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Affinities of bovine photoreceptor cGMP phosphodiesterases for rod and cone inhibitory subunits

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Rods and cones have analogous phototransduction components and cycles, but differ from each other in their physiological response to light. Differences between the affinities of rod and cone phosphodiesterase (PDE) catalytic subunits for their respective inhibitory subunits could potentially contribute to these physiological differences. To test this idea, we expressed both the 13 kDa PDE subunit, unique to a subset of bovine retinal cones [(1990) J. Biol. Chem. 265, 11259–11264], and the rod PDE 11 kDa inhibitory subunit in *E. coli*, purified them, and compared their abilities to inhibit rod and cone PDE catalytic subunits. Rod PDE has similar K_i values (~80 pM) for both the rod and cone recombinant inhibitory subunits. Activated cone PDE has K_i values of 200 pM for the cone 13 kDa subunit and 600 pM for rod PDEγ.

Retinal phosphodiesterase; Inhibition; Cyclic GMP; Bovine retina

1. INTRODUCTION

In the outer segments of vertebrate rod photoreceptors, cGMP phosphodiesterase (PDE) hydrolyzes cGMP in response to light [1]. This causes cation channels in the outer segments to close, and the cell to hyperpolarize [2]. The phototransduction cascade begins when light-activated rhodopsin binds transducin (T$\alpha\beta\gamma$), enabling it to exchange its bound GDP for GTP [3]. Tα-GTP dissociates from T$\beta\gamma$ [4] and activates the PDE. Whether Tα-GTP actually binds and removes the PDE inhibitory subunits [5] or whether it binds and remains associated with the PDE [6,7] is still a matter of controversy. Within seconds of PDE activation, Tα hydrolyzes GTP to GDP [8] and dissociates from the PDE, allowing PDEγ to recombine with PDE$\alpha\beta$. An analogous cascade exists in cones. Thus PDEγ has two main interactions in photoreceptors, one with Tα and one with the PDE catalytic subunits. It is this latter interaction which we explore in this study.

The catalytic complex of the membrane-associated bovine rod PDE consists of a homo- or heterodimer of α (88 kDa) and β (84 kDa) subunits [1,9]. The catalytic dimer is associated with at least two 11 kDa inhibitory (γ) subunits [5]. This holoenzyme can be activated by trypsin which selectively degrades the PDEγ subunits [1,10]. A more soluble form of rod PDE also exists. It is similar to the membrane-associated form except that it co-purifies with a 15 kDa protein which is also present in preparations of cone PDE [11]. The catalytic complex of cone PDE is a homodimer of α' (94 kDa) subunits. Two smaller subunits (11 and 13 kDa) purify with the cone PDE catalytic subunit. The 11 kDa subunit may be rod PDEγ that co-purifies with cone PDE [12]; however, the 13 kDa subunit is unique to cones [2,11]. Purified cone PDE exhibits higher basal activity and is activated by lower concentrations of rod transducin than the rod PDE isozyme [11].

Components of the rod phototransduction cascade can be readily isolated from bovine retina since rods are more abundant than cones. Cones have unique forms of opsin [13], transducin [14,15] and phosphodiesterase [12,16], and differ greatly from rods in their response to light. A rod cell can respond to a single photon; cones require 100-fold more photons to achieve the same signal [17]. Cones are quick to respond, quick to recover [18] and can adapt over a far wider range of light intensities than can rods [17]. Here we explore the hypothesis that differences in the interaction between rod and cone PDEs and their respective inhibitory subunits may account in part for these physiological differences.

2. MATERIALS AND METHODS

2.1. *Materials*

[8-^{3}H]cGMP and [U-^{14}C]GMP were obtained from Amersham ([U-^{14}C]GMP is currently unavailable). Factor Xa, aprotinin, leupeptin,

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Abbreviations: cGMP, guanosine 3′,5′-cyclic monophosphate; HPLC, high pressure liquid chromatography; PAGE, polyacrylamide gel electrophoresis; PDE, phosphodiesterase; PMSF, phenylmethylsulfonyl fluoride; SDS, sodium dodecyl sulfate.

bestatin, and pepstatin were ordered from Boehringer-Mannheim. Trypsin, soybean trypsin inhibitor, PMSF, and benzamidine came from Sigma. Aquapore RP-300 Brownlee columns came from Rainin.

2.2. *Purification of rod and cone PDE holoenzymes*

Rod and cone PDE holoenzymes were purified as described by Gillespie and Beavo [11] and Gillespie et al. [19]. Concentrations of these PDEs were determined by Bradford assay and were multiplied by a factor of 0.8 to yield concentrations consistent with amino acid analyses [19].

2.3. *Expression and purification of recombinant proteins*

The bovine 11 kDa and cone PDE 13 kDa inhibitory subunits were expressed using a method reported by Brown and Stryer [20]. Each subunit is expressed as a λcII N-terminal fusion with a Factor Xa protease cleavage site between the two proteins. Treatment with Factor Xa generates a protein with the native amino acid sequence. The plasmid containing the λcII–Factor Xa–Rod PDEγ fusion cDNA (pLcIIFXSG) was a generous gift of L. Brown and L. Stryer (Stanford University, Stanford, CA) and was used to express the rod PDEγ described here. The bovine cone 13 kDa PDEγ construct was made by PCR amplification of clone 14-6 [12] using primers that added appropriate restriction enzyme sites. The Factor Xa–cone 13 kDa PDEγ construct replaced the Factor Xa–rod PDEγ in pLcIIFXSG and was sequenced to avoid PCR artifacts.

The plasmids described above were transformed into *E coli* AR68 cells (gift of L. Brown and L. Stryer) before each expression experiment. Methods for expression and crude protein purification are described in Brown and Stryer [20]. All buffers contained 10 μg/ml leupeptin, 1 μg/ml aprotinin, 10 μg/ml pepstatin, 1 mM benzamidine, 2 mM PMSF, and 3 μg/ml bestatin. Fusion protein was quantitated by the Bradford method, digested with Factor Xa, and isolated by reverse-phase HPLC using an Aquapore RP-300 Brownlee column and conditions described in Brown and Stryer [20]. Fractions containing PDEγ were identified using an antibody (PDE B) raised against the basic mid-region (amino acids 24–45 in rod PDEγ) common to both rod 11 kDa and cone 13 kDa PDEγ's. These fractions were dried in siliconized microfuge tubes and dissolved in 30 μl distilled water.

Rod PDEγ co-eluted with a 9 kDa degradative product. Additional protease inhibitors and cell lysis by boiling or sonication failed to prevent degradation. To purify the non-degraded form, the rod PDEγ was extracted from an 18% preparative mini-SDS-gel slice (2–3 mm) by the method of Wensel and Stryer (Fig. 9 in [7]). A preparative SDS-gel loaded with water was treated similarly for control purposes. Cone 13 kDa PDEγ showed little degradation but was SDS-PAGE-purified for purposes of comparison.

Protein concentrations in gel extracts were routinely determined by Bradford assay. BSA standards were spiked with a volume of control gel extract to control for any bias introduced by SDS-PAGE components. Concentrations were more accurately determined by amino acid analysis of duplicate aliquots. Concentrations determined by Bradford assay were corrected by multiplying by a factor of 0.54 for 11 kDa PDEγ and 0.73 for 13 kDa PDEγ.

2.4. *PDE assays*

PDE assays follow the [^{3}H]cGMP protocol of Gillespie [21], although final assay volumes were 0.1 ml. Maximally activated PDE was produced by treatment with trypsin for 7 min on ice followed by the addition of 6X trypsin inhibitor.

In experiments determining the amount of active subunit, activities were assayed using 1 nM trypsin-activated rod PDE and 20 mM cGMP. In assays estimating K_i values, the final PDE concentration was 1 pM and the cGMP concentration was 5 μM. Results were corrected for ^{14}C recovery and blanks were subtracted.

3. RESULTS AND DISCUSSION

Recombinant rod 11 kDa and cone 13 kDa PDE subunits were expressed in *E. coli* and purified in order to compare their abilities to inhibit photoreceptor PDE catalytic subunits. Both subunits were expressed as λcII–PDEγ fusion proteins by a previously described method [20]. The cII fragment was cleaved from the N-terminus of each PDEγ-fusion product by Factor Xa. Expressed PDEγ subunits were extracted with urea and purified using a CM-Sephadex C-50 column followed by reverse-phase HPLC. Both rod and cone PDEγ's were further purified by SDS-PAGE and their concentrations determined by amino acid analysis. An SDS gel stained with Coomassie blue (Fig. 1) shows the purified recombinant PDEγ subunits and the cone and soluble rod PDE holoenzymes.

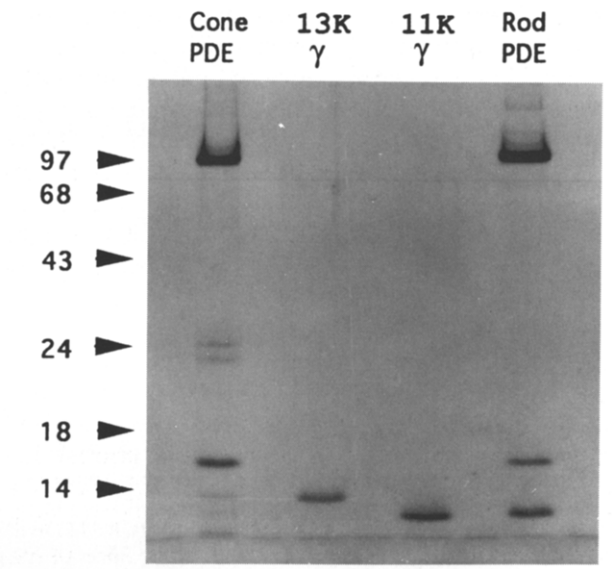

Fig. 1. An SDS-PAGE gel (15% acrylamide/0.16% bis-acrylamide) stained with Coomassie blue showing purified bovine cone and (soluble) rod PDEs in the outer lanes, and recombinant cone 13 kDa and rod 11 kDa PDE subunits in the inner lanes. Equal amounts (0.1 μg) of the 11 and 13 kDa PDEγ's were loaded. A 2-fold excess of cone PDE (3.5 μg) compared to rod PDE (1.8 μg) was loaded to show the band at 13 kDa in cone PDE.

3.1. *Activity of expressed PDEγ subunits*

The activity of each purified subunit was determined by titrating against purified trypsin-activated rod PDE. The concentration of activated PDE used in these experiments was verified by measuring average specific activity, 1,830 ± 250 μmol/min/mg (five experiments, each in triplicate), which falls between published values ranging from 1,200–1,500 μmol/min/mg [10] to 2,300 μmol/min/mg [11].

To estimate the percent of active PDEγ in our preparations, the amount needed to fully inhibit 1 nM trypsin-activated rod PDE was determined. At this concentration (well above the believed K_i of interaction), the relationship between inhibition of PDE activity and concentration of PDEγ should remain linear until the concentration of free catalytic subunits approaches the K_i. The concentration of PDEγ needed for 100% inhibi-

tion was determined by extrapolating the linear portion of the titration curve to 0% activity. The validity of this approach was confirmed by computing inhibition curves expected for 1 nM total PDE exhibiting theoretical K_i values of 800 pM, 80 pM, and 8 pM for PDEγ (Fig. 2, inset). At least two inhibitory subunits are believed necessary for full inhibition of rod PDE [5], so x-intercept values were divided by two to determine the concentration of active PDEγ. The number of inhibitory subunits needed to fully inhibit the cone PDE has not been reported. We have assumed that two are necessary.

Fig. 2 is an example of titration results from a representative experiment using trypsin-activated rod PDE and bacterially expressed rod PDEγ (solid circles). Extrapolation of the data shown in Fig. 2 suggests that 4 nM rod PDEγ completely inhibited 1 nM PDE catalytic complex. If this inhibitory subunit were fully active and the stoichiometry of PDEγ subunits per PDE catalytic complex is 2:1, a value of 2 nM would have been obtained. Since complete inhibition requires a 2-fold higher concentration of rod PDEγ than predicted, only

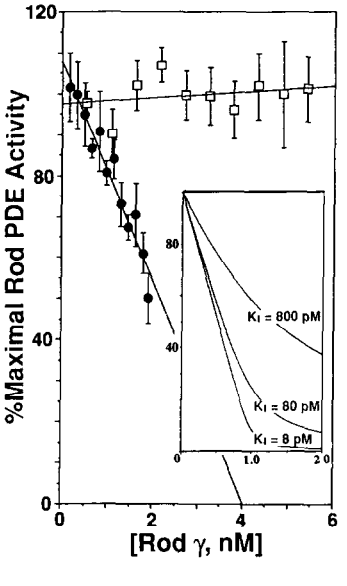

Fig. 2. Titration of trypsin-activated soluble rod PDE with recombinant rod 11 kDa PDEγ (●). Error bars represent the S.D. of triplicate analyses. Complete inhibition was extrapolated to a total rod PDEγ concentration of 4 nM. Assuming two inhibitory subunits per holoenzyme, inhibition of the 1 nM PDE used in the experiment should be complete at 2 nM of fully active rod PDEγ. Since a 2-fold higher concentration of rod PDEγ is required than predicted, the SDS-gel-purified inhibitor is ~50% active. Trypsin treatment of this rod PDEγ followed by addition of trypsin inhibitor destroyed all inhibitory activity (□). (Inset) Theoretical inhibition curves computed for 1 nM total PDE. Curves were calculated by an iterative procedure using the formula $K_i = \{[P](I_t - P_t + [P])\}/(P_t - [P])$ and three hypothetical K_i values (800 pM, 80 pM, 8 pM) for interaction between PDE and PDEγ. An estimate of the concentration of active inhibitor can be determined by extrapolating the linear portion of the curve where the concentration of free PDE is greater than K_i. (P = free PDE, P_t = total PDE, I_t = total PDEγ.)

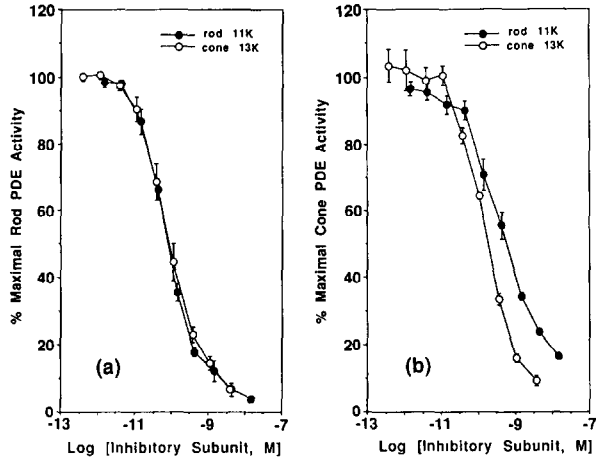

Fig. 3. Inhibition curves of trypsin-activated rod and cone PDEs (1 pM) with recombinant rod and cone PDE inhibitory subunits. Error bars represent S.E.M. calculated from three experiments, each done in triplicate. (a) K_i values of 79 pM and 83 pM are shown for inhibition of activated rod PDE with recombinant rod and cone PDE inhibitory subunits, respectively. (b) K_i values of 200 pM and 600 pM are shown for inhibition of activated cone PDE with recombinant cone 13 kDa PDEγ and rod PDEγ, respectively.

50% of the rod PDEγ appears to be active. To prove that the inhibition observed is due to PDEγ, an aliquot of rod PDEγ was digested with trypsin; the results (open squares) show that trypsin destroys the inhibition. The activity of the SDS-PAGE-purified PDEγ was stable for several weeks, but was only 9% active after 6 months. Identical dilutions and volumes of extracted control gel added to activated rod PDE showed no inhibition (data not shown).

The concentration of cone 13 kDa PDEγ required for 100% inhibition was 3.7 ± 0.5 nM (n = 3; data not shown). Thus, 3–4-fold higher concentrations were required than predicted, indicating that the purified cone PDEγ was 27% active, on average. Trypsin also destroyed the inhibitory properties of the 13 kDa PDEγ.

3.2. K_i values for the inhibitory subunits

Inhibition constants for inhibition of trypsin-activated rod and cone PDE catalytic subunits by rod and cone inhibitory subunits were compared at a catalytic subunit concentration (1 pM) at which IC_{50} values should approximate the K_i. Increasing concentrations of inhibitory subunits were added to activated PDE and equilibrated on ice for 15–30 min before assaying. Results of these experiments are presented in Fig. 3.

Activated rod PDE exhibited virtually identical K_i values for the recombinant rod PDEγ (79 pM) and recombinant cone 13 kDa PDEγ (83 pM, Fig. 3a). K_i values for the interaction of activated cone PDE with recombinant cone 13 kDa PDEγ and rod PDEγ were 200 pM and 600 pM (Fig. 3b), respectively. Similar experiments were conducted using rod and cone PDE inhibitory subunits purified from bovine retinas [21].

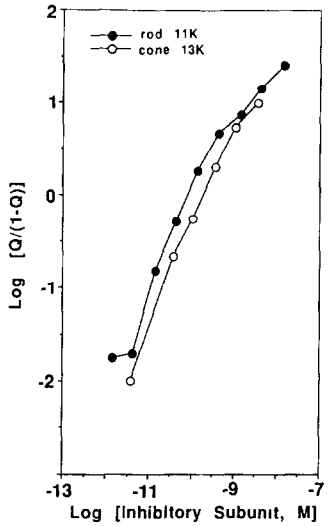

Fig. 4. Hill plots for interactions between trypsin-activated rod PDE and its inhibitor (●) and trypsin-activated cone PDE and the cone 13 kDa PDEγ (□), using the purified recombinant inhibitory subunits. Slopes are approximately 1.1, indicating that inhibitor binding is not cooperative.

The affinities of trypsin-activated rod PDE for retinal rod and cone inhibitory subunits was 6 pM and 48 pM, respectively. Cone PDE showed affinities of 23 pM and 91 pM for retinal rod and cone inhibitory subunits [21].

The lower affinities obtained with the recombinant proteins may reflect a structural difference between retinal vs. bacterially expressed PDEγ subunits. For instance, the N-terminus of rod PDEγ is reported to be acetylated [22]. Perhaps such a modification is necessary to achieve optimal interactions or protein folding. In addition, the 13 kDa subunit isolated from bovine retinal cone PDE may contain a mixture of red/green and blue cone PDEγ subunits which may co-elute during isolation by HPLC and co-migrate on SDS-PAGE. In contrast, the recombinant 13 kDa protein is strictly specific to a subset of cones [12]. The titers of purified recombinant subunits are higher than those for isolated retinal PDEγ's (~12% active) [23]. The recombinant PDEγ's also appear to be more stable. The inhibitors isolated from bovine retina lose 40–75% of their inhibitory activity over a two week period [23]. These differences may account for the range of K_i values. The large quantities of stable recombinant PDEγ subunits make them preferable for use in future phototransduction studies.

Regardless of whether retinal or recombinant PDEγ is used, rod PDE has a higher affinity for PDE inhibitory subunits than does cone PDE. If PDE K_i values were the only factor determining physiological differences between rod and cone responses, we might actually expect rod PDE to have a weaker affinity for inhibitory subunits since they are 100-fold more sensitive to light. The concentration of PDE in rod photoreceptors is ~30 μM [24]. Assuming this value is similar in cones and that conditions of steady state are attained, the concentration of PDEγ in vivo is far above any of the observed K_i values. The differences we observe within the picomolar range may therefore be of little consequence within the cell. However, absolute rates of PDEγ association and dissociation could play a role in response variations. Alternatively, there may be a mechanism which decreases the effective concentration of PDEγ available for inhibiting the catalytic subunits; for instance, bovine rod PDEγ can be phosphorylated in vitro [25].

Wensel and Stryer [6] examined the effects of dilution on the activity of native PDE in ROS homogenates and purified PDE in solution. They estimated that the dissociation constant for the rod inhibitor from the catalytic complex is less than 10 pM. This number falls at the low end of our K_i range, perhaps because our catalytic subunits were modified by trypsin digestion. Trypsin is known to nick the $\alpha\beta$ complex of rod PDE [26] and the α' subunit of cone PDE [23], removing a small C-terminal fragment from these proteins.

3.3. *Cooperativity*

We used Hill plots [27] to examine our data for evidence of cooperative interaction between the PDE catalytic and inhibitory subunits. At concentrations of PDE below the presumed K_i of interaction with PDEγ, 'sites occupied' (Q) is analogous to percent PDE inhibited and 'sites vacant' (1−Q) is represented by percent PDE uninhibited. Fig. 4 is a Hill plot for the inhibition of rod and cone PDEs with recombinant rod 11 kDa and cone 13 kDa PDE subunits. The mid-portions of both plots yield slopes of 1.1, indicating that the binding of the second PDEγ is most probably independent of the binding of the first.

Interactions of PDEγ's with molecules other than the PDE catalytic subunits could contribute to differences between rod vs. cone responses. For example, Gillespie and Beavo [11] reported that a 50-fold higher concentration of rod Tα was required for half-maximal stimulation of rod PDE vs. cone PDE. In addition, purified cone PDE has a higher basal activity than rod PDE [11]. These properties could arise from the lower affinity of cone PDE catalytic subunits for the inhibitory subunits. The recombinant PDEγ subunits may now be used to explore (a) the rates of dissociation within the PDE holoenzymes and (b) the interactions of the PDE inhibitory subunits with transducins.

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