MOLECULAR PHARMACOLOGY, 10, 855–860

The Role of Adenosine Cyclic 3′,5′- Monophosphate in Reserpine-Initiated Adrenal Medullary Tyrosine Hydroxylase Induction

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(Received April 1, 1974)

SUMMARY

MUELLER, R. A., OTTEN, U., AND THOENEN, H.: The role of adenosine cyclic 3′,5′-monophosphate in reserpine-initiated adrenal medullary tyrosine hydroxylase induction. *Mol. Pharmacol.* **10**, 855–860 (1974).

An intact splanchnic nerve is required for the initiation of trans-synaptic induction of tyrosine hydroxylase in rat adrenal medulla. Severing of the splanchnic nerve fibers supplying the left adrenal 4 hr after reserpine administration completely abolished the induction of tyrosine hydroxylase observed 48 hr later on the intact side. Transection of the splanchnic nerves 8 hr after drug administration partially prevented tyrosine hydroxylase induction, whereas transection after 12 hr had no effect on induction. Adrenal medullary cyclic AMP contents were measured at various times up to 12 hr after reserpine. The rapid initial increase in cyclic AMP returned to normal by 90 min and remained there for the next 12 hr. When cyclic AMP had returned to control levels after reserpine, administration of aminophylline (theophylline ethylenediamine) produced a slower rate of increase in cyclic AMP than observed in controls. Therefore it appears either that cyclic AMP is not involved in the trans-synaptic induction of tyrosine hydroxylase observed after reserpine or that the intact splanchnic nerve is required for some part of the inductive process after the cyclic AMP response has terminated.

After administration of reserpine to rats the activity of tyrosine hydroxylase (EC 1.14.3.a), the enzyme which catalyzes the rate-limiting step in the biosynthesis of catecholamines (1), increases in sympathetic ganglia and in the adrenal chromaffin cells (2, 3). The increase in tyrosine hydroxylase activity is due to an increase in the number of specific enzyme molecules (4, 5). Transection of the splanchnic nerve supplying the adrenal medulla prior to drug treatment prevents the increase in tyrosine hydroxylase, suggesting that the inductive signal is trans-synaptic (6). Since administration of high doses of acetylcholine in combination with atropine and eserine (7) or high doses of carbachol (8) to animals with denervated adrenals can also initiate an increase in tyrosine hydroxylase activity *in vitro*, acetylcholine appears to represent the immediate inductive signal.

It has been suggested by Costa and

This study was supported by Grant 3.653.71 from the Swiss National Foundation for Scientific Research and Grant GM 19088 from the United States Public Health Service.

Guidotti (8, 9) that increased trans-synaptic activity after reserpine elicits a neuronally dependent increase of cyclic AMP concentration in the adrenal medulla of the rat. These authors proposed that the increased medullary cyclic AMP could transmit information from the cholinergic receptor to the cell processes which finally elevate tyrosine hydroxylase activity; i.e., cyclic AMP may once again act as a "second messenger."

However, recent studies in our laboratory have revealed marked dissociations between the rate of cyclic AMP elevation and the subsequent increase in tyrosine hydroxylase activity in both superior cervical ganglia and adrenal medulla (10, 11). In the present investigation severing of preganglionic splanchnic nerve fibers 4 hr after reserpine administration blocked tyrosine hydroxylase induction. Since the cyclic AMP changes which followed reserpine administration were already complete for more than 2 hr at this time, either cyclic AMP is not involved in tyrosine hydroxylase induction or an additional trans-synaptic factor is required to permit expression of the cyclic AMP effect.

Male Sprague-Dawley rats (Süddeutsche Versuchstierfarm, Tuttlingen, Germany) weighing 150–180 g were kept under constant laboratory conditions at 24° with a light-dark cycle of 12 hr and had free access to food and water. Experimental animals received an intraperitoneal injection of 10 mg/kg of reserpine and/or 200 μmoles/kg of theophylline (administered as aminophylline). Control animals were given 0.9% NaCl injections.

Denervation of the left adrenal medulla was performed either just before injection of reserpine or various times after reserpine administration. The operation was performed in 5–8 min under ether anesthesia (6). Completeness of denervation was controlled in each adrenal gland by determination of choline acetyltransferase activity (12) with modifications described by Oesch *et al.* (13). Complete denervation diminished choline acetyltransferase activity to less than 10% of the contralateral control adrenal; denervation of adrenals did not cause a reduction in catecholamine content, confirming earlier data (6).

At appropriate time intervals following the last treatment (Fig. 1) the animals were killed by a blow on the head and adrenal glands were rapidly removed. Both adrenal medullae were dissected from the cortex at 0°, using a dissecting microscope. Two adrenal medullae (contaminated with less than 5% cortex tissue as verified histologically) were homogenized in 300 μl of 5% trichloracetic acid. After centriguation at 5000 × *g* for 20 min the supernatant fractions were extracted three times with 7 ml of ether and cyclic AMP concentrations were determined in 50-μl aliquots of the aqueous fractions (14). The protein of the trichloracetic acid precipitate was dissolved in 1 N NaOH and determined according to Lowry *et al.* (15).

All animals were killed 48 hr after reserpine treatment. Each adrenal was homogenized in 2 ml of 0.25 M sucrose. A sample of the sucrose homogenate was then acidified with 0.4 N $HClO_4$ for later estimation of catecholamines, expressed as epinephrine (16, 17), and another was rehomogenized in 0.5% Triton–1 mM EDTA for assay of choline acetyltransferase activity. Tyrosine hydroxylase activity was determined by a modification (2) of the procedure of Levitt *et al.* (18). All enzyme activities were ascertained to be first-order with respect to protein and linear with time for each experimental condition. Radioactivity was determined in toluene containing 6 g of (2,4″ - *tert* - butylphenyl) - 5 - (4″ - biphenyl-1,3,4-oxidazole) (Ciba) and 300 ml of Triton X-100 per liter.

Student's *t*-test was used to establish the significance of differences between means, with $p < 0.05$ as the level of significance (19). The measure of variation in this study is the standard error of the mean. The method of least squares was used to calculate regression coefficients and equations to describe rates of change.

[*ring*-3,5-^{3}H]L-Tyrosine (specific radioactivity, more than 30 Ci/mmole) was purchased from the Radiochemical Centre (Amersham, England); [*G*-^{3}H]adenosine 3′, 5′-monophosphate (10 Ci/mmole), from New England Nuclear Corporation; 6,7-dimethyl-5,6,7,8-tetrahydropteridine HCl, B grade, from Calbiochem; and reserpine (Serpasil), from Ciba-Geigy.

Figure 1 shows the rapid increase and abrupt fall of medullary content of cyclic AMP after the intraperitoneal injection of 10 mg/kg of reserpine. The maximal increase in cyclic AMP was observed 30 min after reserpine. Between 60 and 90 min after reserpine administration cyclic AMP levels again approached control values. From 90 min to 12 hr after treatment with reserpine medullary concentrations of cyclic AMP remained at control levels.

Tyrosine hydroxylase activity was determined 48 hr after reserpine administration, since previous studies had shown that the maximum increase in enzyme activity *in vitro* occurred 48–72 hr after reserpine injection (2). Reserpine produced a similar increase in enzymatic activity in the intact adrenal glands of rats operated 4, 8, and 46 hr after reserpine treatment (Fig. 2). Denervation did not change adrenal tyrosine hydroxylase activity in control animals (6), and operation in addition to reserpine did not produce a greater increase in enzymatic activity in the innervated right adrenal than that produced by reserpine alone. Splanchnicotomy 4 hr after injection of reserpine completely abolished the rise in enzyme activity in the denervated adrenal, whereas cutting the splanchnic nerve 8 hr after reserpine markedly reduced hydroxylase induction, although the small increase in enzymatic activity was still statistically significant ($p < 0.05$). Denervation performed at 12 hr (not shown) or 46 hr did not interfere with the rise in hydroxylase activity. Thus the surgical denervation procedure itself did not produce a decrease in adrenal medullary tyrosine hydroxylase once the signal had been received.

It could be proposed that although the cyclic AMP levels returned to normal 90 min after reserpine, there was still an increased rate of synthesis of cyclic AMP or accumulation of cyclic AMP in a small, active compartment of the cell, but that this increase was no longer evident in the whole adrenal medulla because of elevated phosphodiesterase activity (20). To examine this possibility control and reserpine-treated rats were given 200 μmoles/kg intraperitoneally of theophylline, an inhibitor of medullary phosphodiesterase activity (8), 4 hr after NaCl or reserpine administration

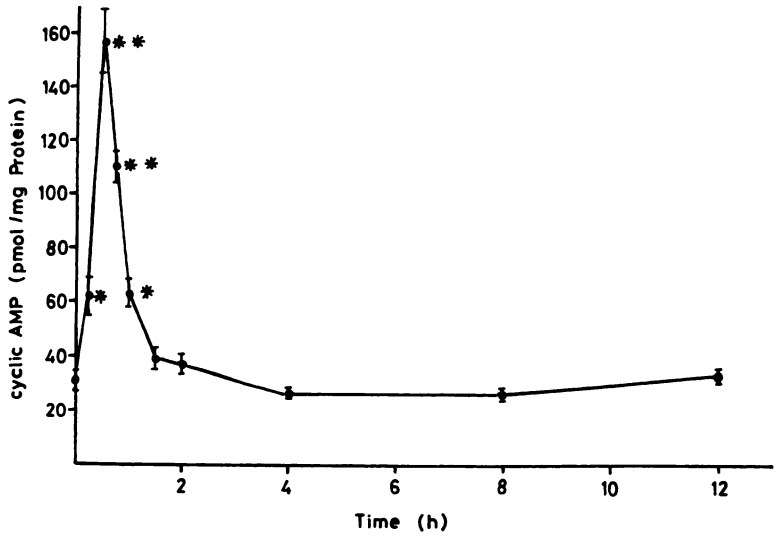

FIG. 1. *Effect of reserpine on concentration of cyclic AMP in rat adrenal medulla*

Cyclic AMP was determined at the time intervals indicated after intraperitoneal injection of 10 mg/kg of reserpine. Values represent means ± standard errors of at least six determinations. The concentration of cyclic AMP in adrenal medullae of control animals was 33 pmoles/mg of protein.

* $p < 0.05$.

** $p < 0.01$.

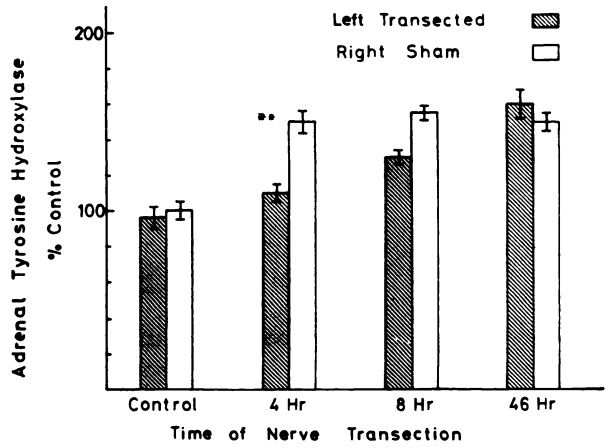

FIG. 2. *Effect of splanchnic nerve transection on reserpine-induced tyrosine hydroxylase activity in rat adrenals*

Tyrosine hydroxylase activity *in vitro* was determined in unilaterally denervated and contralaterally innervated adrenal glands 48 hr after the intraperitoneal administration of 10 mg/kg of reserpine. Values represent mean ± standard errors of at least seven determinations. Surgical denervation was performed in control animals 4, 8, and 46 hr after injection of reserpine. Completeness of splanchnicotomy was controlled by measuring choline acetyltransferase activity and adrenal catecholamine concentrations as described in detail in the text. The activity of tyrosine hydroxylase in intact control adrenal glands was 9.8 ± 0.5 nmoles of dopa per pair of adrenals per hour, and in denervated adrenals of control animals it was 9.5 ± 0.7 nmoles of dopa per pair of adrenals per hour.

** $p < 0.001$.

(Fig. 3). This dose of theophylline has previously been shown to produce a maximal rate of increase in adrenal medullary cyclic AMP in control rats (21). Interestingly, the rate of accumulation was significantly slower (4.6 pmoles of cyclic AMP per milligram of protein per minute; $y = 4.6x + 26.5$, $p < 0.05$) in adrenal medullae of animals previously treated with reserpine than in adrenal medullae of control animals (15.3 pmoles/mg of protein/min; $y = 15.3x + 31$).

The trans-synaptic induction of tyrosine hydroxylase in the peripheral sympathetic nervous system is a slow adaptive process enhancing the capacity for catecholamine synthesis. A consistent increase in the level of this enzymatic activity cannot be recorded earlier than 16–24 hr after beginning a treatment, which leads to enhancement of nerve impulse traffic in the preganglionic cholinergic nerves supplying the terminal adrenergic neurons and the adrenal medullary cells (2, 22–24). The neuronally mediated increase in enzyme activity is the result of augmented synthesis of specific enzyme protein (4) and is entirely attributable to an accumulation of more enzyme molecules, as revealed by immunochemical titration (5). The regulatory events taking place at the level of transcription are terminated by 12–24 hr after the administration of insulin or termination of a 2-hr swim stress (3, 7, 22). However, little is known about the chain of events by which changes in the chromaffin cell membrane effected by enhanced acetylcholine liberation alter the regulation of tyrosine hydroxylase synthesis at the transcription level.

The present study shows that after reserpine injection a rapid, brief increase in cyclic AMP content indeed occurs. After 60–90 min the cyclic AMP content of the adrenal medulla is again normal and remains there for at least the next 10 hr. Cutting the splanchnic nerve 4 hr after reserpine, when the cyclic AMP response is completed, but before tyrosine hydroxylase activity has begun to change, totally blocks induction of the enzyme measured 48 hr later. Although splanchnic nerve transection 8 hr after reserpine still reduces the

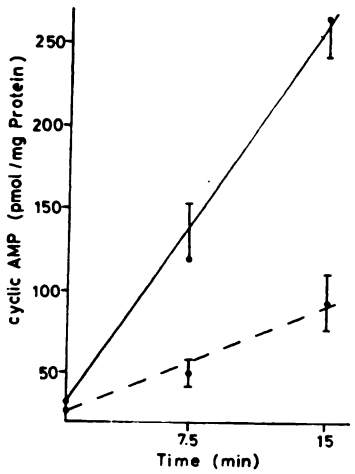

FIG. 3. *Linear rate of accumulation of cyclic AMP in adrenal medullae after intraperitoneal injection of 200 μmoles/kg of theophylline 4 hr after NaCl (control, O——O) or reserpine, 10 mg/kg (O- - -O)*

The values given are means ± standard errors of six determinations. The concentration of cyclic AMP in controls was 31 pmoles/mg of protein, and in reserpine-treated animals it was 26 pmoles/mg of protein. The calculated slopes of the regression lines are 15.3 pmoles of cyclic AMP per milligram of protein per minute for control and 4.6 pmoles/mg of protein per minute for reserpine-treated rats. The correlation coefficients of the regression lines are 0.89 for controls and 0.82 for reserpine-treated animals. The slopes are significantly different ($p < 0.005$).

rise in tyrosine hydroxylase, it can no longer completely prevent a small subsequent increase in enzymatic activity in the adrenals. Thus it appears that trans-synaptic impulses are required between 4 and 12 hr. Since Costa and Guidotti (8) have shown that the increase in cyclic AMP after reserpine can be inhibited by prior splanchnic nerve transection, this response may also be related to increased nerve activity. However, this increased efferent activity either is not related to tyrosine hydroxylase induction or does not exist long enough to induce the adrenal enzyme.

Although the administration of theophylline has previously been used to measure the turnover rate of adrenal medullary cyclic AMP (21), this application assumes not only that phosphodiesterase is completely inhibited but that cyclic AMP is not destroyed by other pathways or lost from the cell. If these assumptions are valid for control and reserpine-treated rats, the present studies suggest that the turnover of cyclic AMP is actually decreased during the critical interval of splanchnic nerve stimulation in the adrenal medullae of reserpine-treated rats. However, since the above assumptions were not directly tested, the lower rate of accumulation of cyclic AMP in reserpine-treated rats may reflect either an impairment by reserpine of central actions of theophylline which increase adrenal medullary cyclic AMP (8, 10) or an increase in disappearance of cyclic AMP after reserpine administration. Other recent studies in our laboratory also question the involvement of cyclic AMP in tyrosine hydroxylase induction. Marked differences were observed between changes in cyclic AMP and subsequent induction of the enzyme both in the adrenal medullae and in superior cervical ganglia. Prior treatment of rats with propranolol nearly abolished (an increase of 40% vs. +410%) the rise in medullary cyclic AMP content produced by reserpine, while the reserpine-induced increase in tyrosine hydroxylase activity was not diminished (12).

For these reasons it seems unlikely that the brief increase in cyclic AMP content after reserpine is the relevant biochemical signal initiating subsequent induction of tyrosine hydroxylase. It is more likely that, as in many other cells, cyclic AMP is simply one manifestation of cell stimulation, but not causally linked to induction of this enzyme.

ACKNOWLEDGMENT

We wish to thank Miss Petra Tengzelius for her excellent technical assistance.

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