Catecholamine-stimulated cyclic GMP accumulation in the rat pineal: Apparent presynaptic site of action

(I-norepinephrine/nerve ending/cyclic AMP/denervation/α-receptor)

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ABSTRACT Guanosine 3’5’-cyclic monophosphate (cGMP) increased 7-fold in rat pineal glands incubated in the presence of I-norepinephrine. This response consisted of two components—one was stereospecific and inhibited by α-adrenergic antagonists while the other was not stereospecific and not readily inhibited by antagonists. Although D-isoproterenol was more potent than L-norepinephrine it had less intrinsic activity and its action was not stereospecific. The increase in cGMP caused by these catecholamines, unlike that of adenosine 3’5’-cyclic monophosphate (cAMP), was dependent upon extra-cellular calcium. Ouabain and high levels of potassium produced a marked, calcium-dependent increase in pineal cGMP, without affecting cAMP. There was no effect of cholinergic agonists on cGMP. Surgical denervation markedly reduced the cGMP response to stimulation by I-norepinephrine, potassium, or ouabain. This was in contrast to the enhanced response of cAMP in denervated glands. The nonspecific increase in cGMP caused by D-isoproterenol, however, was not affected by denervation. These data demonstrate the existence of a calcium-dependent presynaptic mechanism for the generation of cGMP which may be mediated by an α-adrenergic-like receptor. In addition, the mechanisms regulating pineal cGMP appear to be physiologically distinct from those regulating cAMP.

The physiologic role of guanosine 3’5’-cyclic monophosphate (cGMP) has remained elusive despite numerous observations that several biologically active agents can increase its concentration in a variety of cell types (1). Adenosine 3’5’-cyclic monophosphate (cAMP) is a second messenger in the action of several hormones (2), and a similar role has been sought for cGMP. It has been proposed that cGMP functions as an independent biological mediator whose effects oppose those of cAMP (3). Contrasting and often antagonistic regulatory influences of cGMP and cAMP have been reported in several biological systems (3). Since the initial demonstration that acetylcholine raises myocardial cGMP in the perfused, isolated rat heart (4), cholinergic stimulation of cGMP accumulation has been observed in uterine myometrium (5), brain tissue (6–8), and superior cervical ganglia (9). More recently the adrenergic neurotransmitter, norepinephrine, has been shown to elevate cGMP in vas deferens (10) and cerebellar tissue (11).

The pineal gland has proven to be a useful organ in the study of adrenergic neurotransmission and its effects upon responsive cells (12). Located between the cerebral hemispheres, the rat pineal gland is innervated exclusively by noradrenergic nerves whose cell bodies lie in the superior cervical ganglia (13). Beta adrenergic stimulation of the pineal leads to the induction of serotonin N-acetyltransferase (arylamine acetyltransferase; acetyl CoA:arylamine N-acetyltransferase, EC 2.3.1.5) via a cAMP-mediated mechanism (14, 15), and ultimately to the synthesis and secretion of the hormone melatonin (14, 16). Increased release of norepinephrine from nerve terminals caused by prolonged exposure of the animals to light or by surgical denervation of the gland leads to a heightened (i.e., supersensitive) response to subsequent β-adrenergic stimulation—in terms of adenylyl cyclase activation, cAMP accumulation, protein kinase activity, and N-acetyltransferase induction (17–19, M. Zatz and R. F. O’Dea, submitted for publication).

Because tissue cGMP is elevated by norepinephrine (10, 11), the regulation of this cyclic nucleotide was examined in intact and denervated rat pineal glands. Our experiments demonstrate the existence of an apparent presynaptic mechanism for the generation of cGMP, which may be mediated by an α-adrenergic-like receptor.

MATERIALS AND METHODS

Chemicals. Tritium-labeled cGMP (specific activity 10 Ci/mmole) was purchased from Schwarz/Mann. The [3H]cGMP was purified free of a guanosine-like contaminant by application to a Dowex AG 1X-10 (200–400 mesh) formate column. The Dowex 1-formate column was then washed and the cGMP in the eluate was eluted as previously described (20). The formic acid was removed during lyophilization and the [3H]cGMP was dissolved in water.

Cyclic GMP and cyclic AMP were obtained from Sigma. Standard solutions of cGMP were prepared from material purified as above and their concentrations were determined spectrophotometrically utilizing an extinction coefficient of 13.7 × 10³ at 252 nm. Antisera to cGMP and [125I]-labeled cGMP derivative (succinyl-cGMP methyllyrosine) were purchased from Schwarz/Mann.

l- and d-Norepinephrine-d-bitartrate, l- and d-isoproterenol-d-bitartrate, and phenylephrine hydrochloride were provided by Sterling-Winthrop Laboratories. Methoxamine hydrochloride was obtained from Burroughs-Wellcome Inc. d- and l-Propranolol hydrochloride and dl-sotalol hydrochloride were provided by Ayerst Labs and Regis Chemical Co., respectively. Phenoxylbenzamine hydrochloride was obtained from Smith, Kline and French. Phentolamine hydrochloride was provided by Ciba. Other drugs and chemicals were purchased from commercial sources.

Animals. Male Sprague–Dawley rats (150–175 g) were purchased from Zivic-Miller Laboratories (Allison Park, Pa.). All animals were housed under diurnal lighting conditions for at least 6 days before each experiment. Animals were killed after exposure to light for 24 hr or to darkness for 12 hr as previously described (21). Rats were killed by decapitation and their pineal glands were removed immediately (within 15 sec after decapitation) and either homogenized in perchloric acid or placed into organ culture. Rats with denervated pineal glands were prepared by bilateral superior cervical ganglionectomy at least 1 month prior to use. Surgically prepared and their re-
Cyclic GMP was assayed by acetylation radioimmunoassay in neutralized, buffered perchloric acid containing either cGMP standard or tissue extract from l-norepinephrine-stimulated glands. Both standard and tissue extract, where shown, received a 400-fold excess of cAMP to assess the crossreactivity with this cyclic nucleotide. In addition, both standard and tissue extract were preincubated with 5 μg of purified beef heart phosphodiesterase (PD) for 60 min at 30°C prior to the assay of cGMP. The values shown are the average of triplicate determinations in a representative experiment. cGMP was assayed also in unpurified or Dowex 1-formate-purified pooled extract from gland tissue after stimulation with l-norepinephrine. The values shown are the means of six separate column purifications.

Results

Time Course of cGMP Response to l-Norepinephrine. l-Norepinephrine and other adrenergic agonists, such as l-isoproterenol, provoke a rapid increase in rat pineal cAMP levels, which reaches a maximum after 10–20 min (15, 25). l-Norepinephrine can also stimulate increases in cGMP in cerebellum (11) and ductus deferens (10). Therefore, the effects of catecholamines on cGMP in cultured pineals were examined. The addition of 10−5 M l-norepinephrine produced an 8– to 10-fold increase in cGMP within 10 min (Fig. 1). Thereafter, a relatively stable level, three to four times control, was maintained for at least 2 hr. A 60 min incubation was routinely used in later experiments. The basal level of pineal cGMP fell from 400–500 fmol per gland immediately after decapitation to a stable level of 150–200 fmol per gland (150 nmol/kg of tissue wet weight) in vitro after 15 min of preincubation. These levels are similar

![Graph](image-url)
FIG. 2. Dose-response of l-norepinephrine (O—O) and l-isoproterenol (●—●) on pineal cGMP. After 15 min preincubation in organ culture, pineal glands were exposed to varying concentrations of drugs as indicated. Sixty minutes after exposure to either agonist, pineals were removed from culture, homogenized, and assayed for cGMP as described in Materials and Methods. Values shown are the means ±SEM of triplicate determinations in six or more glands. * Signifies greater than control with \( P < 0.001 \), by Student's \( t \)-test. ** Signifies greater than \( 10^{-7} \) l-isoproterenol with \( P < 0.001 \).

Table 2. Effects of agonists and antagonists on pineal cGMP

<table>
<thead>
<tr>
<th>Agonists (mol/liter)</th>
<th>cGMP (fmol/pineal)</th>
<th>Antagonists (mol/liter)</th>
<th>cGMP (fmol/pineal)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>139 ± 14 (23)</td>
<td>+ dl-Propranolol (2 × 10^{-4}) 147 ± 42 (6)</td>
<td></td>
</tr>
<tr>
<td>l-Norepinephrine (10^{-4})</td>
<td>733 ± 61 (44)</td>
<td>+ l-Propranolol (10^{-4}) 869 ± 162 (12)</td>
<td></td>
</tr>
<tr>
<td>d-Norepinephrine (10^{-4})</td>
<td>378 ± 38 (6)</td>
<td>+ dl-Propranolol (10^{-4}) 613 ± 112 (11)</td>
<td></td>
</tr>
<tr>
<td>l-Isoproterenol (10^{-4})</td>
<td>440 ± 24 (23)</td>
<td>+ dl-Sotalol (10^{-4}) 1020 ± 274 (6)</td>
<td></td>
</tr>
<tr>
<td>d-Isoproterenol (10^{-4})</td>
<td>452 ± 74 (6)</td>
<td>l-Norepinephrine (10^{-4}) 147 ± 42 (6)</td>
<td></td>
</tr>
<tr>
<td>Phenylephrine (10^{-4})</td>
<td>112 ± 7 (6)</td>
<td>+ l-Propranolol (10^{-4}) 400 ± 18 (12)</td>
<td></td>
</tr>
<tr>
<td>Methoxamine (10^{-4})</td>
<td>184 ± 18 (6)</td>
<td>+ dl-Propranolol (10^{-4}) 483 ± 22 (12)</td>
<td></td>
</tr>
<tr>
<td>Histamine (10^{-4})</td>
<td>361 ± 15 (6)</td>
<td>+ dl-Sotalol (10^{-4}) 559 ± 33 (6)</td>
<td></td>
</tr>
<tr>
<td>Serotonin (10^{-4})</td>
<td>319 ± 70 (12)</td>
<td>l-Norepinephrine (10^{-4}) 395 ± 76 (6)</td>
<td></td>
</tr>
<tr>
<td>Dopamine (10^{-4})</td>
<td>244 ± 30 (6)</td>
<td>+ Phenoxybenzamine (10^{-4}) 397 ± 70 (6)</td>
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</tr>
</tbody>
</table>

Pineal glands were homogenized and cGMP assayed as described after a 60 min exposure to the various agonists listed above. Adrenergic antagonists were present in the culture media during the 15 min preincubation prior to the addition of agonist. Values shown above are the means ±SEM for triplicate determinations in the number of separate experiments indicated in parentheses.
Biochemistry: O’Dea and Zatz

FIG. 3. Effect of denervation on the pineal cGMP response to catecholamines, potassium, and ouabain. Bilateral superior cervical ganglioneutomized (■) or sham-operated (□) animals were surgically prepared 1 month prior to use. Pineal glands were removed and placed into organ culture. After 15 min preincubation, the various agents were added to each group and incubations continued for an additional 60 min. Individual pineals were homogenized and assayed for cGMP as described in Materials and Methods. Each value shown represents the mean ± SEM of triplicate determinations on six or more glands. l-NE, l-norepinephrine; l-Iso, l-isoproterenol. *Signifies greater than denervated with P < 0.01, by Student’s t-test. ** Signifies greater than denervated with P < 0.001.

tissues. Despite the lack of cholinergic input to the rat pineal (29), the effects of acetylcholine and carbamylcholine were examined. Neither compound (at 10 µM) increased pineal cGMP. Cholinergic agonists also failed to increase cGMP in mouse brain (30). Melatonin, which raises cGMP levels in peripheral blood monocytes (31), also failed to affect cGMP levels in rat pineal.

Effects of Ouabain, Potassium, and Calcium. Calcium-dependent increases in cGMP have been shown in response to l-norepinephrine, ouabain, and high concentrations of potassium in brain tissue (30). Incubation of intact rat pineals with 1 mM ouabain or 80 mM KCl produced marked increases in cGMP levels (Fig. 3). Although both agents increase cAMP in brain slices (30, 32), neither compound affected pineal cAMP (ref. 33; data not shown). There was negligible response of cGMP to l-norepinephrine, ouabain, or 80 mM KCl in the absence of calcium (data not shown). In contrast, the response of pineal cAMP to l-norepinephrine was not blocked by the absence of calcium. In addition, 10 µM phenolamine completely blocked the increase in cGMP observed after high potassium. Neither K+-free or Ca++-free medium altered the basal levels of cGMP. Therefore, in contrast to pineal cAMP, the elevation of pineal cGMP by all agents examined is Ca++-dependent.

Effects of Denervation on Pineal cGMP. The possibility that the α-adrenergic-like and nonspecific components of the cGMP response to l-norepinephrine reside in discrete cellular compartments was examined by comparing responses in intact and denervated glands. The rat pineal gland is innervated by postganglionic sympathetic fibers originating in the superior cervical ganglia (13). Bilateral ganglioneuctomy results in the degeneration of the presynaptic nerve terminals and the development of a supersensitive response to β-adrenergic stimulation in terms of adenylate cyclase activation (17), cAMP ac-
cumulation (18), and protein kinase activity (M. Zatz and R. F. O’Dea, submitted for publication). In contrast, the accumulation of cGMP in response to l-norepinephrine is markedly reduced after ganglioneuctomy (Fig. 3). Indeed, the response is quantitatively identical to the nonspecific effect of d-norepinephrine (Table 2) or of l-isoproterenol (Fig. 3). These data indicate that the stereospecific, α-adrenergic-like component of the noradrenergic stimulation of cGMP depends upon the presence of intact nerve terminals. Furthermore, the response of cGMP to high concentrations of potassium or to ouabain is abolished after ganglioneuctomy (Fig. 3). Thus, the pineal nerve terminals may be a site of cGMP accumulation.

DISCUSSION

Catecholamines elevate cGMP in cultured rat pineal glands. l-Norepinephrine has a similar effect in ductus deferens (10) and cerebellum (11). The accumulation of cGMP in these tissues appears to be related to the stimulation of the α-adrenergic receptor. In the pineal, examination of the dose-dependent effects of l-norepinephrine and l-isoproterenol suggested that both α- and β-adrenergic receptors might have a role in the regulation of cGMP. However, experiments with catecholamine stereoisomers and β-agonists ruled against the participation of the stereospecific β-receptor. The effect of l-isoproterenol, like that of certain other compounds, appears to be nonspecific. In contrast to l-isoproterenol, l-norepinephrine affected both the nonspecific and another, stereospecific, component. This stereospecific component is susceptible to blockade by α-adrenergic antagonists. Thus, as in certain other tissues, cGMP in the pineal gland responds to catecholamines via an α-adrenergic-like mechanism.

The possibility that the nonspecific and stereospecific components reflect pre- and postsynaptic compartments was examined by comparing the increases in cGMP accumulation in denervated and sham-operated pineals. The nonspecific component was unaffected by denervation. It appears that this component does not require intact nerve endings and, therefore, may be postsynaptic.

In contrast to cGMP, the accumulation of cAMP in response to noradrenergic stimulation is greatly enhanced by denervation (18). Thus, postsynaptic adenylyl cyclase, which is closely linked to the β-adrenergic receptor (21), becomes supersensitive after denervation (17). In addition, the cAMP response to l-norepinephrine is enhanced after prolonged exposure to light (18), whereas alteration in lighting had no effect on either basal or stimulated cGMP levels. Furthermore, 1 mM dibutyryl cGMP had no effect on N-acetyltransferase induction in vitro, under several assay conditions (R. F. O’Dea and M. Zatz, unpublished observation). Therefore, the mechanisms which enhance the sensitivity of the postsynaptic β-adrenergic receptor do not participate in the regulation of cGMP, nor does cGMP appear to influence the function of this receptor.

The stereospecific component of the response of cGMP to l-norepinephrine was abolished by denervation, as was the response to high potassium and ouabain. Thus, the stereospecific, α-adrenergic-like component in the generation of cGMP depends upon the presence of intact nerve endings. A presynaptic role for cAMP also appears likely in view of the recent report that it can activate tyrosine hydroxylase in synaptosomal fractions prepared from rat brain (34). High potassium and ouabain were as effective as l-norepinephrine in elevating cGMP. Although both agents produce depolarization of nervous tissue, each does so by a different mechanism. High levels of K+ depolarize by decreasing the difference between intracellular and extracellular K+ con-
centrations (35) whereas ouabain inhibits (Na\(^+\) – K\(^+\))-adenosine triphosphatase, thereby preventing extrusion of Na\(^+\) from cells (36). Since it has been reported that l-norepinephrine hyperpolarizes pinealocyte membranes, whereas potassium and ouabain reverse this effect (33), l-norepinephrine and the “depolarizing” agents may elevate cGMP via dissimilar mechanisms. However, the observation that each agent requires extracellular calcium and is inhibited by \(\alpha\)-adrenergic antagonists, suggests that they may stimulate the generation of cGMP through a common mechanism. It is tempting to speculate that the presynaptic generation and action of cGMP might be related functionally to the presynaptic \(\alpha\)-adrenergic receptor which modulates neurotransmitter release (37).

The response of cGMP to l-norepinephrine in the pineal gland depends on extracellular calcium and consists of a stereospecific, presynaptic, \(\alpha\)-adrenergic-like component and a second component which appears to be nonspecific, postsynaptic, and unrelated to either adrenergic receptor. The data presented here may serve to clarify some of the complexities observed in cGMP regulation in other nervous tissue (11) and elsewhere (1). In conclusion, this report provides evidence for a catecholamine-sensitive mechanism for the generation of cGMP, which is dependent upon intact nerve terminals.

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