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ACTIVATION OF TYROSINE 3-MONO-OXYGENASE IN PHEOCHROMOCYTOMA CELLS BY LASALOCID

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Abstract—Tyrosine 3-mono-oxygenase (TH) activity was measured in extracts of cells prepared from a transplantable rat pheochromocytoma. Incubation of the cells with the ionophore lasalocid (X-537A) results in an increase in TH activity. The activation of TH by lasalocid is associated with an increase in the V_{max} of the enzyme, but is not accompanied by a change in the apparent K_m of the enzyme for its pteridine cofactor or for tyrosine. In these respects, the activation of TH by lasalocid resembles that produced by incubation of the cells in media containing 56 mM K^+. This effect of lasalocid is not dependent upon the presence of extracellular Ca^{2+}. It is proposed that the activation of TH by lasalocid may be mediated by the ionophore-induced release of Ca^{2+} from some intracellular store.

Stimulus–secretion coupling in chromaffin cells and in adrenergic neurons is accompanied by an acute increase in the rate of catecholamine synthesis [1–5]. We have recently reported a similar stimulus-coupled increase in catecholamine synthesis in cell suspensions prepared from a transplantable rat pheochromocytoma [6]. The increase in catecholamine synthesis produced by incubation of these cells in media containing 56 mM K^+ is apparently mediated by the activation of tyrosine 3-mono-oxygenase (EC 1.14.16.2, TH). The ionophore lasalocid (previously called X-537A) stimulates the release of catecholamine from pheochromocytoma cells [7]. We now report that lasalocid also activates TH in these cells.

MATERIALS AND METHODS

Tyrosine 3-mono-oxygenase activity. TH activity was assayed by measuring the conversion of L[3,5-^{3}H]tyrosine to ^3H_2O, by a modification of the method of Nagatsu *et al.* [8]. Cell suspensions were prepared by mechanical disruption of a transplantable rat pheochromocytoma [7], and were pre-incubated for one 30-min period at 37° in Krebs–Ringer phosphate glucose buffer (KRPG), under an atmosphere of 100% O_2. Aliquots of the cell suspensions were then incubated for 10 min at 37° in KRPG or 56 mM K^+-KRPG, containing lasalocid as indicated. After incubation, the cells were rapidly chilled, and were then centrifuged for 1 min at 3000 *g*. The cell pellets were resuspended in 0.5 ml of 10 mM sodium phosphate, 1 mM EDTA, pH 7.0, and were lysed by freeze–thawing three times in a dry ice–acetone bath. The lysates were centrifuged for 10 min at 20,000 *g*, and the supernatant fractions were passed over 0.5 × 1.0 cm columns of Dowex 50W-X8, 100–200 mesh, Na^+ form, which had previously been equilibrated with the sodium phosphate–EDTA buffer. Passage of these extracts over Dowex 50 removes virtually all of the catecholamine from the samples, but does not otherwise affect TH activity. The pellet fractions were washed with 0.5 ml of the sodium phosphate–EDTA buffer, and these supernatants were used to wash the Dowex 50 columns. The combined effluent fractions from the Dowex columns were assayed for protein [9] and for TH activity. Three hundred μl of the cell extracts (containing approximately 0.5 mg protein) was added to 200 μl of reaction mixture containing 150 nmoles 6,7-dimethyl-5,6,7,8-tetra-hydropterin ($DMPH_4$), 50 nmoles brocresine, 1 mg (8400 units) catalase, 5 nmoles L[3,5-^{3}H]tyrosine (20,000 cpm/nmole), 250 nmoles NADPH, excess dihydropteridine reductase, and 50 μmoles potassium phosphate, pH 6.8, and were incubated for 15 min at 30°, in air. The reactions were stopped by the addition of 50 μl of 1.5 M trichloroacetic acid, and the samples were centrifuged for 5 min at 3000 *g*. The ^3H_2O produced in the TH reaction was collected by passing the samples over 0.5 × 1.0 cm columns of Dowex 50W-X4, 200–400 mesh, H^+ form [8]. TH activity is calculated after subtraction of the radioactivity found in control, enzyme-free incubations, and is expressed as pmoles product produced/min/mg of protein, mean ± S. E. M. of experiments performed in triplicate. The apparent K_m of TH for $DMPH_4$ was determined in experiments in which the $DMPH_4$ concentration was varied between 50 and 1000 μM. The apparent K_m of TH for tyrosine was determined in experiments in which the tyrosine concentration was varied between 5 and 100 μM, and in which tetrahydrobiopterin (300 μM) was used in place of $DMPH_4$. Apparent K_m values were calculated by the least-squares analysis of $1/V$ vs $1/S$.

Catecholamine secretion. Catecholamine secretion, and the release of lactate dehydrogenase were measured as previously described [7].

Catecholamine synthesis. Catecholamine synthesis was measured by determining the incorporation of L[^{14}C]tyrosine into dopamine and norepinephrine, as previously described [6]. In these experiments, cells were pre-incubated for two 30-min periods at 37° in KRPG. During the first 30-min period, this buffer contained 10 μM pargyline; this treatment results in an almost complete inhibition of amine oxidase (flavin containing) (EC 1.4.3.4). Aliquots of the cell suspensions were then incubated for 30 min at 37° in

KRPG (or 56 mM K^+–KPRG) containing 10 mM sodium ascorbate, 50 μM L[^{14}C]tyrosine, and, when indicated, 5 μM lasalocid. After incubation, the cells were separated from the medium by centrifugation through a layer of silicone oil, catecholamines were separated by high-voltage electrophoresis, and the radioactivity incorporated into the different catecholamines was determined [6]. Calculations of catecholamine synthesis are based on the specific activity of L[^{14}C]tyrosine in the medium.

Materials. KRPG contained 118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.25 mM $CaCl_2$, 16 mM $Na \cdot PO_4$, pH 7.4, and 10 mM glucose. 56 mM K^+–KRPG was prepared by substituting the appropriate amount of KCl for NaCl. Ca^{2+}-free buffers were prepared by omitting $CaCl_2$, and adding 0.1 mM ethyleneglycol-bis-(*β*-aminoethyl-ether)-*N,N'*-tetraacetic acid. All buffers were equilibrated with 100% O_2 before use. Diphenylhydantoin and beef liver catalase were obtained from Sigma Chemical Co., St. Louis, MO. Diphenylhydantoin was dissolved in dimethyl sulfoxide. L[^{14}C]tyrosine (uniformly labeled) and L[3,5-^{3}H]tyrosine were obtained from New England Nuclear Corp., Boston, MA. The L[3,5-^{3}H]tyrosine was purified by the method of Creveling and Daly [10] before use. Sheep liver dihydropteridine reductase was a generous gift from Dr. S. Kaufman and Dr. S. Milstien, National Institutes of Health, Bethesda, MD. Pargyline was provided by Dr. R. Rando, Harvard Medical School, Boston, MA. Lasalocid sodium and tetrahydrobiopterin were gifts from Dr. W. E. Scott, Hoffmann-LaRoche, Inc., Nutley, NJ. A23187 was a gift from Dr. R. J. Hosley, Eli Lilly & Co., Indianapolis, IN. Lasalocid and A23187 were dissolved in methanol. The final concentration of methanol was never greater than 0.5%; control experiments showed that this concentration of methanol had no effect on TH activity. Brocresine was provided by Dr. E. W. Cantrall, Lederle Laboratories, Pearl River, NY. All other chemicals were reagent grade. Glass-distilled water was used throughout.

RESULTS

We have previously reported that lasalocid stimulates the release of catecholamine from pheochromocytoma cells [7]. Figure 1 illustrates the concentration dependence of this effect. Catecholamine release is stimulated by low concentrations of lasalocid (0.5 μM), plateaus at lasalocid concentrations between 0.5 and 2 μM, and then increases further in response to higher concentrations of the ionophore. Lasalocid does not cause the release of lactate dehydrogenase from the cells.

Incubation of pheochromocytoma cells with lasalocid also results in an increase in TH activity in these cells (Fig. 2). The concentration dependence of TH activation is strikingly similar to that of catecholamine secretion. At concentrations up to 10 μM, lasalocid has no effect on TH activity in cell-free extracts of pheochromocytoma cells.

The stimulation of catecholamine release by lasalocid is not dependent upon the presence of extracellular Ca^{2+} [7]. Lasalocid-induced activation of TH is also not dependent upon extracellular Ca^{2+} (Table 1). Further, this effect of lasalocid is not inhibited

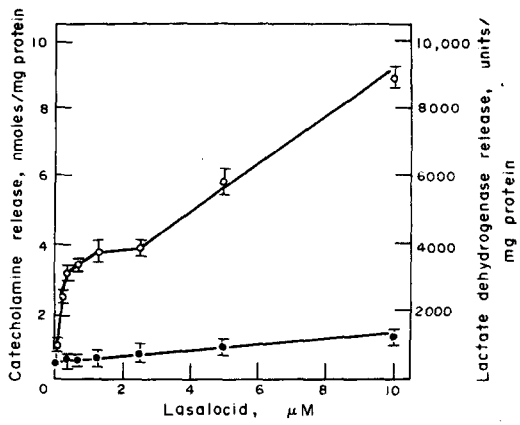

Fig. 1. Stimulation of catecholamine secretion by lasalocid. Pheochromocytoma cells were incubated for 10 min at 37° as previously described [7], in the presence of the indicated concentrations of lasalocid. After incubation, the cells were removed by centrifugation, and the catecholamine content (○) and lactate dehydrogenase activity (●) of the incubation medium were measured. Catecholamine secretion is expressed as nmoles catecholamine released/mg of protein, mean ± S. E. M. of triplicate determinations; lactate dehydrogenase activity is expressed as units (μmoles product formed/min)/mg of protein, mean ± S. E. M.

by diphenylhydantoin, which blocks the uptake of extracellular Ca^{2+} into these cells [11]. In these respects, the activation of TH produced by lasalocid differs from that produced by incubation of pheochromocytoma cells in media containing 56 mM K^+ (Table 1; M. Chalfie and R. L. Perlman, manuscript submitted for publication). Another divalent cation ionophore, A23187, does not increase TH activity. A23187 prevents the activation of TH by 56 mM K^+ and by lasalocid (not shown).

In the experiments reported in Fig. 2 and in Table 1, cells were incubated with lasalocid in KRPG. Lasalocid also activates TH in cells incubated in a buffer

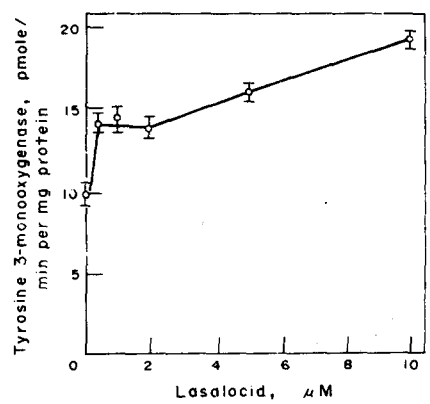

Fig. 2. Activation of tyrosine 3-mono-oxygenase by lasalocid. Pheochromocytoma cells were incubated for 10 min at 37° in KRPG containing various concentrations of lasalocid, as indicated. After incubation, the cells were chilled, and collected by centrifugation, and TH activity was measured as described in the text. TH activity is expressed as pmoles product formed/min/mg of protein, mean ± S. E. M. of triplicate determinations.

Table 1. Activation of tyrosine 3-mono-oxygenase by lasalocid*

Incubation conditions	Tyrosine 3-mono-oxygenase activity		
	Ca^{2+} (1.25 mM)	EGTA† (100 μM) without calcium	Ca^{2+} (1.25 mM) and DPH‡ (100 μM)
Control	13.2 ± 0.2	11.7 ± 1.0	14.0 ± 0.4
K^+ (56 mM)	30.9 ± 2.2	13.2 ± 1.2	16.2 ± 0.4
Lasalocid	29.5 ± 0.9	34.3 ± 2.0	31.9 ± 1.0
A23187	10.9 ± 0.4		

* Pheochromocytoma cells were incubated for 10 min at 37° in KRPG (control) or 56 mM K^+–KRPG, with the modification indicated. When present, lasalocid and A23187 were added to a final concentration of 5 μM. After incubation, the cells were centrifuged, and TH activity was determined as described in the text. Activity is expressed as pmoles product formed/min/mg of protein, mean ± S. E. M. of triplicate determinations.

† Ethyleneglycol-bis-(β-aminoethylether)-*N*,*N*′-tetraacetic acid.

‡ Diphenylhydantoin.

composed of 0.3 M sucrose–10 mM HEPES (*N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid), pH 7.4. Cells incubated for 10 min at 37° in this buffer, in the absence of lasalocid, had a TH activity of 17.2 ± 1.1 pmoles/min/mg of protein, whereas enzyme activity in cells that had been incubated with 10 μM lasalocid was increased to 34.6 ± 0.9 pmoles/min/mg of protein.

It was of interest to determine the effects of lasalocid on the kinetic parameters of TH activity. Under the conditions of our experiments, the apparent K_m of TH for tyrosine was 26 ± 3 μM (N = 4), and was not changed by incubation of the cells with lasalocid or with 56 mM K^+ (not shown). Figure 3 presents the results of an experiment in which the apparent K_m of TH for $DMPH_4$ was estimated. The apparent K_m of the enzyme in control cells for $DMPH_4$ is about 160 μM. Incubation of the cells with lasalocid, or in media containing 56 mM K^+, results in a 70–90 per cent increase in the V_{max} of the enzyme, but causes no significant change in its apparent K_m for $DMPH_4$. In four separate experiments, the apparent K_m of TH for $DMPH_4$ varied between 140 and 200 μM; in no experiment did lasalocid or 56 mM K^+ produce a significant change in the K_m.

Because lasalocid causes the activation of TH in pheochromocytoma cells, we examined the effects of this ionophore on catecholamine synthesis in these cells (Table 2). As previously reported, pheochromo-

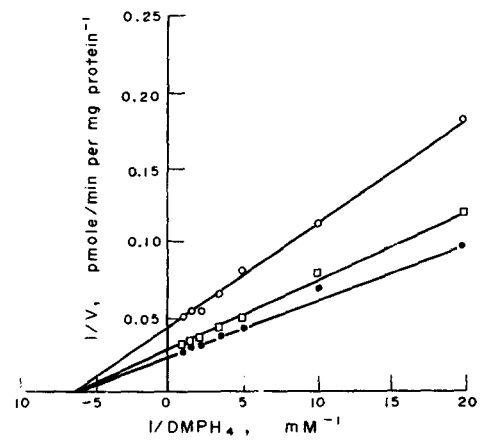

Fig. 3. Apparent K_m of tyrosine 3-mono-oxygenase for $DMPH_4$. Pheochromocytoma cells were incubated for 10 min at 37° in KRPG (○), 56 mM K^+-KRPG (□), or KRPG containing 10 μM lasalocid (●). After incubation, the cells were chilled, and collected by centrifugation, and the apparent K_m of TH for $DMPH_4$ was determined as described in the text. The values shown are for one representative experiment; in this experiment, the apparent K_m of TH for $DMPH_4$ was 158 μM in control cells, 161 μM in 56 mM K^+-stimulated cells, and 154 μM in lasalocid-stimulated cells. Three other experiments produced similar results.

Table 2. Effect of lasalocid on catecholamine synthesis*

Incubation conditions	Dopamine		Norepinephrine		
	Cells	Medium	Cells	Medium	Total
Control	3.3 ± 0.1	1.2 ± 0.1	7.5 ± 0.5	2.2 ± 0.1	14.2 ± 0.6
K^+ (56 mM)	4.9 ± 0.2	3.3 ± 0.1	10.4 ± 0.3	6.3 ± 0.2	24.9 ± 0.5
Lasalocid	3.3 ± 0.2	8.8 ± 0.7	1.7 ± 0.2	1.9 ± 0.2	16.0 ± 1.0

* Pheochromocytoma cells were incubated for 30 min at 37° in KRPG (control) or in 56 mM K^+–KRPG, containing 10 mM sodium ascorbate, 50 μM [^{14}C]L-tyrosine, and lasalocid (5 μM) as indicated. The incubations were terminated by centrifuging the samples through silicone oil, and catecholamine synthesis was measured as described in the text. Total catecholamine synthesis represents the sum of dopamine and norepinephrine in the cells and in the incubation medium. Activity is expressed as pmoles product accumulated/min/mg of protein, ± S. E. M. of triplicate determinations.

cytoma cells synthesize catecholamines from L[^{14}C]tyrosine. In cells incubated under control conditions for 30 min, the accumulation of [^{14}C]norepinephrine is greater than the accumulation of [^{14}C]dopamine, and most of the newly synthesized catecholamine is found within the cells [6]. Incubation of the cells in media containing 56 mM K^+ results in an increase in catecholamine synthesis, but does not change the ratio of norepinephrine to dopamine accumulation. Incubation of the cells with lasalocid results in a large increase in the accumulation of [^{14}C]dopamine. Furthermore, in the presence of lasalocid, most of the newly synthesized dopamine (73 per cent) is found in the incubation medium. However, lasalocid does not stimulate total catecholamine synthesis (norepinephrine + dopamine) in these cells. These effects of lasalocid on catecholamine synthesis are also seen in the absence of extracellular Ca^{2+} (not shown).

DISCUSSION

Lasalocid stimulates the release of catecholamine from sympathetic neurons [12], intact adrenal glands [13,14], and isolated adrenal cells [15], as well as from pheochromocytoma cells [7]. In no case has the mechanism of this action of lasalocid been clearly defined. Lasalocid stimulates the release of catecholamine and of dopamine β-mono-oxygenase, but not the release of lactate dehydrogenase, from pheochromocytoma cells [7]. These results are consistent with the hypothesis that lasalocid stimulates exocytosis in these cells. Lasalocid also causes the activation of TH in pheochromocytoma cells. The similar dose–response curves for the stimulation of catecholamine release and the activation of TH by lasalocid suggest that these two effects may result from a common action of the ionophore.

Incubation of pheochromocytoma cells in media containing 56 mM K^+ also results in the stimulation of catecholamine secretion and the activation of TH. The actions of 56 mM K^+ are dependent upon the presence of extracellular Ca^{2+}, and are inhibited by diphenylhydantoin, which blocks the uptake of Ca^{2+} into the cells [11]. It seems likely that the effects of 56 mM K^+ on catecholamine secretion and TH activity in pheochromocytoma cells are mediated by the uptake of Ca^{2+} into the cells. In contrast, the effects of lasalocid on catecholamine release and on TH activation are not dependent upon extracellular Ca^{2+}. Lasalocid is a Ca^{2+} ionophore [16], and has been shown to cause the release of Ca^{2+} from sarcoplasmic reticulum [17,18] and from mitochondria [19]. It is likely that lasalocid also causes the release of Ca^{2+} from some intracellular store in pheochromocytoma cells. The hypothesis that lasalocid mobilizes an intracellular Ca^{2+} store would rationalize the effects of this ionophore on TH activation and catecholamine secretion by phoechromocytoma cells. A rise in intracellular Ca^{2+} may be a common intermediate step in the activation of TH produced by 56 mM K^+ and by lasalocid. Some workers have reported that Ca^{2+} can activate TH in cell-free preparations of the enzyme [20,21]. However, this result has not been confirmed [22]. We have also been able to detect direct effects of TH Ca^{2+} on TH activity *in vitro*. If Ca^{2+} is involved in the activation of TH by 56 mM K^+ and by lasalocid, the mechanism by which Ca^{2+} increases TH activity is not clear.

Cochrane and Douglas [23] have shown that lasalocid depolarizes frog skeletal muscle fibers, and have proposed that depolarization plays a role in the actions of this ionophore. However, because lasalocid activates TH in pheochromocytoma cells incubated in a medium consisting of 0.3 M sucrose–10 mM HEPES, pH 7.4, it is unlikely that depolarization plays an inportant role in the activation of TH produced by lasalocid.

The ionophore A23187, which would also be expected to raise intracellular Ca^{2+} levels, does not activate TH. The lack of effect of A23187 appears to be incompatible with the hypothesis that TH activity is regulated by intracellular Ca^{2+} levels. However, A23187 causes the lysis of pheochromocytoma cells [7], and its inability to activate TH may be due to the disruption of cellular integrity produced by this ionophore.

In our experiments, the stimulus-coupled activation of TH is associated with an increase in the V_{max} of the enzyme, but is not accompanied by changes in the apparent K_m values of the enzyme for tyrosine or for its pteridine cofactor. In other tissues, the stimulus-coupled activation of TH is accompanied by a decrease in the K_m of the enzyme for its pteridine cofactor [21, 24, 25]. However, the stimulation of guinea pig vas deferens also leads to an increase in the V_{max} of TH in this tissue [21]. The differences in the kinetic properties of TH in our experiments and those of others presumably reflect differences in the TH assay conditions, or differences in the composition of the crude tissue extracts used for TH assay.

The effects of lasalocid on catecholamine synthesis are complex. In cells incubated in control media and in media containing 56 mM K^+, the accumulation of [^{14}C]norepinephrine is greater than the accumulation of [^{14}C]dopamine, and most of the newly synthesized catecholamine is found within the cells. In contrast, in cells incubated with lasalocid, the accumulation of [^{14}C]dopamine is greater than the accumulation of [^{14}C]norepinephrine, and most of the newly synthesized catecholamine is found in the incubation medium. These effects of lasalocid are probably due to its action as a catecholamine ionophore. Lasalocid promotes the release of dopamine from synaptosomes [26], and the release of catecholamine from chromaffin granules [27], and may cause the release of newly synthesized catecholamine from the cytoplasm of pheochromocytoma cells. We have not ruled out the possibility that lasalocid may also inhibit dopamine β-mono-oxygenase activity, and so may block the conversion of dopamine to norepinephrine. However, the observation that lasalocid causes a greater release of newly synthesized catecholamine than does 56 mM K^+ (Table 2), whereas these two stimuli are about equipotent in promoting the release of stored catecholamine [7], suggests that lasalocid causes the release of newly formed catecholamine directly from the cytoplasm into the incubation medium.

The action of lasalocid as a catecholamine ionophore may also account for its inability to stimulate total catecholamine synthesis. If lasalocid causes the release of catecholamine from chromaffin granules in-

side the cells, it will raise the intracellular catecholamine concentration, and so may increase the feedback inhibition of TH by catecholamines. The net effect of lasalocid on catecholamine synthesis will reflect both the activation of TH produced by this ionophore and the feedback inhibition of this enzyme produced by a rise in intracellular catecholamine levels.

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