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Activation of Tyrosine Hydroxylase from Central Noradrenergic Neurons by Calcium

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SUMMARY

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Addition of $CaCl_2$ to soluble preparations of tyrosine hydroxylase from rat medulla pons produces a marked activation of the enzyme assayed with subsaturating concentrations of tyrosine (10 μM) and pteridine cofactor (2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine, 100 μM). While some increase in activity occurs with Ca^{++} concentrations as low as 10 μM, activation is maximal at 50 μM Ca^{++} and remains unchanged up to 1.0 mM. $BaCl_2$ produces similar although less pronounced effects. $MgCl_2$ prevents the activation of the enzyme if added to the reaction mixture before $CaCl_2$. Alone $MgCl_2$ has no effect in concentrations up to 1 mM. Ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid has no direct effects on the enzyme but completely antagonizes the activation produced by Ca^{++}. The activation of tyrosine hydroxylase by Ca^{++} is reflected in changes in the kinetic properties of the enzyme. The K_m for tyrosine decreases from 58.1 to 10.3 μM, the K_m for pteridine cofactor decreases from 673 to 125 μM, and the K_i for norepinephrine increases almost 20-fold, from 0.34 to 6.27 mM, in the presence of Ca^{++}. Thus norepinephrine is a much less effective inhibitor of the Ca^{++}-activated enzyme. The proposal is made that Ca^{++} which enters the nerve terminal during nerve stimulation may enhance norepinephrine synthesis by activating tyrosine hydroxylase in a manner similar to the activation observed *in vitro* with $CaCl_2$. Similar findings are reported for tyrosine hydroxylase isolated from rat cerebral cortex.

INTRODUCTION

Stimulation of peripheral sympathetic neurons has long been known to enhance the formation of the transmitter, norepinephrine, in isolated, sympathetically innervated organs (1-8). In the intact animal an increase in sympathetic activity also results in an increase in transmitter formation. In the central nervous system it has been more difficult to obtain direct evidence for the existence of such a regulatory process. Nonetheless, recently it has been established that both the turnover and

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metabolism of norepinephrine are increased in the central nervous system of the rat when central noradrenergic neurons originating in the locus ceruleus are stimulated electrically (9–12) or when activity in these neurons is enhanced by stress (13). For example, stimulation of the cells of the locus ceruleus leads to a more rapid decline in norepinephrine levels in the presence of the tyrosine hydroxylase inhibitor α-methyl-*p*-tyrosine (10) and to a dramatic increase in the levels of the norepinephrine metabolite, 3-methoxy-4-hydroxyphenylglycol sulfate (11, 12), in the cerebral cortex on the stimulated side. Studies on catecholamine formation in slices of rat brain also indicate that depolarization enhances the synthesis of catecholamines from radiolabeled tyrosine precursor (14). Taken together, these results have been interpreted to mean that central noradrenergic neurons, like those of the peripheral sympathetic nervous system, respond to nerve stimulation by increasing the rate at which transmitter is formed and utilized.

The enhanced formation of transmitter in peripheral sympathetic neurons results from an increase in the apparent activity of the enzyme which is rate-limiting in the formation of norepinephrine, tyrosine hydroxylase (tyrosine-3-monoxygenase, EC 1.14.16.2) (15). However, the precise mechanism whereby the activity of this enzyme is increased dramatically from one minute to the next remains unresolved. One hypothesis, which is based on the observation that catechols and catecholamines inhibit tyrosine hydroxylase by competitively antagonizing the binding of the pteridine cofactor to the apoenzyme (16, 17), proposes that a pool of "free" norepinephrine present in the neuronal cytoplasm may inhibit its own synthesis by feedback inhibition (see ref. 18). In this scheme enzyme activity is believed to be modulated by changes in the size of this regulatory pool of norepinephrine which occur secondary to norepinephrine release. Recently an attempt was made to test this hypothesis of feedback inhibition by using a large excess of pteridine cofactor in the incubation medium (19). It was argued that if enzyme activity is regulated in a simple competitive way by the concentration of norepinephrine in some small, strategically located pool, then it should be possible to bring basal rates of norepinephrine synthesis up to stimulated rates simply by making a vast excess of the pteridine cofactor available. The fact that under these conditions control rates of norepinephrine synthesis never matched those of stimulated tissue (19) suggested that some other activation mechanism may be involved. New evidence obtained in the peripheral nervous system indicates that Ca^{++} can activate tyrosine hydroxylase directly (20, 21). This raises the possibility that the influx of Ca^{++} which occurs at the noradrenergic nerve ending during depolarization (22) and is intimately linked to the release process (22, 23) may also be involved in regulating the activity of tyrosine hydroxylase. The object of the present study was to investigate whether tyrosine hydroxylase present in noradrenergic neurons of the central nervous system can also be activated by Ca^{++}.

METHODS

Materials. L-[3,5-^{3}H]tyrosine, 30 Ci/mmole (New England Nuclear), was purified by passage through aluminum oxide (British Drug Houses, Ltd.) at pH 8.4 and diluted to a final specific activity of 0.1 Ci/mmole with unlabeled L-tyrosine. Synthetic cofactor, $DMPH_4$,[2] was obtained from Calbiochem. Purified bovine catalase (lyophilized powder form), NADPH, ammonium sulfate (enzyme grade), and Tris base (ultrapure), were products of Schwarz/Mann. EGTA and L-norepinephrine HCl were purchased from Sigma Chemical Company; brocresine was a gift from Lederle Laboratories. All other reagents were of maximal commercially available purity.

Preparation of tissue. Male Sprague-Dawley rats (Charles River Breeding Laboratories) weighing 250–300 g were killed by decapitation. The brains were removed

[2] The abbreviations used are: $DMPH_4$, 2-amino-4-hydroxy-6,7-dimethyl-5,6,7,8-tetrahydropteridine; EGTA, ethylene glycol bis(β-aminoethyl ether)-*N,N'*-tetraacetic acid; brocresine, 3-hydroxy-4-bromobenzyloxyamine dihydrogen phosphate.

and immediately placed on ice. The medulla pons and cortex were rapidly dissected out, frozen on Dry Ice, and stored at −70° until assayed (up to 2 weeks). At the time of assay, depending on the number of experimental samples needed, tissues from an appropriate number of animals were pooled, weighed, and homogenized (5–10 strokes) in 10 volumes of ice-cold 0.05 M Tris-acetate buffer, pH 6.0, with a Kontes Duall glass homogenizer (0.010-cm clearance). The homogenate was centrifuged at 104,000 × *g* for 90 min, and a clear supernatant was obtained. This supernatant served as the source of soluble tyrosine hydroxylase.

Preparation of dihydropteridine reductase. Dihydropteridine reductase (EC 1.6.99.7) was prepared from sheep liver through the first ammonium sulfate fractionation according to the method of Kaufman (24). The sheep livers were obtained frozen from Pel-Freez Biologicals and maintained at −20° until the time of preparation. The enzyme preparation was dissolved in a volume of 0.025 M Tris-HCl buffer, pH 7.4, equivalent to one-seventh the original volume and dialyzed overnight against 0.01 M Tris-HCl, pH 7.4. The dialyzed preparation was quick-frozen in 4.0-ml batches and stored at −20° until use.

Tyrosine hydroxylase assay. Tyrosine hydroxylase was assayed by a modification of the methods of Shiman, Akino, and Kaufman (25) and Coyle (26). The supernatant preparation (100 μl) was added to a 15 × 100 mm glass test tube containing a reaction mixture of 1.0 M acetate buffer, pH 6.0 (200 μl), 0.1 mM brocresine (50 μl), sheep liver dihydropteridine reductase in 0.025 M Tris-HCl buffer, pH 7.4 (100 μl), 0.01 M NADPH (100 μl), 3300 units of catalase in glass-distilled water (200 μl), and an appropriate concentration of $DMPH_4$ in ice-cold 0.005 N HCl (100 μl). Catalase, $DMPH_4$, and NADPH were prepared fresh just prior to use. Test solutions or water were added in 100 μl. Blanks consisted of complete incubation mixtures to which 50 μl of glacial acetic acid had been added. After a 5-min incubation at 37° the reaction was initiated by adding the substrate, tyrosine (0.1 Ci/mmole). The reaction was terminated after a 45-min incubation period at 37° by addition of glacial acetic acid (50 μl). The reaction mixture was put over a Dowex column (0.6 × 3 cm) which drained into a scintillation vial containing 13 ml of DTE scintillation fluid (27). After the reaction mixture had passed through the column completely, the Dowex resin was washed with 0.5 ml of distilled water and the eluate plus wash were counted in a Packard scintillation counter, model 3375. In initial experiments L-dopa formation was determined directly by cation-exchange chromatography and shown to be equivalent to tritiated water production. One mole of tritiated water was formed in the reaction per mole of [^{3}H]dopa formed. Protein was determined according to Lowry *et al.* (28), using bovine serum albumin as standard, and tyrosine hydroxylase activity was expressed as picomoles of dopa per milligram of protein per minute. All calculations were performed with a Hewlett-Packard programmable calculator, model 9810A. Statistical estimations of K_m were made according to Wilkinson (29). Kinetics was determined on linear portions of the curves of time course and protein concentration. The rate of formation of tritiated water was linear for at least 1 hr and from 20 to 800 μg of protein.

RESULTS

Effect of Ca^{++} and other divalent cations on activity of tyrosine hydroxylase from rat medulla pons. Figure 1 summarizes the effect of different concentrations of $CaCl_2$ on the activity of tyrosine hydroxylase prepared from rat medulla pons. The enzyme was incubated with the different Ca^{++} concentrations for 5 min prior to addition of the substrate, L-tyrosine. The concentrations of both tyrosine (10 μM) and $DMPH_4$ (100 μM) were subsaturating. Maximal activation, 394% of control, occurred at a Ca^{++} concentration of 6 μM and remained at this level with Ca^{++} concentrations as high as 1 mM.

In Table 1 it can be seen that another divalent cation, Ba^{++} (100 μM), also increased the activity of soluble tyrosine

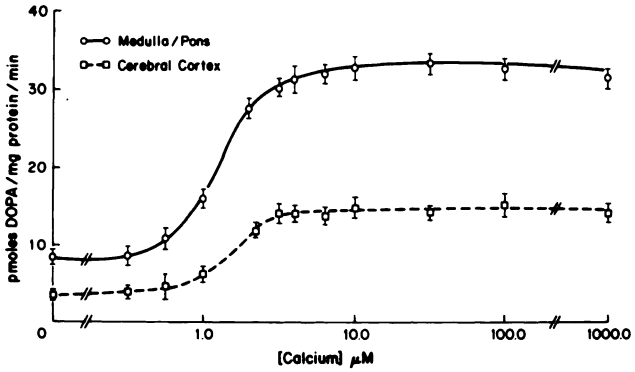

FIG. 1. *Effect of calcium on activity of tyrosine hydroxylase from central noradrenergic neurons*
Tyrosine hydroxylase activity was measured in high-speed supernatants of homogenates of rat medulla pons and cerebral cortex at a tyrosine concentration of 10 μM and a $DMPH_4$ concentration of 100 μM. The results are expressed as the means ± standard errors of six determinations.

TABLE 1

Effect of various divalent ions on tyrosine hydroxylase activity from medulla pons and cerebral cortex

Divalent metals were added to the incubation media 5 min prior to the addition of substrate. Tyrosine hydroxylase activity was determined at a tyrosine concentration of 10 μM and a $DMPH_4$ concentration of 100 μM and is expressed as picomoles of dopa formed per milligram of protein per minute ± standard error of the mean.

Additions to assay medium	Tyrosine hydroxylase activity			
	n	Medulla pons	*n*	Cortex
None	36	8.2 ± 0.9	6	3.6 ± 0.9
Calcium (50 μM)	24	33.2 ± 2.9	6	13.8 ± 2.1
Barium (100 μM)	12	28.7 ± 3.9	6	11.7 ± 1.3
Magnesium (1.0 mM)	12	8.6 ± 1.5		
EGTA (50 μM)	12	8.4 ± 1.1	6	7.4 ± 1.2
EGTA (1.0 mM)	12	8.0 ± 0.7		
Calcium (50 μM) + EGTA (50 μM)[a]	12	8.3 ± 1.5	6	7.1 ± 1.8
Calcium (50 μM) + magnesium (100 μM)[b]	12	30.4 ± 3.8		
Magnesium (100 μM) + calcium (50 μM)[c]	12	15.6 ± 2.1		

[a] EGTA (50 μM) was added to the reaction mixture 5 min after calcium and just prior to substrate.

[b] Magnesium (100 μM) was added to the reaction mixture 5 min after calcium. Magnesium (100 μM) alone had no effect on tyrosine hydroxylase activity.

[c] Calcium (50 μM) was added to the reaction mixture 5 min after magnesium. When calcium (50 μM) was added together with substrate, in the absence of magnesium, tyrosine hydroxylase activity was 31.6 ± 3.7 pmoles of dopa per milligram of protein per minute.

hydroxylase from rat medulla pons. The effect was not, however, as dramatic as the one produced by 50 μM Ca^{++} (404% of control compared with 350% of control). Mg^{++}, which blocks many Ca^{++}-dependent processes [e.g., release of catecholamines from sympathetic nerve terminals (23) and also from the adrenal medulla (23, 30)], had no effect on tyrosine hydroxylase activity up to 1 mM. However, Mg^{++} (100 μM), when added to the incubation mixture prior to or together with equimolar Ca^{++}, partially antagonized the activation of tyrosine hydroxylase produced by Ca^{++} alone. This inhibitory effect was not seen if Mg^{++} was added to the enzyme after incubation with Ca^{++} for 10 min.

The Ca^{++} chelator EGTA caused no change in tyrosine hydroxylase activity in

concentrations up to 1 mM. However, EGTA completely blocked the activation of the enzyme by Ca^{++}, suggesting that the effect of Ca^{++} is a specific one.

Effect of Ca^{++} on kinetics of tyrosine hydroxylase from rat medulla pons. In order to determine the mechanism of the activation of tyrosine hydroxylase produced by Ca^{++}, the effect of Ca^{++} on the kinetic properties of tyrosine hydroxylase was studied. The K_m of the enzyme for the substrate, tyrosine (Fig. 2), was determined by the method of Lineweaver and Burk (31) at a $DMPH_4$ concentration of 1 mM. The control value of the K_m for tyrosine of 58.1 μM fell to 10.3 μM in the presence of 100 μM Ca^{++}. Under these conditions there was no significant change in the maximal velocity of the enzyme reaction. In another series of experiments we studied the effect of Ca^{++} on the K_m of tyrosine hydroxylase for $DMPH_4$. In the presence of a saturating tyrosine concentration (100 μM) the K_m for $DMPH_4$ decreased from 673 μM in the untreated enzyme to 125 μM in the presence of Ca^{++}. Here again there was no significant change in the V_{max}.

The K_i of tyrosine hydroxylase from rat medulla pons for norepinephrine was determined at three concentrations of $DMPH_4$—1 μM, 10 μM, and 100 μM—and a saturating concentration of tyrosine (100 μM). Norepinephrine concentrations ranged from 0.01 to 10 mM. From Fig. 3, in

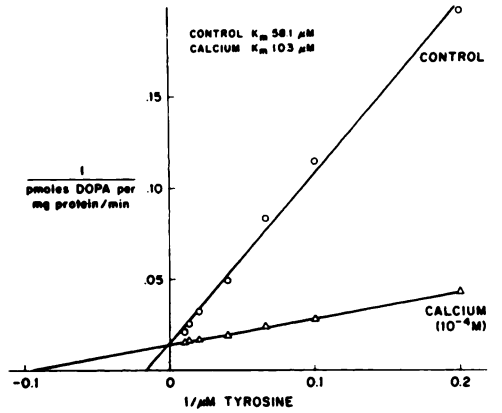

FIG. 2. *Effect of calcium on K_m of tyrosine hydroxylase from rat medulla pons for tyrosine*

The K_m for tyrosine was determined according to the method of Lineweaver and Burk (31) at a $DMPH_4$ concentration of 1.0 mM and seven tyrosine concentrations ranging between 10 and 100 μM. Each K_m value is the mean of intercepts generated from six separate lines.

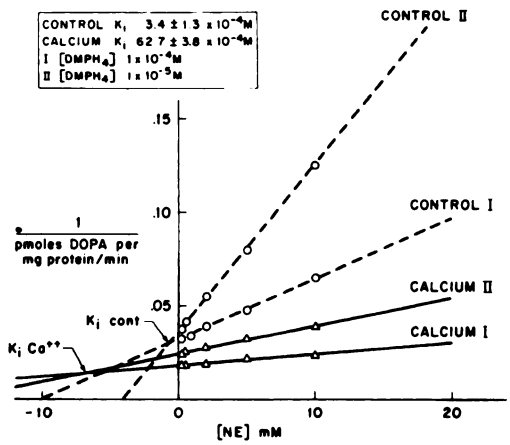

FIG. 3. *Effect of calcium on K_i of tyrosine hydroxylase from rat medulla pons for norepinephrine*

The K_i for norepinephrine (NE) was determined according to the method of Dixon (32) at two $DMPH_4$ concentrations, 10 and 100 μM, and five norepinephrine concentrations ranging from 0.05 to 10 mM. The K_i is expressed as the mean ± standard error of six determinations.

TABLE 2

Effects of known tyrosine hydroxylase inhibitors on activity of calcium-activated enzyme from medulla pons

Tyrosine hydroxylase activity was determined at a tyrosine concentration of 10 μM and a $DMPH_4$ concentration of 100 μM. The tyrosine hydroxylase activities of the control and calcium (100 μM)-activated enzyme were 7.3 ± 1.6 and 34.4 ± 3.0 pmoles of dopa formed per milligram of protein per minute, respectively.

Inhibitor	*n*	Inhibition	
		Control enzyme	Calcium-activated enzyme
		%	%
3-Iodo-L-tyrosine (1 μM)	6	51 ± 3	46 ± 8
α,α-Dipyridyl (0.1 mM)	6	48 ± 5	49 ± 5
3,4-Dihydroxyphenyl-propylacetamide (20 μM)	6	57 ± 3	17 ± 2

which the results have been plotted according to Dixon (32), the K_i of the Ca^{++}-activated enzyme for norepinephrine increased from 0.34 mM to 6.27 mM. In other words, the affinity of the enzyme for norepinephrine in the presence of Ca^{++} is only one twentieth of that of the control enzyme.

Effect of inhibitors of tyrosine hydroxylase. Table 2 compares the effects of three tyrosine hydroxylase inhibitors on the Ca^{++}-activated and control enzyme. In this experiment subsaturating concentrations of both tyrosine (10 μM) and cofactor $DMPH_4$ (100 μM) were used. Under these assay conditions the percentage inhibition produced by 3-iodotyrosine (1 μM) and α,α-dipyridyl (100 μM) were not significantly different in control and Ca^{++}-activated enzymes. On the other hand, 3,4-dihydroxyphenylpropylacetamide (20 μM), a catechol derivative, was a far less effective inhibitor of the Ca^{++}-activated enzyme than of the control enzyme.

Activation of tyrosine hydroxylase of rat cerebral cortex by Ca^{++}. The cortex of the rat brain is an area which is rich in noradrenergic terminals but contains some dopaminergic neurons as well (33, 34). The tyrosine hydroxylase from this region was found to be activated by Ca^{++} in a manner which resembled that seen with tyrosine hydroxylase isolated from the rat medulla pons (Fig. 1). This activation by Ca^{++} could be partially reversed by EGTA (Table 1). The effects of Ca^{++} on the kinetics of tyrosine hydroxylase from rat cortex were similar to those for the enzyme from medulla pons (Table 3). The K_m of tyrosine hydroxylase for tyrosine decreased from 50.1 μM to 9.5 μM in the presence of Ca^{++} (50 μM), and the K_m of the enzyme for $DMPH_4$ decreased from 790 μM to 250 μM with Ca^{++} (50 μM). Finally, a similar change in the K_i of the enzyme for norepinephrine was noted in the presence of Ca^{++}. An unusual feature of the tyrosine hydroxylase isolated from the cortex was that, in contrast to the tyrosine hydroxylase obtained from the medulla pons, its activity was significantly increased by the addition of EGTA alone (50 μM).

DISCUSSION

In this paper the effects of Ca^{++} have been examined on the activity of tyrosine hydroxylase from two distinct regions of the rat brain, the medulla pons, in which a large number of norepinephrine-containing cell bodies are present together with numerous terminals presumed to originate from noradrenergic cells clustered in the midbrain [A-4, 5, and 6 according to Dahlström and Fuxe (35, 36)], and the cortex, which contains norepinephrine terminals coming from cell bodies in the locus ceruleus as well as dopamine-containing fibers of unknown origin (36).

TABLE 3

Effect of Ca^{++} and EGTA on kinetic properties of tyrosine hydroxylase from cerebral cortex

K_m for tyrosine was determined according to the method of Lineweaver and Burk (31) at a $DMPH_4$ concentration of 1.0 mM and seven tyrosine concentrations ranging from 1.0 to 100 μM. The K_m values are expressed as the means ± standard errors of the intercepts from six separate lines. K_m for $DMPH_4$ was determined according to the method of Lineweaver and Burk (31) at a tyrosine concentration of 100 μM and seven $DMPH_4$ concentrations ranging from 0.05 to 1.0 mM. The K_m values are expressed as the means ± standard errors of the intercepts from three separate lines. K_i for norepinephrine was determined according to the method of Dixon (32) at three $DMPH_4$ concentrations (1, 10, and 100 μM) a tyrosine concentration of 100 μM and seven norepinephrine concentrations ranging from 0.05 to 10 mM. The K_i values are expressed as the means ± standard errors of six determinations.

Addition to assay medium	K_m tyrosine	K_m $DMPH_4$	K_i norepinephrine
	μM	mM	mM
Control	50.1 ± 1.5	0.79 ± 0.04	0.16 ± 0.02
Calcium (50 μM)	9.5 ± 1.9	0.25 ± 0.02	3.94 ± 0.17
EGTA (50 μM)	28.9 ± 3.8	0.64 ± 0.07	5.66 ± 0.95
Calcium + EGTA	45.8 ± 3.9	0.77 ± 0.05	0.14 ± 0.05

Tyrosine hydroxylase is the rate-limiting step in the conversion of tyrosine to dopamine and norepinephrine (15), and for this reason it has been the target of investigations aimed at determining the mechanism regulating norepinephrine and dopamine synthesis in both peripheral sympathetic and central noradrenergic and dopaminergic neurons. Clearly any change in the activity or levels of this enzyme must have a profound effect on the rate at which norepinephrine is formed. In the present experiments Ca^{++} was found to activate soluble preparations of tyrosine hydroxylase from both regions of the rat brain examined. In the case of the enzyme from the medulla pons, the effect was reversed by the Ca^{++}-chelating agent EGTA. It was also found that Ba^{++}, which can substitute for Ca^{++} in certain Ca^{++}-dependent processes (e.g., stimulus-secretion coupling) (30, 37), activated tyrosine hydroxylase, while Mg^{++}, which antagonizes many of the effects of Ca^{++} (30), prevented Ca^{++} from activating tyrosine hydroxylase when added to the incubation mixture before Ca^{++}. These results suggest that Ca^{++}, which is known to be essential for the release of transmitter from peripheral sympathetic neurons (22, 38, 39), brain slices (40), and synaptosomes (23) during nerve stimulation or depolarization, may also be directly involved in modulating norepinephrine formation during nerve activity.

The evidence presented in this paper indicates that Ca^{++} produces alterations in the kinetic properties of tyrosine hydroxylase. This effect could account for the increase in the formation of norepinephrine, which has been shown indirectly to take place in central noradrenergic neurons stimulated *in vivo* (9–12), and more directly in slices of rat cortex depolarized by a potassium-rich medium (13). Not only is the affinity of the enzyme increased for both its substrate, tyrosine, and the artificial cofactor, $DMPH_4$, in the presence of Ca^{++}, but norepinephrine, which can reduce its own synthesis by feedback inhibition of tyrosine hydroxylase, becomes a much poorer inhibitor of this enzyme. Another catechol inhibitor, 3,4-dihydroxyphenylpropylacetamide, is also a less effective inhibitor in the presence of Ca^{++}.

At present it is unclear whether the changed affinity of the enzyme for substrate and pteridine cofactor seen *in vitro* is an important factor in bringing about the apparent increase in enzyme activity that is seen during stimulation of central noradrenergic neurons *in vivo* or following depolarization of noradrenergic neurons present in slices of rat cortex. The reason for this is that tissue tyrosine concentrations approach 0.1 mM and thus may be saturating, although this has recently been disputed (41). Also, the identity and concentrations of the natural cofactor are not known. However, regardless of the actual tissue concentrations of substrate and cofactor, the change in the K_i of the enzyme for norepinephrine in the presence of Ca^{++} may account fully for the alterations in the synthesis of norepinephrine observed in the central nervous system *in vivo* during periods of increased nerve activity.

A reduction in end product inhibition has been postulated to explain the apparent increase in tyrosine hydroxylase activity which occurs in intact tissues during nerve activity (18). The concept of feedback inhibition arose from the finding that catecholamines inhibit purified adrenal tyrosine hydroxylase by competing with the pteridine cofactor for the enzyme (17); it is supported by the pronounced inhibition of norepinephrine synthesis which is seen when intraneuronal levels of norepinephrine are raised by the use of monoamine oxidase inhibitors (18, 42), by certain drugs, such as reserpine or bretylium, which disrupt intraneuronal binding of norepinephrine (18, 43), or by high concentrations of norepinephrine added to the incubation medium (5, 18). Based on these findings it was proposed that the apparent *increase* in tyrosine hydroxylase activity seen during nerve stimulation results from a *decrease* in end product inhibition brought about by norepinephrine release (18). However, since endogenous levels of norepinephrine actually change very little during physiological rates of stimulation, the question arose as to the localization of the norepinephrine involved in the regula-

tion of tyrosine hydroxylase. The storage vesicles from which norepinephrine is released by exocytosis (44) were ruled out, because tyrosine hydroxylase does not appear to be associated with these organelles (45). Accordingly, the suggestion was made that the regulatory pool of norepinephrine must be a small, soluble one with access to tyrosine hydroxylase and readily reduced in size by ongoing transmitter release.

The present results suggest that this decrease in end product inhibition of tyrosine hydroxylase, which is believed to occur during nerve stimulation, may in fact be brought about by an entirely different mechanism. When nerve impulses depolarize the noradrenergic nerve terminal, Ca^{++} is believed to pass across the membrane into the terminal (22) and, in so doing, to bring about transmitter release. What now seems likely is that this same influx of Ca^{++}, in addition to initiating transmitter release, may also produce an activation of tyrosine hydroxylase in the nerve terminal similar to the one seen when Ca^{++} is added to the isolated enzyme. This hypothesis seems even more plausible in view of the observation that the concentration of Ca^{++} required to activate the isolated enzyme is similar to estimates of ionized Ca^{++} present in the depolarized nerve terminal (46). Thus, during nerve stimulation, the enhanced formation of norepinephrine in the intact nerve terminal may arise from an altered affinity of the enzyme for the end product inhibitor, the substrate, and the pteridine cofactor rather than from a change in the availability of the feedback inhibitor. Whether the affinity of tyrosine hydroxylase for molecular oxygen is also increased in the presence of Ca^{++} has not been determined, but this could also enhance enzyme activity if tissue oxygen levels were of the order of the K_m of this enzyme for oxygen.

In summary, the evidence presented in this paper suggests that Ca^{++} may play a direct role in the activation of tyrosine hydroxylase of central noradrenergic neurons which occurs during nerve stimulation. Further experiments will be needed to determine whether the activation of the isolated enzyme by Ca^{++} is mimicked by comparable changes in the enzyme produced by nerve stimulation *in situ* or *in vivo*. The evidence from peripheral sympathetic tissues (20, 21) and preliminary results on the rat hippocampus (47) indicate that nerve stimulation produces a rather stable activation of tyrosine hydroxylase which is unaffected by the procedures used to isolate the enzyme and which resembles kinetically the increase in activity produced by incubating the untreated, isolated enzyme with Ca^{++}. Thus it seems very likely that Ca^{++} not only may be involved in stimulus-secretion coupling but may also play a role in the regulation of the synthesis of at least one group of transmitter substances, the catecholamines.

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