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ACTIVATION OF TYROSINE HYDROXYLASE IN THE CENTRAL NERVOUS SYSTEM BY ANAEROBIOSIS*

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Abstract—Subjecting either P_2 fraction or purified synaptosomes isolated from rat brain to periods of anoxic incubation at 30° resulted in activation of dopamine synthesis from tyrosine. This activation was approximately 2.5-fold when the anoxic incubation was carried out at pH 6.2 but was not significant when the pH was 7.4. Measurements of the tyrosine hydroxylase activity at pH 6.2 in Triton X-100-treated preparations of the P_2 fraction showed that, after 20 min of anaerobiosis, the K_m for pterine cofactor decreased by 39% and the K_i for dopamine increased by 44%; there was no change in the K_m for tyrosine. Half-maximal activation of dopamine synthesis occurred in 10 min of anaerobic incubation, and the reversal upon addition of oxygen had a half-time of 15 min. Addition of forskolin or dibutyryl cyclic AMP to anaerobic incubations of P_2 fraction did not result in significant activation of dopamine synthesis. Either the removal of calcium or the addition of calmodulin inhibitor, trifluoperazine, substantially decreased the activation of dopamine synthesis induced by periods of anaerobiosis. It appears that during anoxic incubation tyrosine hydroxylase underwent an activation which occurred over a period of minutes, was stable to detergent treatment, and was fully reversed over a period of minutes following reoxygenation. This activation was, at least in part, dependent on the presence of calcium and was sensitive to the calmodulin antagonist trifluoperazine.

Tyrosine hydroxylase is a monoxygenase that catalyzes formation of L-3,4-dihydroxyphenylalanine from L-tyrosine in central and peripheral catecholaminergic neurons [1, 2]. The reaction is commonly considered rate-limiting in catecholamine synthesis [3] and is subject to multiple modes of regulation (for review see Ref. 4). Long-term regulation includes activation of the enzyme by prolonged stress [5], nerve stimulation [6] and treatment with drugs (see, for example, Refs. 7 and 8). Short-range control involves modulation of enzyme activity by a variety of factors: activation by phospholipids [9] and polyanions [10] and feed-back inhibition by catecholamine end products [11, 12]. Moreover, tyrosine hydroxylase is directly activated by protein phosphorylation which utilizes both the cAMP-dependent [13–20] and cAMP-independent kinases [21–23].

We have shown previously [24] that 30 min of *in vivo* brain ischemia causes an increase in dopamine synthesis by particulate fractions of brain homogenates. The enhancement in dopamine production was observed to continue for 2 hr after the ischemic insult (i.e. during the post ischemic recovery) before returning to control values and was attributed to an activation of tyrosine hydroxylase. In the current work we have investigated the mechanism(s) of this phenomenon using an *in vitro* model system consisting of crude (P_2) and purified synaptosomal preparations incubated for various periods of time in the absence of oxygen.

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MATERIALS AND METHODS

Male Sprague–Dawley rats (200–250 g) were used throughout the study.

Preparation of P_2 and synaptosomal fractions. In the majority of experiments, crude synaptosomal preparations (P_2 fraction) from either striatum or combined fore- and midbrains (cerebral hemispheres, corpus striatum and a small part of rostral midbrain) of rats were used. In some cases, purified synaptosomes were isolated essentially as described by Booth and Clark [25]. The P_2 fraction was prepared from tissue homogenate by centrifugation for 20 min at 20,000 *g* of the initial low speed supernatant fraction (3 min at 1000 *g*). Both the synaptosomes and the P_2 fraction were suspended in the modified Krebs–Henseleit saline (140 mM NaCl, 5 mM KCl, 5 mM $NaHCO_3$, 1.3 mM $MgSO_4$, 1 mM Tris-phosphate and either 10 mM Tris-HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), or 10 mM Tris-MES (2[*N*-morpholino]ethanesulfonic acid), buffered to the appropriate pH and supplemented with 10 mM glucose and 1.3 mM $CaCl_2$.

Incubations. All incubations were carried out at 25° under either aerobic (control) or anaerobic conditions. In the latter, the oxygen was partially (about 80%) removed by gently shaking 5 ml of suspension in a 25-ml Erlenmeyer flask for 2–3 min in a nitrogen atmosphere. The content was then transferred to a sealed chamber without a gas phase. Synaptosomal

respiration was allowed to consume the remaining dissolved oxygen until anaerobiosis was attained (the concentration of oxygen in the medium was monitored continuously with a Clark-type O_2 electrode), and the incubations were continued in the absence of oxygen for the desired time intervals. The closed vessel was stirred with a small magnetic bar. Recovery from anaerobiosis was studied by removing samples incubated anaerobically and continuing the incubations under aerobic conditions for an additional 10–40 min. The appropriately preincubated synaptosomal (or P_2 fraction) suspensions were either used directly to measure dopamine synthesis or were treated with a 10% aqueous solution of Triton X-100 (final concentration 0.2%) and used to assay tyrosine hydroxylase activity.

Measurement of dopamine synthesis. Samples containing 5–6 mg protein in a final volume of 250 μl were incubated aerobically at 30° with shaking after addition of 40 μM L-[1-^{14}C]tyrosine (New England Nuclear, Boston, MA; 58.1 mCi/mmole). In some experiments, 6-methyl-5,6,7,8-tetrahydropterine (6-MPH_4) was also added to final concentrations of 25 μM to 1 mM from a stock solution in 1 M 2-mercaptoethanol (final mercaptoethanol concentration, 0.125 to 20 mM). The cofactor was prepared fresh daily, 10–20 min before its addition to the incubation mixture. The reaction was terminated at time intervals from 5 to 30 min by addition of 1 ml of 4% perchloric acid solution containing 0.2% Triton X-100, and the CO_2 liberated was trapped with filter paper treated with phenylethylamine in methanol (20%, v/v). This technique determines dopamine synthesis by measuring the $^{14}CO_2$ produced by decarboxylation of the L-[1-^{14}C]dihydroxyphenylalanine which is formed by hydroxylation of L-[1-^{14}C]-tyrosine. The rates were calculated from the first 15–20 min of incubation for which CO_2 evolution was a linear function of time.

Measurements of tyrosine hydroxylase activity in detergent-treated samples. Tyrosine hydroxylase activity was assayed by a modification of the method of Waymire *et al.* [26]. The standard incubation mixture contained 0.25 M Tris-HEPES at pH 7.4 (or Tris-MES at pH 6.2), 1300 units catalase, 2000 units sheep liver pteridine reductase, 1 mM NADPH, 40 or 80 μM L-[1-^{14}C]tyrosine and the indicated concentrations of 6-MPH_4, added from a stock solution in 1 M 2-mercaptoethanol (final mercaptoethanol concentration 0.125 to 20 mM), together with the protein sample (1 to 1.5 mg protein of the P_2 fraction containing Triton X-100, final concentration 0.2%) in a final volume of 250 μl. The assay mixture was incubated for 0–30 min at 30° in a Dubnoff metabolic shaker with air as the gas phase. The reaction was terminated at the indicated times by addition of 3-iodotyrosine (final concentration 3 mM) to inhibit the enzyme. Excess of bovine adrenal L-aromatic amino acid decarboxylase, partially purified according to Waymire *et al.* [26], was added together with 0.13 mM pyridoxal phosphate. Radioactive $^{14}CO_2$ was then liberated and measured as described above for the dopamine synthesis assay. The rates were calculated from the first 15–20 min for which the release of CO_2 was a linear function of time.

Measurements of dopamine levels. Samples (300 μl, containing 2–3 mg protein) of appropriately incubated preparations were treated in two different ways. In the first, aliquots were rapidly centrifuged through a layer of silicone oil into 100 μl of cold 1% trichloroacetic acid (TCA) in 4% NaCl. The top layer, which contained the extracellular medium, was pipetted off, acidified with TCA to a final acid concentration of 1%, and used for amino acid determination. The middle silicone oil layer was removed by aspiration, the pellet was agitated with a glass stirring rod in the TCA medium, and the sample was recentrifuged as above. The pellet was then discarded and the extract used for amino acid analysis. In the second procedure, samples were quenched directly by the addition of cold TCA to a final concentration of 1% for the measurements of total neurotransmitter levels. The amount of dopamine was measured directly in the TCA extracts by high pressure liquid chromatography (HPLC) using a 25 cm Whatman Paristil-10 SCX column. The elution was carried out with 25 mM monochloroacetic acid, pH 2.85 (neutralized with NaOH), containing 100 μM EDTA at a flow rate of 2 ml/min. Detection was by a Bioanalytical Systems TL-5 thin-layer transducer with a glassy carbon electrode, and the concentrations were calculated from a standard curve. The retention time was 6 min.

RESULTS

Effect of periods of anaerobic incubation on dopamine synthesis. When the P_2 fraction isolated from rat striatum was incubated anaerobically for 30 min at pH 6.2 and then reoxygenated, the measured rate of synthesis of dopamine was 1.1 ± 0.12 pmoles/min/mg protein in control (aerobically incubated) suspensions compared to 2.6 ± 0.45 pmoles/min/mg ($N = 5$) in the anaerobically incubated samples. This is a mean increase of 2.4-fold with respect to the control values and is statistically significant to $P < 0.001$ by the paired *t*-test. Since the amount of tissue in the striatum is too small to permit extensive characterization of the phenomenon without killing a large number of rats, further experiments were undertaken using the P_2 fraction from forebrains and midbrains. The effect of anaerobic incubation on dopamine synthesis by the P_2 fraction isolated from rat forebrains and midbrains (Table 1) was essentially the same as that observed in the striatum, although the absolute rates were 2.5-fold higher in the latter. Since tyrosine hydroxylase isolated from different regions of the brain has been reported to exhibit very similar characteristics ([27, 28] but see Ref. 29 to the contrary), it was decided to use the P_2 fractions from combined forebrains and midbrains in all subsequent studies described in this paper. Experiments with purified synaptosomes gave results very similar to those using the P_2 fraction and, where they were used, it is noted in the table legend.

The rates of dopamine synthesis were investigated in the presence and absence of both 10 μM forskolin and 1 mM 6-MPH_4 in samples incubated either aerobically or anaerobically for 40 min at three different pH values, 6.2, 6.7 and 7.4. The results, presented in Table 1, can be briefly summarized as follows: (1) The rates of dopamine synthesis by aerobically

Table 1. Dopamine synthesis by P_2 fraction under various experimental conditions*

	Dopamine synthesis (pmoles/min/mg protein)							
	Aerobic				Anaerobic			
pH	Control	Forskolin	6-MPH_4	Forskolin + 6-MPH_4	Control	Forskolin	6-MPH_4	Forskolin + 6-MPH_4
6.2	0.42 ± 0.03	0.50 ± 0.04 $P < 0.05$	0.92 ± 0.04 $P < 0.001$	1.08 ± 0.14 $P < 0.001$	1.10 ± 0.17 $P < 0.001$	1.09 ± 0.15 $P < 0.001$ NS	1.45 ± 0.27 $P < 0.001$ NS	1.33 ± 0.11 $P < 0.001$ NS
6.7	0.32 ± 0.01	0.48 ± 0.01 $P < 0.001$	0.77 ± 0.06 $P < 0.001$	0.95 ± 0.04 $P < 0.001$	0.51 ± 0.02 $P < 0.001$	0.67 ± 0.02 $P < 0.001$ $P < 0.001$	0.99 ± 0.01 $P < 0.001$ $P < 0.001$	1.18 ± 0.06 $P < 0.001$ $P < 0.001$
7.4	0.21 ± 0.02	0.30 ± 0.02 $P < 0.005$	0.51 ± 0.01 $P < 0.001$	0.73 ± 0.09 $P < 0.001$	0.22 ± 0.02 NS	0.33 ± 0.06 $P < 0.01$ $P < 0.01$	0.55 ± 0.08 $P < 0.001$ $P < 0.001$	0.75 ± 0.01 $P < 0.001$ $P < 0.001$

* Dopamine synthesis was measured in parallel in P_2 fractions immediately after 40 min of either aerobic or anaerobic preincubation as described in Materials and Methods. The concentration of 6-MPH_4 was 1 mM and that of forskolin 10 μM. All measurements were made in duplicate, and the given values are means ± S.D. for three to six experiments. P values were calculated with respect to aerobic controls at each pH (upper row) and, in the anaerobic groups, also with respect to anaerobic controls (bottom row); *t* statistics for two means was used. NS = not significant.

incubated (control) preparations were about 2-fold higher at pH 6.2 than at pH 7.4. (2) Addition of forskolin stimulated the rate of dopamine synthesis by about 20% at pH 6.2 and 40% at pH 7.4, while the pterine cofactor enhanced it over 2-fold at all pH values. The effect of both added together was less than additive at pH 6.2 and almost additive at pH 7.4. (3) Periods of anaerobic incubation led to enhanced dopamine production only at acid pH, and the effect was larger at pH 6.2 than at pH 6.7. No stimulation was observed at pH 7.4. (4). Anaerobic incubation at pH 6.2 enhanced catecholamine synthesis by 2.6-fold in control samples, by 2.4-fold with forskolin, and by 1.6-fold when 1 mM 6-MPH_4 was present.

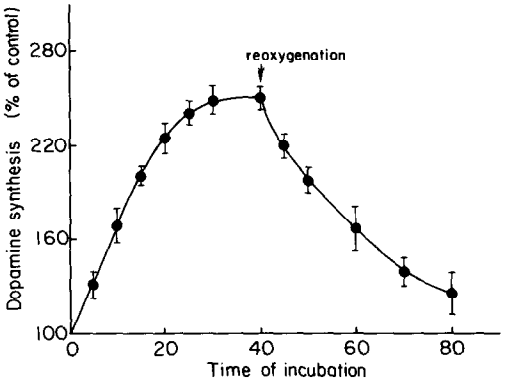

Fig. 1. Time courses for activation and deactivation of dopamine synthesis. Dopamine synthesis from ^{14}C-labeled tyrosine was measured as described in Materials and Methods with no added 6-MPH_4. In "activation" experiments, the P_2 fraction was incubated anaerobically for 5, 10, 15, 20, 25 and 40 min at 30°, and dopamine synthesis was measured immediately thereafter. In "reoxygenation" experiments, the P_2 fractions were first incubated for 40 min in the absence of oxygen and then transferred to aerobic conditions for 5, 10, 20, 30 and 40 min before dopamine production was determined. In each experiment, the measurements were carried out in duplicate, and the presented values are the average of three experiments.

Dibutyryl-cAMP (1 mM) stimulated the rate of synthesis of dopamine at all pH values but the amount of stimulation was even less than that by forskolin. Forskolin concentrations higher than 10 μM did not lead to greater increases in dopamine production than that shown in Table 1.

Time dependence of activation of dopamine production by anaerobic incubation and of its reversal following reoxygenation. The time dependence of the activation of dopamine synthesis was investigated by varying the duration of anaerobic incubation. Figure 1 shows that 30 min of anaerobiosis was sufficient for maximum effect. To determine the reversibility of this phenomenon, the anaerobically preincubated P_2 fractions were subsequently transferred to aerobic conditions and reincubated for 20–40 min. Figure 1 shows that, when the anaerobic incubation was carried out for 40 min, a subsequent 40 min of aerobic incubation (prior to the measurement of dopamine synthesis) largely reversed the activation of dopamine synthesis. Essentially the same behavior was observed when samples were incubated anaerobically for only 20 min before reoxygenation.

Effect of calcium withdrawal and of trifluoperazine on the rate of dopamine synthesis. The effect of calcium on dopamine synthesis was evaluated by incubating the P_2 fraction aerobically or anaerobically in the presence of either 1.3 mM Ca^{2+} or 10 mM Mg^{2+}. Table 2 shows that, whereas substitution of Ca^{2+} with Mg^{2+} had no influence on the rate of dopamine production in aerobically-incubated suspensions, it had a marked effect in samples subjected to anaerobiosis. In the latter, the rate was about 40% lower for P_2 fractions incubated in medium containing Mg^{2+} than for those incubated in medium containing Ca^{2+}. Even larger inhibition of anaerobiosis-induced stimulation of dopamine synthesis was observed with trifluoperazine, a phenothiazine which binds to calmodulin and inhibits its effects [30].

Table 2. Calcium dependence of the activation of dopamine synthesis by anaerobic incubation at pH 6.2*

Conditions	Dopamine synthesis (pmoles/min/mg protein)	
	Aerobic	Anaerobic
Control	0.39 ± 0.01	1.25 ± 0.03
Trifluoperazine	0.35 ± 0.01†	0.62 ± 0.14‡
$-Ca^{2+}$, $+Mg^{2+}$	0.43 ± 0.05†	0.79 ± 0.02‡

* P_2 fractions were suspended in either normal medium containing 1.3 mM $CaCl_2$, with or without 100 μM trifluoperazine, or medium in which the calcium salt was omitted and replaced with 10 mM $MgCl_2$. They were then incubated either aerobically or anaerobically for 40 min at 30°, pH 6.2. At the end of the 40-min incubations, aliquots were used to measure dopamine synthesis at pH 6.2 as described in Materials and Methods. In each experiment, duplicate measurements were made and averaged. The data given in the table are the means ± S.D. for three to six experiments.

† Not significantly different from aerobic control.

‡ $P < 0.001$, with respect to aerobic control (t statistics for two means).

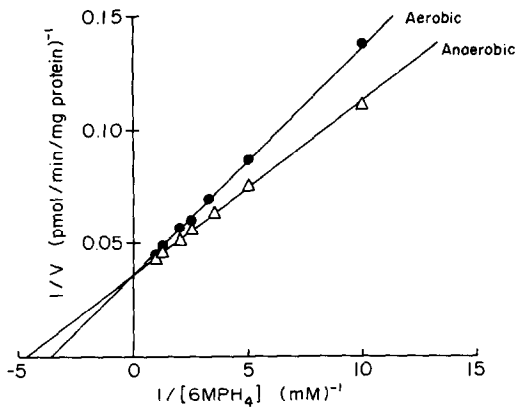

Fig. 2. Dependence of the tyrosine hydroxylase activity of aerobically and anaerobically preincubated P_2 fraction on the concentration of 6-MPH_4. The P_2 fractions were subjected to 20 min of either aerobic or anaerobic incubation at pH 6.2 and 30°, and then tyrosine hydroxylase activity was measured as described in Materials and Methods. The tyrosine concentration was 40 μM; the data are plotted as the reciprocal of the rate of decarboxylation (ordinate) against the reciprocal of the cofactor concentration (abscissa).

Effect of anaerobic incubations on the kinetic parameters of tyrosine hydroxylase. Since the rate-limiting enzyme in dopamine synthesis is tyrosine hydroxylase, experiments were carried out to determine the effect of anaerobic incubation of the P_2 fraction on the activity of this enzyme. To measure tyrosine hydroxylase activity under well controlled conditions, the preparations were treated with Triton X-100 at a final concentration of 0.2%. This level of detergent destroys synaptosomal membranes and removes the permeability barriers for substrate, cofactor and product. Analysis of the cofactor dependence of tyrosine hydroxylase activity was carried out at pH 6.2 by varying the concentration of 6-MPH_4 in the presence of two concentrations of tyrosine, 40 and 80 μM. The results of a typical experiment (tyrosine concentration 40 μM) are presented in Fig. 2 and a summary of the data is given in Table 3. The K_m values for 6-MPH_4 were found to be independent of tyrosine concentration, whereas the V_{max} values increased at the higher tyrosine concentration. Activation of the enzyme during anaerobic incubation appears to have involved a decrease in the K_m for 6-MPH_4 with no change in the V_{max}.

The K_m for tyrosine was determined at pH 6.2 or

Table 3. Effect of anaerobic incubation on the K_m and V_{max} of tyrosine hydroxylase for 6-MPH_4*

Conditions	K_m (μM)	V_{max} (pmoles/min/mg protein)
(A) 40 μM Tyrosine		
Aerobic, pH 7.4	177 ± 25 (4)	8.7 ± 1.6 (4)
Anaerobic, pH 7.4	165 ± 17 (3)	8.5 ± 2.9 (3)
Aerobic, pH 6.2	307 ± 49 (6)	29.8 ± 6.0 (6)
Anaerobic, pH 6.2	223 ± 19† (3)	29.0 ± 4.3 (3)
(B) 80 μM Tyrosine		
Aerobic, pH 7.4	144 ± 22 (3)	16.4 ± 2.4 (3)
Anaerobic, pH 7.4	119 ± 23 (4)	15.7 ± 3.1 (3)
Aerobic, pH 6.2	300 ± 67 (3)	57.0 ± 8.4 (3)
Anaerobic, pH 6.2	198 ± 23† (3)	51.4 ± 7.1 (3)

* P_2 fractions were incubated for 40 min at 30° either aerobically or anaerobically at pH 7.4 or pH 6.2. Aliquots were then treated with Triton X-100, and the activity of tyrosine hydroxylase was measured as described in Materials and Methods and in the legend of Fig. 2. In each experiment the measurements were made in duplicate; the values given in the table are means ± S.D. for the number of experiments indicated in parentheses.

† $P < 0.05$ with respect to control (t statistics for two means), or $P < 0.02$ when each measurement was compared to its control and the paired t-test used.

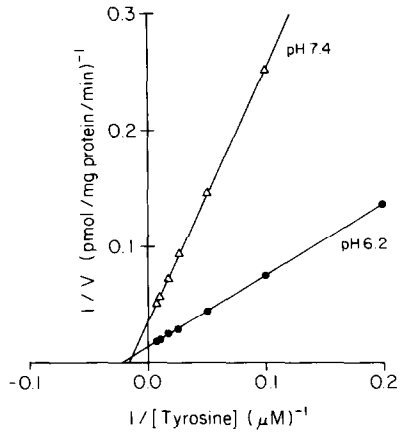

Fig. 3. Dependence of tyrosine hydroxylase activity on tyrosine concentration at pH 6.2 and pH 7.4. The P_2 fractions were incubated aerobically for 20 min at 30° and the indicated pH. The preparation was then treated with Triton X-100, and tyrosine hydroxylase activity was measured at the same pH and 30° as described in Materials and Methods. The concentration of 6-MPH_4 was 1 mM, while tyrosine concentrations of 5–120 μM were used. The data are plotted as the reciprocal of the reaction rate (ordinate) against the reciprocal of tyrosine concentration (abscissa).

7.4 by assaying tyrosine hydroxylase activity with various concentrations of tyrosine in the presence of 1 mM 6-MPH_4. A typical experiment is shown in Fig. 3. The kinetic constants for tyrosine were dependent on pH: the K_m was smaller and the V_{max} was larger at pH 6.2 than at pH 7.4. The respective values (±S. D.) determined from three to five independent experiments were: at pH 6.2, K_m 45 ± 4.9 μM and V_{max} 67.3 ± 5 pmoles/min/mg protein and at pH 7.4, K_m 60 ± 11 μM and V_{max} 27 ± 4.3 pmoles/min/mg protein. (These differences were statistically significant at the level of $P < 0.05$ for the K_m and $P < 0.001$ for the V_{max}.) Subjecting the P_2 fractions to periods of anaerobic incubation affected neither the K_m nor the V_{max} for tyrosine.

The K_i for inhibition of tyrosine hydroxylase by dopamine was determined at pH 6.2 in the presence of various concentrations of 6-MPH_4 (0.1 to 1 mM) and 40 μM tyrosine. The results of a typical experiment, graphed as Dixon plots, are shown in Fig. 4. The convergence of the curves obtained at various 6-MPH_4 concentrations indicates that dopamine is a competitive inhibitor of the pterine cofactor. The K_i value generated from the intersections of lines on the Dixon plots was found to be 34 ± 1.7 μM for four independent experiments. After 40 min of anaerobic incubation, the K_i for dopamine was increased to 48.6 ± 4.0 μM ($N = 4$, $P < 0.001$).

It is worth pointing out that the values for the kinetic constants for tyrosine hydroxylase reported above are in excellent agreement with the results of other authors (see, for example, Refs. 18 and 20).

Effect of forskolin on the kinetic parameters of tyrosine hydroxylase. The P_2 fraction was incubated aerobically with 10 μM forskolin at either pH 6.2 or 7.4 for 10–40 min, treated with Triton X-100, and appropriately diluted for the measurements of tyrosine hydroxylase activity. Analysis of both the 6-MPH_4 dependence of the enzyme activity and inhibition of the enzyme by dopamine showed them to be indistinguishable experimentally from samples incubated aerobically in the absence of forskolin.

Effect of periods of anaerobic incubation on the levels of dopamine in synaptosomes. Since dopamine is a known feed-back inhibitor of tyrosine hydroxylase, the levels of this catecholamine were measured in the synaptosomes and in the extrasynaptosomal

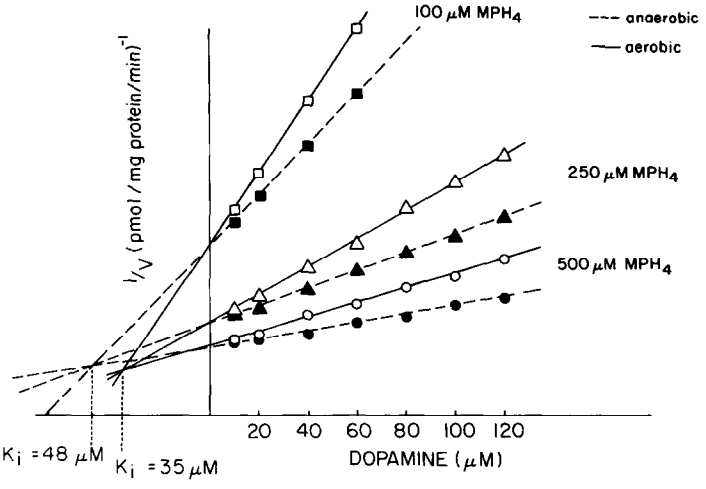

Fig. 4. Effect of 20-min anaerobic incubation at pH 6.2 on the inhibition of tyrosine hydroxylase by dopamine. The P_2 fractions were incubated either aerobically (solid lines) or anaerobically (dashed lines) for 20 min at 30°, pH 6.2, and then tyrosine hydroxylase activity was measured in detergent-treated samples as described in Materials and Methods. The assay was carried out at 30° and pH 6.2 using a tyrosine concentration of 80 μM. The data are presented as Dixon plots (reaction rate on the ordinate and the reciprocal of dopamine concentration on the abscissa) to facilitate calculation of the inhibitor constant (K_i) for dopamine.

medium, as well as their "total" amounts, during aerobic and anaerobic incubation at pH 6.2, 6.7 and 7.4. The results are summarized in Table 4. The following points can be made: (1) the levels of dopamine in the synaptosomes (and the total amounts expressed as pmoles/mg protein) increased with decreasing pH, (2) during anaerobic incubation, there was a decrease in the intrasynaptosomal level of dopamine and a concomitant increase in its concentration in the external medium, and (3) incubations at pH 7.4 caused a gradual decrease in the total amount of dopamine, whereas at pH 6.2 there was an increase in its level with time.

DISCUSSION

The data presented in this paper show that anaerobic incubation of synaptosomes at acid pH resulted in an increase in dopamine synthesis. This increase in the capacity for catecholamine production was due primarily to activation of tyrosine hydroxylase, the rate-limiting enzyme on its biosynthetic pathway. Activation of tyrosine hydroxylase, as measured in detergent-solubilized preparations, was manifested by a decrease in the K_m for the pterine cofactor and by an increase in the K_i for dopamine, with no change in the kinetic parameters for tyrosine. The decrease in the K_m for 6-MPH_4 was 39% and the increase in the K_i for dopamine was 44%. Since the concentration of the pterine cofactor in synaptosomes is likely to be much below its saturation value [31, 32] while cytosolic dopamine is above its K_i, the observed changes in K_m and K_i should cause an activation of dopamine synthesis by a factor of 1.39×1.44, i.e. 2.0, accounting for most of the 2.4- (Table 1) to 3.2-fold (Table 2) stimulation caused by anaerobic incubation. The much smaller stimulation of catecholamine production measured in the presence of 1 mM 6-MPH_4 (1.6 fold—see Table 1) is close to that expected from the change in K_i for dopamine, assuming a constant amine concentration that is above the K_i.

Activation of tyrosine hydroxylase can occur through a variety of mechanisms, the most important of which are: cAMP-dependent phosphorylation [13–20], cAMP-independent, Ca^{2+}-independent phosphorylation [22], cAMP-independent, Ca^{2+}-dependent phosphorylation [23], limited proteolytic digestion [33], and release of end-product inhibition [11, 12]. The pattern of activation of tyrosine hydroxylase by anaerobic incubation resembles that caused by cAMP-dependent phosphorylation. The inability of either 1 mM dibutyryl-cAMP or the diterpene forskolin, a potent stimulator of adenylate cyclase in neuronal [34] and other tissues [35], to enhance the extent of the anaerobic effect would tend to support such an interpretation. On the other hand, several lines of evidence argue against the cAMP-dependent mechanism being solely responsible for the observed increase in dopamine synthesis. First, the effect of anaerobiosis was much larger than that of the saturating concentrations of

Table 4. Effect of anaerobic incubation and pH on synaptosomal dopamine levels*

Condition	Dopamine		
	Pellet (pmoles/mg protein)	Supernatant (10^{-9} M)	Total (pmoles/mg protein)
pH 7.4			
10-min Aerobic	16.7 ± 1.0	10.1 ± 0.6	17.6 ± 0.9
40-min Aerobic	14.5 ± 0.2	5.0 ± 0.35	15.2 ± 0.9
40-min Anaerobic	10.8 ± 1.0	101 ± 1.3	19.1 ± 0.8
40-min Anaerobic + 20-min recovery	11.7 ± 1.2	6.0 ± 0.1	12.2 ± 0.9†
pH 6.7			
10-min Aerobic	19.6 ± 0.4	12.5 ± 0.5	20.8 ± 0.8
40-min Aerobic	19.6 ± 0.2	8.0 ± 0.7	20.6 ± 0.2
40-min Anaerobic	11.0 ± 1.0	104 ± 2.3	21.7 ± 1.6
40-min Anaerobic + 20-min recovery	17.2 ± 1.2	18.5 ± 1.0	19.2 ± 1.4
pH 6.2			
10-min Aerobic	27.3 ± 1.9	13.5 ± 1.1	29.6 ± 2.3
40-min Aerobic	28.6 ± 0.4	9.3 ± 0.9	29.8 ± 0.4
40-min Anaerobic	14.0 ± 1.1	150 ± 22	34.4 ± 2.0
40-min Anaerobic + 20-min recovery	29.0 ± 2.8	58.5 ± 8.1	37.2 ± 1.2‡

* Purified synaptosomes were suspended as described in Materials and Methods and then incubated either aerobically or anaerobically for 40 min at 30° and pH values of 7.4, 6.7 or 6.2. After anaerobic incubation for 40 min, part of the suspension was reoxygenated and incubation was continued for 20 min. At each point the synaptosomal suspension was analyzed for its rate of dopamine synthesis as described in Materials and Methods. The assays in each experiment were in duplicate, and the values in the table are the mean ± S.D. for three experiments.

† $P < 0.002$ (t statistics for two means).

‡ $P < 0.01$.

dibutyryl-cAMP or forskolin; second, anaerobic incubation caused relatively stable (covalent?) activation of the enzyme which reversed slowly (half-time of about 15 min), whereas parallel incubation with forskolin did not have such an effect; third, both replacement of calcium with magnesium and addition of trifluoperazine substantially decreased the extent of activation during anaerobiosis; and fourth, activation by anaerobiosis was observed only at acid pH values, predominantly at pH 6.2 with no activation at pH 7.4, whereas cAMP-dependent phosphorylation induces a second optimum, at pH 7.4 (see, for example, Refs. 19, 20 and 36).

These arguments indicate that another mechanism, most likely involving calcium, may operate during anaerobic incubations. Anaerobiosis results in membrane depolarization [37–40] which, in turn, may increase calcium influx. The existence of calcium-dependent protein kinases in nerve terminals, which are activated during depolarization, has indeed been reported by Schulman and Greengard [41]. Moreover, tyrosine hydroxylase has also been shown to be activated by depolarization [42–45], and the involvement of calcium in this process has been postulated [23, 46]. Our own results are consistent with this possibility.

In addition to the requirement for the presence of extracellular calcium, stimulation of dopamine synthesis caused by anaerobiosis was blocked by the calmodulin antagonist trifluoperazine [30]. The mechanisms which link calmodulin to activation of tyrosine hydroxylase can be numerous since calmodulin is known to be involved in a number of intracellular functions and is present in large amounts in synapses (for review see Ref. 47). Calmodulin can activate a protein kinase, it can modulate calcium fluxes between the intracellular and extracellular environment, and it can also mediate intracellular effects of this cation. Any one of these or a combination thereof could be responsible for the observed effect.

The two other mechanisms listed above, if operative under our conditions, are probably of minor importance. Limited proteolysis would, most likely, yield irreversible activation of tyrosine hydroxylase, whereas in our system the effects of anaerobics were reversed by subsequent aerobic incubation. Feedback inhibition by dopamine is also unlikely to have contributed significantly to the reversible activation of dopamine synthesis for the following reasons: (1) Stimulation of tyrosine hydroxylase activity by anaerobiosis was still present in P_2 fractions treated with detergent at a level sufficient to destroy integrity of the plasma membrane. Under such conditions, the concentration of dopamine in the incubation mixture (approx. 30 pmoles/mg protein, Table 4, or 0.2×10^{-6} M) was considerably below the K_i of the enzyme for this catecholamine. Moreover, since the total levels of dopamine in aerobically- and anaerobically-incubated samples were essentially the same (Table 4), the final concentrations of the amine in detergent-containing incubations must also have been the same. (2) Dopamine synthesis in intact P_2 (or synaptosomal) fractions was found to be activated only by anaerobic incubations at pH 6.2, whereas declines in catecholamine concentrations occurred at both pH 7.4 and pH 6.2. Hence, if a decrease in intrasynaptosomal dopamine were a significant factor in stimulation of tyrosine hydroxylase, very similar effects should have been seen at the acid and neutral pH values.

It is finally important to mention the possible physiological implications of our observations. The constraints on catecholamine synthesis *in vivo* are imposed by ambient pH and the concentrations of pterine cofactor and dopamine. Activation of tyrosine hydroxylase by anaerobiosis at acid pH may represent a response under conditions resembling those of tissue hypoxia and ischemia in which cellular acidosis is accompanied by membrane depolarization and leakage of neurotransmitters. In such situations, transient activation of the enzyme would make resupply of the catecholamines faster and more efficient. However, when the stimulus subsided in post-anaerobic recovery, tyrosine hydroxylase would revert to a less active state adequate to maintain normal neurotransmitter production.

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