EVIDENCE FOR THE LACK OF DIRECT PHOSPHORYLATION OF BOVINE CAUDATE TYROSINE HYDROXYLASE FOLLOWING ACTIVATION BY EXPOSURE TO ENZYMATIC PHOSPHORYLATING CONDITIONS

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SUMMARY: Highly purified bovine caudate tyrosine hydroxylase can be activated by exposure to enzymatic phosphorylating conditions. This activation is due to both a decrease in the K_m for the pterin cofactor and to some increase in V_{max}. The K_m for the enzyme's substrate, tyrosine, is unchanged by activation. After tyrosine hydroxylase was activated in the presence of [γ-^{32}P]-ATP, no incorporation of ^{32}P into the enzyme was observed by either immunoprecipitation studies or by sucrose gradient studies.

INTRODUCTION: Several recent reports have described a cAMP[1]-dependent activation of tyrosine hydroxylase in brain extracts and synaptosomal preparations (1-3). Since this enzyme plays a rate-limiting role in the biosynthesis of the neurotransmitter, norepinephrine, any modulation of its activity may be of physiological significance in the regulation of the adrenergic nervous system. We have recently described the stimulation of bovine caudate tyrosine hydroxylase by phosphatidyl-L-serine, a phospholipid present in neuronal tissue (4). The stimulation by phosphatidyl-L-serine was found to be due to a decrease in the enzyme's K_m for the pterin cofactor. We now report that activation of the same enzyme by enzymatic phosphorylating conditions is also due to a reduction in the K_m for the pterin cofactor and that these two types of stimulation are non-independent.

MATERIALS AND METHODS: The ^{3}H-3,5-L-tyrosine (1.0 Ci/mmole) and [γ-^{32}P]-ATP, (5.0 Ci/mmole)were purchased from New England Nuclear. Catalase was obtained from Boehringer-Mannheim, cAMP-dependent muscle kinase was from Sigma and dihydropteridine reductase was prepared in this laboratory as previously described (5). Bovine caudate tyrosine hydroxylase was

[1]The abbreviations used are as follows: NADPH, nicotinamide adenine dinucleotide phosphate, reduced form; 6-MPH4, 6-methyltetrahydropterin; DMPH4, 6,7-dimethyltetrahydropterin; pterin, 2-amino-4-hydroxypteridine; cAMP, adenosine 3':5' cyclic monophosphate; DOPA, L-3,4-dihydroxy phenylalanine; TRIS, tris(hydroxymethyl)amino methane.

purified by an extension of the procedure previously described (4). (The details of this purification will be published elsewhere) The tyrosine hydroxylase used in these studies was estimated by disc-gel electrophoresis to be greater than 75% pure and had a specific activity of greater than 19 nmoles DOPA/mg/min at 37° C. The components of the enzymatic phosphorylating system were as follows: Tris-HCl, pH 7.0, 40 mM; $MgCl_2$, 4 mM; aminophylline, 4.0 mM; protein kinase, 0.4 mg/ml; ATP, 3.0 mM; cAMP, 2.5 μM; and bovine caudate tyrosine hydroxylase, up to 1.50 mg/ml; and water to a final volume of either 0.125 ml or 0.25 ml. The activating system contained all of the above components and the control system lacked only the kinase. The preincubation of tyrosine hydroxylase with the control or activating system was at 30° C for 15 min. After the preincubation, the samples were placed in ice, the tyrosine hydroxylase assay system added and the samples were incubated again for 10 min at 37° C. The tyrosine hydroxylase assay system was a modification of that described by Nagatsu et al. (6) where formation of ^3H-OH from ^3H-3,5-L-tyrosine was used as a measure of tyrosine hydroxylase activity. The following components were used for this assay system: potassium phosphate, pH 6.8, 100 mM, ^3H-3,5-L-tyrosine, 50 μM (containing 800,000 cpm/assay); sheep liver dihydropteridine reductase in excess, NADPH, 0.35 mM; catalase, 2000 units per assay, Na_2 EDTA, 10 mM; 6-MPH_4, 50 μM, 100-200 μl preincubation mixture (containing 20-30 μg tyrosince hydroxylase preparation), and water to a final volume of 0.5 ml.

Antiserum to bovine adrenal tyrosine hydroxylase was prepared as previously described (7). Protein was measured by the methods of Lowry et al. with bovine serum albumin as a standard (8) and radioactivity was determined by liquid scintillation spectrometry.

RESULTS: We have observed that after activation by exposure to phosphorylating conditions, bovine caudate tyrosine hydroxylase displays the same kinetic changes which we previously reported after stimulation of this enzyme by phosphatidyl-L-serine. The nature of these changes are shown in Table 1 and include the following: (1) neither activating condition causes a change in the K_m for the substrate, tyrosine; (2) both activating conditions cause a reduction in the K_m for the tetrahydrobiopterin; (3) both activating conditions lead to a shift in the pH optimum from 6.0 to above 6.8 or 7.4. Furthermore, the decrease in the K_m of the cofactor with both activating conditions is not dependent upon the structure of the cofactor, i.e., it is also seen with $DMPH_4$ and 6-MPH_4.

The two methods for activating tyrosine hydroxylase lead to similar changes in some of the kinetic properties of the enzyme. The extent of similarity of these activations was examined by determining whether or not the enzyme could be further activated by phosphatidyl-L-serine after having been activated by exposure to enzymatic phosphorylating conditions. The data shown in Table II demonstrate that under the conditions used, a three-fold stimu-

Table I. A Comparison of Stimulation of Tyrosine Hydroxylase by Phosphatidyl-L-Serine and by Exposure to Enzymatic Phosphorylating Conditions.

	Control	Phosphatidyl-L-serine (1.0 mM)	Phosphorylating Conditions Control	Activated
Tyrosine K_m	57 μM	57 μM	60 μM	60 μM
Cofactor K_m (tetrahydrobiopterin)	220 μM	67 μM	250 μM	60 μM
pH optimum	6.0	6.8	6.0	7.4

The above determinations were made using 10-20 μg highly purified bovine caudate tyrosine hydroxylase per sample resulting in production of 0.042 to 0.89 nmoles ^{3}H-OH/10 min. The tyrosine K_m studies were performed with constant specific activity ^{3}H-3,5-L-tyrosine; phosphatidyl-L-serine was sonicated in water immediately before use and the pH optimum studies were carried out in both Tris-chloride and potassium phosphate buffers with a pH range of 5.5 to 9.5. The control and activating phosphorylating conditions used were as described in the Methods with the preincubation being at 30° C for 15 min. The tyrosine hydroxylase assay system is described in the Methods and contained 50 μM 6-MPH$_4$ for the tyrosine K_m and pH optimum studies and all tyrosine hydroxylase incubations were at 37° C for 10 min.

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lation by phosphatidyl-L-serine could be acheived either in the absence of any phosphorylating conditions (A), or after exposure to control phosphorylating conditions (B). A three-fold activation was also observed after exposure to complete phosphorylating conditions, but the addition of phosphatidyl-L-serine to the enzymatically-activated enzyme produced no significant further activation (C). Thus, it would appear that the two activations are not independent effects.

Since specific antibodies to tyrosine hydroxylase were available in this laboratory, we attempted to determine whether or not tyrosine hydroxylase was directly phosphorylated under the conditions used to achieve activation by enzymatic phosphorylating conditions. In several experiments [γ-^{32}P]-ATP was used in the control (minus kinase) and activating conditions, the tyrosine hydroxylase was removed from the mixture by addition of the appropriate titer of antibody, and the resulting immunoprecipitate was washed repetitively. Finally, the immunoprecipitate

Table II. Stimulation of Purified Bovine Caudate Tyrosine Hydroxylase by Phospholipids and/or Enzymatic Phosphorylating Conditions.

Sample	Phosphatidyl-L-Serine	nmoles ^{3}H-OH / 10 min
(A) tyrosine hydroxylase only	-	0.27
	1.0 mM	0.78
(B) tyrosine hydroxylase and control phosphorylating conditions (- kinase)	-	0.29
	1.0 mM	0.77
(C) tyrosine hydroxylase and complete phosphorylating conditions	-	0.90
	1.0 mM	1.09

For each of the above samples 20 µg highly purified bovine caudate tyrosine hydroxylase was preincubated for 15 min at 15° C in (A) 0.02 M TRIS/HCl pH 7.0, 8% sucrose, (B) control phosphorylating conditions (as described in the Methods) or (C) complete phosphorylating conditions. The samples were then placed on ice, phosphatidyl-L-serine added as indicated, the tyrosine hydroxylase assay mixture (as described in Methods) was added to each and the samples incubated for 10 min at 37° C. The final concentration of 6-MPH_4 was 50 µM.

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was examined for incorporation of ^{32}P into the protein.

Typical results from one such experiment are presented in Table III where it can be seen that the amount of ^{32}P remaining in the activated samples treated with either control or immune γ-globulin was not significantly different. Furthermore, tyrosine hydroxylase which had been subjected to control phosphorylating conditions and then treated with either control or immune γ-globulin showed a similar, but insignificant amount of ^{32}P in the immunoprecipitate pellets. Since the specific activity of the [γ-^{32}P]-ATP in the phosphorylating system was about 10 µCi/nmole and since approximately 0.15 nmole bovine tyrosine hydroxylase (molecular weight approximately 200,000) was used, one would have expected about 3.0×10^6 cpm ^{32}P to have been incorporated into the immune pellet if tyrosine hydroxylase had been phosphorylated with a stoichiometry of 1.0 mole phosphate/mole tyrosine hydroxylase.

Table III. Immunoprecipitation of Tyrosine Hydroxylase by Specific Anti-Tyrosine Hydroxylase γ-Globulin After Exposure of Tyrosine Hydroxylase to Phosphorylating Conditions using [γ-^{32}P]-ATP.

Sample	CPM/Sample			
	Control Phosphorylating Conditions		Activating Phosphorylating Conditions	
	γ-Globulin		γ-Globulin	
	Control	Immune	Control	Immune
Incubation Supernatant	36 X 10^6	35.4 X 10^6	36 X 10^6	34.4 X 10^6
1st precipitate washing	583000	792000	723600	643200
2nd precipitate washing	36800	43610	47940	52090
3rd precipitate washing	2940	4680	4900	6280
4th precipitate washing	1015	1820	970	1210
5th precipitate washing	1063	2460	880	1160
6th precipitate washing	830	990	1030	1300
Immunoprecipitate pellet dissolved in 1% sodium dodecyl sulfate	2770	2210	4110	3410

Each of the above samples contained 40 µg of highly purified bovine caudate tyrosine hydroxylase and was incubated with either control (- kinase) or complete phosphorylating conditions (as described in the Methods) for 15 min at 30° C. The specific activity of the [γ-^{32}P]-ATP in the preincubation mixture was approximately 10 µCi/nmole. Parallel samples were incubated in control and activating conditions and assayed for tyrosine hydroxylase activity as described in the Methods; the activation was 6.5-fold relative to the activity of the control. Either 100 µl control or 100 µl immune anti-tyrosine hydroxylase γ-globulin was added to each of the [γ-^{32}P]-ATP incubated samples, a second incubation at 30° for 30 min performed and the samples placed in ice for 12 hours. Each sample was then centrifuged and the resulting immunoprecipitate lattice washed with 1.0 ml aliquots of a previously described buffer (9) containing 20 mM potassium phosphate (pH 7.4), 140 mM KCl, 3 mM $MgCl_2$, 0.2% triton X-100, and 0.2% bovine serum albumin. The immunoprecipitate was collected each time by centrifugation and after 7 washings, the pellets were dissolved in 1.0 ml 1% sodium dodecyl sulfate.

The possibility that tyrosine hydroxylase could be directly phosphorylated by the conditions of enzymatic phosphorylation which lead to activation of tyrosine hydroxylase was also examined by sucrose gradient experiments. The results of a typical experiment are presented in Figure 1, which shows that after exposure to enzymatic phosphorylating conditions activated and control tyrosine hydroxylase have approximately the same

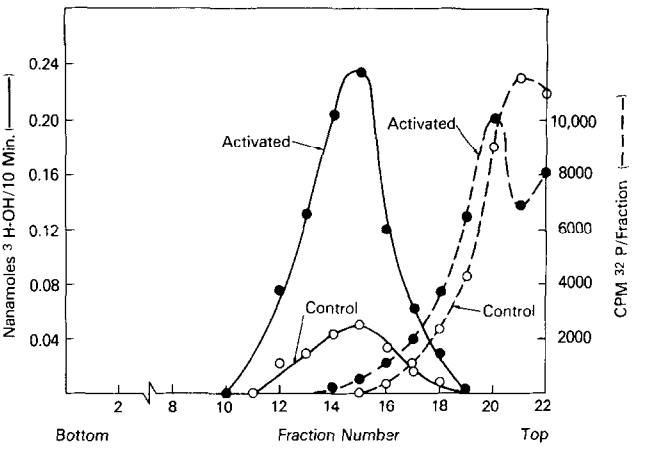

Fig. 1. Tyrosine Hydroxylase Activity and ^{32}P-Bound Protein Following Exposure to Phosphorylating Conditions and Sucrose Gradient Centrifugation.

Four 60 µg samples of highly purified bovine caudate tyrosine hydroxylase were subjected to either control (- kinase) or complete phosphorylating conditions as described in the Methods. After a preincubation of 15 min at 30° C, each 200 µl sample was layered onto a 5.0 ml 8 to 28% linear sucrose gradient which contained 0.02 M Tris/HCl pH 8.3 and 1 mM dithiothreitol. All samples were centrifuged in a Beckman SW 50.1 rotor in a Beckman L2-65B centrifuge for 4 hours at an average centrifugal force of 234,000 X g and each fractionated into twenty 250 µl fractions by elution from the bottom of the tubes. Tyrosine hydroxylase activity was measured in 125 µl aliquots of a control and activated sample as described in Methods. The ^{32}P incorporation into protein was measured on the entire 250 µl fractions from samples that had been exposed to control and complete phosphorylating conditions in the presence of 10 µCi/nmole [γ-^{32}P]-ATP. To each 250 µl fraction was added 5.0 mg bovine serum albumin in 100 µl H_2O, followed by 500 µl 10% trichloroacetic acid, the samples were well mixed, then filtered under suction onto Gelman glass fiber filters, (Type A, 25 mm diameter), washed four times with 1.0 ml 10% trichloroacetic acid, twice with 2.0 ml H_2O, and the filters counted by liquid scintillation spectrometry.

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sedimentation pattern (four hour centrifugation). Duplicate samples of control and activated tyrosine hydroxylase were examined for incorporation of ^{32}P into protein by addition of carrier protein to each of the 20 sucrose gradient fractions, precipitating protein with trichloroacetic acid, filtering and washing the precipitate on glass fiber filters and finally counting the filters by liquid scintillation spectrometry. It is apparent that ^{32}P is not incorporated into either control or activated tyrosine

hydroxylase. Some acid precipitable ^{32}P was found near the top of both the control and activated gradients and may represent incorporation of ^{32}P into a less dense protein. The data in Table III and Fig. 1 strongly suggest that bovine tyrosine hydroxylase is not directly phosphorylated after exposure to enzymatic phosphorylating conditions which result in activation of the enzyme.

In view of the similar nature of activation of tyrosine hydroxylase which we have observed with phosphatidyl-L-serine and after exposure to phosphorylating conditions, as described in Table I, and the non-independence of these two activations as shown in Table II, it is possible that the phosphorylating conditions produce a phosphorylated protein which then interacts with tyrosine hydroxylase as a polyanion, in a manner similar to that observed with phosphatidyl-L-serine (4). This putative activator could be present as a contaminant in the protein kinase preparation, or as a small dissociable subunit of tyrosine hydroxylase, itself. Further studies are in progress to elucidate the nature of this activator.

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