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ACTIVITY OF AROMATIC-L-AMINO-ACID DECARBOXYLASE IN PHEOCHROMOCYTOMA CELLS*

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Abstract—The activity of aromatic-L-amino-acid decarboxylase in pheochromocytoma cells was assayed by measuring the formation of $^{14}CO_2$ by cells incubated with L-[1-^{14}C]dopa. The V_{max} of dopa decarboxylation in intact cells was about 3 nmoles CO_2 produced/min per mg protein, and the apparent K_m of this process was approximately 32 μM. The V_{max} of decarboxylase activity was at least an order of magnitude greater than the rate of dopa synthesis in these cells. Incubation of the cells with cholera toxin or with 56 mM K^+ caused an activation of tyrosine 3-monooxygenase and an increase in the rate of dopa production, but it did not affect the activity of the decarboxylase in the cells. The pheochromocytoma cells contained about 12 pmoles dopa/mg protein. Treatment of the cells with cholera toxin or with high K^+ increased the dopa content of the cells. Aromatic-L-amino-acid decarboxylase was not the rate-limiting enzyme in the pathway of catecholamine synthesis in these cells; moreover, it appeared that the activities of this enzyme and of tyrosine 3-monooxygenase were not regulated coordinately.

The enzyme aromatic-L-amino-acid decarboxylase (EC 4.1.1.28, AADC) catalyzes the conversion of dopa to dopamine [1] and, therefore, plays an essential role in the pathway of catecholamine biosynthesis [2]. Because AADC is thought not to regulate catecholamine synthesis [3], there have been relatively few studies of the activity of this enzyme in catecholamine-synthesizing tissues. Our laboratory has been studying the regulation of catecholamine synthesis in cell suspensions prepared from a transplantable rat pheochromocytoma. These pheochromocytoma cells contain the enzymes required for the synthesis of norepinephrine [4], and they convert [^{14}C]tyrosine to [^{14}C]norepinephrine [5]. Tyrosine 3-monooxygenase (EC 1.14.16.2) appears to regulate the pathway of catecholamine synthesis in these cells, as it does in normal chromaffin cells [6]. Cholera toxin, high concentrations of K^+, and the carboxylic ionophore lasalocid, all cause an activation of tyrosine 3-monooxygenase and increase the rate of catecholamine synthesis in the cells [7, 8]. We have now studied the activity of AADC in pheochromocytoma cells.

MATERIALS AND METHODS

AADC activity was assayed by measuring the formation of $^{14}CO_2$ by cells incubated with L-[1-^{14}C]dopa, according to a modification of the methods of Chiriboga and Roy [9] and of Christenson *et al.* [10]. Pheochromocytoma cells were prepared as previously described [4]. The cells were preincubated for 20 min at 37° before measurement of AADC activity. The cells were then incubated in 1 ml of medium containing L-[1-^{14}C]dopa. Unless otherwise indicated, incubations were carried out for 30 min, at 37°, in the presence of 100 μM L-[1-^{14}C]dopa (50 mCi/mole). These incubations were carried out in 17 × 100 mm plastic tubes, sealed with rubber caps from which plastic wells were suspended. At the end of the incubations, the tubes were placed on ice, 0.2 ml of phenylethylamine was injected with a syringe into the plastic well, and 0.25 ml of 1.5 M perchloric acid was injected with another syringe into the incubation medium. The tubes were then incubated for an additional 30 min at 37°, to trap all of the $^{14}CO_2$ in the wells. Finally, the tubes were uncapped, the wells were placed in scintillation vials containing 3.5 ml Scintiverse, and the amount of radioactivity that had accumulated in the wells was measured. The counting efficiency was approximately 70 per cent. Enzyme activity was calculated after subtraction of the amount of radioactivity present in samples that had been incubated in the absence of cells and is expressed as nmoles CO_2 produced/min per mg protein, the mean ± S.E.M. of experiments performed in triplicate. Because the rate of uptake and decarboxylation of extracellular dopa was very much greater than the rate of formation of endogenous dopa (see below), we have based our calculations of AADC activity on the specific activity of dopa in the incubation medium. In these calculations, we have assumed that all of the radioactivity in the [^{14}C]dopa was in the 1 position. The V_{max} and K_m of enzyme activity were estimated by least squares analysis of $1/v$ vs $1/s$ graphs of enzyme activity [11]. Samples that were incubated at 0°, or samples that contained boiled cells, had about the same amount of radioactivity as did the samples incubated in the absence of cells. The radioactivity in the experimental samples was typically twenty to thirty times greater than the radioactivity in these

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blanks. All experiments were repeated several times, with similar results; for convenience, only the results of individual experiments are presented.

The activity of AADC was also determined in cell-free extracts of pheochromocytoma cells. In these experiments, the cells were incubated for 15 min at 0° in 0.1 M potassium phosphate, pH 7.0, containing 1 mg/ml digitonin. This treatment lysed the cells and caused the complete release of AADC (and the release of approximately 50 per cent of the cell protein) into the incubation medium. Following this incubation, membranes and debris were removed by centrifugation for 10 min at 20,000 *g*, and aliquots of the cell-free extracts were assayed for AADC activity in 1 ml of 0.08 M potassium phosphate buffer, pH 7.0, containing 10 μM pyridoxal phosphate. Enzyme activity in these extracts was also expressed as nmoles CO_2 produced/min per mg protein; to facilitate comparison of the AADC activity in cell-free extracts with that in intact cells, these calculations were based on the total protein content of the cell suspensions, rather than on the protein content of the extracts.

The dopa content of the cells was measured by a modification of the method of Kehr *et al.* [12]. Cells were incubated for 30 min at 37° in medium containing 100 μM L-tyrosine. At the end of this incubation, the cells were collected by centrifugation at 3,000 *g* for 10 min at 4° and resuspended in 1 ml of 0.15 M trichloroacetic acid containing 0.1 mM EDTA. Precipitated protein was removed by centrifugation at 13,000 *g* for 10 min at 4°. The dopa in the supernatant solutions was isolated by chromatography on Dowex-50 columns [12] and was assayed by liquid chromatography with electrochemical detection, according to the method of Felice *et al.* [13] as modified by Erny *et al.* [14]. Measurements were corrected for the recovery of dopa from the columns, which was approximately 60 per cent. The dopa content of the cells is expressed as pmoles/mg protein.

Protein was determined by the method of Bradford [15], with bovine serum albumin as a standard.

Materials. The standard medium used in these experiments contained 118 mM NaCl, 4.7 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 10 mM sodium ascorbate, 10 mM glucose, and 25 mM Hepes [4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid], titrated to pH 7.4 with NaOH. Medium containing 56 mM K^+ was prepared by the substitution of KCl for NaCl. In experiments in which the dopa content of the cells was measured, 0.4 units/ml adenosine deaminase (EC 3.5.4.4) was added to this medium [14], and sodium ascorbate was omitted. L-[1-^{14}C]dopa was purchased from the Amersham Corp., Arlington Heights, IL. Scintiverse and scintillation-grade phenylethylamine were obtained from Fisher Scientific, Pittsburgh, PA. The rubber caps and plastic wells used in the AADC assays were products of Kontes, Vineland, NJ. Brocresine (NSD-1055) was provided by Dr. E. W. Cantrall, Lederle Laboratories, Pearl River, NY. Lasalocid (X-537A) was a gift from Dr. E. W. Scott, Hoffmann-LaRoche Inc., Nutley, NJ. All other chemicals were obtained from commercial sources. Glass-distilled water was used throughout.

RESULTS

Pheochromocytoma cells catalyze the decarboxylation of dopa. The formation of $^{14}CO_2$ from L-[1-^{14}C]dopa was a linear function of time, up to 60 min, and of enzyme concentration (i.e. cell density), as along as no more than 25–30 per cent of the substrate was metabolized. To ensure that the AADC assays were performed under appropriate conditions, the protein content of the cell suspensions was kept below 0.3 mg protein/ml, and the incubations were routinely carried out for 30 min. In eleven separate experiments, AADC activity measured under standard conditions (100 μM dopa) was 2.16 ± 0.55 nmoles CO_2 produced/min per mg protein (mean ± S.D.), with a range of 1.13 to 3.34 nmoles/min per mg protein. The decarboxylation of dopa was actually taking place inside the cells, since no detectable enzyme activity was released from the cells into the incubation medium during a 30-min incubation. The addition of 1 μM pyridoxal phosphate to the incubation medium had no effect on the activity of AADC in the cells (data not shown). Figure 1 shows the dependence of AADC activity on the concentration of dopa in the incubation medium. Over the range of 5–200 μM dopa, this process can be described by Michaelis–Menten kinetics. The V_{max} of dopa decarboxylation in intact cells was approximately 3 nmoles/min per mg protein, and the apparent K_m of this process was 32 μM.

Brocresine has been used extensively as an inhibitor of AADC activity. The experiment reported in Fig. 2 demonstrates that brocresine is a potent inhibitor of dopa decarboxylation in pheochromocytoma cells. In the presence of 100 μM dopa, the IC_{50} for brocresine was approximately 0.7 μM. This result is similar to the IC_{50} of 0.27 μM reported for the inhibition by brocresine of AADC from guinea pig kidney [16].

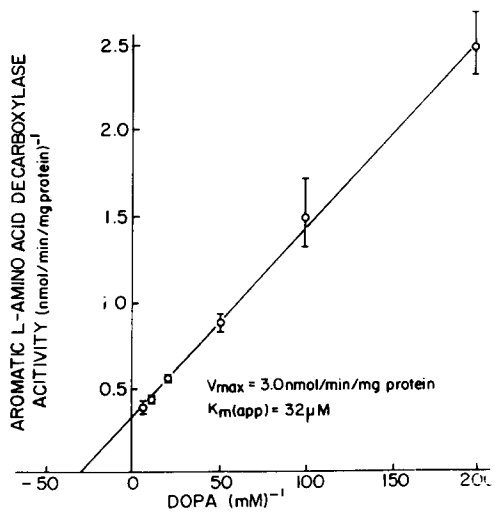

Fig. 1. Effect of extracellular dopa on the activity of aromatic-L-amino-acid decarboxylase in intact pheochromocytoma cells. Cells were incubated for 30 min at 37° with various concentrations of L-[1-^{14}C]dopa. Enzyme activity was determined as described in the text and is presented as mean ± S.E.M. of an experiment performed in triplicate.

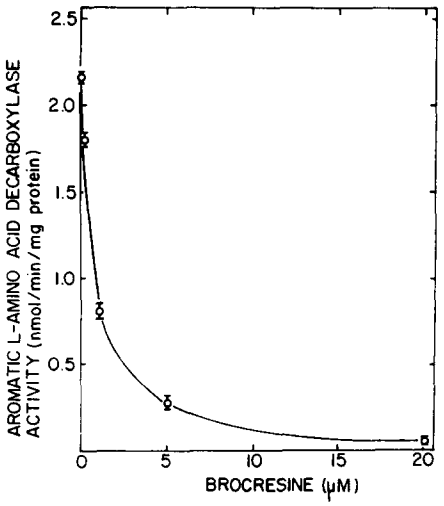

Fig. 2. Inhibition of the activity of aromatic-L-amino-acid decarboxylase by brocresine. Cells were incubated for 20 min at 37° with 100 μM L-[1-^{14}C]dopa, in the presence of various concentrations of brocresine. Enzyme activity was determined as described in the text and is presented as mean ± S.E.M. of an experiment performed in triplicate.

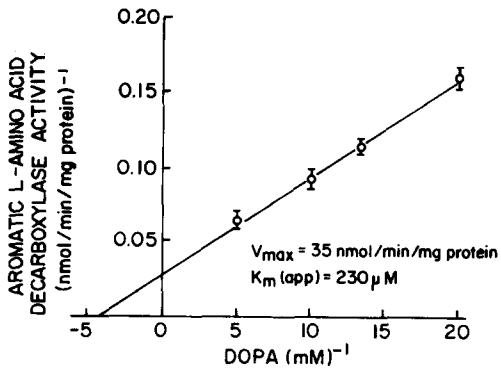

Fig. 3. Activity of aromatic-L-amino-acid decarboxylase in extracts of pheochromocytoma cells. Extracts of pheochromocytoma cells were prepared by treatment of the cells with digitonin, as described in the text. Aliquots of these extracts were incubated for 30 min at 37° with various concentrations of L-[1-^{14}C]dopa. Enzyme activity was determined as described in the text and is presented as mean ± S.E.M. of an experiment performed in triplicate.

Incubation of pheochromocytoma cells with cholera toxin or with 56 mM K^+ leads to the activation of tyrosine 3-monooxygenase and to an increase in the rate of catecholamine synthesis in the cells [8]. It was of interest to determine whether these treatments lead to a change in AADC activity in the cells. As shown in Table 1, these treatments did not affect the activity of AADC in the cells. Incubation of the cells with lasalocid also activates tyrosine 3-monooxygenase [7]; in contrast, this ionophore decreased the activity of AADC in pheochromocytoma cells. This inhibition of AADC by lasalocid appeared to be specific, since other ionophores (monensin and ionomycin) had little effect on the activity of this enzyme (data not shown).

We also measured the kinetic parameters of AADC activity in extracts of pheochromocytoma cells. Enzyme activity in cell-free extracts was much greater than it was in intact cells (Fig. 3). The V_{max} of AADC activity in cell-free extracts was approximately 35 nmoles/min per mg protein, and the apparent K_m of the enzyme was about 230 μM. Pyridoxal phosphate (1–10 μM) caused approximately a 2-fold increase in AADC activity in cell-free extracts (data not shown); for this reason, pyridoxal phosphate was included in all AADC assays in these extracts. The data in Table 1 indicate that incubation of the cells with cholera toxin, 56 mM K^+, or lasalocid, did not cause a stable change in AADC activity that could be detected in cell-free extracts.

In other experiments, we measured the dopa content of pheochromocytoma cells (Table 2). Cells incubated under control conditions had a dopa content of approximately 12 pmoles/mg protein. Pheochromocytoma cells contain approximately 11 μl intracellular water/mg protein [6]. If the dopa content of the cells is distributed uniformly throughout

Table 1. Activity of aromatic-L-amino-acid decarboxylase in pheochromocytoma cells*

Incubation conditions	AADC activity	
	Intact cells (nmoles/min per mg protein)	Extracts (nmoles/min per mg protein)
Control	2.21 ± 0.07	6.06 ± 0.13
56 mM K^+	1.78 ± 0.25	5.90 ± 0.35
Cholera toxin (1 μg/ml)	2.18 ± 0.27	6.81 ± 0.32
Lasalocid (10 μM)	1.08 ± 0.03	6.10 ± 0.31

* In one experiment, pheochromocytoma cells were incubated for 30 min in the presence of 100 μM L-[1-^{14}C]dopa, under the conditions described above, and the activity of AADC in the cells was determined. In a separate experiment, cells were incubated for 15 min under the conditions described above, and then the activity of AADC in extracts of the cells was determined. In both experiments, cells that were incubated with cholera toxin were also exposed to this agent during a 30 min preincubation period. Results shown are the means ± S.E.M. of experiments performed in triplicate.

Table 2. Intracellular dopa content in pheochromocytoma cells*

Incubation conditions	Dopa content (pmoles/mg protein)
Control	12
56 mM K^+	16
Cholera toxin (1 μg/ml)	24

* Pheochromocytoma cells were incubated for 30 min at 37° in the presence of 100 μM L-tyrosine, under the conditions indicated above. The dopa content of the cells was then determined by liquid chromatography with electrochemical detection, as described in the text. The data shown are the averages of two separate experiments.

the intracellular water, the concentration of dopa in the cells would be approximately 1.1 μM. Incubation of the cells with cholera toxin or with 56 mM K^+ increased the dopa content of the cells; the dopa content of cholera toxin-treated cells was approximately 2-fold greater than that of control cells.

DISCUSSION

Pheochromocytoma cells contain all of the enzymes required to convert tyrosine to norepinephrine [4]. We have found recently that the rate of dopa formation in these cells, as measured by amino acid analysis [6] or by liquid chromatography with electrochemical detection [14], is approximately 100 pmoles/min per mg protein. The maximal activity of AADC in these cells—3 nmoles/min per mg protein as measured in intact cells, or 35 nmoles/min per mg protein as measured in cell-free extracts—is therefore very much greater than is the rate of endogenous dopa formation. Thus, AADC is not the rate-limiting enzyme in the pathway of catecholamine synthesis in these cells.

The V_{max} of AADC activity in extracts of pheochromocytoma cells was very much greater than in intact cells. The apparent K_m of AADC in cell-free extracts (230 μM) was also much greater than in intact cells (32 μM). The most likely reason for these differences is that the rate of dopa uptake limited the rate of decarboxylation of extracellular dopa in intact cells. It is difficult to measure dopa uptake precisely enough to test this idea directly. In the absence of an AADC inhibitor, virtually all of the [^{14}C]dopa taken up into the cells was decarboxylated; in the presence of an AADC inhibitor, dopa uptake was linear for only a few seconds (data not shown). Nonetheless, the observation that cells incubated with [^{14}C]dopa did not accumulate a significant amount of intracellular [^{14}C]dopa is consistent with the hypothesis that the rate of dopa uptake limits the decarboxylation of extracellular dopa. Alternatively, however, the higher activity of AADC in cell-free extracts than in intact cells may reflect the different conditions used in the two assays. The apparent K_m of AADC in extracts of pheochromocytoma cells was similar to the K_m that has been reported for AADC purified from hog kidney (190 μM, Ref. 10).

Incubation of pheochromocytoma cells with cholera toxin, 56 mM K^+, or lasalocid results in the activation of tyrosine 3-monooxygenase in the cells [7, 8]. None of these treatments caused an activation of AADC in the cells; in fact, lasalocid actually inhibited AADC activity. The lack of effect of 56 mM K^+ on AADC activity is consistent with the finding that this treatment does not affect the conversion of [^{3}H]dopa to catecholamines [5]. These experiments indicate that the activities of tyrosine 3-monooxygenase and of AADC are not regulated coordinately. The mechanism of inhibition of AADC by lasalocid is not understood. Since lasalocid is an aromatic acid, it might inhibit AADC directly. Alternatively, the inhibition of AADC activity could be secondary to a lasalocid-induced change in the intracellular ionic content. The finding that lasalocid does not inhibit the activity of AADC in cell-free extracts supports the latter possibility.

In the steady state, the cells do not accumulate dopa; under these conditions, the rate of dopa decarboxylation must equal the rate of dopa production. The concentration of dopa in the cells will be determined by the ratio between the flux through the pathway of catecholamine synthesis (v) and the V_{max} of AADC, according to the Michaelis–Menten equation:

$$\frac{v}{V_{max}} = \frac{S}{K_m + S}$$

In pheochromocytoma cells, as in normal catecholamine-synthesizing tissues, the V_{max} of AADC is very much greater than is the rate of catecholamine synthesis. Under these conditions, the dopa concentration in the cells is very small in comparison to the K_m of AADC, and:

$$S \approx \frac{v \cdot K_m}{V_{max}}$$

This equation can be used to estimate the dopa concentration in the cells. The intracellular dopa concentration corresponding to a rate of catecholamine synthesis of 100 pmoles/min per mg protein is estimated to be 1.1 μM from measurements of AADC activity in intact cells and 0.7 μM from measurements of enzyme activity in cell-free extracts. Our determination that the dopa content of the cells is approximately 1.1 μM agrees well with both of these estimates. In addition, our measurements confirm the prediction that an increase in the rate of catecholamine synthesis is accompanied by an increase in the dopa content of the cells.

One consequence of the high AADC activity in pheochromocytoma cells is the maintenance of a low concentration of dopa in these cells. It may be important that the concentration of dopa is kept low in order to conserve the solvent capactiy of intracellular water [17] or to prevent side reactions that might occur if the dopa concentration was increased. When AADC activity is inhibited by brocresine, most of the dopa formed in the cells is released into the incubation medium [6]. The maintenance of a low intracellular dopa concentration may prevent this loss of dopa from the cells. When the substrate of an enzyme-catalyzed reaction is much lower than the K_m, the ratio K_m/V_{max} is a measure of the lifetime of the substrate. The lifetime of dopa in pheochromocytoma cells (estimated from enzyme activity in intact cells or in cell-free extracts and on the basis that the cells contain 11 μl water/mg protein) is on the order of 0.1 min. Dopa is a labile compound; its rapid turnover may help to minimize its oxidation.

In summary, the experiments reported here show that AADC is not the rate-limiting enzyme in the pathway of catecholamine synthesis in pheochromocytoma cells. The function of the high AADC activity in these cells is presumably to maintain a low intracellular concentration of dopa, rather than to regulate the rate of catecholamine synthesis. Finally, the activities of tyrosine 3-monooxygenase and of AADC are not regulated coordinately.

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