Stimulation by Dopamine of Adenylate Cyclase in Retinal Homogenates and of Adenosine-3':5'-Cyclic Monophosphate Formation in Intact Retina

(adrenergic receptors/epinephrine/norepinephrine/protein kinase/ouabain)

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ABSTRACT A catecholamine-sensitive adenylate cyclase system is present in homogenates of both calf and rat retinas. Dopamine is a more potent activator of the bovine enzyme than is norepinephrine or epinephrine. Cyclic AMP concentrations in intact bovine retina are increased by dopamine, as well as by other catecholamines, and by depolarizing agents. Studies with adrenergic blocking agents suggest that the stimulation of retinal adenylate cyclase by catecholamines cannot be clearly defined in terms of the characteristics of alpha or beta adrenergic receptors. Bovine retina also contains a protein kinase that is stimulated 20-fold by cyclic AMP. It is proposed that dopamine is the major activator of a retinal adenylate cyclase, and that this activation is related to its role as a neurotransmitter.

It has recently been demonstrated that rod outer segments from vertebrates contain an adenylate cyclase of high specific activity that is inactivated by light in proportion to the bleaching of rhodopsin, suggesting a role for cyclic AMP in sensory processes (1, 2). The retina is functionally, histologically, and embryologically part of the central nervous system, with a limited number of discretely defined cell types. The predominant amine in the retina is dopamine (3, 4), and it fulfills most of the criteria required to classify it as a neurotransmitter in this tissue (5-8). The formation of cyclic AMP in incubated brain slices is stimulated by certain putative central nervous system transmitters (norepinephrine, epinephrine, histamine, and serotonin) (9, 10), as well as by depolarizing agents (11) and by electrical stimulation (12). Adenylate cyclase activity is localized primarily in the synaptosomal fraction of brain (13). Several lines of evidence suggest a postsynaptic localization and indicate that the activation of this enzyme and the concomitant changes in cyclic AMP concentrations may mediate some of the effects of neurotransmitters (14-16). Attempts to demonstrate dopaminergic activation of brain adenylate cyclase have been unsuccessful (9, 11, 17). It was hoped that the retina, by virtue of its unique properties, might provide a more useful model to attempt to link the role of dopamine, both generally and as a neurotransmitter, with the adenylate cyclase system.

Preliminary reports from this laboratory had shown rat retinal homogenates to contain an epinephrine-sensitive adenylate cyclase*. In the present study, dopamine is shown to increase cyclic AMP concentrations in intact bovine retinal tissue, as well as to directly activate adenylate cyclase of calf and rat retinas. The influence of other catecholamines, as well as certain depolarizing agents and adrenergic blocking agents, on these parameters is also described.

MATERIALS AND METHODS

Calf eyes were removed within 1 hr after slaughter and kept on ice for 1-2 hr until the retinas were removed. Male Sprague-Dawley rats were killed by decapitation and the eyes were enucleated immediately. The anterior section of the eye was removed by cutting slightly behind the equator, and the retina was isolated by gently teasing it away from the choroid and severing its connection at the optic nerve head.

For assay of adenylate cyclase activity, tissue was homogenized in an all-glass homogenizer in about 10 volumes of cold 20 mM glycylglycine buffer (pH 7.8)-5 mM MgSO4. Assay was by incubation of aliquots of homogenates containing 100-300 μg of tissue protein with 0.625 mM [α-32P]-ATP (about 1.5 × 106 dpm) for 20 min at 30°C, followed by chromatographic separation of labeled cyclic AMP. Assay conditions were as described (18), with the following modifications: The incubation mixture also contained 0.15 mM unlabeled cyclic AMP, an ATP-regenerating system consisting of 2 mM phosphoenolpyruvate and 10 μg/ml of pyruvate kinase (EC 2.7.1.40), and 0.5 mM disodium ethylenediaminetetraacetic acid (EDTA). Ouabain was included in the assay at 20 μM where indicated. Labeled cyclic AMP was separated by thin layer chromatography on polyethyleneimine (PEI)-cellulose with isopropanol-ammonium hydroxide-water 7:1:3. In some instances, the quantity of [α-32P]ATP remaining at the end of the incubation was determined by chromatography on PEI-cellulose with 1 M LiCl as the developing solvent.

For the experiments with intact retinas, a bicarbonate-buffered medium containing glucose (19), pre-equilibrated at 35°C with 5% CO2-95% O2, was used. Tissue was removed and weighed within 60 sec, placed in beakers containing 10 ml of medium, and incubated at 35°C with continuous gassing with 5% CO2-95% O2. At the end of a 40-min incubation, retinas were transferred to beakers containing fresh medium, 5 mM caffeine, and hormones or other agents. Incubations with hormones were terminated after 10 min by rapid removal of the retina and homogenization in 1 ml of 50% acetic acid. An aliquot of the acetic-acid homogenate was removed for protein determination, and the remainder of the homogenate was heated for 3 min at 90°-95°C and centrifuged,

**RESULTS AND DISCUSSION**

Adenylate cyclase activity in homogenates of calf and rat retinas was stimulated to about the same level by dopamine, norepinephrine, and epinephrine at a concentration of 0.2 mM, as shown in Table 1. In other experiments, rat retinal preparations tested without ouabain showed small, but consistent, increases above basal levels (<10%) in the presence of hor-

TABLE 1. Stimulation of adenylate cyclase of calf and rat retinas by catecholamines

<table>
<thead>
<tr>
<th></th>
<th>Calf enzyme</th>
<th>Rat enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+ Ouabain</td>
<td>+ Ouabain</td>
</tr>
<tr>
<td>− Ouabain</td>
<td>(20 μM)</td>
<td>(20 μM)</td>
</tr>
<tr>
<td></td>
<td>Ac−tivity</td>
<td>% Increase</td>
</tr>
<tr>
<td>Control</td>
<td>66.6</td>
<td>74.0</td>
</tr>
<tr>
<td>0.2 mM Dopamine</td>
<td>101.0</td>
<td>148.3</td>
</tr>
<tr>
<td>0.2 mM Norepinephrine</td>
<td>91.4</td>
<td>141.3</td>
</tr>
<tr>
<td>0.2 mM Epinephrine</td>
<td>91.9</td>
<td>137.8</td>
</tr>
<tr>
<td>8 mM NaF</td>
<td>182.9</td>
<td>181.3</td>
</tr>
</tbody>
</table>

Activity is expressed as nanomol of \(^{32}\)Pcyclic AMP formed per 100 mg of protein under standard assay conditions as described in the text, except for omission of EDTA from the assay of rat enzyme.

% Increase is above basal level, and represents the mean value for either 4 (calf) or 2 (rat) determinations; standard errors are all less than 15%.

and the supernatant was removed. For assay of cyclic AMP concentrations, 10-μl aliquots of the acetic-acid supernatants were added to small tubes, dried at 80°C, and assayed in these tubes by a modification of the isotope-dilution method of Gilman (20). A binding protein from brain, purified through the ammonium sulfate step (21), rather than a binding protein from muscle was used, and the incubation was performed at pH 3.5. Titration with unlabeled cyclic AMP was similar to that described by Gilman, allowing detection of less than 0.2 pmol, and displacement by nucleotides other than cyclic AMP was the same or less than that reported. The capacity of the retinal extracts to displace \(^{3}H\)cyclic AMP from the binding protein was destroyed by prior incubation of the sample with purified beef-heart cyclic nucleotide phosphodiesterase. Homogenization of tissue in trichloroacetic acid and extraction with ether gave equivalent results to those obtained with the acetic acid extract.

Protein kinase was prepared from frozen bovine retina through the ammonium sulfate fractionation, as described by Miyamoto, Kuo, and Greengard (21). Enzyme activity was measured by the method of Kuo and Greengard (22) with 2.5 μM [γ-\(^{32}\)P]ATP (about 10⁶ dpm), unfractionated calf-thymus histone as substrate, and with 10 mM NaF and 2 mM theophylline included in the assay mixture.

Proteins were determined by the method of Lowry et al. (23). l-Norepinephrine d-bitartrate was purchased from Mann Research Laboratories, l-epinephrine d-bitartrate from Nutritional Biochemicals Corp., and dopamine HCl from Sigma.
Table 2. Effects of alpha and beta adrenergic blocking agents on catecholamine-stimulated adenylate cyclase activity in calf retina

<table>
<thead>
<tr>
<th>Hormone (20 μM)</th>
<th>None</th>
<th>Phentolamine (380 μM)</th>
<th>Phenoxymethazin (100 μM)</th>
<th>Dichloroisoproterenol (100 μM)</th>
<th>Propranolol (70 μM)</th>
<th>Propranolol (7 μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. Dopamine</td>
<td>31.1</td>
<td>5.8</td>
<td>21.9</td>
<td>27.3</td>
<td>27.2</td>
<td>—</td>
</tr>
<tr>
<td>B. Dopamine</td>
<td>31.9</td>
<td>9.5</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>34.9</td>
</tr>
<tr>
<td>A. Epinephrine</td>
<td>15.8</td>
<td>2.7</td>
<td>4.4</td>
<td>4.9</td>
<td>5.8</td>
<td>—</td>
</tr>
<tr>
<td>B. Epinephrine</td>
<td>20.1</td>
<td>3.0</td>
<td>—</td>
<td>—</td>
<td>—</td>
<td>24.1</td>
</tr>
</tbody>
</table>

Activity is expressed as (nanomol of [3H]cyclic AMP formed per 100 mg of protein in the presence of hormone) minus (basal activity with appropriate blocking agent) under standard assay conditions.

A and B represent separate experiments. Each point is the mean of two determinations; standard errors are all less than 10%.

basal activity seen here. Further studies will be necessary to evaluate this possibility.

Fig. 1 shows a dose-response curve for activation of bovine adenylate cyclase in the presence of ouabain. At 200 μM hormone concentrations, dopamine, norepinephrine, and epinephrine were equally effective in stimulating enzyme activity, while at 20 μM and 2 μM, dopamine was significantly more potent than either norepinephrine or epinephrine (P < 0.01). Activation by dopamine was maximal at 20 μM, whereas maximal activation by norepinephrine and epinephrine occurred at 200 μM. Activation by 2 mM norepinephrine and epinephrine were equivalent to that observed at the 200 μM concentration (not shown). Based on effective concentrations for half-maximal stimulation, dopamine was about 10 times as potent as the other catecholamines. The same pattern of stimulation and the same relative potencies of the catecholamines were seen when the adenylate cyclase reaction was terminated at 5 min instead of at 20 min.

Stimulation of adenylate cyclase by dopamine has been studied in detail in the rat erythrocyte, the only system in which dopamine has been reported to be an effective activator (24). Stimulation of the erythrocyte cyclase by dopamine was maximal at 1 mM, and was only half that found with maximally activating concentrations of norepinephrine (0.1 mM). N-methyl-DL-propranolol, however, was about as potent as norepinephrine, and the stimulation by both of these agents was blocked to some extent by phenolamine, but much more effectively by propranolol. Additional evidence was given to support the hypothesis that the responses to the catecholamines lacking a β-hydroxyl group were mediated through a beta receptor system.

The effects of alpha and beta adrenergic blocking agents on catecholamine stimulation of bovine adenylate cyclase in the presence of ouabain are seen in Table 2. Phenolamine produced the strongest blockade of both dopamine- and epinephrine-stimulated enzyme activity. This is in contrast to the pattern seen in the rat erythrocyte. Since phenoxymethazin is relatively weak as a competitive blocking agent, its more effective blockade of epinephrine- than of dopamine-stimulated enzyme activity can probably be explained in terms of the lower agonistic potency of epinephrine. The retinal adenylate cyclase exhibits at least some of the properties of an alpha adrenergic system, in terms of the relative potency of agonists and the effects of alpha blockade. However, stimulation of the enzyme by epinephrine appeared to be blocked by beta blocking agents as well. The apparent beta adrenergic properties are much less marked, since lower concentrations of propranolol were incapable of blocking stimulation by epinephrine and since the more potent agonism of dopamine could not be overcome by the beta blockers.

The existence of a single hormone-sensitive adenylate cyclase, with a greater affinity for dopamine than for other catecholamines, is suggested by the fact that the catecholamines all produced about equivalent maximal enzyme stimulation. The receptor would appear to be one that cannot be rigorously defined in terms of classic adrenergic mechanisms, but might instead be a more-specific dopaminergic receptor. Some characteristics of dopaminergic receptors in the central nervous system have been described (25, 26). Behavioral responses elicited by dopamine can be mimicked by apomorphine and blocked selectively by certain tranquilizers, such as haloperidol and perphenazine. Further studies that use criteria such as these may be useful in an attempt to determine whether the differences in blockade of dopamine and epinephrine seen in Table 2 reflect the properties of a single adenylate cyclase receptor with a greater affinity for dopamine or of two types of receptors, one of which responds exclusively to dopamine.

The studies on adenylate cyclase activation in broken-cell preparations demonstrate that a potential exists for modulation by this enzyme of the actions of catecholamines in the retina. In order to assess the physiological importance of this mechanism we examined the effects of catecholamines on cyclic AMP levels in intact bovine retina (Table 3). At 0.1 mM hormone concentrations, dopamine, norepinephrine, and epinephrine all increased cyclic AMP concentrations in bovine retina about 2-fold, with no clear differences in relative potency. Concentrations in retina incubated without hormones are very similar to those measured in brain (9). It has been reported that depolarizing agents, including ouabain and high concentrations of potassium ions, increase cyclic AMP concentrations in brain slices (11). These increases are believed to be mediated by the release of adenosine and are blocked by the methylxanthines, and the depolarizing agents potentiate the effects of several hormones on cyclic AMP formation (11, 27). The changes in cyclic AMP concentrations

† Recently, we have been informed that dopamine also stimulates adenylate cyclase in homogenates of superior cervical ganglion. (Kebabian, J. W. & Greengard, P., Science, in press).
Table 3. Effects of catecholamines and depolarizing agents on cyclic AMP concentrations in intact calf retina

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Cyclic AMP (picomol per mg of protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.1 (6)</td>
</tr>
<tr>
<td>0.1 mM Dopamine</td>
<td>13.2 (4)</td>
</tr>
<tr>
<td>0.1 mM Norepinephrine</td>
<td>13.5 (3)</td>
</tr>
<tr>
<td>0.1 mM Epinephrine</td>
<td>12.7 (3)</td>
</tr>
<tr>
<td>0.01 mM Ouabain</td>
<td>14.1 (2)</td>
</tr>
<tr>
<td>83 mM K⁺</td>
<td>13.9 (2)</td>
</tr>
<tr>
<td>0.01 mM Ouabain + 0.1 mM Dopamine</td>
<td>23.0 (2)</td>
</tr>
<tr>
<td>83 mM K⁺ + 0.1 mM Dopamine</td>
<td>21.6 (2)</td>
</tr>
</tbody>
</table>

Numbers in parentheses are the number of separate determinations; mean values are all significantly different from control ($P < 0.01$).

For incubations with high K⁺, NaCl in the medium was replaced with an equivalent amount of KCl.

produced by incubation of retina with high concentrations of potassium ions and with ouabain are of smaller magnitude than those seen in brain. Although this could be due to the inclusion of caffeine, adenosine did not alter cyclic AMP concentrations, even when caffeine was omitted. This would suggest that the mechanisms involving adenosine that have been proposed to function in brain slices may not be functional in the retina. The multiplicity of possible actions of potassium and ouabain on an intact nervous tissue make it impossible to draw further conclusions. It should be noted that the effects of these agents and those of dopamine appear to be additive; thus, they presumably exert their effects by different mechanisms. Serotonin and histamine were tested and did not alter cyclic AMP concentrations in intact retina.

It has been proposed that cyclic AMP may exert all of its functions through stimulation of a cyclic AMP-dependent protein kinase (28). We have isolated a protein kinase from bovine retina that is stimulated more than 20-fold by the addition of 1 μM cyclic AMP. Control activity was 49 pmol of 32P transferred to histone per mg of enzyme protein, while enzyme activity in the presence of 1 μM cyclic AMP was 1226 pmol/mg of protein.

Histochemical and chemical evidence indicate that virtually all of the catecholamine in the retina of rabbit, guinea pig, and rat is dopamine (3, 4). It is localized in fine cell processes with strongly fluorescent varicosities that resemble the catecholamine-rich synaptic terminals found in parts of the central nervous system (3). Evidence has been given for the presence of synthetic and degradative enzymes for dopamine in retina and for the release of dopamine after stimulation by light (5). Neuropsychological studies have shown exogenously applied dopamine and norepinephrine to inhibit the firing of retinal ganglion cells (7, 8). It is interesting in this context that the inhibition of cerebellar Purkinje cells by norepinephrine is enhanced by theophylline and is mimicked by cyclic AMP (16), and that the inhibitory effect of a dopaminergic interneuron in the superior cervical ganglion was postulated to be mediated through postsynaptic increases in cyclic AMP concentrations (15). By analogy to these systems, it is likely that the stimulation of adenylate cyclase by dopamine is closely related to its role as an inhibitory retinal neurotransmitter. It is also of interest that cyclic AMP was reported to induce glutamine synthetase activity in embryonic-chick retina (29). The normal development of retinal glutamine synthetase activity rises sharply in parallel with the final morphological and functional maturation of the visual apparatus (30, 31), suggesting an interdependence of the biochemical and neurological capacities. Such a relationship might be mediated through postsynaptic increases in cyclic AMP concentrations elicited by synaptic activation. Alternatively, the appearance of glutamine synthetase could relate more directly to metabolic changes coincident with retinal development.

The myriad functions demonstrated to be subserved by cyclic AMP in other tissues leave much room for speculation as to its role in the retina. Our data indicate that some of the effects of the catecholamines might be translated via an adenylate cyclase system. The greater potency of dopamine, and its unique role as a neurotransmitter, suggest that these effects may be related to synaptic events, while our results with various adrenergic blocking agents suggest that we may be dealing with a specific dopaminergic receptor. The existence of a dopaminergic-specific adenylate cyclase in retina should provide a useful model for study of the actions of dopamine in the central nervous system, as well as a tool with which to evaluate the functions of this neurohormone in retinal processes.

We thank Dr. H. L. Kern for his advice and assistance during the course of these studies, Mr. Michael Jacob for his technical assistance, and Dr. O. Rosen for providing a purified preparation of beef-heart cyclic nucleotide phosphodiesterase. This work was supported by grants S701 GM00065 and AM-09213 from the National Institutes of Health and was performed in partial fulfillment of the requirements of J.H.B. for the doctoral degree.