Response and Level of \(\beta\)-Adrenergic, Vasoactive Intestinal Peptide, and PACAP Receptors During the Circadian Cycle

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Purpose. To determine whether a nocturnal increase of ciliary process \(\beta\)-adrenergic receptor responsiveness can explain the observation that timolol decreased the aqueous flow rate and intraocular pressure (IOP) during the night but not during the day in rabbits.

Methods. Rabbits were housed in alternating 12-hour periods of light and dark. In vitro stimulation of tissue cyclic adenosine monophosphate (cAMP) levels by isoproterenol (ISO), vasoactive intestinal peptide (VIP), pituitary adenylate cyclase activating polypeptide (PACAP), or a soluble derivative of forskolin (sFSK) was measured in ciliary processes harvested at mid-light phase and early and late dark phase. Inhibition of ISO and VIP stimulation of ciliary process cAMP by an \(\alpha_2\)-adrenergic agonist and maximal binding of \([^{125}\text{I}]\)Pindolol, \([^{125}\text{I}]\)VIP, and \([^{125}\text{I}]\)PACAP in ciliary process membranes were measured at the same three times.

Results. Although there may have been a nocturnal increase in the sensitivity of ciliary process cAMP levels to stimulation by ISO, this was not observed consistently. VIP, but not PACAP, stimulation increased at night, but there was no change in the response to sFSK. Inhibition by apraclonidine of elevated ciliary process cAMP levels was constant at all three times. Ligand-binding studies showed little change in ciliary process \(\beta\)-adrenergic, VIP-, or PACAP-receptor levels at the three times.

Conclusions. There is no convincing evidence for a nocturnal increase in \(\beta\)-adrenergic receptor sensitivity in rabbit ciliary processes that could explain the difference between day and night effects of timolol on aqueous flow and IOP. Invest Ophthalmol Vis Sci. 1997;38:1708–1718.

Rabbits have circadian rhythms of intraocular pressure (IOP), aqueous flow, and concentration of nor-epinephrine in the aqueous (NE\(_{\text{aq}}\)) that increase at night.\(^1\)–\(^6\) The range of the rhythm of aqueous flow accounts for a major portion of the rhythm of IOP.\(^3\)\(^,\)\(^4\)\(^,\)\(^7\) The rhythm of NE\(_{\text{aq}}\) is consistent with a nocturnal increase in ocular sympathetic tone and was abolished by superior cervical ganglionectomy (CGX) or preganglionic section of the cervical sympathetic trunk (decentralization; DX).\(^5\)\(^,\)\(^6\) Major portions of the rhythms of IOP and aqueous flow depend on sympathetic input to the eye, and both rhythms were significantly blunted by CGX or DX.\(^5\)\(^,\)\(^7\)\(^,\)\(^9\) Furthermore, low-frequency stimulation of the cervical sympathetic trunk increased IOP and NE\(_{\text{aq}}\).\(^10\) These observations are consistent with the theory that increased release of norepinephrine from ocular sympathetic nerves at night increases aqueous flow, and therefore IOP. \(\beta\)-adrenergic receptors mediate part of the nighttime increases of IOP and aqueous flow that depend on ocular sympathetic input. Timolol reduced IOP and flow when applied at night\(^7\)\(^,\)\(^11\)\(^,\)\(^12\) and blocked the nocturnal circadian increase in the concentration of cyclic adenosine monophosphate (cAMP) in the aqueous.\(^6\)\(^,\)\(^13\) Sympathectomy abolished the effect of timolol on IOP and eliminated the nocturnal increase in the concentration of cAMP in the aqueous.\(^6\)\(^,\)\(^11\)

However, the relation between ocular sympathetic tone and \(\beta\)-adrenergic receptor control of IOP during the day is less clear. DX decreased NE\(_{\text{aq}}\) during day and night to the same low level, and blockade of prejunctional \(\alpha_2\)-adrenergic receptors with rauwolscine increased NE\(_{\text{aq}}\) during both day and night.\(^6\)\(^,\)\(^12\) There-
Ciliary Process Receptors During the Circadian Cycle

fore, there is significant ocular sympathetic tone during the day in normal rabbits. However, timolol had no effect on either IOP or aqueous flow when applied during the day. In contrast, apraclonidine (p-aminoclonidine, PAC), an \(\alpha_2\)-adrenergic agonist, was equally effective at decreasing IOP during day and night in rabbits.

There is a circadian rhythm of melatonin synthesis in the mammalian pineal. The nocturnal increase in pineal melatonin results from increased release of noradrenaline from sympathetic nerves from the superior cervical ganglion at night and is mediated by noradrenaline stimulation of pineal \(\alpha_1\)- and \(\beta\)-adrenergic receptors in rats. There are also daily rhythms of pineal \(\beta\)-adrenergic, but not \(\alpha_1\)-adrenergic, receptor density and of the response of pineal cAMP to stimulation by the \(\beta\)-adrenergic agonist isoproterenol (ISO); these rhythms increase during the day, peak at the onset of dark, and then fall during the night.

These observations of daily changes in \(\beta\)-adrenergic receptor density and response to agonist stimulation in the pineal led us to perform similar experiments with rabbit ciliary processes in an attempt to reconcile the lack of a response of IOP and aqueous flow to \(\beta\)-adrenergic blockade during the day with the observation that there is significant ocular sympathetic tone during the day. However, we postulated that changes in \(\beta\)-adrenergic receptor sensitivity in ciliary processes are somewhat phase-delayed relative to those in the pineal—in other words, that the sensitivity of ciliary process \(\beta\)-adrenergic receptors is low during most of the day, increases late in the day, peaks early in the night, and then decreases during the night in a pattern similar to the daily rhythm of aqueous flow.

We examined the in vitro response of ciliary process cAMP levels to stimulation with ISO and measured \(\beta\)-adrenergic receptor density at three times during the circadian cycle: mid-light phase, early dark phase, and late dark phase. IS0 stimulation of ciliary process cAMP levels and \(\beta\)-adrenergic receptor density depressed at mid-light phase (when IOP, aqueous flow, and \(\text{NE}_{\text{aq}}\) are all low) relative to early dark phase (when IOP and aqueous flow are at or near maximum and \(\text{NE}_{\text{aq}}\) is approaching maximum)? Does ISO stimulation of ciliary process cAMP levels at late dark phase (when aqueous flow has fallen and IOP has begun to decrease, but \(\text{NE}_{\text{aq}}\) remains high) show any evidence for desensitization of ciliary process \(\beta\)-adrenergic receptors?

We compared the ISO responses of ciliary process cAMP and \(\beta\)-adrenergic receptor density with the response and receptor density of vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP), two peptide hormones that also stimulate adenylate cyclase and are known to have receptors in rabbit ciliary processes. We also measured the cAMP response to a water-soluble derivative of forskolin (sFSK), a direct activator of adenylate cyclase. PACAP is a recently discovered hypothalamic neuropeptide with two molecular forms, one with 27 amino acid residues (PACAP27) and one with 38 residues (PACAP38), which have significant structural homology with VIP. There are two high-affinity receptors for PACAP. One has a higher affinity for PACAP than for VIP (type I, selective); the other has a similar affinity for PACAP and VIP (type II, nonselective). The type II, nonselective PACAP receptor is thought to be identical to the VIP receptor. The anterior uvea of the rabbit is reported to have primarily type II PACAP receptors.

VIP immunoreactivity has been localized in the rabbit iris ciliary body to parasympathetic nerve fibers of the facial nerve that originate from the pterygopalatine ganglion. The distribution of binding sites for VIP has been studied by autoradiography, and the ciliary epithelial cells were one of the sites of highest binding density in this species. Furthermore, the density of VIP receptors in the rabbit ciliary body is greater than or comparable to that of any of the adrenergic receptors. VIP has been reported to increase aqueous flow in monkeys and to decrease IOP in rabbits. Because VIP may play a role in the control of aqueous flow and IOP, we attempted to determine the concentration of VIP in the aqueous as a measure of the rate of VIP release from ocular VIP-containing nerves at three times during the circadian cycle to correlate VIP release and the aqueous flow rate during the circadian cycle.

**MATERIALS AND METHODS**

ISO, VIP, PACAP, and sFSK Cyclic Adenosine Monophosphate Responses

ISO and sFSK were dissolved in and diluted with Hank’s balanced salt solution (BSS). VIP, PACAP27, and PACAP38 were dissolved in 1 mM acetic acid, and aliquots of the resulting solution were lyophilized in small (0.4-ml) polypropylene tubes and stored at −20°C. Immediately before each experiment, VIP was dissolved in 1 mM acetic acid to produce a 216-μM stock solution that was diluted to the concentrations desired. The highest concentration of acetic acid in any assay mixture (at the highest concentration of VIP, 5.4 μM) was approximately 25 μM. Lyophilized PACAP aliquots were dissolved in water to produce 200-μM stock solutions that were diluted to the concentrations desired. All VIP and PACAP dilutions were conducted in polypropylene test tubes with BSS containing 1.0 mM bacitracin, 1.0 mM phenylmethylsulfonyl fluoride (PMSF), and 0.01% soybean trypsin inhibitor (STI) as diluent.
Excised ciliary processes from four eyes were distributed as equally as possible among 21 or 24 Potter–Elvehjem glass homogenizers (1 ml) (Kontes, Vineland, NJ) on ice containing BSS. The processes were preincubated at 30° for 2.5 minutes under 300 µl of BSS; then, 100 µl ISO, porcine VIP, PACAP27, PACAP38, or 7β-deacetyl-7β-(γ-N-methylpiperazino)-butyryl forskolin dihydrochloride (sFSK) was added. The incubation was terminated 2.5 minutes (ISO), 5 minutes (VIP), 15 minutes (PACAP), or 10 minutes (sFSK) later by adding trichloroacetic acid (final concentration 7%) containing approximately 10,000 dpm [3H]cAMP (to monitor cAMP recovery). Immediately afterward, the tissue was homogenized with a motor-driven PTFE pestle (Kontes, Vineland, NJ). The optimal time for tissue incubation was taken from earlier work (ISO,6 VI)PACAP21) or by determining the time course of agonist-induced cAMP increase (sFSK, PACAP). Homogenized tissue samples were centrifuged at 2000g for 10 minutes, and the supernatants (containing CAMP) were frozen at −20°C. The pellets were dissolved in 1 N NaOH, and the resulting solutions were assayed for protein by the method of Lowry et al63 using bovine serum albumin (BSA) as standard. Cyclic AMP in the supernatants was assayed using radioimmunoassay kits (Biomedical Technologies, Stoughton, MA). After trichloroacetic acid supernatants were extracted with diethyl ether to remove trichloroacetic acid, aliquots were diluted with 50 mM acetic acid (pH = 6.2) and assayed in duplicate using the acetylated procedure.64 Cyclic AMP levels were corrected for recovery of [3H]cAMP and expressed as pmoles cAMP/mg protein.

Ligand-Binding Studies

Crude Membrane Preparation. Crude membrane fractions were prepared from ciliary processes using previously published procedures for human retina65 and rabbit ciliary processes66 with slight modifications. Ciliary processes from six rabbit eyes were pooled in a Potter–Elvehjem glass homogenizer (3 ml) on ice containing homogenizing medium (0.25 M sucrose, 5.0 mM EDTA, 1.0 mM PMSF, 1 mM bacitracin, 5 µM leupeptin, and 1 µg/ml aprotinin). The tissue was homogenized in 2.5 ml of homogenizing medium with 10 strokes of a motor-driven PTFE pestle; the homogenizer was immersed in ice water during homogenization. The homogenate was centrifuged in a Sorvall SS34 rotor (Dupont Co., Wilmington, DE) at 3000g for 5 minutes at 4°C. The supernatant was centrifuged in a Beckman Ti70.1 rotor at 18,000g for 30 minutes at 4°C.

For [125I]I-pindolol (I-PIN) binding studies, the pellet was resuspended in a glass homogenizer with four strokes of a motor-driven PTFE pestle in sufficient (2 ml) membrane buffer (33.3 mM Tris-HCl, 6.7 mM Tris, and 145 mM NaCl [pH = 7.5]) to give a protein concentration of approximately 0.15 mg/ml. The resuspended pellet was transferred to a polypropylene tube, frozen in liquid nitrogen, and stored at −80°C.

For [125I]I-VIP and [125I]I-PACAP binding studies, the pellet was resuspended in a glass homogenizer in 1.5 ml of 25 mM Hepes and 104 mM NaCl (pH = 7.4) with four strokes of a motor-driven PTFE pestle. The membrane suspension was sampled for protein determination by the method of Bradford,27 and then an equal volume of 25 mM Hepes, 104 mM NaCl, 10 mM MgCl2, 0.5% BSA, 0.02% STI, 2 mM PMSF, 2 µg/ml aprotinin, 10 µM leupeptin, 2 mM bacitracin, and 4 mM EDTA (pH = 7.4) was added. The membrane suspension was transferred to a polypropylene tube, frozen in liquid nitrogen, and stored at −80°C.

[125I]I-PIN Binding. Binding of [125I]I-PIN to crude membrane fractions was performed as described by Wax.68,69 Aliquots (100 µl) of crude membrane fractions prepared from ciliary processes containing 15 to 28 µg protein were incubated at 37°C for 20 minutes in 17 × 100-mm polypylene test tubes with 150 µl of a mixture of [125I]I-PIN, guanosine triphosphate, sodium ascorbate, and BSA. Final concentrations of components in the binding assay mixtures were 24 mM Tris/Tris-HCl (pH = 7.5), 87 mM NaCl, 0.1 mM guanosine triphosphate, 1.1 mM ascorbate, 0.0004% BSA, and approximately 2.5 to 350 pM [125I]I-PIN. Nonspecific binding was defined as binding of [125I]I-PIN not displaced by 0.1 mM ISO. After 20 minutes of incubation at 37°C, binding mixtures were diluted with 10 ml of 10 mM Tris/Tris-HCl and 145 mM NaCl (pH = 7.5) (dilution buffer) at room temperature and passed through a glass fiber filter (Schleicher & Schuell Inc., Keene, NH; 25-mm circles, #30 glass). The filters were washed with 10 ml of dilution buffer and then counted in a CompuGamma 1282 gamma counter (LKB Instruments, Turku, Finland). A single membrane preparation was used for each [125I]I-PIN binding experiment. Preliminary ligand binding experiments showed that maximum I-PIN binding was attained within 10 to 15 minutes (data not shown). Therefore, the 20-minute incubation used for [125I]I-PIN binding was long enough for binding equilibrium to be attained.

[125I]I-VIP and [125I]I-PACAP27 Binding. Lyophilized [125I]I-VIP and [125I]I-PACAP27 were reconstituted by adding 1 ml of distilled water to the vial containing [125I]I-VIP or [125I]I-PACAP27. The solution was removed and the vial was rinsed with 1 ml of HME (25 mM Hepes, 104 mM NaCl, 5 mM MgCl2, 0.25% BSA, 0.01% STI, 1 mM PMSF, 1 µg/ml aprotinin, 5 µM leupeptin, 1 mM bacitracin, and 2 mM EDTA [pH = 7.4]). The aqueous and HME solutions were pooled and aliquots stored frozen at −20°C in polypropylene tubes. The protein concentration of crude membrane
preparations was adjusted to approximately 0.1 mg/ml immediately before use by adding HMS. Aliquots (100 μl) of crude membrane fractions containing 10 to 15 μg protein were incubated in polypropylene microcentrifuge tubes with 100 μl of a solution of [125I]VIP in HMS for 3 hours or with 100 μl of a solution of [125I]PACAP27 for 90 minutes at room temperature (19°C to 21°C). Binding mixtures were then diluted with 200 μl of cold HMS and centrifuged at 12,000g for 3 minutes in a refrigerated microfuge (Tomy MTX-150 (Peninsula Laboratories, Inc., Belmont, CA) or Sorvall RMC 14 (Dupont Co., Wilmington, DE)) at 4°C. The supernatants were decanted and the pellets were washed by gently adding 400 μl of cold HMS to the microcentrifuge tubes and recentrifuging them at 12,000g for 1 minute. The supernatants were decanted and the pellets were counted in a Compugamma 1282 gamma counter (LKB Instruments). Nonspecific binding was defined as binding of [125I]VIP or [125I]PACAP27 not displaced by 30 nM of unlabeled peptide. A relatively low concentration of unlabelled ligand was selected to minimize binding to low-affinity receptors for VIP and PACAP. Ligand binding was measured from approximately 10 to 1000 pM of [125I]VIP or [125I]PACAP27. A single membrane preparation was used for each binding experiment. Maximum binding was attained for [125I]VIP after 2 to 3 hours of incubation and for [125I]PACAP27 after 1 to 2 hours; there was no evidence of decreased binding of either ligand for up to 6 hours. Therefore, to ensure that binding equilibrium was attained, 3-hour and 90-minute incubations were used for [125I]VIP and [125I]PACAP27 binding experiments, respectively.

Kd and Bmax for [125I]PIN, [125I]VIP, and [125I]PACAP27 were estimated by Scatchard plots and nonlinear regression using the computer program LiGAND® (MacLiGAND, version 4.96) kindly provided by Dr. Peter J. Munson (NIH, Bethesda, MD).

VIP and PACAP Assays

The concentrations of VIP and PACAP38 in aqueous humor were measured using radioimmunoassay kits from Peninsula Laboratories, Belmont, CA. Proteolysis of VIP and PACAP38 was blocked in aqueous samples by adding 15 μl of a mixture containing 33 μg/ml aprotinin, 0.33% STI, 33 mM bacitracin, 0.17 mM leupeptin, and 33 mM EDTA, and then 5 μl of 100 mM PMSF in ethanol. Aqueous samples were frozen in liquid nitrogen and then stored at −80°C until assayed. Known amounts of VIP or PACAP38 were added to some aqueous samples to confirm recovery of VIP and PACAP38.

Statistical Analysis

Data are expressed as the mean ± the standard error of the mean. Statistical significance was determined by Student's t-test for unpaired samples; P < 0.05 was considered significant. Vertical lines in the figures represent the standard error.

Animals and Animal Surgery

All experimental procedures adhered to the ARVO Resolution on the Use of Animals in Research. Male New Zealand white rabbits weighing 2 to 2.5 kg were entrained to a lighting schedule of alternating 12-hour periods of light and dark (12L:12D) for at least 2 weeks before they were used.2,8 “Lights on” was defined as 00:00 circadian time (CT). Rabbits subjected to unilateral CGX or DX were entrained to 12L:12D for at least 2 weeks after surgery before being used.9

Rabbits were killed by intravenous injection ( marginal ear vein) of Beuthanasia-D (Schering-Plough Animal Health, Kenilworth, NJ; active ingredients: pentobarbital sodium [390 mg/ml] and phenytoin sodium [50 mg/ml]) at mid-light phase (06:00 CT), 2 hours after lights off (14:00 CT), or 2 hours before lights on (22:00 CT). Rabbits were killed in the dark under a dim red light.2,8 Eyes were enucleated immediately and immersed in ice-cold saline. The eyes were cut open posteriorly, the vitreous and lens were removed, the anterior segment was covered with ice-cold BSS, and the ciliary processes were dissected from the anterior segment.

Chemicals

ISO-HCl, bacitracin, PMSF, STI, leupeptin, aprotinin, BSA, and BSS were purchased from Sigma Chemical (St. Louis, MO). Porcine VIP, PACAP27, PACAP38, and neuropeptide Y were purchased from Bachem California (Torrance, CA) or Peninsula Laboratories (Belmont, CA). Secretin and glucagon were purchased from Peninsula Laboratories. sFSK was purchased from Calbiochem (San Diego, CA). [125I]lodo(-)-pindolol was purchased from New England Nuclear Research Products (Boston, MA). 3-[125I]iodotyrosylVIP was purchased from Amersham (Arlington Heights, IL). [125I]I- PACAP27 was purchased from DuPont New England Nuclear (Boston, MA). PAC was generously provided by Dr. Louis DeSantis (Alcon Laboratories, Fort Worth, TX).

RESULTS

Ciliary Process Cyclic Adenosine Monophosphate

Figure 1 shows the response of cAMP levels in ciliary processes from rabbits killed at 06:00, 14:00, and 22:00 CT to stimulation by increasing concentrations of ISO. Although CAMP levels at the same ISO concentration were not statistically significantly different at the three times, tissue from rabbits killed at 14:00 CT appeared
FIGURE 1. Stimulation of cyclic adenosine monophosphate levels by ISO in ciliary processes excised at three different circadian times. Incubations of ciliary processes with ISO were performed in triplicate at each concentration of ISO for each dose–response curve, and each curve was performed in triplicate (06:00 and 14:00 CT) or ×5 (22:00 CT). All data were combined to produce a single curve at each circadian time.

to produce more cAMP in response to ISO than at 06:00 CT. Because EC50 for ISO (approximately 150 nM) did not appear to change, we accumulated additional data at only two concentrations of ISO to determine whether this difference was real. These additional data and data from Figure 1 were combined and are presented in Figure 2.

Stimulation of ciliary process cAMP by ISO showed statistically significant increases during the dark. To what extent is this nocturnal increase unique to ISO stimula-

FIGURE 2. Stimulation of cyclic adenosine monophosphate levels by 0.1 and 1.0 μM ISO in ciliary processes excised at three different circadian times. Data from Figure 1 were combined with data from an additional experiment at each circadian time performed with six tissue incubations at each ISO concentration. Statistically significantly different (P < 0.05) from *06:00 or #22:00 Ct at same [ISO].

FIGURE 3. Stimulation of cyclic adenosine monophosphate levels by VIP in ciliary processes excised at three different circadian times. Incubations of ciliary processes with VIP were performed in triplicate at each concentration of VIP for each dose–response curve, and each curve was performed in triplicate (06:00 and 14:00 CT) or ×4 (22:00 CT). All data were combined to produce a single curve at each circadian time. Statistically significantly different from 22:00 CT (P < 0.05) *06:00; #14:00.

FIGURE 4. Stimulation of cyclic adenosine monophosphate levels by sFSK in ciliary processes excised at three different circadian times. Incubations of ciliary processes with sFSK were performed in triplicate at each concentration of sFSK for each dose–response curve, and each curve was performed in triplicate. All data were combined to produce a single curve at each circadian time.
VIP (approximately 60 nM) did not appear to change. We measured the response of ciliary process cAMP to ISO. The ISO response of tissue from CGX eyes tended to increase at night (Table 2). Processes at 06:00, 14:00, and 22:00 CT. The enhanced nocturnal responses of ciliary process cAMP levels to the same extent at all three times (Fig. 3). Although there are some statistically significant differences of basal ciliary process cAMP levels, there are no convincing trends at the three times; the increase of basal cAMP at 14:00 and 22:00 CT relative to 6 AM CT after 2.5-minute in vitro incubations is not apparent after 5- or 15-minute incubations (Table 1).

As an initial approach to identifying the mechanisms underlying the nocturnal increase in the cAMP response to ISO, we measured the response to a maximally effective ISO concentration of ciliary processes from rabbits that had been previously subjected to unilateral DX or CGX and killed at 06:00, 14:00, or 22:00 CT. Table 2 provides data for cAMP response to ISO or VIP by PAC, an α₂-adrenergic agonist, to explore the possibility that α₂-adrenergic receptors might participate in control of the responses of ciliary process cAMP to hormonal stimulation and also to confirm the nocturnal increase in the responses to ISO and VIP. Inhibition of the responses to ISO and VIP by PAC were the same at the three times (Table 3). VIP stimulation of ciliary process cAMP was higher at 22:00 CT than at 06:00 or 14:00 CT; however, as was the case in tissue from contralateral eyes of rabbits subjected to unilateral CGX, ISO stimulation did not increase at night.

Because the type II, nonselective PACAP receptor is thought to be identical to the VIP receptor, we determined the response of ciliary process cAMP to stimulation by maximally effective concentrations of PACAP27 and PACAP38 (determined in preliminary dose–response experiments) at 06:00, 14:00, and 22:00 CT. In contrast to what we observed with VIP, PACAP stimulation of ciliary process cAMP levels was the same at all three times; there were no differences between the responses of PACAP27 and PACAP38 (Table 4).

### Ligand-Binding Studies

K<sub>d</sub> and B<sub>max</sub> for [125I]PIN, [125I]VIP, and [125I]PACAP27 were determined in membranes prepared from ciliary processes at 06:00, 14:00, and 22:00 CT. Figure 5A shows the results of one binding experiment with [125I]PIN. K<sub>d</sub> and B<sub>max</sub> for [125I]PIN binding were different at the three times (Table 5). Binding data for [125I]PIN were consistent with binding to a single receptor site, and our estimates of K<sub>d</sub> and B<sub>max</sub> for [125I]PIN are comparable to those reported recently for membranes prepared from rabbit iris ciliary body (39 pM and 100 fmol/mg, respectively). 27

Figures 5B and 5C show the results of single bind-

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### TABLE 1. Basal Cyclic Adenosine Monophosphate Levels in Rabbit Ciliary Processes at 06:00, 14:00, and 22:00 CT

<table>
<thead>
<tr>
<th>Incubation Time (min)</th>
<th>06:00 CT (pmol/mg)</th>
<th>14:00 CT (pmol/mg)</th>
<th>22:00 CT (pmol/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.5</td>
<td>16.1 ± 1.3</td>
<td>21.8 ± 1.7</td>
<td>22.6 ± 1.4</td>
</tr>
<tr>
<td></td>
<td>(n = 21)</td>
<td>(n = 21)</td>
<td>(n = 25)</td>
</tr>
<tr>
<td>5</td>
<td>20.3 ± 2.4</td>
<td>18.2 ± 1.3</td>
<td>24.0 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>(n = 18)</td>
<td>(n = 18)</td>
<td>(n = 21)</td>
</tr>
<tr>
<td>15</td>
<td>12.4 ± 0.7</td>
<td>14.3 ± 0.6</td>
<td>11.4 ± 0.7</td>
</tr>
<tr>
<td></td>
<td>(n = 16)</td>
<td>(n = 16)</td>
<td>(n = 15)</td>
</tr>
</tbody>
</table>

CT = circadian time. Values for cyclic adenosine monophosphate are given as pmol/mg.

* Statistically significantly different from 06:00: P < 0.01; † P < 0.005.

*† Statistically significantly different: P < 0.025.

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### TABLE 2. ISO (1.0 μM) Stimulation of Cyclic Adenosine Monophosphate in Ciliary Processes From Rabbits After Unilateral DX or CGX

<table>
<thead>
<tr>
<th>Circadian Time</th>
<th>DX</th>
<th>Contralateral</th>
<th>CGX</th>
<th>Contralateral</th>
</tr>
</thead>
<tbody>
<tr>
<td>06:00</td>
<td>81.7 ± 5.2</td>
<td>82.3 ± 4.7</td>
<td>70.3 ± 4.7</td>
<td>84.2 ± 6.0</td>
</tr>
<tr>
<td>14:00</td>
<td>93.8 ± 5.5</td>
<td>99.5 ± 2.6*</td>
<td>64.3 ± 4.4†</td>
<td>88.6 ± 7.2</td>
</tr>
<tr>
<td>22:00</td>
<td>105 ± 8*</td>
<td>111 ± 6*</td>
<td>67.6 ± 5.6</td>
<td>81.6 ± 5.3</td>
</tr>
</tbody>
</table>

Data are from six experiments performed in triplicate (n = 18). Values for cyclic adenosine monophosphate are given as pmol/mg.

DX = decentralization; CGX = cervical ganglionectomy.

* Statistically significantly different from 06:00: P < 0.02.
† Statistically significantly different from contralateral control: P < 0.01.
VIP, PACAP38, PACAP27, glucagon, and secretin, all binding was evaluated by comparing the abilities of these ligands to increase during the night (Table 5). Kₐ for [¹²⁵I]I-PACAP27 binding were less than the sensitivity of the radioimmunoassay used (VIP, <2 pg/200 μl aqueous; PACAP38, <10 pg/100 μl aqueous).

**TABLE 3. PAC (10 μM) Inhibition of ISO (1.0 μM) and VIP (0.9 μM) Stimulated Ciliary Process Cyclic Adenosine Monophosphate (Total cAMP, Uncorrected for Basal)**

<table>
<thead>
<tr>
<th>Circadian Time</th>
<th>ISO</th>
<th>ISO + PAC</th>
<th>%I</th>
<th>VIP</th>
<th>VIP + PAC</th>
<th>%I</th>
</tr>
</thead>
<tbody>
<tr>
<td>06:00</td>
<td>189 ± 9</td>
<td>61 ± 3</td>
<td>67</td>
<td>476 ± 32*</td>
<td>304 ± 19*</td>
<td>36</td>
</tr>
<tr>
<td>14:00</td>
<td>188 ± 10</td>
<td>51 ± 3</td>
<td>72</td>
<td>445 ± 35*</td>
<td>251 ± 19*</td>
<td>41</td>
</tr>
<tr>
<td>22:00</td>
<td>184 ± 12</td>
<td>55 ± 3</td>
<td>70</td>
<td>616 ± 35</td>
<td>404 ± 19</td>
<td>34</td>
</tr>
</tbody>
</table>

Data are from three (06:00 and 22:00) or four (14:00) experiments each performed in replicates of six. Values for cyclic adenosine monophosphate are given as pmol/μg.

ISO = isoproterenol; PAC = p-aminoclonidine; VIP = vasoactive intestinal peptide.

* Statistically significantly different from 22:00: P < 0.01.

**DISCUSSION**

Exposing β-adrenergic receptors to agonists generally leads to rapid desensitization in which phosphorylation of receptors by β-adrenergic receptor kinase, followed by binding of β-arrestin, and by protein kinase A results in their uncoupling from Gₛ. The receptors are also rapidly sequestered, rendering them inaccessible to hydrophilic ligands, and this process may be required for dephosphorylation and receptor resensitization. Agonist exposure also results in a slower process—termed downregulation—in which receptor number decreases. Previous studies have demonstrated desensitization of β-adrenergic receptors in rabbit iris ciliary body after pretreatment with ISO in vitro and also after treatment with epinephrine in vivo. Pinel β-adrenergic receptor density decreased and ISO stimulation of pinel cAMP showed marked desensitization during the night in response to increased nocturnal sympathetic input. Because NEₐq (and presumably sympathetic tone) increases at night and remains high for most of the 12-hour dark phase in rabbits, one might also expect rabbit ciliary process β-adrenergic receptors to become increasingly desensitized during the night. In contrast to results obtained in rat pineal, this does not appear to be the case. ISO stimulation of ciliary process cAMP did not decrease at night and in two of four experiments may have increased (see Fig. 2 and Table 2, DX rabbits) as we had postulated to explain the nocturnal response of aqueous flow and IOP to β-adrenergic blockade with timolol. However, these increases were small (always less than twofold) and were not observed consistently. We have no explanation for this inconsistency. It may be that nocturnal increases of β-adrenergic receptor sensitivity to stim-
Ciliary Process Receptors During the Circadian Cycle

<table>
<thead>
<tr>
<th>Orcadian Time</th>
<th>I-PIN</th>
<th>I-VIP</th>
<th>I-PACAP27</th>
</tr>
</thead>
<tbody>
<tr>
<td>06:00 (pM)</td>
<td>22.9 ± 0.7</td>
<td>59.8 ± 3.5</td>
<td>100 ± 5</td>
</tr>
<tr>
<td>14:00</td>
<td>23.6 ± 1.0</td>
<td>58.0 ± 4.1</td>
<td>125 ± 13</td>
</tr>
<tr>
<td>22:00</td>
<td>25.3 ± 0.9</td>
<td>54.8 ± 5.3</td>
<td>120 ± 12</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Bmax (pmol/mg) (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>06:00</td>
</tr>
<tr>
<td>14:00</td>
</tr>
<tr>
<td>22:00</td>
</tr>
</tbody>
</table>

*† Statistically significantly different: * P < 0.05.

By agonist occur, but the experimental protocol we used was not optimal to measure such changes consistently. Nor did Bmax for [125I]I-PIN show any evidence of nocturnal downregulation of β-adrenergic receptors observed in rat pineal. Perhaps these differences between ciliary process and pineal β-adrenergic receptors reflect the richer sympathetic innervation of the pineal compared with ciliary processes. 48,49 Bausher and McNellig 32 showed that the in vitro increase in cAMP levels in response to ISO in ciliary processes from rabbits killed during the day did not change relative to controls 2 to 6 weeks after CGX. Abdel-Latif et al 50 confirmed this observation 2 weeks after CGX and also showed by ligand-binding studies that CGX had no effect on α2- or β-adrenergic receptor density in ciliary processes. Our failure to detect an increase in ciliary process ISO sensitivity after surgical sympathectomy during either daytime or night supports these earlier failures to demonstrate that postjunctional denervation supersensitivity of β-adrenergic receptors in ciliary processes develops after long-term CGX.

The α2-adrenergic receptors in some tissues and cells are subject to agonist-mediated desensitization. Short-term exposure to agonist resulted in an increased EC50 for epinephrine inhibition of forskolin-stimulated adenylate cyclase activity that was associated with phosphorylation of the α2-adrenergic receptor; longer exposure to epinephrine resulted in both increased EC50 and decreased maximum inhibition by epinephrine associated with decreased levels of the inhibitory G protein, Gi. 51 In another study, persistent

**FIGURE 5.** Ligand binding to membranes from rabbit ciliary processes. Specific binding is the difference between total and nonspecific binding. (A) [125I]I-PIN. (B) [125I]I-VIP. (C) [125I]I-PACAP27.
stimulation of adenylate cyclase by forskolin or β-adrenergic receptor agonist was reported to enhance Gs-mediated inhibition of cAMP production by increasing Gs levels. The data presented in Table 3 do not support changes in maximum inhibition by α2-adrenergic agonists during the circadian cycle mediated by changes in levels of Gs, which have been observed in other systems.

Although we have no evidence to suggest circadian changes of VIP release from ocular VIP-containing nerves (the sensitivity of the assay we used was not sufficient to estimate the concentration of VIP, or PACAP, in aqueous humor), our observation that the VIP-mediated cAMP response of ciliary processes increases late in the dark phase, and may reflect increased receptor density, suggests the possibility that VIP plays a role in controlling the circadian rhythm of IOP in rabbits. Melatonin synthesis in rat pineal is stimulated by VIP receptors in addition to adrenergic receptors, and the cAMP response to VIP peaked at the beginning of the dark phase and decreased through the night.

Nilsson et al reported that the anterior uvea of the rabbit has primarily type II, non-selective PACAP receptors. VIP and PACAP displacement of bound [125I]VIP and [125I]PACAP27 showed that high-affinity binding of VIP is 79% that of PACAP in the rabbit ciliary body; the additional 21% reflects PACAP binding to the type I, high-affinity, PACAP receptor, which does not bind VIP. The specificity of [125I]PACAP27 binding demonstrated in Figure 6B is consistent with Nilsson et al’s data. Low concentrations of VIP, PACAP27, or PACAP38 displaced bound [125I]PACAP27; the bound [125I]PACAP27 not displaced by VIP probably represents [125I]PACAP27 bound to type I PACAP receptors. Based on Nilsson et al’s estimate of the relative amounts of selective and nonselective PACAP receptors in ciliary processes, Bmax for [125I]VIP should be approximately 80% that for [125I]PACAP27. Furthermore, changes in Bmax during the circadian cycle for one of these ligands should be paralleled by changes in Bmax for the other. Bmax for [125I]VIP was 77%, 66%, and 80% that for [125I]PACAP27 at 06:00, 14:00, and 22:00 CT, respectively. Therefore, our ligand-binding data are consistent with Nilsson et al’s data showing that in rabbit ciliary body, VIP and PACAP bind to the same nonselective receptor, which is about four times more abundant than the type I PACAP receptor. Because the type II PACAP receptor is thought to be identical to the VIP receptor, we anticipated that PACAP stimulation of ciliary process cAMP levels would increase at 22:00 CT, as did VIP stimulation. However, PACAP did not show an increase in stimulation in late dark phase. This observation is difficult to reconcile with the idea that the VIP and type II PACAP receptors are identical. However, PACAP is a newly discovered regulatory peptide, and its properties and those of its receptors are not yet fully understood.

In summary, we have no convincing evidence for circadian changes of ciliary process β-adrenergic or α2-adrenergic receptor sensitivity or β-adrenergic receptor density. The nocturnal increases in ISO stimulation of ciliary process cAMP levels that we observed were small and inconsistent. Therefore, we conclude...
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that the circadian rhythms of aqueous flow and IOP are likely to be driven in part by input from the central nervous system through the ocular sympathetic nerves and by other neural and humoral pathways that remain to be identified, and are unlikely to reflect changes in responsiveness of these adrenergic receptors in the ciliary processes.

Key Words

β-adrenergic, ciliary process, circadian, pituitary adenylate cyclase activating polypeptide, vasoactive intestinal peptide

References

32. McNellis EL, Bausher LP. Stimulatory and inhibitory


