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Potassium-Induced Acceleration of Catecholamine Biosynthesis in Brain Slices

I. A Study on the Mechanism of Action

JANE E. HARRIS[1] AND ROBERT H. ROTH

Departments of Pharmacology and Psychiatry, Yale University School of Medicine, New Haven, Connecticut 06510

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SUMMARY

Incubation of cortical or striatal slices in a K^+-enriched medium (53 mM) enhanced the release of newly synthesized catecholamines and accelerated the synthesis of catecholamines at the rate-limiting tyrosine hydroxylase step. The specific activity of the ^{14}C-tyrosine found in the tissues after incubation in a K^+-enriched medium was similar to that of control tissues. Thus the enhancement of catecholamine synthesis observed in the K^+-enriched medium did not appear to arise from an increase in the specific activity of the precursor. No evidence was obtained for any alteration in the level of tyrosine hydroxylase activity in homogenates of tissues previously incubated in a K^+-enriched medium. Conditions which block the release of catecholamines, such as absence of calcium or high magnesium concentrations, blocked the release of newly synthesized catecholamines and simultaneously antagonized the K^+-induced acceleration of catecholamine biosynthesis. On the other hand, the catecholamine synthesis rate of tissues incubated in normal Krebs-Ringer-phosphate buffer was enhanced by initial incubation of the slices in a K^+-enriched medium. Furthermore, when catecholamines were added to the medium at a concentration as low as 10^{-7} M, the catecholamine synthesis rate was inhibited; this inhibition occurred intraneuronally, since the inhibitory effect of exogenous norepinephrine was antagonized by cocaine, even under conditions in which tissue levels of exogenous amines remained the same. These results support the hypothesis that neuronal depolarization enhances the release of newly formed catecholamines, which, in turn, relieves the rate-limiting enzyme, tyrosine hydroxylase, from end-product inhibition and thereby accelerates the catecholamine synthesis rate.

INTRODUCTION

In peripheral sympathetically innervated tissues, the maintenance of a constant norepinephrine concentration, despite varying degrees of nervous activity, has been postulated to be the result of changes in the local biosynthesis of norepinephrine. In several species and tissues, the electrical stimulation of sympathetic nerves which innervate various effector organs has been observed to accelerate norepinephrine biosynthesis. Electrical stimulation of the guinea pig hypogastric nerve (1, 2), the rat stellate ganglion (3), and the rabbit recurrent cardiac nerve (4) enhanced the conversion of tyrosine

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to norepinephrine in the vas deferens, heart, and pulmonary artery, respectively. In the rat submaxillary gland, the synthesis of norepinephrine *in vivo* from ^{14}C-tyrosine was accelerated 5–6-fold by electrical stimulation of the cervical sympathetic chain, while the formation of norepinephrine was reduced approximately 50% with decentralization (5). In additional experiments, results showed that newly synthesized ^{14}C-norepinephrine was released more readily by nerve impulses than exogenous ^{3}H-norepinephrine that was taken up by adrenergic nerve terminals (5, 6).[2] Thus, in peripheral sympathetic neurons, it has been postulated that the acceleration of norepinephrine synthesis produced by sympathetic activation might be due to the release of some strategic norepinephrine pool which normally gives rise to partial inhibition of tyrosine hydroxylase.

In the central nervous system the indirect stimulation of central neurons, either by environmental stress or by drugs, has also been observed to increase catecholamine biosynthesis. By indirectly increasing neuronal activity with electronconvulsive shock (7) or exposure to cold (8), or with blockade of catecholaminergic receptors by phenoxybenzamine (9) or chlorpromazine (10, 11), an enhancement of tyrosine hydroxylase activity has been demonstrated in certain brain regions.

The purpose of the present investigation was twofold: first, to examine the effect of direct neuronal depolarization by potassium on the rate of catecholamine biosynthesis and on the release of newly formed catecholamines from rat cortical and striatal slices and, second, to attempt to define the mechanism or mechansims responsible for the observed acceleration of catecholamine biosynthesis induced by potassium depolarization.

A preliminary report of some of these results has been published (12).

METHODS

Male Sprague-Dawley rats (200–250 g), obtained from Charles River Laboratories, were killed by decapitation, and their brains were rapidly removed and placed in cold, oxygenated Krebs-Ringer-phosphate buffer, pH 7.4. Slices from the cerebral hemispheres were prepared with a bow slicer and a glass slide, recessed about 0.3 mm, according to the technique described by McIlwain and Rodnight (13). Slices were then floated into cold, oxygenated medium, subsequently drained briefly on filter paper, weighed (approximately 150–200 mg), and then transferred to a 20-ml beaker containing 5.0 ml of KRP buffer.[3] The medium contained 128 mM NaCl, 4.8 mM KCl, 0.75 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM glucose, and 16 mM Na_2HPO_4, at pH 7.4. When a K^+-enriched medium was employed, NaCl was replaced with equimolar quantities of KCl.

Incubations were carried out in a Dubnoff metabolic shaker at 37° under saturation with 95% O_2–5% CO_2. Following a 10-min initial incubation, unless indicated otherwise, a total of 18 μg of uniformly labeled L-tyrosine-^{14}C (specific activity, 12.5 mCi/mmole) was added to the medium, producing a final tyrosine concentration of 2×10^{-5} M. In some cases the decarboxylase inhibitor NSD-1055 (4-bromo-3-hydroxybenzyloxyamine) was employed to inhibit the conversion of tyrosine to catecholamines at the decarboxylation step, resulting in the accumulation of labeled dopa. Preliminary experiments indicated that NSD-1055 at a concentration of 10^{-4} M caused a 91 ± 1% blockade of the conversion of newly formed dopa to catecholamines. After the slices had been incubated for an additional 15–30 min, the beakers were chilled on ice and slices were separated from the medium by filtration, collected from the filter paper, and homogenized in 10% trichloracetic acid; each incubation medium was acidified with 0.5 ml of 50% trichloracetic acid. Unlabeled NE, dopamine, dopa, and tyrosine (50 μg each) were added to both tissue homogenates and medium, and the precipitated protein was then removed by centrifugation at 10,000 rpm for 10 min. To the supernatant solution were added 0.2 ml of 1.0 M Tris buffer (pH 8.2), 1 ml of 10% EDTA, and

[2] J. Hughes and R. H. Roth, unpublished observations.

[3] The abbreviations used are: KRP, Krebs-Ringer-phosphate; dopa, 3,4-dihydroxyphenylalanine; NE, norepinephrine.

2.0 ml of 0.5 M KH_2PO_4. Each sample was brought to a final volume of 10 ml and neutralized to pH 8.4 by titration with NaOH. Samples were then passed twice over a (14 × 4 mm column of aluminum oxide (British Drug Houses) in order to retain the catechol compounds selectively (14). The effluent and the first 10 ml of H_2O wash, containing the labeled tyrosine and noncatechols, were saved for subsequent analysis by ion-exchange chromatography, and the columns were washed with an additional 35 ml of distilled water. The catecholamine compounds were eluted with 2 ml of perchloric acid (1.0 ml of 0.2 M and 1.0 ml of 0.1 M), and the deaminated catecholamines were subsequently eluted with 2.0 ml of 1.0 N HCl; 0.4 ml of the 2.0-ml elutes was pipetted into 20 ml of scintillation fluid (15.0 g of 2,5-diphenyloxazole, 300 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, 240 g of naphthalene, 1.0 liter of toluene, 1.0 liter of dioxane, and 1.0 liter of ethanol). The procedure provided a NE recovery of 83.9% ± 4% and a dopamine recovery of 84.9 ± 3.4% (mean ± standard deviation).

The figures presented in the following tables have not been corrected for recoveries on alumina. Since 80–90% of the labeled catechols were shown to be catecholamines (method described in the next section), the catecholamine synthesis rate (nanomoles per gram, wet weight, per hour) was calculated by dividing the disintegrations per minute of catecholamines formed per gram tissue per hour by the specific activity of the tyrosine added to the medium and multiplying by 1.14 to correct for the label lost during the decarboxylation reaction. Tissue blanks were run by incubating cortical slices which were initially precipitated with 15% trichloracetic acid, with ^{14}C-tyrosine. *O*-Methylated catecholamine metabolites represented less than 5% of the newly formed catecholamines.

In experiments utilizing the striatum, slices of tissue were prepared from rat brain as follows. A coronal section was made about 4 mm from the anterior pole of the cerebral hemispheres, and a coronal slice about 5.0 mm thick was cut from the posterior portion. The slice was placed on a piece of wet filter paper, and the striata (weighing about 50–60 mg) were dissected out. Slices of the striatum were made with a Stadie blade and placed in 5.0 ml of KRP solution; other conditions were identical with those described above for cortical slices.

Isolation of catechol metabolites. To determine the percentage of newly synthesized catecholamines (NE and dopamine) in the alumina perchloric acid eluate, the pH was adjusted to 4.0 with KOH, and the supernatant solution was chromatographed on Dowex 50W-X4 (Na^+), 100–200 mesh (12 cm), by a modification of the procedure of Rutledge and Weiner (15). The resin was washed with 15 ml of 0.1% disodium EDTA, and the dopa was collected by elution with 15 ml of 0.1 M Na_2HPO_4 buffer, pH 6.5, and 10 ml of water; the NE was then eluted with 30 ml of 1.0 M HCl (three 10-ml fractions), and the dopamine, in 30 ml of 2.0 N HCl. The effluent, dopa, NE, and dopamine fractions were lyophilized to dryness, taken up in 2.0 ml of 0.25 M HCl, and placed in 20 ml of scintillation fluid. The recoveries of NE and dopamine were about 60% each, and that of dopa was about 77%. In the alumina perchloric acid eluates of cortical tissues, newly formed NE and dopamine represented 29% and 51% of the total labeled catechols, respectively; in the perchloric acid eluates of the KRP medium, 36% was NE and 55% dopamine. The percentages of NE and dopamine in cortical tissues incubated in KRP medium containing 53 mM K^+ were similar to those incubated in the normal medium, whereas in the K^+-enriched medium 19% was NE and 78% dopamine.

In the experiments utilizing D,L-dopa-2-^{14}C (specific activity, 4.5 mCi/mmole), following incubation tissue proteins were precipitated with 0.4 N perchloric acid, homogenized, and centrifuged for 10 min at 10,000 rpm. The supernatant fluid was then adjusted to pH 4.0 with KOH, and the precipitate was removed by centrifugation. To each tissue and medium supernatant fraction were added 50 μg each of nonradioactive NE, dopamine and dopa, and the same procedure as that described above was employed to separate catecholamines from dopa. The data have been corrected for recoveries on

Dowex 50W-X4 (Na^+). Tissue blanks were run by incubating cortical slices with labeled dopa and a decarboxylase inhibitor, NSD-1055, at a final concentration of 10^{-4} M.

Assay of tyrosine hydroxylase in vitro. After a 30-min incubation of cortical slices in either normal KRP or 53 mM K^+ KRP medium at 37°, the tyrosine hydroxylase activity was determined in tissue homogenates by a modification of the method of McGeer *et al.* (16). Following incubation of slices, tissues were homogenized in 0.25 M sucrose to yield 10–20 mg (wet weight)/0.1 ml of sucrose. Tissue homogenates (0.1 ml) were then incubated for 5–30 min at 37° in a Dubnoff shaker with 0.1 ml of ^{14}C-tyrosine (specific activity, 32 μCi/μmole; final concentration, 5×10^{-5} M), the decarboxylase inhibitor NSD-1055 (final concentration, 1.5×10^{-4} M), and 0.1 ml of 0.28 M KH_2PO_4 buffer, pH 6.2. The reaction was stopped with 2.0 ml of 15% trichloracetic acid, and to each sample were added 50 μg of non-radioactive tyrosine, dopa, dopamine, and NE. The samples were then centrifuged at 10,000 rpm for 10 min, and the supernatant fractions were poured into 20-ml beakers containing 0.6 ml of 10% EDTA. The contents of the centrifuge tubes were rinsed with 1.5 ml of 0.4 M KH_2PO_4 and 8.4 ml of H_2O and transferred to the 20-ml beakers, to which was added 0.2 ml of 1 M Tris buffer, pH 8.2. The pH was raised to 8.4 by titration with NaOH, and the samples were applied to the alumina column twice, as described previously. The alumina was washed with 40 ml of H_2O, and the dopa was eluted with 4 ml of 0.5 N HCl; 1 ml was counted in 20 ml of scintillation fluid. Tissue blanks were run by incubating tissue homogenates (initially heated to 80–90° for 12–15 min and then cooled in ice) with tyrosine-^{14}C. The V_{max} of tyrosine hydroxylase was determined by varying both the incubation time and the quantity of tissue present in the assay (16). The tyrosine hydroxylase activity was calculated in terms of nanomoles of dopa synthesized per gram, wet weight, per hour, by dividing the disintegrations per minute of dopa per gram per hour by the specific activity of tyrosine added to the medium.

Tyrosine assay. Following a 30-min incubation of cortical slices in either normal KRP or 53 mM K^+ KRP medium with ^{14}C-tyrosine (specific activity, 12.5 mCi/mmole; final concentration 2×10^{-5} M), the specific activity of tyrosine was determined in both tissues and medium. The catechols were separated from the tyrosine on alumina columns by the method described previously. The tyrosine was collected in both the alumina effluent and the first 10 ml of water wash. The pH was brought down to 2.0 with HCl, and the effluent was applied to a 4-cm column of Dowex 50W-X8 (H^+), 100–200 mesh, and washed with 10 ml of 0.1% EDTA and 20 ml of H_2O in order to remove any labeled deaminated non-catechols. The tyrosine was eluted with 5 ml of 1 M NH_4OH, evaporated, and brought up in 1 ml of water; of this 1 ml, 25 μl were counted in 20 ml of scintillation fluid, and the remainder was used to assay for tyrosine by the nitroso-naphthol fluorescent method (17, 18).

RESULTS

Increased synthesis and release of newly formed catecholamines with K^+ in cortical and striatal slices. In an attempt to determine the effects of neuronal activation on the synthesis and release of newly formed catecholamines, the use of a K^+-enriched medium provided us with a simple reliable method for depolarizing excitable membranes of central adrenergic neurons (19). The catecholamine synthesis rate was determined *in vitro* by separately analyzing the medium and tissues for labeled catecholamines after incubation of rat brain slices for 30 min in oxygenated Krebs-Ringer-phosphate buffer, pH 7.4, at 37°, with ^{14}C-tyrosine. The catecholamine synthesis rate (total nanomoles of catecholamines synthesized per hour per gram of tissue, wet weight) was determined by adding the nanomoles of newly formed catecholamines released into the medium to the nanomoles of newly formed catecholamines remaining in the tissues. By measuring the labeled catecholamines formed in cortical slices following a 30-min incubation with various concentrations of tyrosine, the apparent K_m for the over-all reaction was determined to be 3.5 μM, and the apparent V_{max} to be 0.80 nmole/g, wet weight, per

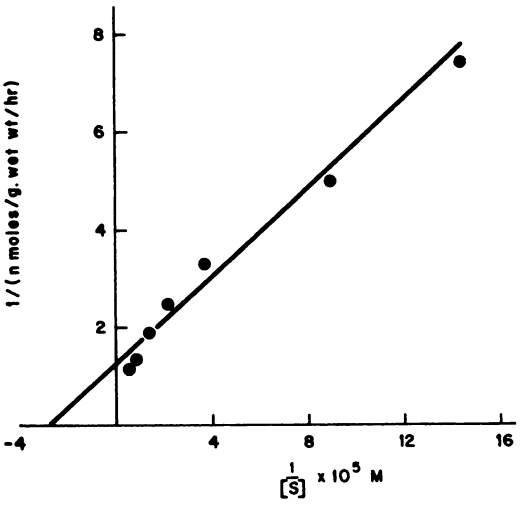

FIG. 1. *Kinetic analysis of catecholamine biosynthesis in cortical slices*

Cortical slices were incubated for 30 min in KRP medium at 37° with various concentrations of ^{14}C-tyrosine, and total catecholamine synthesis was determined as described under METHODS. A Lineweaver-Burk plot was constructed, and the line giving the best fit produced an apparent K_m of 3.5 μM and an apparent V_{max} of 0.80 nmole/g, wet weight, per hour, with a linear regression coefficient of 0.992.

hour (Fig. 1). With a saturating concentration of labeled tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole), the synthesis of labeled catecholamines was found to be linear for at least 30 min. Elevation of the K^+ concentration in the medium to 53 mM resulted in a 6-fold increase in the release of newly formed catecholamines, and the total ^{14}C-catecholamines synthesized in cortical and striatal slices increased by about 80% and 55%, respectively (Table 1). When the K^+ concentration was elevated to 80 mM, the release of labeled catecholamines from cortical slices was increased about 10-fold, while the synthesis rate was doubled. In additional experiments, this K^+ effect was shown not to be dependent on the Na^+ concentration in the medium or on permeability of the tissues to sodium, since neither restoring the Na^+ concentration to normal nor prior treatment with tetrodotoxin (10^{-6} g/ml) antagonized the stimulation of catecholamine biosynthesis by K^+.

Since K^+ may alter the membrane permeability of brain slices to tyrosine, it was important to determine at the outset whether the acceleration of synthesis observed in the above experiments could be explained by an

TABLE 1

Synthesis and release of ^{14}C-catecholamines from brain slices in K^+-enriched KRP medium

Cortical or striatal slices were incubated for 30 min in either normal or K^+-enriched medium with ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole). Following incubation, the tissues and medium were analyzed separately for labeled catecholamines as described under METHODS. Values for catecholamine synthesis are presented as means ± standard errors; the ratio of labeled catecholamines remaining in the tissues to those released into the medium is represented in the last column as T:M.

Medium	n[a]	Catecholamine synthesis			T:M
		Tissue	Medium	Total	
		nmoles/g/hr			
Cortical slices					
KRP	11	0.73 ± 0.03	0.10 ± 0.01	0.83 ± 0.03	7.3
53 mM K^+ KRP	17	0.87 ± 0.03	0.65 ± 0.03	1.52 ± 0.05[b]	1.3
80 mM K^+ KRP	4	0.62 ± 0.01	1.05 ± 0.05	1.67 ± 0.05[b]	0.6
Striatum					
KRP	5	13.0 ± 0.7	0.5 ± 0.1	13.5 ± 0.7	26.0
53 mM K^+ KRP	3	17.8 ± 1.0	3.1 ± 0.2	20.9 ± 0.9[c]	5.8

[a] n = number of separate experiments.
[b] $p < 0.01$ when compared to KRP controls.
[c] $p < 0.05$ when compared to KRP controls.

alteration in the specific activity of labeled tyrosine precursor in the slices. The possibility of an altered rate of tyrosine uptake into the slices was ruled out by demonstrating that the specific activity of tyrosine was not altered in slices at the end of the incubation in 53 mM K^+ KRP medium compared to slices incubated in normal medium. However, the possibility that 53 mM K^+ might selectively alter the ability of the sympathetic neurons to accumulate ^{14}C-tyrosine could not be resolved.

Increase in catecholamine synthesis by K^+, and localization at the tyrosine hydroxylase step. In order to demonstrate that the K^+-induced acceleration of catecholamine synthesis occurred at the rate-limiting tyrosine hydroxylase step and was not a result of some effect on an event beyond the formation of dopa, the following experiments were performed. ^{14}C-dopa was used to bypass the tyrosine hydroxylase step, and an analysis of the labeled dopamine and NE contents in the medium and cortical slices showed that high K^+ concentrations enhanced the release of newly synthesized catecholamines but that the total amount of newly synthesized catecholamines did not significantly increase (Table 2). Furthermore, when the decarboxylase inhibitor NSD-1055 was utilized to prevent the further conversion of dopa to catecholamines, the direct effect of K^+ stimulation on the hydroxylation of tyrosine to dopa was substantiated (Table 3).

Tyrosine hydroxylase activity unaltered in tissue homogenates following incubation in high-K^+ KRP medium. One important mechanism which was considered is that the persistent depolarization by K^+ might alter the ability of catecholamine-containing

TABLE 2

Synthesis of catecholamines from ^{14}C-dopa in cortical slices incubated in K^+-enriched medium

Cortical slices were incubated for 30 min with dopa-2-^{14}C (2.2×10^{-5} M; specific activity, 4.5 mCi/mmole) in either normal or 53 mM K^+ KRP medium. The labeled catecholamines were determined in both the tissues and medium as described under METHODS. Values for catecholamine synthesis are presented as means ± standard errors; the tissue to medium ratio for labeled catecholamines is shown in the last column as T:M.

Medium	n[a]	Catecholamine synthesis			T:M
		Tissue	Medium	Total	
		nmoles/g/hr			
KRP	3	11.7 ± 0.4	6.1 ± 1.7	17.8 ± 1.5	1.9
53 mM K^+ KRP	3	7.6 ± 0.1	10.0 ± 1.8	17.6 ± 1.8	0.8

[a] n = number of separate experiments.

TABLE 3

Synthesis of ^{14}C-dopa from ^{14}C-tyrosine in cortical slices incubated in 53 mM K^+ KRP medium

Cortical slices were incubated for 10 min with NSD-1055 (10^{-4} M), and then for an additional 30 min with ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole) in either normal or 53 mM K^+ KRP medium. The ^{14}C-dopa content was analyzed in both the tissues and medium by the same method described for catecholamine determinations. Values for dopa synthesis are presented as means ± standard errors; the tissue to medium ratio for labeled dopa is shown in the last column as T:M.

Medium	n[a]	Dopa synthesis			T:M
		Tissue	Medium	Total	
			nmoles/g/hr		
KRP + NSD-1055 (10^{-4} M)	6	0.50 ± 0.05	0.34 ± 0.04	0.84 ± 0.02	1.5
53 mM K^+ KRP + NSD-1055 (10^{-4} M)	6	0.78 ± 0.07	1.00 ± 0.03	1.78 ± 0.07[b]	0.8

[a] n = number of separate experiments.

[b] Significantly different from control ($p < 0.01$).

neurons to form catecholamines by increasing the amount of tyrosine hydroxylase or pteridine reductase in the neuron. This possibility was tested by determining whether crude synaptosomes prepared from slices incubated in 53 mM K^- KRP buffer had an increased capacity to hydroxylate tyrosine. In this study, cortical slices were incubated in either normal medium or medium containing 53 mM K^+ for 30 min; the slices were then removed and homogenized in sucrose, and the tyrosine hydroxylase activity in the homogenate was estimated by monitoring the conversion of labeled tyrosine to dopa in the presence of a decarboxylase inhibitor. Each value in Table 4 represents the V_{max} of tyrosine hydroxylase, determined in separate experiments by varying both the incubation time and the quantity of tissue present in the assay. In both the first and second sets of experiments, there was no significant increase in the tyrosine hydroxylase activity in tissue homogenates following incubation of slices in 53 mM K^+ KRP medium. Thus the ability of K^+ to cause an increase in the conversion of tyrosine to dopa, as observed in cortical slices incubated in high-K^+ medium, is lost when the tissue is homogenized.

TABLE 4

Tyrosine hydroxylase activity in tissue homogenates following incubation of cortical slices in 53 mM K^+ KRP medium

Following a 30-min incubation in either normal or 53 mM K^+ KRP medium, cortical slices were homogenized and the tyrosine hydroxylase activity was measured as described under METHODS. Each value represents a separate determination.

Medium	Expt	Dopa formation
		nmoles/g/hr
KRP	I	1.8
		1.8
	II	1.2
		1.2
53 mM K^+ KRP	I	2.1
		2.0
	II	1.2
		1.1

Effect of Ca^{++} and Mg^{++} on catecholamine biosynthesis. In order to determine whether the K^+-induced acceleration of catecholamine synthesis might be dependent on the increased release of catecholamines, the Mg^{++} and Ca^{++} concentrations were manipulated to inhibit the release of labeled catecholamines. In previous investigations, Baldessarini and Kopin (20) observed that the release of exogenously labeled catecholamines from brain slices was Ca^{++}-dependent and that, by removing the Ca^{++} from the medium, the stimulus-induced release of labeled catecholamines was antagonized. The effect of K^+ on the synthesis and release of labeled catecholamines from cortical slices in the absence of Ca^{++} or in the presence of high Mg^{++} concentrations is shown in Table 5. In the absence of Ca^{++}, the K^+-induced release of newly formed catecholamines was reduced by 76% while the K^+-accelerated catecholamine synthesis rate was inhibited by 86%. In fact, the synthesis rate in the Ca^{++}-free, K^+-enriched medium was not significantly different from the rate observed in normal KRP medium with or without Ca^{++}. Likewise, when the Mg^{++} concentration was increased 10-fold to 12 mM, the K^+-induced acceleration of catecholamine release and synthesis was depressed by 64% and 49%, respectively.

Increased synthesis of catecholamines following incubation in K^+-enriched KRP medium. If increased release of endogenous or newly formed catecholamines is, indeed, the triggering mechanism for the acceleration of catecholamine biosynthesis in cortical tissue, then the release of tissue catecholamines during preliminary incubation in a K^+-enriched medium should result in at least a temporary increase in catecholamine biosynthesis when the tissues are incubated further in normal KRP medium. Thus, cortical slices were first incubated for 30 min in either normal KRP or 53 mM K^+ KRP medium (a condition observed to accelerate the release of newly synthesized catecholamines 6-fold); tissues were then removed, rinsed, and incubated for an additional 15 min in normal KRP medium containing labeled tyrosine (Table 6). The preliminary incubation in 53 mM K^+ KRP medium increased the content of labeled catecholamines retained in the tissue by 64% while

TABLE 5

Synthesis and release of ^{14}C-catecholamines from cortical slices incubated in Ca^{++}-deficient or Mg^{++}-enriched 53 mM K^+ KRP medium

Cortical slices were incubated for 30 min with ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole) in either 53 mM K^+ KRP or K^+-enriched medium which had been made Ca^{++}-deficient or enriched with 12 mM Mg^{++}. Tissues and medium were analyzed separately for labeled catecholamines as described under METHODS. Values for catecholamine synthesis are presented as means ± standard errors; in the last column the tissue to medium ratios for labeled catecholamines are shown as T:M.

Medium	n[a]	Catecholamine synthesis			Change	T:M
		Tissue	Medium	Total		
		nmoles/g/hr			%	
Normal KRP	11	0.73 ± 0.03	0.10 ± 0.01	0.83 ± 0.03		7.3
KRP, Ca^{++}-free	4	0.69 ± 0.03	0.13 ± 0.02	0.82 ± 0.03[b]	0	5.3
53 mM K^+ KRP	17	0.87 ± 0.03	0.65 ± 0.03	1.52 ± 0.05[c]	+83	1.4
53 mM K^+ KRP, Ca^{++}-free	8	0.66 ± 0.04	0.26 ± 0.03	0.92 ± 0.03[b]	+11	2.5
53 mM K^+ KRP + 12 mM Mg^{++}	5	0.88 ± 0.03	0.30 ± 0.03	1.18 ± 0.02[c]	+42	2.9

[a] n = number of separate experiments.
[b] Not significantly different from control ($p > 0.05$).
[c] Significantly different from control ($p < 0.01$).

TABLE 6

Effect of prior incubation in K^+-enriched KRP medium on catecholamine synthesis in cortical slices during incubation in normal medium

Cortical slices were first incubated for 30 min in either 53 mM K^+ KRP or normal KRP medium; the slices were then removed, washed, and incubated again in normal KRP medium with ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole) for an additional 15 min. Tissues and medium were analyzed separately for ^{14}C-catecholamines as described under METHODS. Values are presented either as individual determinations or as means ± standard errors.

Medium	n[a]	Catecholamine synthesis		
		Tissue	Medium	Total
			nmoles/g/hr	
KRP (normal incubation)	11	0.73 ± 0.03	0.10 ± 0.01	0.83 ± 0.03
KRP	2	0.63, 0.75	0.22, 0.14	0.85, 0.89
53 mM K^+ KRP (prior incubation for 30 min)	3	1.13 ± 0.07	0.14 ± 0.04	1.27 ± 0.09[b]

[a] n = number of individual experiments.
[b] Significantly different from control ($p < 0.05$).

enhancing the over-all catecholamine synthesis rate by 45%.

Effect of cocaine on NE- and dopamine-induced inhibition of catecholamine biosynthesis. The results presented thus far seem to indicate that in central neurons potassium depolarization increases catecholamine biosynthesis, as a consequence of decreased end-product inhibition of tyrosine hydroxylase by NE and/or dopamine. McGeer and McGeer (21) have demonstrated that tyrosine hydroxylase activity in rat brain homogenates is inhibited by 40% and 69% when incubated with 10^{-4} M *dl*-NE and dopamine, respectively. Thus it was of interest to determine whether small amounts of catecholamines added to the medium could inhibit tyrosine hydroxylase in intact brain slices. Cortical slices were incubated *in vitro* with NE or dopamine at concentra-

tions ranging from 10^{-7} to 10^{-5} M, and the percentage inhibition of catecholamine synthesis was calculated. Catecholamine synthesis was inhibited at a concentration of catecholamines in the medium as low as 10^{-7} M (Table 7). Similarly, in striatal slices, the conversion of tyrosine to dopa was inhibited 25% and 58% at dopamine concentrations of 10^{-6} and 10^{-5} M, respectively.

To determine whether exogenous catecholamines act intraneuronally to inhibit catecholamine synthesis, cocaine, a drug which blocks the neuronal uptake of catecholamines into brain slices, was utilized (22). In these experiments, cortical slices were incubated for 10 min with 10^{-5} M cocaine HCl, and then for 30 min with 10^{-5} M cocaine Hcl, 5×10^{-7} or 2.5×10^{-7} M ^{3}H-NE, and ^{14}C-tyrosine. The percentage inhibition of the catecholamine synthesis rate by NE is presented in Table 8. In the presence of 10^{-5} M cocaine HCl the blockade of neuronal uptake of NE almost completely antagonized the inhibition of catecholamine synthesis by NE. However, incubation of cortical slices in KRP medium containing 10^{-5} M cocaine HCl alone had no effect on the conversion of ^{14}C-tyrosine to labeled catecholamines.

DISCUSSION

The maintenance of constant levels of catecholamines, despite wide variations in sympathetic nerve activity, was postulated some years ago by von Euler (23) to be the result of rapid adjustments in the rate of catecholamine biosynthesis. In several sympathetically innervated organs, electrical stimulation of the sympathetic nerves has been observed to enhance the release of newly formed NE and, furthermore, to increase the total amount of NE synthesized. This greater release of NE seemingly acts to produce a positive feedback effect on NE biosynthesis, by rapid changes in end-product inhibition of the rate-limiting hydroxylation of tyrosine. In brain, the increase in catecholamine levels produced with monoamine oxidase inhibitors has been demonstrated to inhibit the turnover of catecholamines *in vivo*, and from such evidence a similar feedback inhibitory mechanism has been proposed to control catecholamine biosynthesis in central adrenergic neurons (24). Therefore, our experiments were designed to determine whether an increased release of newly formed catecholamines, with direct neuronal stimulation by potassium, is associated with an enhancement of the brain catecholamine synthesis rate.

Since our investigations of the interrela-

TABLE 7

Inhibition of catecholamine biosynthesis in cortical slices incubated with norepinephrine or dopamine

Cortical slices were incubated for 10 min with NE or dopamine (10^{-7}–10^{-5} M), and for an additional 30 min with ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole). Values are presented as means ± standard errors.

Catecholamine concentration	Inhibition of catecholamine synthesis	
	NE	Dopamine
M	%	%
10^{-7}	27.6 ± 3.2 (6)[a]	27.1 ± 2.3 (6)
10^{-6}	44.5 ± 2.8 (4)	54.0 ± 2.4 (6)
10^{-5}	63.3 ± 3.4 (4)	58.3 ± 2.9 (4)

[a] Numbers in parentheses represent the number of experiments.

TABLE 8

Inhibition by NE of catecholamine biosynthesis in cortical slices following incubation with cocaine HCl

Cortical slices were incubated for 10 min with or without cocaine HCl (10^{-5} M); subsequently ^{3}H-NE (2.5×10^{-7} M or 5×10^{-7} M) and ^{14}C-tyrosine (2×10^{-5} M; specific activity, 12.5 mCi/mmole) were added, and the tissues were incubated for an additional 30 min. Tissues and medium were analyzed separately for ^{14}C-catecholamines and ^{3}H-NE as described under METHODS. Values are presented as means ± standard errors of three experiments.

Medium NE	Cocaine HCl	NE uptake into tissues	Inhibition of catecholamine synthesis
M × 10^7	*M*	*nmoles/g*	%
5	0	7.6 ± 1.2	44.9 ± 4.2
5	10^{-5}	3.3 ± 0.1	4.6 ± 1.9
2.5	0	3.1 ± 0.1	33.1 ± 2.2
2.5	10^{-5}	1.8 ± 0.1	2.3 ± 1.4

tionship between catecholamine synthesis and release were carried out in brain slices, a brief discussion of this technique seems warranted. Brain slices provided a system in which the effects of ionic stimulation on the synthesis and release of newly formed catecholamines could be analyzed more directly than with techniques *in vivo*. By using a sufficiently high concentration of exogenous tyrosine (2×10^{-5} M) to saturate the rate-limiting enzyme, tyrosine hydroxylase, and a short enough incubation period (30 min) to produce linearity in the formation of labeled catecholamines, an "apparent" rate of synthesis and release of newly formed catecholamines could be determined. However, since only about 50% of the exogenous tyrosine (at 2×10^{-5} M) was equilibrated with the endogenous tyrosine in the tissues during the period of incubation, and insofar as the specific activity of the tyrosine at the site of its conversion to ^{14}C-dopa in catecholamine-containing neurons cannot be expermentally determined, the catecholamine synthesis rate, based upon the specific activity of exogenous tyrosine, can only be assumed to be "apparent" and not a "true" or "absolute synthesis rate."

It is evident from out data (Table 1) that the incubation of either cortical or striatal slices in a K^+-enriched medium results in an increase in the release of newly formed catecholamines and, in addition, increases the sum total of catecholamines synthesized. The acceleration of catecholamine biosynthesis by a K^+-enriched medium was localized at the tyrosine hydroxylase step by two methods. First, when the rate-limiting step was bypassed with ^{14}C-dopa, the K^+-induced enhancement of catecholamine synthesis could no longer be demonstrated (Table 4). However, since the transformation of dopa to dopamine can be catalyzed in non-catecholamine-containing neurons by the nonspecific aromatic L-amino acid decarboxylase, and inasmuch as the NE formed represented only about 5% of the catecholamines synthesized from ^{14}C-dopa, a second method was employed to verify localization of the K^+ stimulation at the tyrosine hydroxylase step. By preventing the conversion of dopa to dopamine with the decarboxylase inhibitor NSD-1055 (Table 5), the K^+-induced acceleration in the formation of dopa from tyrosine was directly confirmed. In another set of experiments, the incubation of cortical slices in 53 mM K^+ KRP medium was shown not to alter the specific activity of tissue tyrosine. Since the tyrosine utilized for catecholamine biosynthesis probably represents only a small fraction of the total tissue tyrosine pool, it is quite possible that a significant alteration in the specific activity of tyrosine in this pool may not have been reflected in the specific activity of the total tissue tyrosine. However, the fact that the K^+-induced acceleration of catecholamines could be completely blocked by the removal of Ca^{++} from the medium argues against the possibility that this acceleration might have been due to an increase in the specific activity of neuronal tyrosine. Furthermore, if there was an alteration in amino acid uptake with K^+, one might expect a similar increase in catecholamine biosynthesis when slices were incubated with another amino acid precursor, ^{14}C-dopa, in a K^+-enriched medium. However, the synthesis of catecholamines from ^{14}C-dopa in cortical slices was not affected by the K^+-enriched medium, and therefore one may assume that uptake of dopa into the slice may not be significantly influenced by the K^+ concentration in the medium. It also seems evident that the K^+ effect is not dependent on the concentration of or permeability to Na^+, since neither restoring the Na^+ concentration to normal nor prior treatment with tetrodotoxin antagonized the K^+ stimulation of catecholamine biosynthesis. Finally, prior incubation of cortical slices for 30 min in 53 mM K^+ KRP buffer did not alter the tyrosine hydroxylase activity in tissue homogenates (Table 6); thus, an increase in the formation of enzyme (tyrosine hydroxylase) or protection of existing enzyme by potassium, during the short time course of our experiment, is unlikely.

Another possible mechanism to explain the K^+ effect, analogous to that postulated in peripheral sympathetic neurons (25, 26), is that the acceleration of synthesis might be due to the release of some strategic pool of catecholamines, which normally acts to

partially inhibit tyrosine hydroxylase. This seems a likely possibility for two reasons. First, we have observed that catecholamine synthesis in cortical slices can be inhibited by relatively low concentrations of NE and dopamine in the medium (Table 7). Furthermore, cocaine HCl, which blocks neuronal NE uptake, antagonized the feedback inhibition of catecholamine synthesis by NE in cortical slices even when the exogenous NE content in the tissues was maintained at similar concentrations (Table 8). This supports the hypothesis that the product inhibition of their own synthesis by catecholamines in brain slices occurs intraneuronally. Second, Baldessarini and Kopin have demonstrated that K^+ efficiently releases exogenously labeled catecholamines from brain slices (20). Thus the release of endogenous or newly synthesized catecholamines might be expected to relieve tyrosine hydroxylase from the normal end-product inhibition occurring in the neuron. If such a mechanism were operable in cortical tissue, any agent which interferes with the release of catecholamines might also antagonize the potassium-induced increase in synthesis. Previous experiments in both the adrenal medulla (27) and peripheral sympathetic neurons (28) have demonstrated that the release of catecholamines is Ca^{++}-dependent and that removal of the Ca^{++} or increasing the Mg^{++} concentration of the medium antagonizes the K^+-induced release of catecholamines. Furthermore, Baldessarini and Kopin have reported that the electrically induced release of catecholamines from brain slices is also Ca^{++}-dependent (20). In our experiments, the removal of Ca^{++} from the K^+-enriched medium resulted in a marked reduction in the K^+-induced release of newly formed catecholamines and, in addition, inhibited the K^+-stimulated increase of catecholamine biosynthesis (Table 5). Likewise, when the Mg^{++} concentration in the high-K^+ KRP medium was increased 10-fold to 12 mM, the release of newly synthesized catecholamines from cortical slices was reduced and the synthesis rate was also depressed (Table 5). The results of these experiments are highly suggestive that the release of catecholamines is a prerequisite for the K^+-induced acceleration of catecholamine biosynthesis observed in brain slices.

If increased release of endogenous and/or newly formed catecholamines is indeed the triggering mechanism for the acceleration of catecholamine biosynthesis in brain slices, the release of tissue catecholamines from slices incubated in a K^+-enriched medium should induce at least a temporary increase in catecholamine biosynthesis when the slices are incubated again in normal Krebs-Ringer-phosphate medium. The data in Table 6 seem to indicate that the catecholamine synthesis rate of cortical slices incubated in normal KRP buffer can be enhanced by first incubating them in a K^+-enriched medium. Furthermore, the acceleration of catecholamine biosynthesis in these experiments, was demonstrated in cortical tissues incubated with ^{14}C-tyrosine in normal KRP medium, which seems to reinforce the concept that an alteration in the tyrosine uptake or precursor specific activity within the neuron does not account for the K^+-induced acceleration of catecholamine biosynthesis. Work is now in progress in our laboratory to determine whether the specific activity of the catecholamines in the tissues and that released into the medium is altered during incubation in K^+-enriched KRP medium; it is hoped that the results from such a study will provide a basis for determining which of the catecholamine pools in the neuron controls the synthesis rate by end-product inhibition.

Observations in both peripheral (25, 26) and now in central nervous tissue indicate that neuronal depolarization by incubation of tissues in a high-potassium medium results in an acceleration of catecholamine biosynthesis. The evidence obtained in the above experiments is consistent with the hypothesis that neuronal depolarization causes the release of a small, strategic pool of catecholamines, which, in turn, relieves the rate-limiting enzyme, tyrosine hydroxylase, from end-product inhibition and thereby accelerates the rate of catecholamine biosynthesis.

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