

the two-kidney form of renal hypertension. However, the results also strongly suggest that one-kidney renal hypertension is maintained by pressor mechanisms different from the renin-angiotensin system.

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Dopamine-Sensitive Adenyl Cyclase: Possible Role in Synaptic Transmission

Abstract. *An adenyl cyclase activated by low concentrations of dopamine has been found in the mammalian superior cervical sympathetic ganglion. The existence of this enzyme may account for the increased amount of adenosine 3',5'-monophosphate associated with synaptic activity in the ganglion. The results suggest that the physiological effects of dopamine in the ganglion, and possibly elsewhere in the nervous system, may be mediated by stimulating the synthesis of adenosine 3',5'-monophosphate.*

Experiments in our laboratory (1) have demonstrated that preganglionic stimulation of the superior cervical sympathetic ganglion of the rabbit, under relatively physiological conditions, produced a severalfold increase in the content of adenosine 3',5'-monophosphate (cyclic AMP) in the ganglion. In contrast, postganglionic stimulation produced no increase in the cyclic AMP content of the ganglion. From these and other observations, it was concluded that the increased amount of cyclic AMP was associated with the process of synaptic transmission within the ganglion, and that the increase occurred primarily in postsynaptic cells. This and a variety of other evidence, summarized elsewhere (2, 3), suggests that the cyclic AMP system may be intimately associated with the physiology of synaptic transmission. It therefore seemed of considerable importance to clarify the mechanism responsible for the increase in cyclic AMP associated with synaptic transmission in the ganglion.

Catecholamines have been shown to increase the cyclic AMP content of

many tissues, including brain. There is evidence that suggests adrenergic regulation of synaptic transmission in the superior cervical ganglion (4-6). Superior cervical ganglia from several species, including bovine and rabbit, contain two catecholamines, dopamine and norepinephrine, in comparable amounts (7); and we have, therefore, studied the ability of these two catecholamines to stimulate the formation of cyclic AMP. Our experiments, with blocks of tissue prepared from bovine ganglia, indicate that dopamine, in low concentrations, and norepinephrine, in higher concentrations, increase the amount of cyclic AMP in intact cells. In addition, in experiments with homogenates of bovine ganglia, it was found that dopamine stimulates adenyl cyclase activity, but does not significantly alter phosphodiesterase activity. These observations support the hypothesis that small chromaffin-like interneurons release dopamine in response to preganglionic stimulation, and that this catecholamine activates adenyl cyclase in postganglionic neurons, thereby mediating the increased

amount of cyclic AMP that follows preganglionic stimulation (1). Our experiments provide experimental evidence for a new type of adenyl cyclase, one with apparent specificity for dopamine, and suggest a mechanism, at the cellular and molecular levels, for the action of dopamine in the ganglion and possibly in other regions of the nervous system.

The amount of cyclic AMP accumulation in blocks of ganglion tissue was determined by means of the prelabeling technique (8) in which the amount of radioactive cyclic AMP formed from prelabeled adenosine triphosphate is determined (9). The relative potencies of dopamine and norepinephrine in causing the accumulation of cyclic AMP in blocks of ganglion tissue were compared (Fig. 1). At low concentrations dopamine was more effective than norepinephrine in causing the accumulation of cyclic AMP. The maximum responses to the two catecholamines were approximately equal, although in some experiments the maximum response to norepinephrine was somewhat greater than to dopamine. In each of several experiments similar to that shown in Fig. 1, a half-maximum increase in cyclic AMP accumulation occurred in the presence of 6 to 10 μ M dopamine. Moreover, in those experiments, 42 μ M norepinephrine produced a response approximately equal to that seen with 7 μ M dopamine.

In some experiments, the absolute amount of cyclic AMP was also determined by means of the protein kinase assay method (11). The results obtained with the two methods were similar. For instance, in one experiment the absolute amount of cyclic AMP in prelabeled but nonincubated tissue was 14.5 pmole per milligram of protein. Incubation in the presence of 10 mM theophylline alone caused a 2.6-fold increase in the absolute amount of cyclic AMP and a 3.2-fold increase in the amount of radioactive cyclic AMP. Incubation in the presence of 30 μ M dopamine plus 10 mM theophylline caused a 9.7-fold increase in the absolute amount of cyclic AMP and a 9.1-fold increase in the amount of radioactive cyclic AMP.

We have studied the effect of agents that antagonize the actions of catecholamines in other tissues on the accumulation of cyclic AMP in the superior cervical ganglion. Phentolamine, an α -adrenergic antagonist, prevented the increase in cyclic AMP produced by

7 μ M dopamine, whereas it did not appreciably alter the accumulation of cyclic AMP in the absence of added dopamine (Fig. 2). The response to 7 μ M dopamine was reduced by about 50 percent in the presence of 28 μ M phentolamine. Another α -adrenergic antagonist, phenoxybenzamine, at a concentration of 70 μ M, abolished the increase in cyclic AMP produced by 7 μ M dopamine. In contrast, propranolol, the β -adrenergic antagonist, was ineffective, at concentrations from 7 μ M to as high as 210 μ M, in preventing the increase in cyclic AMP mediated by 7 μ M dopamine. Interestingly, propranolol (120 μ M) caused a substantial reduction of the increase in cyclic AMP mediated by 40 μ M norepinephrine. Two other β -adrenergic antagonists, dichloroisoproterenol and MJ 1999 [4'-(2-isopropylamino-1-hydroxyethyl) methanesulfonanilide], each tested at concentrations as high as 400 μ M, were also ineffective in preventing the accumulation of cyclic AMP mediated by 7 μ M dopamine; in contrast, each of these agents, tested at a dose of 400 μ M, reduced the increase in cyclic AMP mediated by 40 μ M norepinephrine.

It is important to know whether the accumulation of cyclic AMP observed in response to dopamine results from an increased synthesis or from a decreased degradation of the cyclic nu-

Table 1. The effects of catecholamines and theophylline on adenylyl cyclase activity and phosphodiesterase activity of homogenates of the bovine superior cervical ganglion. Activity is expressed as nanomoles per milligram of protein per minute. Each value represents the mean of determinations on two samples.

Additions	Activity (nmole/min)	Percent of control
<i>Adenylyl cyclase</i>		
None	33×10^{-3}	100
Dopamine (7 μ M)	59×10^{-3}	179
Norepinephrine (7 μ M)	56×10^{-3}	170
<i>Phosphodiesterase</i>		
None	17.1	100
Dopamine (7 μ M)	16.7	97
Dopamine (30 μ M)	17.4	100
Theophylline (10 mM)	2.2	13
Theophylline (10 mM) plus dopamine (30 μ M)	2.8	16

cleotide. In order to distinguish between these two possibilities, we studied the effects of dopamine on the adenylyl cyclase activity and phosphodiesterase activity of homogenates of ganglia (12). Dopamine and norepinephrine each stimulated adenylyl cyclase activity of the ganglion homogenate (Table 1). In contrast, dopamine did not inhibit phosphodiesterase activity of the ganglion homogenate, nor did it increase the inhibition observed in the presence of theophylline. These observations indi-

cate that catecholamine-sensitive adenylyl cyclase activity exists within the superior cervical ganglion, and that the dopamine-mediated accumulation of cyclic AMP is the result of an increase of adenylyl cyclase activity rather than an inhibition of phosphodiesterase activity.

The present data provide the first evidence, in any biological system, that the effects of dopamine are mediated through activation of adenylyl cyclase (13). In previous studies, in which slices of intact mammalian nervous tissue were used, dopamine had little or no effect on the amount of cyclic AMP (14). Moreover, although dopamine was able to stimulate the adenylyl cyclase activity of rat erythrocyte ghosts, the concentration of dopamine required for half-maximum activation of the enzyme was 17 to 350 times greater than that required for other catecholamines, and the maximum activation by dopamine was only 50 percent of the stimulation by other catecholamines (15). However, on the basis of our studies, we propose as a working hypothesis that the other biological actions of dopamine, including those within the basal ganglia, are also mediated by the activation of adenylyl cyclase in the responsive tissues.

It is of interest to consider our results in relation to the increase in cyclic AMP that occurs in the supe-

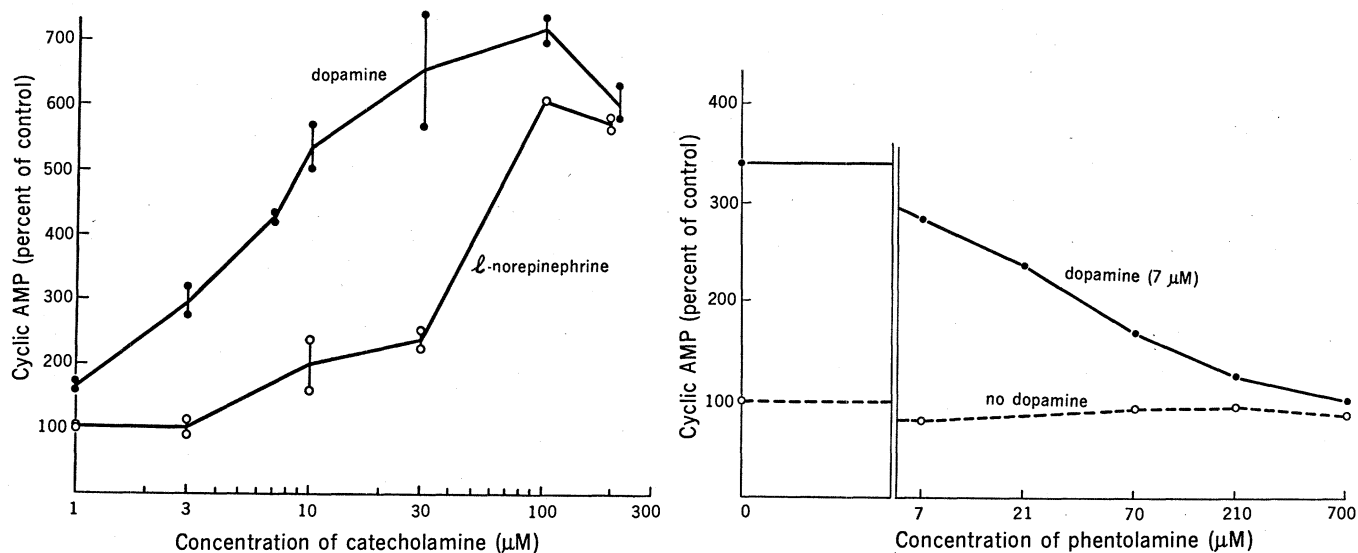


Fig. 1 (left). Stimulation by dopamine and l-norepinephrine of cyclic AMP accumulation in blocks of bovine superior cervical ganglion. Tissue was incubated for 5 minutes at 37°C in oxygenated Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM theophylline and the indicated amount of catecholamine. Cyclic AMP accumulation during incubation in the presence of catecholamine and theophylline is expressed as the percentage of that observed during incubation in the presence of theophylline alone. The curves are drawn through the average of two data points; each data point represents the mean of duplicate determinations on an individual sample. Fig. 2 (right). Effect of the α -blocker, phentolamine, on cyclic AMP accumulation, in the presence and absence of 7 μ M dopamine, in blocks of bovine superior cervical ganglion. Tissue was incubated for 5 minutes at 37°C in oxygenated Krebs-Ringer bicarbonate buffer, pH 7.4, containing 10 mM theophylline and the indicated amounts of dopamine and phentolamine. Cyclic AMP accumulation is expressed as the percentage of that observed in the presence of theophylline alone. Each point represents the mean of determinations on two to four samples analyzed in duplicate.

rior cervical ganglion in association with the process of synaptic transmission (1). We have attempted (1) to account for this increase in cyclic AMP by the following schema: (i) physiological activity in the preganglionic neurons, in addition to directly activating the postganglionic neurons, also excites small dopamine-containing interneurons; (ii) the excitation of these interneurons causes them to secrete dopamine; and (iii) the dopamine thus released activates dopamine-sensitive adenylyl cyclase in the postganglionic neurons. A variety of anatomical, pharmacological, and biochemical evidence supports this proposal concerning the cell types and neurotransmitter within the superior cervical ganglion that are responsible for the increased amount of cyclic AMP associated with synaptic activity. Although dopamine and norepinephrine, the two catecholamines found within the ganglion, can each cause an accumulation of cyclic AMP, these two substances are found in different cell types. Norepinephrine is found within the postganglionic neurons. Dopamine, on the other hand, is concentrated in small interneurons (16). These interneurons, which possess many of the morphological features of the chromaffin cells of the adrenal medulla, form synapses on the postganglionic neurons (17). Preganglionic, cholinergic fibers synapse on these interneurons as well as directly on the postganglionic neurons. Pharmacological evidence that the increase in cyclic AMP caused by preganglionic stimulation involves the dopamine-containing interneurons comes from studies with both adrenergic and cholinergic blocking agents. Recently, it has been found (18) that low concentrations of phenolamine blocked the increase in cyclic AMP associated with preganglionic stimulation, but MJ 1999, at concentrations up to 200 μ M, did not. Thus, the increase in cyclic AMP caused by stimulation of preganglionic nerve fibers was antagonized by adrenergic blocking agents in a manner similar to the increase in cyclic AMP caused by exogenous dopamine. It has also been found that atropine, which appears to block the excitation of the interneurons (5), also prevents the increase in cyclic AMP that follows preganglionic stimulation (18). In contrast, hexamethonium, which blocks the action of acetylcholine on the postganglionic neurons but does not appear to affect the interneurons (5), does not prevent

the increase in cyclic AMP that follows preganglionic stimulation (18). Finally, the biochemical data, which demonstrate the stimulation by dopamine of cyclic AMP synthesis in the ganglion, support the schema outlined above.

The physiological significance of the increase in cyclic AMP associated with synaptic activity in these ganglia is a matter of considerable interest. It is possible that this increase in cyclic AMP may be responsible for the slow hyperpolarization (slow inhibitory postsynaptic potential) of the ganglion that follows preganglionic stimulation. Recent observations support this idea. Exogenous dopamine (6, 18) is able to hyperpolarize the postganglionic neurons of the rabbit superior cervical ganglion. As described above, the increase in cyclic AMP seen in response either to preganglionic stimulation or to exogenous dopamine can be antagonized by α -adrenergic blocking agents. The slow hyperpolarization that follows preganglionic stimulation (5), as well as that caused by exogenous dopamine (6), are also antagonized by α -adrenergic blocking agents. Moreover, 1 mM theophylline when added to the medium bathing the rabbit superior cervical ganglion causes a substantial increase in the magnitude of the slow hyperpolarization that follows preganglionic stimulation (18), implicating cyclic AMP in the production of this physiological response.

The biochemical events by which cyclic AMP might induce the hyperpolarization of the postganglionic cell membrane are not known. However, it has been suggested that the diverse effects of cyclic AMP in various tissues may be mediated through regulation of the activity of protein kinases (2, 19). A hypothesis worthy of consideration [compare (1, 2)] is that the ability of cyclic AMP to hyperpolarize the nerve cell membrane results from the activation of a protein kinase, with the consequent phosphorylation of a protein constituent of the nerve cell membrane, leading to a change in membrane properties concerned with ion movement.

The hyperpolarization of the membrane of the postganglionic neuron alters the responsiveness of the neuron to subsequent activity in the presynaptic fibers. Thus, the role of the increase in ganglionic cyclic AMP that follows preganglionic stimulation may be to mediate dopaminergic transmission and thereby to modulate cholinergic

transmission. The extent to which cyclic AMP may also be involved in synaptic transmission elsewhere in the nervous system is an important area for future investigation.

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9. Superior cervical ganglia from young calves of both sexes were removed immediately after death, at a local abattoir, and placed in ice-cold oxygenated Krebs-Ringer bicarbonate buffer that contained (in millimoles per liter): NaCl, 122; KCl, 3; MgSO₄, 1.2; CaCl₂, 1.3; KH₂PO₄, 0.4; NaHCO₃, 25; and D-glucose, 10. This buffer had been previously equilibrated with a gas mixture of 95 percent O₂ and 5 percent CO₂ and had a pH of 7.4 at 25°C. The ganglia were then desheathed, and were cut freehand with a razor blade into thin slices. These slices were rapidly brought back to the laboratory in oxygenated Krebs-Ringer bicarbonate buffer at 4°C and maintained at that temperature until they were cut into small blocks with a McIlwain tissue chopper (settings 0.52 mm by 0.26 mm). On sectioning, the blocks were placed in an oxygenated Krebs-Ringer bicarbonate buffer at 37°C. The blocks were then preincubated for 45 minutes in 32 ml of oxygenated Krebs-Ringer bicarbonate buffer at 37°C, containing 11.9 μ M [8-¹⁴C]adenine (specific activity 41.6 mc/mmole) and then washed extensively with oxygenated Krebs-Ringer bicarbonate buffer at 37°C, to remove extracellular adenine. For incubation, portions of tissue were transferred to homogenizers in which there were 5.0 ml of oxygenated Krebs-Ringer bicarbonate buffer (37°C), containing 10⁻²M theophylline and appropriate concentrations of test agents. Unless otherwise stated incubation time was 5 minutes. Throughout the period of preincubation and incubation, blocks of tissue were prevented from settling by the gas mixture bubbling through the solution. At the end of the incubation, the Krebs-Ringer bicarbonate buffer was removed by aspiration, 1.5 ml of ice-cold 6 percent trichloroacetic acid was added, and the tissue was immediately homogenized. Tritiated carrier cyclic AMP (0.1 μ moles) was added to each sample and the homogenate was centrifuged. The supernatant was decanted for cyclic AMP assay. The precipitate was suspended in 2.0 ml of 1.0N NaOH and protein was determined by the method of O. H. Lowry, N. J. Rosebrough, A. L. Farr, R. J. Randall, *J. Biol. Chem.* **193**, 265 (1951), with bovine serum albumin as the standard. The supernatant solution was extracted three times with 1.0 ml of diethyl ether. Cyclic AMP was isolated by minor modification of the method of Krishna *et al.* (10),

and the amounts of ^3H and ^{14}C in the isolated sample were counted simultaneously in a Packard Tricarb liquid scintillation system. The amount of radioactivity present in the cyclic AMP of the original samples was calculated per milligram of protein with the use of the recovery of tritiated cyclic AMP to correct each sample for loss during the isolation procedure. The data were corrected by subtracting the amount of radioactive cyclic AMP present in prelabeled but non-incubated tissue.

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12. For studies of homogenates, ganglia were prepared by means of a McIlwain tissue chopper, in a manner similar to the procedure used for the prelabeling technique, and then homogenized manually for 60 seconds with 1.4 volumes of 6 mM tris(hydroxymethyl)-aminomethane-maleate buffer, pH 7.4. Adenyl cyclase activity of the homogenates was measured in the presence of 10 mM theophylline, by a slight modification of the method of Krishna *et al.* (10). Phosphodiesterase activity of the homogenates was measured by minor modification of the procedure of J. Beavo, J. Hardman, E. W. Sutherland, *J. Biol. Chem.* **245**, 5649 (1970).
13. The possibility exists that, in our experiments with intact ganglion cells, exogenous dopamine could become concentrated by post-ganglionic neurons and converted into norepinephrine, and that this newly synthesized norepinephrine, rather than the exogenous dopamine per se, would activate the adenyl cyclase. We consider this to be improbable for several reasons. (i) Low concentrations of dopamine were effective in stimulating the formation of cyclic AMP in homogenates of bovine ganglia; these homogenates were unfortified by the addition of cofactors necessary for the enzymatic conversion of dopamine to norepinephrine. (ii) As described above, experiments with the β -adrenergic antagonist, propranolol, have shown that this agent does not affect the dopamine-mediated increase in cyclic AMP, but does reduce the accumulation of cyclic AMP caused by norepinephrine. (iii) We have found that cocaine (210 μM), which has been shown to block the uptake of dopamine and norepinephrine by various tissues, caused a slight increase in the dopamine-mediated accumulation of cyclic AMP in blocks of bovine ganglia, whereas a decrease would be expected if the intracellular accumulation of dopamine and its conversion to norepinephrine were required. Thus, these data indicate that exogenous dopamine caused the accumulation of cyclic AMP in the ganglion by direct stimulation of an adenyl cyclase sensitive to low concentrations of dopamine.
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Proportional Release of Norepinephrine and Dopamine- β -Hydroxylase from Sympathetic Nerves

Abstract. Dopamine- β -hydroxylase (DBH), the enzyme that catalyzes the conversion of dopamine to norepinephrine, is localized in the vesicles containing catecholamine in sympathetic nerves. This enzyme is released with norepinephrine when the nerves to the guinea pig vas deferens are stimulated *in vitro*, and the amount of enzyme discharged increases as the length of stimulation periods increases. The amount of DBH released is proportional to the amount of norepinephrine released, and the ratio of norepinephrine to DBH discharged into the incubation medium is similar to that in the soluble portion of the contents of the synaptic vesicles from the vas deferens. These data are compatible with the release of the neurotransmitter norepinephrine and DBH from sympathetic nerves by a process of exocytosis.

Norepinephrine is stored in sympathetic nerve terminals within vesicular structures and is released in response to neural stimulation (1). The mechanism by which this neurotransmitter is released is not known. Norepinephrine might be liberated from nerves by a process of exocytosis, analogous to the mechanism of release of catecholamines from the adrenal medulla. In this release, the chromaffin granule discharges the soluble portion of its contents to the exterior of the cell presumably through an opening in the cell membrane (2). One way to test for a mechanism of release involving exocytosis is to determine whether other soluble molecules in the storage vesicle, especially large molecules, are discharged with norepinephrine in response to stimulation.

Dopamine- β -hydroxylase (DBH), the enzyme that catalyzes the conversion of dopamine to norepinephrine (3), is localized in the vesicles storing catecholamine, both in the adrenal medulla (4) and in sympathetic nerves (5). This enzyme is released with catecholamines when the isolated perfused adrenal gland is stimulated with acetylcholine (6) and when the sympathetic nerves to the isolated perfused spleen are stimulated electrically (7). For the adrenal medulla, the ratio of norepinephrine to DBH released is similar to the ratio in the chromaffin granule, a result that supports exocytosis as the mechanism of release (6). When nerves to the spleen were stimulated, however, the ratio of amine to DBH released was 100 times greater than that found in vesicles isolated from the splenic nerve (7). These data have raised serious questions about exocytosis as the mechanism of release of norepinephrine from sympathetic nerves (8). The development of a sensitive enzymatic assay for DBH activity (9) enabled us to study quantitatively the release of DBH with norepinephrine from sympathetic nerves.

The results of these experiments are compatible with the coupled release of norepinephrine and DBH from sympathetic nerves by a process of exocytosis.

The animals used were male albino guinea pigs, 500 to 800 g. Vasa deferentia and attached hypogastric nerves were dissected after the animals were killed by a blow on the head. The organs were placed in 10-ml baths containing medium (10) aerated with 5 percent CO_2 in O_2 , and were maintained at 37°C . The bath fluid was changed four times and was then replaced by fresh medium containing 0.25 percent bovine serum albumin. This fluid was replaced after 10 minutes with 5 ml of medium containing 0.25 percent albumin, and the organ preparations were allowed to equilibrate for 5 minutes before the start of electrical stimulation, 30 seconds per minute for 30 to 90 minutes (5 to 7 volts, 25 Hz, 5 msec).

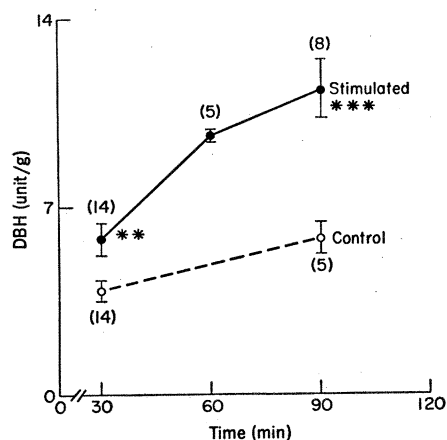


Fig. 1. The release of dopamine- β -hydroxylase after stimulation of hypogastric nerve. Activity of DBH is expressed as nanomoles of octopamine formed from tyramine per gram of tissue per hour. The numbers in parentheses represent the number of vasa deferentia in each group. Symbols are as follows: **, $P < .02$ compared with unstimulated 30-minute control; and ***, $P < .01$ compared with unstimulated 90-minute control.