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Both Nicotinic and Muscarinic Agonists Acutely Increase Tyrosine 3-Monooxygenase Activity in the Superior Cervical Ganglion[1]

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ABSTRACT

The activity of tyrosine 3-monooxygenase in rat superior cervical ganglia *in vitro* was measured by monitoring their rate of dopa production. Cholinergic agonists produce a rapid and reversible increase in dopa synthesis in the ganglia. Carbachol (0.1 mM) causes a 5- to 6-fold increase in dopa synthesis. The action of carbachol is largely inhibited by the nicotinic antagonist hexamethonium (3 mM) and is completely blocked by a combination of hexamethonium and the muscarinic antagonist atropine (6 μM). Dimethylphenylpiperazinium (1 mM), a specific nicotinic agonist, produces a 4-fold increase in dopa synthesis. The action of dimethylphenylpiperazinium is blocked by hexamethonium but not by atropine. Bethanechol (1 mM), a muscarinic agonist, causes a 2-fold increase in dopa synthesis. The action of bethanechol is inhibited by atropine but not by hexamethonium. It is concluded that tyrosine 3-monooxygenase activity in rat superior cervical ganglia can be increased by both nicotinic and muscarinic stimulation, that nicotinic stimulation can produce a greater increase than can muscarinic stimulation and that carbachol increases enzyme activity by a combination of both pathways. These cholinergic mechanisms for the acute regulation of tyrosine 3-monooxygenase may be activated *in vivo* by acetylcholine released from preganglionic neurons and thus may play a role in the physiological regulation of catecholamine synthesis in sympathetic ganglia.

Although there have been many studies of the regulation of TH (EC 1.14.16.2) activity in sympathetic ganglia, most of these studies have focused on the "trans-synaptic induction" of enzyme synthesis after neural or chemical stimulation (for review, see Zigmond and Bowers, 1981). Stimulation of the preganglionic cervical sympathetic trunk leads to a long-term increase in TH activity in the SCG. Because preganglionic nerve stimulation causes the activation of both nicotinic and muscarinic receptors in the ganglion, the involvement of these receptors in the regulation of TH has been examined. The long-term effect of nerve stimulation on TH activity is completely inhibited by nicotinic antagonists such as hexamethonium and chlorisondamine. Thus, this effect appears to be mediated by the release of acetylcholine from preganglionic nerve terminals and the subsequent activation of nicotinic receptors on the principal neurons in the ganglion (Chalazonitis *et al.*, 1980; Chalazonitis and Zigmond, 1980). It has been suggested that the activation of muscarinic receptors in the ganglion can inhibit the induction of TH by nicotine (Hanbauer and Costa, 1976), but Chalazonitis *et al.* (1980) found no evidence that such an inhibitory mechanism occurs during preganglionic nerve stimulation.

In contrast to the extensive studies of TH induction in the SCG, there have been relatively few studies on the acute regulation of the activity of the enzyme in this tissue. Lloyd *et al.* (1979) reported that carbachol increases the activity of TH in SCG incubated *in vitro*. This action of carbachol was blocked by atropine but not by hexamethonium and so was thought to be mediated by the activation of muscarinic receptors. Because of the apparent differences in the type of cholinergic receptors involved in the short-term and the long-term regulation of TH activity, we decided to investigate further the short-term effects of cholinergic agonists on TH activity in intact SCG *in vitro*.

Materials and Methods

Male Sprague-Dawley rats (175–200 g) were purchased from Charles River Laboratories (Wilmington, MA) and were maintained for at least one week before use. Rats were killed by cervical dislocation and their SCGs were removed and desheathed. Ganglia were preincubated for 30 min at 37°C in 0.65 ml of Earle's Balanced Salt Solution (Grand Island Biological Company, Grand Island, NY) supplemented with 0.1 mM tyrosine and 0.1 mM EDTA and equilibrated with 95% O_2-5% CO_2. The

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ABBREVIATIONS: TH, tyrosine 3-monooxygenase; SCG, superior cervical ganglion; DMPP, dimethylphenylpiperazinium.

ganglia were then incubated for up to 120 min at 37°C in 0.65 ml of fresh medium containing 150 μM brocresine and cholinergic agonists as indicated. In experiments with cholinergic antagonists, these antagonists were present in both the preincubation and the incubation media. At the end of the incubation period, the ganglia were homogenized in 65 μl of 1.65 M trichloroacetic acid and 1 mM EDTA. To measure the total amount of dopa produced by the ganglia, the incubation media were added to the homogenates and the combined samples were chilled and then centrifuged for 2 min in a Beckman microfuge. The dopa content of the samples was assayed by liquid chromatography with electrochemical detection, according to a modification of the method of Felice *et al.* (1978), as previously described (Erny *et al.*, 1981). Unless otherwise indicated, results are expressed as picomoles of dopa produced per ganglion per hr, mean ± S.E.M. The differences in means of various groups of ganglia were evaluated by Student's *t* test (two-tailed).

Brocresine was a gift from Dr. David N. Ridge, Lederle Laboratories (Pearl River, NY). Cholinergic agonists and antagonists (carbachol, DMPP iodide, bethanechol chloride, atropine sulfate and hexamethonium bromide) were obtained from Sigma Chemical Co. (St. Louis, MO).

Results

When SCGs are incubated *in vitro* in the presence of brocresine [an inhibitor of aromatic-L-amino-acid decarboxylase (EC 4.1.1.28)], they produce and accumulate dopa. In 10 experiments, the rate of dopa production by ganglia incubated in control medium was 80 ± 4 pmol/ganglion/hr (range 60–100). In the absence of brocresine, the ganglia do not accumulate dopa (data not shown); under these conditions, presumably, all of the dopa that is formed in the ganglia is converted to dopamine. Incubation of the SCG with carbachol results in a large increase in dopa synthesis (table 1). In 10 experiments with 0.1 mM carbachol, the increase in dopa accumulation averaged 5.5 ± 0.4-fold (range 3.4–7.8). 3-Iodotyrosine (2 mM) almost completely abolishes dopa production, both in the presence (table 1) and absence (not shown) of carbachol. Therefore, we believe that dopa synthesis results from, and is a measure of, the activity of TH. In our experiments, we have routinely measured dopa production in a 15- or 30-min incubation period. The rate of dopa production by ganglia incubated with or without carbachol is approximately constant for this period. Moreover, dopa formed by the ganglia is stable for at least 30 min under our incubation conditions. In the experiment reported in table 1, one group of ganglia was incubated for 30 min with 0.1 mM carbachol and another group was first incubated for 30 min with carbachol and then incubated for a second 30-min period in the presence of 2 mM 3-iodotyrosine. The amounts of dopa accumulated by the two groups of ganglia were not significantly different. Thus, dopa formed during the first incubation period was stable during the subsequent incubation with 3-iodotyrosine. Approximately half of the dopa produced by the ganglia is in the ganglia and half is in the incubation medium.

TABLE 1

Dopa synthesis by SCG.

Ganglia were incubated for 30 min in medium without additions (A), in the presence of 0.1. mM carbachol (B) and in the presence of 0.1 mM carbachol and 2 mM 3-iodotyrosine (C). 3-Iodotyrosine inhibited dopa synthesis in the presence of carbachol by more than 90%. To determine whether the dopa formed during an incubation with carbachol was stable, a fourth group of ganglia (D) was incubated for 30 min with 0.1 mM carbachol, after which 3-iodotyrosine was added to a final concentration of 2 mM and incubation was continued for an additional 30 min. Dopa synthesis in this group was similar to that in group B. Dopa synthesis is expressed as picomoles per ganglion, mean ± S.E.M. of three ganglia.

Group	Incubation Conditions	Dopa Production
		pmol/ganglion
A	No additions	30 ± 4
B	Carbachol (0.1 mM)	145 ± 20
C	Carbachol (0.1 mM) + 3-iodotyrosine (2 mM)	10 ± 2
D	Carbachol (0.1 mM) followed by 3-iodotyrosine (2 mM)	166 ± 1

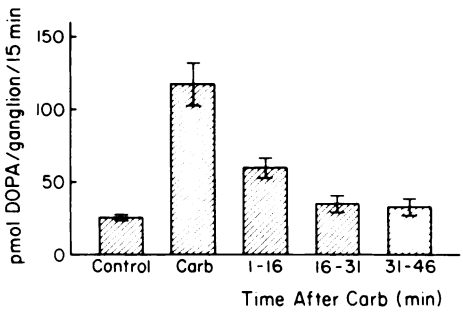

Fig. 1. Reversibility of the carbachol (Carb)-evoked increase in dopa synthesis. Ganglia were incubated for 15 min in the presence or absence of Carb (0.1 mM) and their rate of dopa synthesis was measured. Other ganglia were exposed to Carb for 15 min and then dopa synthesis was measured in 15-min intervals after washing in Carb-free medium for 1, 16 or 31 min. Dopa synthesis is expressed as picomoles per ganglion per 15 min, mean ± S.E.M. of three ganglia.

To study the reversibility of the effect of carbachol on TH activity, ganglia were incubated for 15 min with 0.1 mM carbachol in the absence of brocresine, washed various times in carbachol-free medium and then incubated for 15 min in carbachol-free, brocresine-containing medium for measurement of dopa production (fig. 1). In this experiment, incubation of ganglia with 0.1 mM carbachol in the presence of brocresine resulted in a 4.7-fold increase in dopa synthesis. When ganglia were exposed to carbachol for 15 min and then washed in carbachol-free medium for 1 min, the rate of dopa synthesis (measured over the subsequent 15 min) was greatly reduced. After ganglia were incubated in a carbachol-free medium for 16 min, their rate of dopa production returned to control levels. The time-course of this decrease in dopa synthesis is consistent with the hypothesis that carbachol causes a reversible increase in TH activity and that the half-time for the reversal of this effect is approximately 5 min.

Figure 2 illustrates the concentration-dependence of the action of carbachol. Carbachol (10 μM) significantly increases dopa synthesis; 1 mM carbachol produces a maximal increase (approximately 8-fold) in dopa accumulation.

Carbachol is a mixed cholinergic agonist and activates both nicotinic and muscarinic receptors. To investigate the receptor types that mediate the carbachol-induced increase in dopa synthesis, we examined the effects of specific cholinergic antagonists on this process (table 2). Atropine (6 μM) causes a small decrease in carbachol-stimulated dopa production, which was not statistically significant. Hexamethonium (3 mM) causes a much larger (75–80%), although not complete, inhibition of carbachol action. Hexamethonium and atropine together completely inhibit the carbachol-induced increase in dopa production.

The experiment reported in table 2 indicates that the major portion of the carbachol-induced increase in dopa synthesis is mediated by nicotinic receptors and suggests that a small

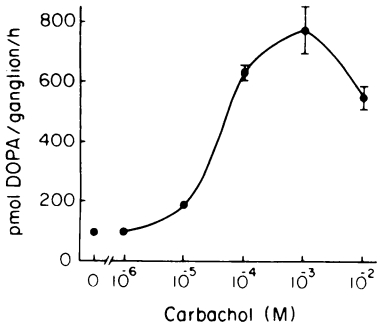

Fig. 2. Effect of carbachol on dopa synthesis in the SCG. Ganglia were incubated for 30 min in the presence of various concentrations of carbachol and the synthesis of dopa was measured. Dopa synthesis is expressed as picomoles per ganglion per hr, mean ± S.E.M. of three ganglia. Where no error bars are shown, the S.E.M.s are smaller than the size of the data points.

TABLE 2

Effects of carbachol and of cholinergic blocking agents on dopa synthesis in the SCG.

Ganglia were incubated for 30 min in the presence or absence of carbachol (0.1 mM) and in the presence of hexamethonium (6 mM) and atropine (6 μM) as indicated. Ganglia that were treated with hexamethonium and with atropine were also exposed to these agents during a 30-min preincubation period. Dopa synthesis was measured and is expressed as picomoles per ganglion per hr, mean ± S.E.M. of three ganglia, except for ganglia treated with carbachol and hexamethonium, where $n = 6$. In ganglia treated with carbachol, hexamethonium produced a significant inhibition of dopa synthesis ($P < .01$). In ganglia treated with carbachol and hexamethonium, atropine produced a significant inhibition of dopa synthesis ($P < .01$).

Incubation Conditions	Dopa Production	
	Control	Carbachol (0.1 mM)
	pmol/ganglion/hr	
No additions	78 ± 14	353 ± 38
Atropine (6 μM)	89 ± 20	289 ± 16
Hexamethonium (3 mM)	91 ± 14	143 ± 17
Hexamethonium (3 mM) + atropine (6 μM)	70 ± 4	67 ± 2

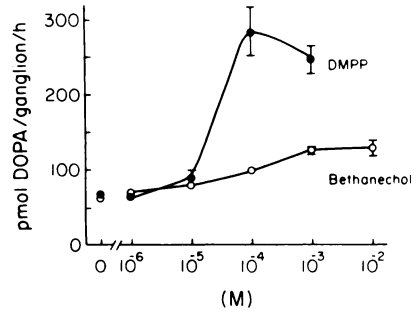

Fig. 3. Effects of DMPP and of bethanechol on dopa synthesis in the SCG. Ganglia were incubated for 30 min in the presence of various concentrations of DMPP or of bethanechol and the synthesis of dopa was measured. Dopa synthesis is expressed as picomoles per ganglion per hr, mean ± S.E.M. of three to six ganglia.

portion of this action is mediated by muscarinic receptors. To clarify the roles of these two receptors in mediating the stimulation of TH activity, we studied the effects of specific nicotinic and muscarinic agonists on dopa production. Both DMPP, a nicotinic agonist, and bethanechol, a muscarinic agonist, produce concentration-dependent increases in dopa synthesis (fig. 3). DMPP produces a larger effect than does bethanechol. In three separate experiments, 0.1 mM DMPP produced on average a 4.5-fold increase, whereas 1 mM bethanechol produced a 2.1-fold increase in dopa production.

Finally, the effects of cholinergic antagonists on the stimulation of dopa production evoked by DMPP and by bethanechol were examined. As shown in table 3, the action of DMPP is almost completely abolished by hexamethonium, but is unaffected by atropine. In contrast (table 4), the action of bethanechol is blocked by atropine, but is not significantly inhibited by hexamethonium.

Discussion

Our studies demonstrate that both nicotinic and muscarinic cholinergic agonists increase the rate of dopa production by SCG *in vitro*, that nicotinic stimulation produces a larger increase in dopa synthesis than does muscarinic stimulation and that mixed cholinergic agonists, such as carbachol, can increase dopa synthesis by a combination of nicotinic and muscarinic pathways.

There have been relatively few studies of the short-term regulation of catecholamine synthesis in the SCG. Ikeno *et al.* (1981) (see also Lloyd *et al.*, 1979) have reported that carbachol causes an acute increase in TH activity in this ganglion. There are, however, several major discrepancies between our results and the results of these workers. First, Ikeno *et al.* (1981) report a much lower rate of TH activity in ganglia incubated under control conditions than we observe (approximately 8 pmol/ganglion/hr, in contrast to approximately 80 pmol/ganglion/hr). Second, these workers observe only a muscarinic activation of tyrosine hydroxylation, whereas we find that dopa synthesis is increased by both nicotinic and muscarinic stimulation. Finally, these workers report a 35 to 80% increase in TH activity in response to 0.1 mM carbachol, whereas we observe a 5-fold

TABLE 3

Stimulation of dopa production by a specific nicotinic agonist.

Ganglia were incubated for 30 min in the presence or absence of DMPP (1 mM) and in the presence of hexamethonium (3 mM) or atropine (6 μM) as indicated. Ganglia that were treated with hexamethonium and with atropine were also exposed to these agents during a 30-min preincubation period. Dopa synthesis was measured and is expressed as picomoles per ganglion per hr, mean ± S.E.M. of the number of ganglia indicated in parentheses.

Incubation Conditions	Dopa Production
	pmol/ganglion/hr
DMPP (1 mM)	246 ± 18 (6)
DMPP + atropine (6 μM)	287 ± 34 (3)
DMPP + hexamethonium (3 mM)	87 ± 3 (3)
Control	63 ± 5 (6)

TABLE 4

Stimulation of dopa production by a specific muscarinic agonist.

Ganglia were incubated for 30 min in the presence or absence of bethanechol (1 mM) and in the presence of hexamethonium (3 mM) or atropine (6 μM) as indicated. Ganglia that were treated with hexamethonium and with atropine were also exposed to these agents during a 30-min preincubation period. Dopa synthesis was measured and is expressed as picomoles per ganglion per hr, mean ± S.E.M. of nine ganglia.

Incubation Conditions	Dopa Production
	pmol/ganglion/hr
Bethanechol (1 mM)	134 ± 7
Bethanechol + hexamethonium (3 mM)	111 ± 10
Bethanechol + atropine (6 μM)	72 ± 3
Control	65 ± 3

increase in enzyme activity in response to this agent. The magnitude of the response to carbachol reported by Ikeno *et al.* (1981) is comparable to the response to muscarinic stimulation that we observe.

We cannot account completely for the differences between our results and those of Ikeno *et al.* (1981). Our experimental protocol differed from theirs in a number of ways: we used Earle's Balanced Salt Solution, while they used BGJ_b medium; we used adult rats, whereas they predominantly used newborn rats; and we measured dopa accumulation in the presence of brocresine by liquid chromatography, while they measured the release of ^3H_2O from [3,5-^{3}H]tyrosine. The control rate of dopa production that we observe, 80 pmol/ganglion/hr, agrees well with the rates of catecholamine synthesis in rat SCG reported by Brown *et al.* (1977) and by Steinberg and Keller (1978). Like Ikeno *et al.* (1981), Steinberg and Keller (1978) used a radioisotopic method to measure catecholamine synthesis and calculated rates of synthesis from the specific activity of tyrosine in the incubation medium. Our control rates of dopa synthesis also agree well with predictions based on steady-state analyses of catecholamine metabolism in the SCG. The rate-constant for the metabolism of norepinephrine in the rat SCG has been estimated at 0.46 (Fischer and Snyder, 1965) and 0.35/hr (Brodie *et al.*, 1966). As the rat SCG contains about 150 pmol of norepinephrine (Brown *et al.*, 1977), this turnover rate corresponds to 50 to 70 pmol/ganglion/hr. Thus, our measurements of dopa synthesis in the SCG *in vitro* are similar to estimates of catecholamine synthesis in the ganglion *in vivo*.

Previous studies have demonstrated that nicotinic receptors mediate the long-term regulation of TH activity in the SCG by nerve stimulation (Chalazonitis *et al.*, 1980) and by cholinergic agonists (Hanbauer and Costa, 1976). Our experiments indicate that nicotinic receptors can also participate in the acute regulation of TH activity. Thus, the activation of nicotinic receptors *in vivo* by acetylcholine may not only mediate synaptic transmission through the ganglion but may also lead to both short- and long-term modifications of the transmitter-synthesizing capacity of ganglionic neurons *via* alterations in TH activity. The functional significance of this acute regulation of TH activity in ganglia remains to be determined. The stimulation of catecholamine synthesis by acetylcholine may be coupled to the secretion of catecholamines within the SCG. Preganglionic nerve stimulation has been reported to cause the release of norepinephrine in the SCG (Noon *et al.*, 1975; Martinez and Adler-Graschinsky, 1980). Catecholamine-containing varicosities have been observed in dendrites of the principal neurons in the ganglion (Jacobowitz, 1970; Kondo *et al*, 1980) and may be the sites from which this norepinephrine is released. Moreover, there are adrenergic receptors in the SCG (Brown and Caulfield, 1979) and it has been proposed that catecholamines may modulate transmission through the ganglion (Haefely, 1969).

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References

Brodie, B. B., Costa, E., Dlabac, A., Neff, N. H. and Smookler, H. H.: Application of steady state kinetics to the estimation of synthesis rate and turnover time of tissue catecholamines. J. Pharmacol. Exp. Ther. **154:** 493–498, 1966.

Brown, D. A. and Caulfield, M. P.: Hyperpolarizing 'α_2'-adrenoceptors in rat sympathetic ganglia. Br. J. Pharmacol. **65:** 435–446, 1979.

Brown, J. H., Nelson, D. L. and Molinoff, P. B.: Organ culture of rat superior cervical ganglia. J. Pharmacol. Exp. Ther. **201:** 298–311, 1977.

Chalazonitis, A., Rice, P. J. and Zigmond, R. E.: Increased ganglionic tyrosine hydroxylase and dopamine β-hydroxylase activities following preganglionic nerve stimulation: Role of nicotinic receptors. J. Pharmacol. Exp. Ther. **213:** 139–143, 1980.

Chalazonitis, A. and Zigmond, R. E.: Effects of synaptic and antidromic stimulation on tyrosine hydroxylase activity in the rat superior cervical ganglion. J. Physiol. (Lond.) **300:** 525–538, 1980.

Erny, R. E., Berezo, M. W. and Perlman, R. L.: Activation of tyrosine 3-monooxygenase in pheochromocytoma cells by adenosine. J. Biol. Chem. **256:** 1335–1339, 1981.

Felice, L. J., Felice, J. D. and Kissinger, P. T.: Determination of catecholamines in rat brain parts by reverse-phase ion-pair liquid chromatography. J. Neurochem. **31:** 1461–1465, 1978.

Fischer, J. E. and Snyder, S.: Disposition of norepinephrine-H^1 in sympathetic ganglia. J. Pharmacol. Exp. Ther. **150:** 190–195, 1965.

Haefely, W. E.: Effects of catecholamines in the cat superior cervical ganglion and their postulated role as physiological modulators of ganglionic transmission. Prog. Brain Res. **31:** 61–72, 1969.

Hanbauer, I. and Costa, E.: Induction of tyrosine hydroxylase in the superior cervical ganglia of rats: Opposite influence of muscarinic and nicotinic receptor agonists. Neuropharmacology **15:** 85–90, 1976.

Ikeno, T., Dickens, G., Lloyd, T. and Guroff, G.: The receptor-mediated activation of tyrosine hydroxylation in the superior cervical ganglion of the rat. J. Neurochem **36:** 1632–1640, 1981.

Jacobowitz, D.: Catecholamine fluorescence studies of adrenergic neurons and chromaffin cells in sympathetic ganglia. Fed. Proc. **29:** 1929–1943, 1970.

Kondo, H., Dun, N. J. and Pappas, G. D.: A light and electron microscopic study of the rat superior cervical ganglion cells by intracellular HRP-labeling. Brain Res. **197:** 193–199, 1980.

Lloyd, T., Montgomery, P. and Guroff, G.: The receptor-mediated, short-term activation of tyrosine hydroxylase in organ cultures of rat superior cervical ganglia. Biochem. Biophys. Res. Commun. **86:** 1058–1062, 1979.

Martínez, A. E. and Adler-Graschinsky, E.: Release of norepinephrine induced by preganglionic stimulation of the isolated superior cervical ganglion of the cat. J. Pharmacol. Exp. Ther. **212:** 527–532, 1980.

Noon, J., McAfee, D. A. and Roth, R. H.: Norepinephrine release from nerve terminals within the rabbit superior cervical ganglion. Naunyn-Schmiedeberg's Arch. Pharmacol. **291:** 139–162, 1975.

Steinberg, M. I. and Keller, C. E.: Enhanced catecholamine synthesis in isolated rat superior cervical ganglia caused by nerve stimulation: Dissociation between ganglionic transmission and catecholamine synthesis. J. Pharmacol. Exp. Ther. **204:** 384–399, 1978.

Zigmond, R. E. and Bowers, C. W.: Influence of nerve activity on the macromolecular content of neurons and their effector organs. Ann. Rev. Physiol. **43:** 673–687, 1981.

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