on rat striatal tyrosine hydroxylase partially purified from the P_2 fraction. Tyrosine hydroxylase was assayed in a mixture containing sodium acetate, pH 6.0, 0.1 M; pteridine reductase (0.05 mg protein); catalase, 2000 units; NADPH 0.5 mM; L[^{14}C-U]tyrosine (0.025 μCi) 0.02 mM; 6-MPH_4, 0.05 mM; and partially purified enzyme preparation (0.009 mg protein). The final volume was 0.2 ml and lipids were added at the start of the reaction. The control activity was 5.0 nmoles dopa/mg of protein/hr. V/V_0 = relative velocity (see Fig. 1 legend. Key: □—□, phosphatidylserine; ■—■, lysolecithin; ○—○, Folch-Fraction VI (crude lipid brain extract); and ▲—▲, phosphatidylethanolamine. Each point represents the average of duplicate determinations.

lecithin (Fig. 3A). However, in contrast to the effect of lysolecithin on cofactor K_m, the K_m for tyrosine was not significantly affected (Fig. 3B).

Since phospholipid micelles have detergent properties, we tested the possibility that phospholipids may cause a dissociation of tyrosine hydroxylase into subunits. Upon sucrose gradient centrifugation (Fig. 4), the enzyme showed identical sedimentation pattern in control and lysolecithin-containing gradients, giving a single peak of activity. A sedimentation coefficient ($S_{20,w}$) of 8.6 was calculated according to Martin and Ames [20]. Using the relation $S_1/S_2 = (MW_1/MW_2)^{\frac{2}{3}}$, a crude estimation of the molecular weight of the enzyme can be calculated [28]. A value of 176,000 ± 5,000 was found (mean value of three centrifugations). Preincubation of the enzyme

Table 1. Effect of lysolecithin on the K_m for 6-MPH_4 of rat striatal tyrosine hydroxylase*

Enzyme	K_m for 6-MPH_4
Soluble	0.34 ± 0.03
Particulate	0.19 ± 0.02†
Soluble + lysolecithin (40 μg/0.2 ml)	0.14 ± 0.01†

* Partially purified soluble and particulate tyrosine hydroxylase from rat striatal P_2 fraction was prepared as described in Methods. The enzyme was incubated in a mixture containing sodium acetate, pH 6.0, 0.1 M; catalase, 2000 units; NADPH, 0.5 mM; L[^{14}C-U]tyrosine, 12 μM (0.25 μCi); and pteridine reductase, 0.07 mg protein. 6-MPH_4 was dissolved in DTT to give a final DTT concentration of 2.5 mM. The range of cofactor concentrations used was 0.025 to 2.0 mM.

† $P < 0.001$ compared to control soluble enzyme.

Table 2. Effect of phosphatidyl-L-serine on the K_i for dopamine*

Addition to the incubation	K_i (mM)
None	0.065
Phosphatidyl-L-serine (40 μg)	0.20

* Partially purified tyrosine hydroxylase from rat striatal P_2 fraction (0.02 mg protein) was incubated at a constant concentration of tyrosine (2.5 μM), three concentrations of 6-MPH_4 (10, 100 and 1000 μM), and six concentrations of dopamine (from 0 to 0.5 mM) for the estimation of the apparent K_i [27]. Dihydropteridine reductase and NADPH were used as reducing systems as described in Methods.

in the presence of lysolecithin at maximal stimulatory concentration at 37° for 15 min and subsequent centrifugation on low-salt sucrose gradients (5–20% sucrose in 10 mM Tris–HCl, pH 7.4), using a Spinco SW 56 (8 hr) or SW 40 rotor (14–18 hr), yielded the same sedimentation coefficient.

The interaction between phospholipid activation and the activation produced by a mixture of cyclic

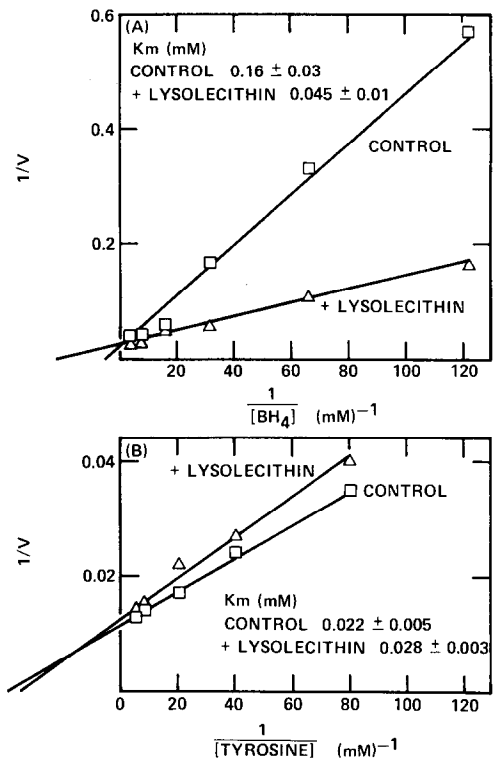

Fig. 3. (A) Effect of lysolecithin on the K_m for tetrahydrobiopterin of rat striatal tyrosine hydroxylase. Tyrosine hydroxylase from rat striatal P_2 fraction was assayed in the presence of sodium acetate, pH 6.0, 0.1 M; catalase, 2000 units; dihydropteridine reductase, 0.08 mg protein; NADPH, 1.0 mM; and L[^{14}C-U]tyrosine (0.25 μCi), 5 μM. The lysolecithin concentration was 40 μg/0.2 ml of assay mix. The K_m in the presence of the phospholipid differs significantly from the control ($P < 0.001$). Velocity is expressed as nmoles dopa/mg of protein/hr. (B) Effect of lysolecithin on the K_m for L-tyrosine in the presence of tetrahydrobiopterin (0.25 mM). Assay conditions are as in section A.

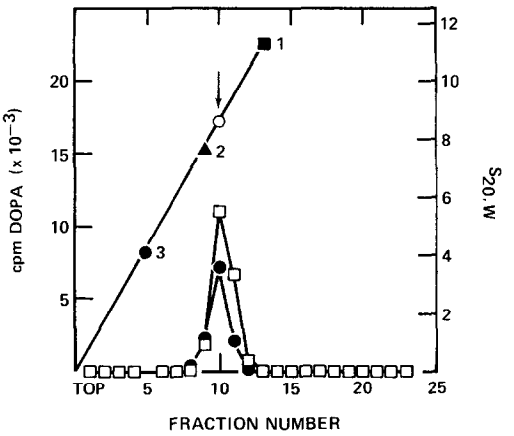

Fig. 4. Sedimentation behavior of striatal tyrosine hydroxylase in sucrose density gradient centrifugation. Partially purified tyrosine hydroxylase from rat striatal P_2 fraction (0.18 mg) was centrifuged together with marker proteins for 14 hr at 40,000 *g*, using a SW 40 rotor. Gradients contained Tris–HCl, pH 7.4, 10 mM; KCl, 0.1 M; and mercaptoethanol, 5 mM. One gradient contained 0.2 mg lysolecithin/ml. After centrifugation, tubes were punctured and fractions of thirty drops (0.54 ml) collected. Tyrosine hydroxylase was assayed as described in Fig. 2. Positions of marker proteins: (1) catalase; (2) yeast alcohol dehydrogenase; and (3) hemoglobin (horse). Arrow indicates the position of tyrosine hydroxylase. The position of markers and the position of tyrosine hydroxylase were the same in the lysolecithin-containing gradient as in control gradients. Key: □·—□, tyrosine hydroxylase in gradient without lysolecithin; ●—●, with lysolecithin.

AMP, EGTA (which was added to chelate any calcium that could possibly inhibit protein kinase), magnesium and ATP (cyclic AMP mix) was studied by two different experimental designs. In the first, lysolecithin was present along with the cyclic AMP mix (with both activators present at maximal stimulating concentrations) during the enzyme preincubation and incubation. Under these conditions the combination of lysolecithin plus cyclic AMP mix produced a stimulation greater than that seen by either agent alone (Table 3). Since the additional stimulation caused by lysolecithin may have been due to an interaction with the cyclic AMP mix activating step(s), a second experiment was performed in which the enzyme was first treated with the cyclic AMP mix, then applied to a Sephadex G-25 column to separate the enzyme from the cyclic AMP mix, and then treated with lysolecithin. Again the lysolecithin treatment produced an activation greater than that seen with the cyclic AMP mix alone (Table 4). It should be noted that in agreement with the findings reported by Lovenberg *et al.* [29], we were unable to demonstrate stimulation by the addition of cyclic AMP alone, and while some stimulation could be observed with ATP plus Mg^{2+}, the combination of the four components in the cyclic AMP mix gave the greatest stimulation.

DISCUSSION

The characterization of the properties of tyrosine hydroxylase from rat brain striatal synaptosomes is of special interest, since this particular preparation is frequently utilized to study the properties of dopamine synthesis regulation in a structurally intact system [30–34]. The present studies show that tyrosine hydroxylase from rat brain striatal synaptosomes can be activated by various phospholipids. The activation is produced by a significant decrease in K_m for reduced pteridine cofactor, without a significant alteration in K_m for tyrosine or in the V_{max}. While these studies were initially prompted by the reports of phospholipid-induced activation of phenylalanine hydroxylase [35], the mechanism of activation of these two enzymes appears to differ, since phospholipids do not decrease the K_m of phenylalanine hydroxylase for cofactor [35]. While these studies were in progress, it was reported that phosphatidylserine could activate bovine caudate tyrosine hydroxylase, also by decreasing the K_m for cofactor [13]. However, there appear to be significant differences between the properties of the bovine caudate and the rat striatal synaptosomal preparations. For instance, it was reported that lysolecithin did not significantly activate bovine caudate tyrosine hydroxylase, while the present studies show that the rat striatal enzyme is markedly stimulated by lysolecithin (Figs. 1 and 2). Also,

Table 3. Effect of lysolecithin on the cyclic AMP-dependent activation of striatal tyrosine hydroxylase*

Addition to the incubation	Tyrosine hydroxylase activity (nmoles dopa/mg protein/hr)
None	0.15 ± 0.004
Lysolecithin	0.44 ± 0.05†
Cyclic AMP mixture	0.35 ± 0.012†
Lysolecithin + cyclic AMP mixture	1.05 ± 0.04‡

* Striatal tissue was homogenized in 5 vol. of 0.05 M potassium-phosphate buffer, pH 6.0, and centrifuged at 80,000 *g* for 45 min. The supernatant (50 μl) was preincubated for 10 min at 37° after addition of 50 μl of the homogenization buffer containing either 40 μg lysolecithin or a mixture of cyclic AMP, 0.1 mM (concentrations in the preincubation mixture); ATP, 0.5 mM; $MgCl_2$, 5 mM; and EGTA, 0.1 mM (referred to as cyclic AMP mixture) or both. The reaction was started by addition of an incubation mixture to give the following final concentrations: potassium-phosphate buffer, pH 6.0, 0.05 mM; L[^{14}C-U]tyrosine (0.25 μCi), 12 μM; NADPH, 0.5 mM; catalase, 2000 units; 6-MPH_4, 0.05 mM; and dihydropteridine reductase, 0.09 mg protein. The dopa synthesis incubation was for 45 min.

† $P < 0.001$ compared to controls.

‡ $P < 0.001$ compared to all other incubation conditions.

Table 4. Effects of lysolecithin on tyrosine hydroxylase after Sephadex G-25 chromatography*

Addition to the incubation		
Before Sephadex G-25	After Sephadex G-25	Tyrosine hydroxylase activity (nmoles dopa/mg protein/hr)
None	None	0.54 ± 0.02
None	Lysolecithin	1.35 ± 0.05†
Cyclic AMP mixture	None	1.10 ± 0.12†
Cyclic AMP mixture	Lysolecithin	2.34 ± 0.06‡

* Striatal tyrosine hydroxylase was prepared and preincubated as described in Table 3. After 10 min the preincubation mixture was cooled in an ice bath and applied to a Sephadex G-25 column (1.5 × 15 cm), equilibrated with 5 mM potassium phosphate buffer, pH 7.0, and eluted with the same buffer. Fifty μl of the void volume containing the enzyme activity was assayed as described in Table 3. Tetrahydrobiopterin (25 μM) was used as cofactor.

† $P < 0.001$ compared to incubation without any additions.

‡ $P < 0.001$ compared to all other incubation conditions.

substrate inhibition by tyrosine is observed for the bovine caudate enzyme when tetrahydrobiopterin is employed as cofactor, but not for the rat striatal enzyme, for up to 0.2 mM tyrosine (Fig. 3B). In addition, no shift in pH optimum of the rat striatal enzyme was observed with either phosphatidylserine or lysolecithin (data not shown), in contrast to the bovine caudate preparation. Whether these differences are due to species differences or to differences in enzyme preparation and/or degrees of enzyme purity remains to be determined.

The effects of phospholipids on rat striatal tyrosine hydroxylase are similar to the effects of the polyanion heparin [5, 6], in that both treatments lower the K_m for cofactor. In addition, both treatments significantly increase the K_i for catecholamine inhibition (Table 2) [5]. It is possible, as suggested by Lloyd and Kaufman [13], that these effects are mediated by the highly negatively charged surfaces provided by heparin and phospholipids. No evidence for phospholipid-induced subunit association or dissociation could be obtained, since phospholipid-treated enzyme behaved identically to untreated enzyme upon sucrose density centrifugation (Fig. 4).

The ability of various phospholipids, including the brain lipid extract Folch-Fraction VI, to alter the kinetic properties of tyrosine hydroxylase suggests that phospholipid–enzyme interactions may play a role in the regulation of catecholamine synthesis *in vivo*. The present study indicates that synaptosomal tyrosine hydroxylase can respond to the addition of phospholipids with a decrease in K_m for pteridine cofactor, a property which could be of physiological significance since various studies have indicated that the amount of cofactor normally present in tissue is not saturating [36–39]. A phospholipid-induced increase in the K_i for dopamine inhibition (Table 2) has not previously been reported, and represents an additional potential regulatory function of phospholipids. Alterations in phospholipid–enzyme interactions may occur during neuronal depolarization, since the depolarization-induced influx of calcium could alter the membrane binding of tyrosine hydroxylase [5]. It is of interest to note in this regard that the addition of phospholipid to the soluble enzyme does in fact lower its K_m for cofactor to a value similar to that seen for the particulate enzyme (Table 1).

The fact that lysolecithin could further activate the cyclic AMP-treated enzyme even after removal of the cyclic AMP-activating mix by Sephadex G-25 chromatography (Table 4) indicates that in this system lysolecithin is not causing additional stimulation by interaction with the cyclic AMP-activating step(s). If depolarization-induced increases in cyclic AMP [40] can activate tyrosine hydroxylase *in vivo* [11], the data presented here suggest that even further stimulation could be achieved by alterations in phospholipid–enzyme interactions.

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