Tyrosine 3-Monoxygenase
Regulates Catecholamine
Synthesis in Pheochromocytoma
Cells*

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Incubation of pheochromocytoma cells with 56 mM K+ or with cholera toxin increases the conversion of [14C]tyrosine to [14C]catecholamines (Chalifé, M., Settipani, L., and Perlman, R. L. (1979) Mol. Pharmacol. 15, 263-270). We have now measured the tyrosine content and the rate of dihydroxyphenylalanine production in these cells. Incubation with 56 mM K+ or with cholera toxin increases the rate of dihydroxyphenylalanine production but decreases the tyrosine content of the cells. We have also measured the uptake of tyrosine into pheochromocytoma cells. The rate of tyrosine uptake is more than 1 order of magnitude greater than the rate of dihydroxyphenylalanine production. Moreover, tyrosine uptake is not affected by cholera toxin and is decreased by approximately 30% in media that contain 56 mM K+. These results provide direct evidence that tyrosine 3-monoxygenase regulates catecholamine synthesis in pheochromocytoma cells and that incubation with 56 mM K+ or with cholera toxin causes the activation of this enzyme in these cells.

We have been studying the regulation of catecholamine synthesis in cell suspensions prepared from a transplantable rat pheochromocytoma. These cells contain tyrosine 3-monoxygenase (EC 1.14.16.2), aromatic L-amino-acid decarboxylase (EC 1.14.17.1), and they convert [14C]tyrosine to [14C]norepinephrine (1-3). Incubation of pheochromocytoma cells with cholera toxin or with 56 mM K+ increases the rate of conversion of [14C]tyrosine to [14C]catecholamines (3). Moreover, these treatments cause a stable increase in tyrosine 3-monoxygenase activity, as assayed in vitro (3). These results are consistent with the hypotheses that tyrosine 3-monoxygenase regulates catecholamine synthesis in pheochromocytoma cells and that cholera toxin and 56 mM K+ increase catecholamine synthesis by increasing the activity of this enzyme. The interpretation of these results, however, is subject to several possible complications. The conversion of radioactive tyrosine to catecholamines may be affected not only by the activity of tyrosine 3-monoxygenase, but also by the rate of tyrosine uptake and by the specific activity of the intracellular tyrosine pool. In addition, the activity of tyrosine 3-monoxygenase as measured in vitro may not accurately reflect its activity in intact cells. Rolleston (4) has emphasized the importance of measuring endogenous concentrations of metabolites in analyzing the regulation of metabolic pathways. Demonstration that the substrate of an irreversible reaction decreases under conditions in which the flux through that reaction increases provides strong evidence for the regulatory role of the enzyme that utilizes that substrate. Such an analysis is based on the "crossover theorem" that was developed by Chance et al. (5) for the study of oxidative phosphorylation.

Carlsson et al. (6) have determined the flux through the tyrosine 3-monoxygenase reaction by measuring the accumulation of dopa-1 in the brains of animals that had been treated with an inhibitor of aromatic L-amino acid decarboxylase (6). We have adopted the strategy used by these workers to measure the rate of dopa production by pheochromocytoma cells. In addition, we have measured the total content of these cells and the uptake of tyrosine into the cells. These studies provide direct evidence that tyrosine 3-monoxygenase regulates catecholamine synthesis in pheochromocytoma cells.

EXPERIMENTAL PROCEDURES

Suspensions of pheochromocytoma cells were prepared as previously described (1). Cells were incubated for 30 min at 37°C in a medium that contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM CaCl2, 1.2 mM MgSO4, 10 μM pargyline, 10 mM glucose, and 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) titrated to pH 7.4 with NaOH. In some experiments, this medium also contained 100 μM L-tyrosine. This 30-min incubation with pargyline results in a complete inhibition of amine oxidase (flavin containing, EC 1.4.3.4) activity (2). When indicated, cholera toxin (Schwarz/Mann, Orangeburg, NY) was added to a concentration of 1 ng/ml. All incubations were carried out under an atmosphere of 100% O2. The cells were centrifuged, washed, resuspended in fresh, pargyline-free medium, and then incubated for an additional 90 mm at 37°C in normal or in medium containing 56 mM K+ (prepared by the substitution of KCl for NaCl). These media contained L-tyrosine at the concentrations specified in the text and were supplemented with cholera toxin as indicated. They did not contain pargyline, but did contain 150 μM bromocresine (Lederle Laboratories, Pearl River, NY) to inhibit aromatic L-amino acid decarboxylase (2). Following this incubation, the cells were removed by centrifugation, and the supernatant (i.e., the incubation medium) was saved for amino acid analysis. The cells were washed two times in ice-cold, tyrosine-free medium, and were then resuspended in 300 μl of 0.15 M lithium citrate, pH 2.2, containing 4% sulfosalicylic acid. The protein was removed by centrifugation, and the supernatant, which contained the acid-soluble intracellular contents, was used for amino acid analysis. Amino acid analyses were performed by the single-column, lithium citrate method on a Beckman model 121MB amino acid analyzer equipped with AA-10 resin and nanobore flow systems. In this procedure, dopa elutes between methionine and isoleucine. Aliquots of the cell suspensions were also assayed for protein by the method of Bradford (7). The intracellular tyrosine content is expressed as nmol/mg of protein, mean ± S.E. Most of the dopa produced by the cells is released into the incubation medium. The total amount of dopa produced during the 90-min incubation was calculated as the sum of the content of

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1 The abbreviation used is: dopa, dihydroxyphenylalanine.

2 121M Application Notes, 121-M-013, Beckman Instruments, Inc., Palo Alto, CA, July, 1976.
Tyrosine 3-Monoxygenase Activity in Intact Cells

When pheochromocytoma cells are incubated under control conditions in the presence of brocresine, they produce dopa at a rate of approximately 100 pmol/min/mg of protein (Table I). Incubation of the cells with 56 mM K⁺ or with cholera toxin results in a 2- to 3-fold increase in the rate of dopa production. These experiments therefore provide direct evidence, not dependent upon radioactive tracers, that these treatments increase the flux through the tyrosine 3-monoxygenase reaction in intact pheochromocytoma cells. The Vₘₐₓ of tyrosine 3-monoxygenase in extracts of pheochromocytoma cells is on the order of 1 nmol/min/mg of protein (1). Thus, the activity of tyrosine 3-monoxygenase in intact cells is only a small fraction of the Vₘₐₓ of the enzyme.

Incubation with 56 mM K⁺ or cholera toxin could increase dopa formation by increasing the concentration of tyrosine or by activating tyrosine 3-monoxygenase in the cells. To distinguish between these possibilities, we measured the tyrosine content of the cells. The tyrosine content of cells incubated under control conditions is approximately 2.1 nmol/mg of protein (Table I). Pheochromocytoma cells contain approximately 11 μl of intracellular water/mg of protein. If the tyrosine content of the cells is distributed uniformly throughout the intracellular water, the concentration of tyrosine in the cells would be approximately 190 μM. This value is very much greater than the apparent Kₘ of tyrosine 3-monoxygenase in these cells for tyrosine, as measured in vitro (25 to 30 μM; Ref. 3). Because the intracellular tyrosine concentration is much higher than the apparent Kₘ of tyrosine 3-monoxygenase for tyrosine, it is unlikely that an increase in the tyrosine concentration would greatly increase the rate of dopa formation. As shown in Table I, incubation of the cells with 56 mM K⁺ or with cholera toxin results in a marked decrease in the content of intracellular tyrosine. The tyrosine content of cells incubated with 56 mM K⁺ is approximately 1.1 nmol/mg of protein, or about one-half that in control cells; the tyrosine content of cells incubated with cholera toxin is approximately 1.5 nmol/mg of protein. Conversion to dopa and incorporation into protein are the major routes of tyrosine metabolism in pheochromocytoma cells (2). Neither cholera toxin nor 56 mM K⁺, however, affects the rate of incorporation of [3,5,6-H]tyrosine into protein in these cells. In other experiments (not shown), we have found that brocresine has no effect on the content of tyrosine in pheochromocytoma cells, and that 56 mM K⁺ and cholera toxin decrease intracellular tyrosine in the absence of brocresine. In the absence of brocresine, however, the cells accumulate very little dopa. Under these conditions, presumably, almost all of the dopa produced by tyrosine 3-monoxygenase is converted to catecholamines.

The activity of tyrosine 3-monoxygenase is inhibited by high concentrations of tyrosine in vitro (9). It was conceivable that the activity of tyrosine 3-monoxygenase is normally restrained by substrate inhibition and that cholera toxin and 56 mM K⁺ might increase the rate of dopa production by decreasing the intracellular tyrosine content and thereby relieving this inhibition. In order to study the effect of intracellular tyrosine on the activity of tyrosine 3-monoxygenase, we measured the tyrosine content and the rate of dopa production by cells that were incubated with various concentrations of extracellular tyrosine (Fig. 1). Cells incubated in tyrosine-free media in containing 100 μM nonradioactive tyrosine and were then incubated at 30°C in media that contained 100 μM L-[U-¹³C]tyrosine (Amersham Corp., Arlington Heights, IL), 0.75 μCi/μmol. Samples were removed after 10, 20, and 30 s of incubation, and the cells were separated from the incubation medium by centrifugation through 50 μl of a silicone oil mixture (60% Dow Corning No. 550, specific gravity, 1.07; 40% Dow Corning No. 510, 50 centistokes, specific gravity, 1.00) in a Beckman Microfuge, as previously described (8). The tips of the centrifuge tubes, which contained the cell pellets, were cut off with a razor blade and the cells were solubilized in 0.5 ml of 0.5 N KOH. The samples were neutralized with acetic acid and the radioactivity associated with the cells was measured by liquid scintillation spectrometry. Tyrosine uptake was estimated after subtraction of the radioactivity in the cell pellet prepared from a sample that had been maintained at 0°C in the presence of 1 nm tyrosine and is expressed as nmol/min/mg of protein, mean ± S.E. The rate of tyrosine uptake was constant during this 30-s incubation period.

### Results and Discussion

Tyrosine content and dopa production in pheochromocytoma cells

| Incubation conditions | Tyrosine content | Dopa production |
|-----------------------|-----------------|-----------------|
| Control (n = 7)        | 2.07 ± 0.39     | 116 ± 24        |
| 56 mM K⁺ (n = 4)       | 1.06 ± 0.33     | 242 ± 46        |
| Cholera toxin, 1 μg/ml (n = 7) | 1.50 ± 0.32 | 306 ± 63        |

Fig. 1. Effect of extracellular tyrosine on tyrosine content and on Dopa production. Pheochromocytoma cells were incubated for 90 min in a tyrosine-free medium, or in media containing 20, 50, 100, 150, or 200 μM tyrosine. Following incubation, the cells were removed by centrifugation, and aliquots of the cells and of the incubation media were taken for amino acid analysis. The tyrosine content of the cells is expressed as nmol/mg of protein, mean ± S.E. Dopa production was calculated as the sum of the content of dopa in the cells plus that in the incubation medium and is expressed as pmol/min/mg of protein, mean ± S.E. The number of experiments is shown in parentheses.

B. T. Liang, unpublished observations.
K. K. Vaccaro, unpublished observations.

### Table I

Tyrosine content and dopa production in pheochromocytoma cells

- **Control (n = 7)**: 2.07 ± 0.39 nmol/min/mg protein, 116 ± 24 pmol/min/mg protein.
- **56 mM K⁺ (n = 4)**: 1.06 ± 0.33 nmol/min/mg protein, 242 ± 46 pmol/min/mg protein.
- **Cholera toxin, 1 μg/ml (n = 7)**: 1.50 ± 0.32 nmol/min/mg protein, 306 ± 63 pmol/min/mg protein.
media that contained 100 μM nonradioactive L-tyrosine and the addition noted in the table. The Na' -free medium was prepared by the substitution of choline chloride for NaCl; the pH of this medium was adjusted with tris(hydroxymethyl)aminomethane instead of with NaOH. The cells were then incubated for 10, 20, and 30 s at 30°C in medium by centrifugation through silicone oil, and the amount of tyrosine taken up into the cells was determined as described in the text. Tyrosine uptake is expressed as nmol/min/mg of protein, mean ± S.E. of three experiments.

| Incubation conditions | Tyrosine uptake (nmol/min/mg protein) |
|-----------------------|--------------------------------------|
| Control               | 3.57 ± 0.16                          |
| Cholera toxin, 1 μg/ml| 3.25 ± 0.15                          |
| 56 mM K'              | 2.57 ± 0.12                          |
| Na'-free              | 1.68 ± 0.08                          |

Tyrosine 3-Monooxygenase Activity in Intact Cells

Pheochromocytoma cells were incubated for 60 min at 37°C in media that contain 100 μM nonradioactive L-tyrosine and the additions noted in the table. The Na' -free medium was prepared by the substitution of choline chloride for NaCl; the pH of this medium was adjusted with tris(hydroxymethyl)aminomethane instead of with NaOH. The cells were then incubated for 10, 20, and 30 s at 30°C in medium by centrifugation through silicone oil, and the amount of media have a low tyrosine content and produce dopa at a rate of approximately 50 pmol/min/mg of protein. The tyrosine content of the cells increases progressively with increasing extracellular tyrosine. Cells incubated with 20 to 100 μM tyrosine have tyrosine contents between 0.5 and 2.5 nmol/mg of protein and exhibit maximal rates of dopa production. Cells that are incubated with greater than 100 μM tyrosine have an intracellular tyrosine content larger than 3 nmol/mg of protein and show a slightly decreased rate of dopa production. This decrease in dopa production in the presence of high intracellular tyrosine is due to substrate inhibition of tyrosine 3-monooxygenase. Nonetheless, the decrease in intracellular tyrosine produced by incubation of the cells with cholel toxin or with 56 mM K' (Table I) is not likely to have a significant effect on the rate of dopa production; the relief of tyrosine 3-monooxygenase from substrate inhibition cannot account for the increased rate of dopa production caused by these treatments.

We have also studied the effects of cholera toxin and of 56 mM K' on the uptake of tyrosine into pheochromocytoma cells. When pheochromocytoma cells are incubated in the presence of 100 μM tyrosine, they take up tyrosine at a rate of approximately 3.6 nmol/min/mg of protein (Table II). This rate of tyrosine uptake is more than 1 order of magnitude greater than the rate of dopa production; thus, tyrosine uptake is not the rate-limiting step in the process of dopa formation in these cells. Cholera toxin has no significant effect on tyrosine uptake, but incubation of the cells with 56 mM K' inhibits tyrosine uptake by approximately 30%. This decrease in tyrosine uptake in media that contain 56 mM K' is probably due to the decreased concentration of Na' in these media; tyrosine uptake is decreased by more than 50% in Na'-free media (Table II). It is likely that the transport of tyrosine into pheochromocytoma cells occurs by at least two mechanisms, one of which is Na'-dependent, and the other of which is independent of Na'. This inhibition of tyrosine uptake in media that contain 56 mM K' provides an explanation for the observation that, although 56 mM K' raises a smaller increase in the rate of dopa production, it results in a greater decrease in intracellular tyrosine than does cholera toxin (Table I).

The experiments reported here demonstrate that the tyrosine content of pheochromocytoma cells decreases under conditions in which the conversion of tyrosine to dopa is increased. These experiments therefore show that incubation with 56 mM K' or with cholera toxin increases the activity of tyrosine 3-monooxygenase in intact pheochromocytoma cells. We have previously reported that both of these treatments lead to an increase in tyrosine 3-monooxygenase activity, as measured in extracts of pheochromocytoma cells in vitro (3). The activation of tyrosine 3-monooxygenase by cholera toxin is probably mediated by cyclic adenosine 3':5'-monophosphate; in contrast, 56 mM K' activates tyrosine 3-monooxygenase by a mechanism that requires extracellular Ca" but that is not accompanied by a change in the content of cyclic adenosine 3':5'-monophosphate in the cells. It is likely that this stable activation of tyrosine 3-monooxygenase underlies the increased activity of the enzyme in the intact cells. In addition, however, the activity of tyrosine 3-monooxygenase in the intact cells may be affected by allosteric modifiers and by the availability of its pteridine cofactor (10). In any event, these experiments provide direct evidence that an increase in tyrosine 3-monooxygenase activity mediates the stimulation of catecholamine synthesis in pheochromocytoma cells produced by 56 mM K' and by cholera toxin.

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