

The Mammalian Pineal Expresses the Cone but Not the Rod Cyclic GMP Phosphodiesterase

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Abstract: To determine the presence of cone or rod cyclic GMP phosphodiesterase (EC 3.1.4.17) in the mammalian pineal, extracts from adult rat and bovine pineals were injected onto a Mono Q anion-exchange HPLC column and eluted with a NaCl linear gradient. Fractions were immunoadsorbed with monoclonal antibodies specific to rod and cone phosphodiesterases (ROS-1) and to calmodulin-phosphodiesterase complexes (ACC). Profiles were assayed with 10 $\mu\text{mol/L}$ [^3H]cyclic GMP in the presence of calcium-calmodulin, histone, or trypsin. Rat and bovine pineals displayed a single peak of activity recognized by ROS-1, which corresponded to the activity of the cone but not to the rod in bovine retina. ROS-1 immunoadsorbed ~80% of the activity in the 60-day-old rat pineal but only 26% of the activity in bovine pineal. ACC immunoadsorbed the remaining activity in both species. Western blot analysis of rat pineal extracts revealed three polypeptides of ~87, 15, and 10 kDa when probed with a rod/cone phosphodiesterase-specific antiserum. The specific activity of the cone-like phosphodiesterase in 10-day-old rat pineals was twice that of this isozyme in the bovine retina and 150 times that in the bovine pineal. The specific activity of phosphodiesterase in rat pineals decreased with age. We conclude that an enzyme with biochemical and antigenic characteristics similar to cone, but distinct from rod phosphodiesterase, is present in bovine and rat pineals. **Key Words:** Pineal—Cone—Photoreceptor—Phototransduction—Phosphodiesterase—Cyclic GMP.

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The phototransduction cascade of vision is the mechanism by which a single photon of light is transformed into a neurosensory signal. Activation of the cascade results in hydrolysis of the second messenger cyclic GMP by a phosphodiesterase (cyclic GMP PDE) that subsequently leads to the closure of a cyclic GMP-gated cation channel and hyperpolarization of the cell (Pugh and Lamb, 1990; Stryer, 1991; Kaupp and Koch, 1992). This biochemical process takes place exclusively in the photoreceptor, a cell type unique to the retina and the pineal. Two types of photoreceptors exist in the retina, the rod and the cone. The rod photo-

receptor is composed of an outer segment, a thin rudimentary cilium, and an inner segment. The rod outer segment contains the machinery for vision transduction, and the inner segment contains the energy-generating and protein-synthesizing organelles. The rod outer segment is a dual membrane system. A stack of ~2,000 flattened membrane disks is surrounded by a plasma membrane. The disks transform light energy into amplified changes in the concentration of second messenger molecules (cyclic GMP). The plasma membrane translates these chemical changes into neurological signals. The cone photoreceptor differs from the rod in that the foldings of the outer segment do not separate from the membrane to form disks. The phototransduction cascade of vision has been extensively studied in the rod photoreceptor; however, because of the paucity of cone photoreceptors in most mammalian retinas, our knowledge of the cone phototransduction cascade is incomplete.

Pineal photoreceptors in lower vertebrates share structural analogies with the cone photoreceptor in the retina (Zimmerman and Tso, 1975). Fish and amphibians have functional pineal photoreceptors capable of initiating neural signals. Light interrupts the dark current of the pineal photoreceptor and disrupts the secretion of hormones and neurotransmitters. This results in hyperpolarization of second-order neurons that convey information on solar radiation and day length via a pineal tract to deeper areas in the brain (Uchida et al., 1992). Chelonians and lacertilians have rudimentary pineal photoreceptors, whereas ophidians, birds, and mammals have predominantly secretory pinealocytes (Underwood, 1989).

Regardless of photoreceptor differentiation, all pinealocytes are capable of synthesizing melatonin dur-

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Abbreviations used: CaM, calmodulin; PDE, phosphodiesterase.

ing darkness (Falcon and Collin, 1989). The pineals of certain fish, lizards, and birds are directly sensitive to light and contain endogenous light-entrained circadian oscillators driving the rhythm of melatonin production. The mammalian pineal receives light input signals indirectly from the retina via a circadian clock in the suprachiasmatic nucleus of the hypothalamus and sympathetic neurons of the superior cervical ganglion. Norepinephrine synthesized and released from postganglionic fibers during the dark cycle interacts with postsynaptic β - and α_1 -adrenergic receptors in the mammalian pinealocyte, resulting in increased levels of melatonin in the circulation (Craft et al., 1984).

Over the last few years, evidence suggests that the mammalian pinealocyte has retained crucial components of the phototransduction cascade. Enucleated neonatal rats exposed to light demonstrated biochemical changes in the pineal that were effectively prevented by applying a lighttight metal cap to the skull (Zweig et al., 1966). Synaptic ribbons associated with electrolucent vesicles and dense-core granules, and outer segment-like membrane changes similar to those seen in the developing photoreceptor in the retina can be seen in the pineal of the neonatal rat, although they are rare in the adult (Zimmerman and Tso, 1975). In addition, several members of the phototransduction cascade of vision including arrestin, opsin, rhodopsin kinase, S-modulin, and phosducin have been identified in the adult mammalian pineal (Korf et al., 1992; Lolley et al., 1992). Most of these proteins appear to be more abundant in the neonatal rat than in the adult (Araki and Taketani, 1992; Babila et al., 1992).

Rods and cones have immunologically related but distinct cyclic GMP PDEs (Hurwitz et al., 1984, 1985). Previous work from our laboratory demonstrated that retinoblastoma has the cone but not the rod cyclic GMP PDE (Hurwitz et al., 1990a). Because retinoblastoma is a primitive neuroectodermal tumor that can arise from both retina and pineal, we hypothesized that the mammalian pineal photoreceptor originates from a stem cell common to both retina and pineal, capable of differentiating along the cone cell lineage. We demonstrate here that, as in retinoblastomas, the adult mammalian pineal expresses an enzyme with biochemical and antigenic characteristics similar to cone cyclic GMP PDE but distinct from rod cyclic GMP PDE. This suggests a common cell of origin for pineal and cone photoreceptors. The mammalian pineal could provide an alternative tissue to study the cone phototransduction cascade.

MATERIALS AND METHODS

Materials

Animal studies were conducted in compliance with the NIH *Guide for the Care and Use of Laboratory Animals* (NIH publication no. 85-23, 1985). Pineals and retinas from 2–60-day-old Zivic-Miller Sprague-Dawley rats were harvested under light conditions and frozen at -80°C or in

liquid nitrogen until ready to use. Pineals from adult cows were harvested under light conditions and saved at -80°C until ready to use. Bovine retinas were obtained frozen at -80°C from Hormel (Omaha, NE, U.S.A.). ROS-1, a monoclonal antibody that specifically recognizes the rod and cone PDEs, has been previously described (Hurwitz et al., 1984). ACC, a monoclonal antibody that specifically recognizes calmodulin (CaM) (Hansen and Beavo, 1986), was a gift from Dr. Joe Beavo, University of Washington. CaM was isolated in our laboratory as the protein product of a chicken CaM cDNA (gift from Dr. Anthony Means, Duke University) expressed in a bacterial system (Putkey et al., 1985). A specific rabbit cone/rod cyclic GMP PDE affinity-purified antiserum was a gift from Dr. Rehwa Lee, Sepulveda VA Hospital (Los Angeles, CA, U.S.A.) (Lee et al., 1985).

Protein determination

Protein concentrations were determined by the method of Bradford (1976) using bovine serum albumin as the standard.

HPLC

All experiments were performed under room light at 4°C unless otherwise specified. Samples were analyzed by anion-exchange HPLC by a modification of a method previously described (Hurwitz et al., 1985). One adult bovine pineal (160 mg of tissue/ml), four adult rat pineals (29 mg/ml), one adult bovine retina (150 mg/ml), and one adult rat retina (50 mg/ml) were each homogenized in 10 mmol/L Tris-HCl (pH 7.5), 1 mmol/L dithiothreitol, 1 mmol/L EDTA, 0.5 mmol/L phenylmethylsulfonyl fluoride. Homogenates were centrifuged twice in a microfuge (Fisher Scientific, Pittsburgh, PA, U.S.A.) at 14,000 g for 10 min and twice in an airfuge (Beckman Instruments, Palo Alto, CA, U.S.A.) at 100,000 g for 10 min. Supernatants were injected onto a Mono Q (Pharmacia Fine Chemicals, Piscataway, NJ, U.S.A.) anion-exchange HPLC column. The protein was eluted with a 0.05–0.5 mol/L NaCl linear gradient in homogenization buffer at a rate of 0.5 ml/min. Fractions were collected at 0.5-min intervals for 60 min.

Immunoabsorption assays

PDE immunoabsorptions were performed as described elsewhere (Hurwitz et al., 1984). Rabbit antimouse IgG₁ (200 μl , Zymed Laboratories, South San Francisco, CA, U.S.A.) was incubated with formalin-fixed *Staphylococcus aureus* (1.8 ml) for 30 min at room temperature. The *S. aureus*–IgG₁ complex was sequentially washed and resuspended in 1.8 ml of 150 mmol/L Tris-HCl (pH 7.5) and 0.5 g/L bovine serum albumin (buffer A) and incubated overnight at 4°C with 200 μl of ROS-1 containing mouse ascites fluid. Formalin-fixed *S. aureus* (1.8 ml) was coupled directly to ACC-containing mouse ascitic fluid (200 μl) with overnight incubation at 4°C . The *S. aureus*–IgG₁–ROS-1 and the *S. aureus*–ACC complexes were washed and resuspended in buffer A (2 ml). Samples from each pineal and retina HPLC fraction (75 μl) were incubated with each of these antibody-coupled cells (25 μl) in the presence of 1 mmol/L CaCl_2 , 1 $\mu\text{mol/L}$ CaM at 4°C , with constant slow motion for 2 h. The samples were centrifuged in a microfuge for 1 min and the supernatants and washed pellets separated.

Cyclic GMP PDE assays

Cyclic GMP PDE was activated either with type VIII-S histone from calf thymus (1 g/L, Sigma Chemical, St. Louis, MO, U.S.A.), a protein that has been shown to activate both

photoreceptor and $\text{Ca}^{2+}/\text{CaM}$ -dependent PDEs (Miki et al., 1975; Hurwitz et al., 1990a) or with trypsin from bovine pancreas (0.1 g/L, Sigma), which activates cyclic GMP PDE by digesting the inhibitory subunit of the photoreceptor enzyme or the CaM-binding domain of the CaM-dependent enzyme (Klee et al., 1979; Hurley and Stryer, 1982). Activation by either method was in the presence of 1 mmol/L EGTA. After 5 min of incubation in ice, trypsin was inactivated with soybean trypsin inhibitor (0.5 g/L, Sigma). Alternatively, PDE was activated with 100 nmol/L CaM in the presence of 1 mmol/L CaCl_2 . Retinas were assayed for 10 min with 10–200 $\mu\text{mol/L}$ [^3H]cyclic GMP (11.2 Ci/mmol, Sigma, ICN Biomedicals, Irvine, CA, U.S.A.). Pineals were assayed for cyclic GMP PDE activity with 10–100 $\mu\text{mol/L}$ [^3H]cyclic GMP for 10 min as indicated. The resulting [^3H]5'-GMP was then incubated for 10 min with *Crotalus atrox* venom (0.23 g/L, Sigma) to release [^3H]guanosine. The reaction mixture was passed through a DEAE-Sephadex column (Pharmacia Fine Chemicals) and the void volume collected in 2.5 ml of aqueous buffer. The recovered nucleoside was mixed with 4 ml of Ultima Gold scintillation cocktail (Packard Instrument, Meriden, CT, U.S.A.) and the samples were counted in a scintillation counter (Packard Instrument). The efficiency of recovery of the nucleoside was monitored by allowing an excess of cyclic GMP PDE to hydrolyze all the cyclic nucleotide in the assay. If <90% of the total nucleoside was recovered, the assays were repeated. The cyclic GMP PDE activity was calculated as nanomoles of cyclic GMP hydrolyzed per minute per milligram of protein.

Calculation of specific activities in PDE profiles and tissue homogenates

To determine the specific activities of the $\text{Ca}^{2+}/\text{CaM}$ and cone-like phosphodiesterases in the various profiles, the total activity immunoadsorbed by ACC in each peak and the total activity not immunoadsorbed by ACC in peak I was calculated (see Figs. 2 and 3). The ACC-adsorbed activity in peak I corresponds to the 61-kDa $\text{Ca}^{2+}/\text{CaM}$ -dependent isozyme, that in peak II to the 63-kDa $\text{Ca}^{2+}/\text{CaM}$ -dependent isozyme, and the activity remaining in peak I to the cone-like PDE (Hurwitz et al., 1990a,b). The Michaelis–Menten equation, $v = V_{\text{max}}/[1 + (K_m/[S])]$, was used to calculate the relative specific activity (V_{max}) of each isozyme knowing the total activity calculated from the profile (v), the substrate concentration ($[S] = 10 \mu\text{mol/L}$), and the K_m for each isozyme [2.7 $\mu\text{mol/L}$ for the 61-kDa enzyme, 1 $\mu\text{mol/L}$ for the 63-kDa enzyme (Sharma and Kalra, 1994), and 20 $\mu\text{mol/L}$ for the cone-like enzyme (see Results)]. These results closely approximated the activities in the bovine and rat retina homogenates assayed at the saturating substrate concentration of 100 $\mu\text{mol/L}$ (data not shown). The V_{max} of each isozyme divided by the sum of the V_{max} values of the three enzymes gave the relative fraction of each isozyme in the profile. To calculate the relative percentage of each isozyme in the tissue homogenates, the relative amounts of each isozyme in the corresponding profile, assayed at 10 $\mu\text{mol/L}$ cyclic GMP, was determined. These percentages were assumed to be the same as in the homogenates assayed at 10 $\mu\text{mol/L}$ cyclic GMP and the activity of each isozyme was then determined. The activity of each isozyme was then used to calculate the specific activities at saturating substrate concentrations (V_{max}) as described in the methods for the HPLC profiles.

The percent cone PDE activity in bovine retinas has pre-

viously been determined to be ~3% (Booth et al., 1991). The percent cone PDE activity in rat retinas was below the sensitivity of the assay system and is reported here to be <2% (see Results).

Western blot analysis

To determine the apparent subunit molecular weight of the pineal cyclic GMP PDE, a western blot was prepared with extracts of 10-day-old rat pineals and retinas (Hurwitz et al., 1990a). Four pineals (3.3 mg) and one retina (40 mg) were homogenized in 0.2-ml Dounce homogenizers with glass pestles and centrifuged as described above. The soluble extracts were mixed at a 2:1 ratio with 25 g/L sodium dodecyl sulfate, 0.6 g/L bromophenol blue, 100 mmol/L dithiothreitol, 25 mmol/L Tris-HCl, 2.5 mmol/L EDTA (pH 8), boiled 5 min at 95°C, and saved on ice. Sample volumes with roughly equal amounts of cyclic GMP PDE enzyme activity were loaded onto a 150 g/L acrylamide, 1 g/L bisacrylamide modified Laemmli gel (Laemmli, 1970) and electrophoresed at 6 mA for 16 h. The gel was then electrophoretically transferred to an Immobilon-P membrane (Bio-Rad, Melville, NY, U.S.A.) at 60 V for 3 h, blocked with 50 g/L nonfat dry milk, and incubated overnight at 4°C with a specific rabbit cone/rod cyclic GMP PDE affinity-purified antisera (gift from Dr. Rehwa Lee). The membrane was developed with a Problot Immunoscreeing System (Promega Biotec, Madison, WI, U.S.A.), as specified in the manufacturer's instructions, and scanned with a densitometer (Molecular Dynamics, Sunnyvale, CA, U.S.A.).

RESULTS

HPLC

The cyclic GMP PDE HPLC Mono Q profile of the bovine retina, assayed at 200 $\mu\text{mol/L}$ cyclic GMP, is shown in Fig. 1A. Two main peaks of histone activatable activity were detected, i.e., a small early peak from fractions 41–44 and a large heterogeneous peak from fractions 50–65. These peaks have previously been shown to immunoadsorb with ROS-1 and to contain the cone and the rod cyclic GMP PDEs, respectively (Hurwitz and Beavo, 1984; Hurwitz et al., 1985), as well as small amounts of $\text{Ca}^{2+}/\text{CaM}$ -dependent cyclic GMP PDE activity (Hurwitz et al., 1984).

The cyclic GMP PDE HPLC Mono Q profile of the bovine pineal assayed at 10 $\mu\text{mol/L}$ cyclic GMP is shown in Fig. 1B. As in bovine retina, two main peaks of histone-activatable enzyme activity are noted, i.e., a large peak from fractions 40–44 and a slightly smaller peak from fractions 46–55. The first peak eluted in the same fraction as the bovine cone PDE; however, $\text{Ca}^{2+}/\text{CaM}$ activated both this peak and the subsequent peak of activity in the profile better than histone/EGTA did (data not shown). Furthermore, the 61- and 63-kDa $\text{Ca}^{2+}/\text{CaM}$ -dependent PDEs have previously been found to elute from HPLC anion-exchange columns in the same fractions as the two peaks in the bovine pineal profile, suggesting that most of the activity may be due to the presence of these two isozymes (Hurwitz et al., 1990b). Immunoadsorption studies were then performed on the bovine pineal profiles to verify these findings (Fig. 2). The profile from

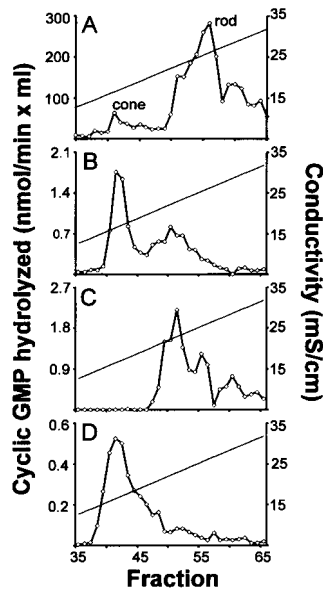


FIG. 1. HPLC Mono Q cyclic GMP PDE activity profiles. Extracts from adult bovine and rat pineals and retina were injected onto a Mono Q anion-exchange HPLC column and the protein eluted with a 0.05–0.5 mol/L NaCl linear gradient, at a rate of 0.5 ml/min. Samples were collected every 0.5 min. The resulting fractions were assayed for cyclic GMP PDE enzyme activity as described in Materials and Methods. **A:** Bovine retina with histone. **B:** Bovine pineal with histone. **C:** Rat retina with trypsin. **D:** Rat pineal with trypsin. (\diamond), Cyclic GMP PDE activity profile; (—), NaCl conductivity measurements.

a bovine retina is shown in Fig. 2A for comparison. The photoreceptor cyclic GMP PDE antibody (ROS-1) removed only a small amount of activity from the first peak (fractions 40–43) and none from the second peak in the profile for the bovine pineal (ROS-1 supernatant, Fig. 2B). The $\text{Ca}^{2+}/\text{CaM}$ -dependent PDE antibody (ACC) removed almost all the activity from the first and second peaks but failed to recognize a small peak of activity from fractions 40–44 (ACC supernatant, Fig. 2C). Some of the activity immunoadsorbed by ROS-1 was recovered in the *S. aureus*–ROS-1 pellet from fractions 40–43 (ROS-1 pellet, Fig. 2B). This activity correlated well with the enzyme activity not recognized by the ACC antibody (ACC supernatant, Fig. 2C) and with the cone cyclic GMP PDE activity in the bovine retina (Fig. 2A). When calculated using the Michaelis–Menten equation as described in Materials and Methods, the activity recognized by ROS-1 but not by ACC represented $\sim 26\%$ of the total activity in the profile. The activity immunoadsorbed by ACC was recovered in the *S. aureus*–ACC pellet (ACC pellet, Fig. 2C). These data indicate that there is a small amount of cyclic GMP PDE activity present in the bovine pineal similar to the cone but not the rod cyclic GMP PDE.

The cyclic GMP PDE HPLC Mono Q profile of the rat retina, assayed at 200 $\mu\text{mol/L}$ cyclic GMP, is shown in Fig. 1C. To activate the photoreceptor cyclic

GMP PDE, the inhibitory subunit was digested with trypsin in the presence of EGTA. Unlike its bovine counterpart, rat retina displayed only one broad, heterogeneous peak of activity from fractions 48–64. This peak correlated well with the second peak in the bovine retina that is known to contain rod cyclic GMP PDE (Hurwitz et al., 1984). There was no activity in the fractions where cone cyclic GMP PDE would have been expected in bovine retina, a finding that has been reproduced in our laboratory (unpublished observation) and that is consistent with the observation that $<1\%$ of photoreceptors in rat retina are cone (Szél and Rohlich, 1992).

The cyclic GMP PDE HPLC Mono Q profile of the rat pineal, assayed at 10 $\mu\text{mol/L}$ cyclic GMP, is shown in Fig. 1D. Enzyme studies were performed with trypsin in the presence of EGTA. When the rat pineal was assayed for cyclic GMP PDE activity, only a single peak of activity was noted from fractions 38–48, which correlated well with the early peak in the bovine retina known to contain the cone cyclic GMP PDE but not with the single peak in the rat retina known to contain the rod cyclic GMP PDE. Furthermore, there was no activity in the fractions where the rod cyclic GMP PDE would be expected to appear. Immunoadsorption profiles of the rat pineal are shown in Fig. 3. The rat retina profile is shown in Fig. 3A for compari-

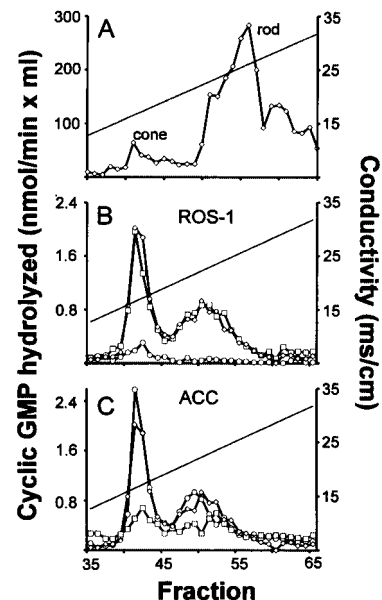


FIG. 2. Immunoabsorption studies in bovine pineal. Fractions were immunoabsorbed overnight either with *S. aureus*–rabbit antimouse IgG₁–ROS-1, with *S. aureus*–ACC, or with *S. aureus* as control. ROS-1 recognizes photoreceptor PDE and ACC recognizes $\text{Ca}^{2+}/\text{CaM}$ -dependent PDEs. The samples were then centrifuged and the supernatants and pellets assayed for PDE activity. **A:** Bovine retina. **B:** Bovine pineal and ROS-1. **C:** Bovine pineal and ACC. (\diamond), *S. aureus* supernatant; (\square), *S. aureus*–antibody supernatant; (\circ), *S. aureus*–antibody pellet; (—), NaCl conductivity measurements.

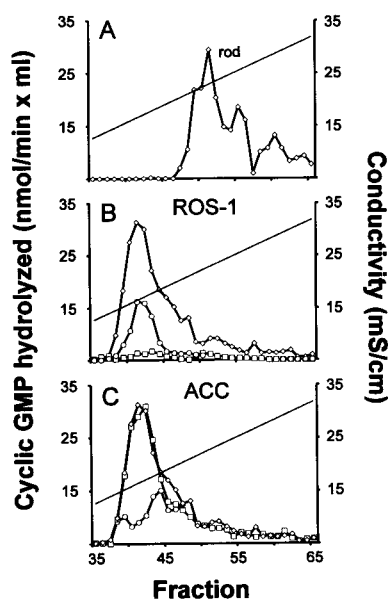


FIG. 3. Immunoadsorption studies in rat pineal and retina. Rat pineal fractions were immunoadsorbed and assayed for PDE activity as described in the legend to Fig. 2. **A:** Rat retina. **B:** Rat pineal and ROS-1. **C:** Rat pineal and ACC. (\diamond), *S. aureus* supernatant; (\square), *S. aureus*-antibody pellet; (\circ), *S. aureus*-antibody supernatant; (—), NaCl conductivity measurements.

son. ROS-1 immunoadsorbed $\sim 90\%$ of the activity in fractions 38–48 of the rat pineal profile, indicating that most of the cyclic GMP PDE enzyme activity present was cone-like in origin (ROS-1 supernatant, Fig. 3B). About 60% of the enzyme activity was recovered in the *S. aureus*-ROS-1 pellet (ROS-1 pellet, Fig. 3B). The missing 30% of activity represents inhibition of the photoreceptor enzyme by the ROS-1 antibody, an effect that has been previously reported (Hurwitz et al., 1984). Immunoadsorption with ACC in the presence of $\text{Ca}^{2+}/\text{CaM}$, on the other hand, removed $\sim 10\%$ of the activity present in the rat pineal in fractions 45–48 (ACC supernatant, Fig. 3C). This activity was recovered in the *S. aureus*-ACC pellet (ACC pellet, Fig. 3C).

Kinetic analysis of pineal photoreceptor PDE

A hypotonic extract of rat pineal was immunoadsorbed with ACC coupled to *S. aureus* to remove calmodulin-dependent PDE activities. The remaining cyclic GMP PDE activity was recognized by ROS-1 as was shown above. The pineal photoreceptor PDE was then assayed in the presence of trypsin-EGTA, and 100, 25, 14.29, and 10 $\mu\text{mol/L}$ cyclic GMP and cyclic AMP. A Lineweaver-Burk double-reciprocal plot was prepared for each substrate (data not shown). The pineal photoreceptor PDE was found to exhibit Michaelis-Menten kinetics with a K_m for cyclic GMP of $20 \pm 2 \mu\text{mol/L}$. This activity was found to be highly specific for cyclic GMP. A K_m for cyclic AMP was

estimated to be $>1 \text{ mmol/L}$. These findings are in close agreement with kinetic values previously obtained for both the rod and cone PDEs (Hurwitz et al., 1984; Gillespie and Beavo, 1988; Srivastava et al., 1995).

Specific activity of cyclic GMP PDE

The specific activity of cyclic GMP PDE in all tissue extracts was determined after digestion with trypsin and in the presence of EGTA. The retinas and adult pineals were assayed with both 10 and 100 $\mu\text{mol/L}$ [^3H]cyclic GMP as substrate. Developmental studies in rat pineal were assayed with 10 $\mu\text{mol/L}$ [^3H]cyclic GMP. The V_{max} (specific activity) was calculated for each activity at 10 $\mu\text{mol/L}$ cyclic GMP by using the Michaelis-Menten equation as detailed in Materials and Methods and by adding these results to obtain the total specific activity. The calculated results were similar to those actually determined at 100 $\mu\text{mol/L}$ cyclic GMP indicating that the specific activity of the neonatal rat pineal assayed at 10 $\mu\text{mol/L}$ cyclic GMP could be calculated with a certain degree of reliability and compared with the specific activity of the phosphodiesterase in the retina and adult pineal. These studies are summarized in Table 1. The specific activities in 60-day-old rat and adult bovine pineals were 14 and 3.1 nmol/min/mg of protein, respectively. The specific activity of 10-day-old rat pineals was nine times more than 2-day-old rat pineals. After the neonatal period, the cyclic GMP PDE specific activity in rat pineals decreased significantly with age ($p < 0.01$). No difference in PDE specific activity was noted in light- and dark-adapted age-matched rat pineals (data not shown).

Cone-like cyclic GMP PDE activity

The total PDE activity in the bovine pineal profile was calculated as described in Materials and Methods. The total cone-like activity was determined from the

TABLE 1. Cyclic GMP PDE activity in retina and pineal

Specific activity	Total	Cone-like	%
Adult bovine retina	$2,500 \pm 200$	75 ± 6	3 ^a
Adult bovine pineal	3.1 ± 0.2	0.8 ± 0.05	26
10-day rat retina	$2,600 \pm 550$	VBS	<2
2-day-old rat pineal	17 ± 4	12 ± 2.7	70
10-day rat pineal	160 ± 34	145 ± 31	90
30-day rat pineal	90 ± 27	81 ± 24	90
60-day rat pineal	14 ± 2.3	11 ± 1.8	80

PDE assays were performed as described in the presence of 10 $\mu\text{mol/L}$ cyclic GMP and trypsin. The relative activities of each isozyme were determined. This information was used to estimate the specific activity (V_{max}) at saturating conditions for each isozyme using the Michaelis-Menten equation (Materials and Methods). Cyclic GMP PDE activity is shown as nanomoles per minute per milligram of protein. Rat pineal and retina data are mean \pm SD values of nine determinations ($p < 0.01$). Bovine data are two determinations. VBS, value below sensitivity.

^a From Booth et al. (1991).

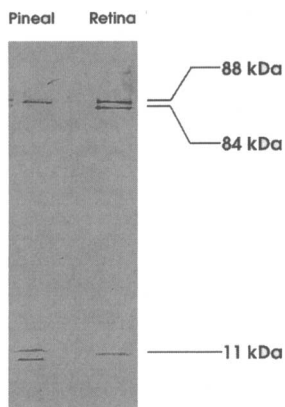


FIG. 4. Subunit composition of the rat pineal cyclic GMP PDE. Protein soluble extracts from 10-day-old rat pineals and retinas were mixed with sodium dodecyl sulfate sample buffer, boiled 5 min at 95°C, loaded onto a 150 g/L total acrylamide, 1 g/L bisacrylamide Laemmli (low bis) gel, and electrophoresed at 4 mA for 16 h. The protein was transferred to an Immobilon-P membrane and probed with a specific rabbit cone/rod cyclic GMP PDE affinity-purified antiserum as described in Materials and Methods.

activity remaining in peak I of the profile after ACC precipitation (Fig. 2). The supernatant of the ACC immunoprecipitation was used because, although all the remaining activity was precipitated by the monoclonal antibody ROS-1, this antibody is known to inhibit phosphodiesterase activity, making accurate quantitation of the cone-like enzyme impossible from those data (Hurwitz et al., 1984). A similar treatment was applied to the rat pineal profiles and to immunoadsorptions of 2-day-old rat pineal extracts assayed with 10 $\mu\text{mol/L}$ cyclic GMP (Fig. 3). In addition, cone-like cyclic GMP PDE activity was measured directly from ACC immunoadsorptions of 30-day-old rat pineal extracts with 100 $\mu\text{mol/L}$ cyclic GMP. Greater than 70% of the cyclic GMP PDE activity present in rat pineals corresponded to a cone-like enzyme as opposed to 26% in bovine pineal, 3% in bovine retina, and <2% in rat retina. The specific activity of the cone-like enzyme in 10-day-old rat pineals was twice that of the enzyme in bovine retina, 150 times that of the bovine pineal, and 12 times that of the 2- and 60-day-old rat pineals. After the neonatal period, cone-like activity in rat pineals decreased with age ($p < 0.01$).

Western blot analysis

Ten-day-old rat pineals and retinas were used to show the subunit molecular composition of the pineal photoreceptor cyclic GMP PDE (Fig. 4). The photoreceptor-specific cyclic GMP PDE antiserum recognized two polypeptides of ~ 88 and 84 kDa in the rat retina known to be the α and β subunits of the enzyme but only a single ~ 87 -kDa polypeptide in the rat pineal. In addition, the antibody recognized two low molecular mass polypeptides in the rat pineal (~ 15 and 10 kDa). Whereas a polypeptide comparable with the ~ 11 -kDa inhibitory γ subunit of the rat rod enzyme was visible, the β subunit was not recognized in the rat pineal.

DISCUSSION

The importance of the rat pineal in the study of the photoreceptor cascade in general has been highlighted

by the recent identification of an arrestin in both the cone photoreceptors of the retina and in a subpopulation of cells in the adult rat pineal (Craft et al., 1994). The additional finding that retinoblastoma, a primitive neuroectodermal tumor of the retina and pineal, has been found to have elements of the cone but not the rod phototransduction cascade further suggests that pinealocytes may derive from a stem cell capable of differentiating along the cone but not the rod photoreceptor lineage (Bogenmann et al., 1988; Hurwitz et al., 1990). In addition, these data strongly suggest that the cone cyclic GMP PDE may be present in the mammalian pineal.

Bovine rod cyclic GMP PDE ($\alpha\beta\gamma_2$) is composed of two similar but genetically distinct catalytic α and β subunits (88 and 84 kDa) and two inhibitory γ subunits (11 kDa) (Baehr et al., 1979; Hurley and Stryer, 1982; Deterre et al., 1988). Bovine cone cyclic GMP PDE is a homodimer of a single catalytic subunit (92 kDa) and three distinct smaller subunits (15, 13, and 11 kDa) (Hurwitz et al., 1985; Gillespie and Beavo, 1988). Besides the photoreceptor enzymes, at least four $\text{Ca}^{2+}/\text{CaM}$ -dependent cyclic GMP PDEs have been characterized, one from the heart (59 kDa), one from the brain (61 kDa), one from the lung (58 kDa), and one from brain and activated bovine lymphocytes (63 kDa) (Hurwitz et al., 1990b; Novack et al., 1991; Sharma and Kalra, 1994). The cone PDE has a deduced amino acid sequence that is $\sim 70\%$ identical with the rod cyclic GMP PDE α and β subunits (Ovchinnikov et al., 1987; Li et al., 1990), and share antigenic determinants with the rod but not with the CaM -dependent cyclic GMP PDE isoenzymes (Hurwitz et al., 1984).

To determine whether the mammalian pineal had cone or rod cyclic GMP PDE, anion-exchange chromatography on rat and bovine retinal and pineal extracts was performed and the eluted fractions were assayed for cyclic GMP PDE activity. This technique has been shown to be a valid method for separating the cone and the rod cyclic GMP PDEs (Hurwitz and Beavo, 1984; Hurwitz et al., 1985). A cyclic GMP PDE activity was found in both bovine and rat pineals that correlated well with the cone but not with the rod cyclic GMP PDE in the retina. The elution of this activity from anion-exchange HPLC columns has been consistent in all species studied so far (Hurwitz et al., 1984, 1985; Booth et al., 1991).

To distinguish photoreceptor from CaM -dependent cyclic GMP PDEs, immunoadsorptions with ROS-1, a monoclonal antibody that specifically recognizes rod and cone photoreceptor cyclic GMP PDEs (Hurwitz et al., 1984, 1985), and with ACC, a monoclonal antibody that specifically recognizes the CaM -dependent isoenzymes (Hansen and Beavo, 1986) were performed. These antibodies do not cross-react with each other (Hurwitz et al., 1990). Immunoadsorptions with ROS-1 demonstrated the presence of photoreceptor cyclic GMP PDE activity in the first peak of the bovine

and rat pineal profiles (~26 and 90% of total activity, respectively) but not in the second peak of the bovine pineal. This indicated the presence of a cone-like activity in the pineal. This activity was not immunoadsorbed by ACC. Furthermore, this cone-like activity was specific for cyclic GMP with a K_m of 20 $\mu\text{mol/L}$. Both of these characteristics are very similar to published reports for both the cone and rod PDE (Hurwitz et al., 1984; Gillespie and Beavo, 1988; Srivastava et al., 1995). The remaining activity not immunoadsorbed by ROS-1 was immunoadsorbed by ACC indicating that most of the activity in the bovine pineal and a small fraction in the rat pineal was Ca^{2+} /CaM-dependent PDE. The two peaks of Ca^{2+} /CaM-dependent PDE activity immunoadsorbed by ACC closely correlate with the elution pattern from anion-exchange HPLC columns of the 61- and 63-kDa isozymes found in brain (Hurwitz et al., 1990). In addition, the enzyme activity immunoadsorbed by ACC hydrolyzed cyclic AMP almost as well as cyclic GMP as is the case with these Ca^{2+} /CaM-dependent isozymes (data not shown; Sharma and Kalra, 1994). The activity immunoadsorbed by ROS-1, however, was not immunoadsorbed by ACC ruling out a Ca^{2+} /CaM-dependent isoenzyme. These findings strongly suggest that the pineal has cone but not rod cyclic GMP PDE activity.

To determine the subunit composition of the pineal photoreceptor cyclic GMP PDE, a western blot from rat pineal and retinal protein extracts was prepared and probed with a photoreceptor-specific cyclic GMP PDE antiserum that recognizes both cone and rod cyclic GMP PDEs (Lee et al., 1985). We were able to demonstrate that the rat pineal cyclic GMP PDE is composed of a large ~87-kDa α' subunit, and two smaller subunits, ~10 and 15 kDa, respectively (Fig. 4). This subunit composition resembles that of the bovine cone cyclic GMP PDE. The presence of an ~87- instead of a 92-kDa α' subunit cyclic GMP PDE in the pineal of the rat can be attributed to species differences in true molecular weight or charge. Species differences have also been reported in the α' subunit of bovine and lizard cones, and in the α and β subunits of bovine, lizard, fish, and frog rods and have not changed the HPLC elution pattern of the cone and rod cyclic GMP PDEs (Hurwitz et al., 1984; Booth et al., 1991). The absence of a β subunit in rat pineal is consistent with the pineal photoreceptor cyclic GMP PDE being of cone and not rod origin because the rod PDE is not active either in vivo or in vitro without the coexpression of this subunit (Pittler and Baehr, 1991; Piriev et al., 1993; Suber et al., 1993; Qin and Baehr, 1994).

This report adds cyclic GMP PDE to the growing number of phototransduction proteins described in the mammalian pineal. The observations that both bovine and rat pineals have an enzyme with biochemical and antigenic characteristics similar to cone cyclic GMP PDE but distinct from rod cyclic GMP PDE, and that the rat retina has rod and almost no cone cyclic GMP PDE, strongly suggests that both the pinealocyte and

the cone originate from a common stem cell capable of differentiating along the cone and not the rod cell lineage.

That cone-like activity in rat pineals is relatively low at day 2 of life, increases significantly by day 10, and decreases after the neonatal period is consistent with morphologic studies that have shown photoreceptor differentiation in rat pineals from days 4–17 of life (Zimmerman and Tso, 1975). It is also consistent with the developmental profile of other photoreceptor proteins found in rat pineal (Araki and Taketani, 1992; Babila et al., 1992). The additional finding that adult rat pineals display a greater absolute and relative amount of cone-like activity than adult bovine pineals suggests a significant role for this photoreceptor enzyme in the rat pineal. Furthermore, these data suggest that a functional cone phototransduction cascade may be present in the pineal of the neonatal rat. Because the phototransduction cascade is well conserved during evolution (Hurwitz et al., 1984; Booth et al., 1991), the pineal of the rat may provide a readily available cone tissue to study the mammalian cone phototransduction cascade.

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