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the evidence from the fluorescence assays, which indicate that Py blocks cGMP binding to the PDE catalytic site, the cross-linking results favor participation of the NKXD motif in the interaction with cGMP.

Acknowledgments

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[43] Kinetics and Regulation of cGMP Binding to Noncatalytic Binding Sites on Photoreceptor Phosphodiesterase

By Rick H. Cote

Introduction

The photoreceptor phosphodiesterase (PDE; EC 3.1.4.35) is the central effector enzyme of the phototransduction pathway in the outer segments of rod and cone photoreceptors. Of the 10 families of phosphodiesterases discovered to date, the photoreceptor enzyme (classified as PDE6) is the only one known to be regulated by interaction with a heterotrimeric G protein, transducin. Activation of transducin following photolysis of the visual pigment stimulates cyclic nucleotide hydrolysis at the catalytic sites of PDE, resulting in a subsecond decrease in cytoplasmic cGMP levels in the outer segment. The rapid drop in cGMP levels during visual excitation induces dissociation of cGMP from allosteric binding sites on the cGMP-gated ion channel in the plasma membrane of the cell, leading to closure of the channels and hyperpolarization of the membrane.1-5

The inactivation of PDE must be as precisely controlled as its activation in order for rod and cone photoreceptors to respond rapidly to changes in light stimulation. One way of controlling PDE inactivation is by regulating the extent and lifetime of activation of the transducin α-GTP subunit that binds to and activates PDE. In addition to upstream mechanisms that control the rate and extent of formation of α-GTP, it is now appreciated

that the inhibitory $\gamma$ subunit of PDE ($\gamma$) can act in concert with a regulator of G-protein signaling (RGS), RGS9, to shorten the lifetime of $\alpha_{i}\text{GTP}$ by accelerating its intrinsic GTPase activity.\textsuperscript{6-8} Furthermore, other processes (e.g., posttranslational modifications of $\gamma$ or changes in membrane localization of PDE\textsuperscript{10}) may also act to reduce the extent and lifetime of PDE activation during the recovery from a light stimulus and/or during light adaptation.

The rod PDE holoenzyme consists of a catalytic dimer ($\alpha\beta$) to which the inhibitory $\gamma$ subunits bind. In addition to containing the catalytic domain where cyclic nucleotides are hydrolyzed, the $\alpha$ and $\beta$ subunits also contain high-affinity noncatalytic cGMP binding sites.\textsuperscript{11} The occurrence of noncatalytic cGMP binding sites in photoreceptor PDE is a feature shared with other PDE isoforms. For the case of PDE2, cGMP binding to these noncatalytic sites allosterically stimulates catalysis at the active site of the enzyme.\textsuperscript{12} For PDE5 and photoreceptor PDE, the role of these noncatalytic sites is less clear. However, it has been demonstrated that occupancy of the noncatalytic sites with cGMP increases the binding affinity of $\gamma$ for $\alpha\beta$,\textsuperscript{13} and conversely, binding of $\gamma$ to $\alpha\beta$ confers high-affinity binding of cGMP to the noncatalytic sites.\textsuperscript{14-16} This reciprocal relationship between $\gamma$ binding and cGMP binding may be important for $\gamma$ to function with RGS9 as an accelerator of transducin GTPase. The noncatalytic sites may also serve additional allosteric functions by altering the conformation of the $\alpha$ and $\beta$ tertiary and quaternary structure, thereby changing the interaction of the $\alpha\beta$ dimer with (1) the transducin $\alpha_{i}\text{GTP}$ subunit, (2) the two distinct domains of interaction of $\gamma$ with $\alpha\beta$, and/or (3) the $\delta$ subunit that has been implicated in promoting release of PDE from its membrane-associated state.

\textsuperscript{7} W. He, C. W. Cowan, and T. G. Wensel, Neuron 20, 95 (1998).
This chapter describes experimental approaches that have been developed to study the regulation of amphibian PDE, with an emphasis on the role of the noncatalytic cGMP binding sites. Other articles in this volume\textsuperscript{16a,b} describe procedures for the study of bovine PDE. There are several advantages to working with the amphibian system, including the ability to obtain fully dark-adapted and highly purified rod outer segments (ROS) at defined times of the circadian photoperiod; sufficient quantities of phototransduction proteins for biochemical studies; the wealth of information on the electrophysiologic responses of amphibian rod photoreceptors during excitation, recovery, and adaptation, and the ability to correlate biochemical and electrophysiologic responses to illumination in the same cell preparation.

Isolation of Purified Rod Outer Segments from Frog Retinas

Solutions

Frog Ringer’s solution consists of (in mM): 105 NaCl, 2.0 MgCl\textsubscript{2}, 2.0 KCl, 1.0 CaCl\textsubscript{2}, 10 HEPES (hemisodium salt), pH 7.5, 232 mOs. Just before use, 5.0 mM glucose is added from a 100× stock.

Percoll (Sigma, St. Louis, MO) is first adjusted to physiologic ionic strength by the addition of 1 volume 10× Ringer’s solution to 9 volumes of Percoll, and then dialyzed 3 hr in Spectra/Por tubing (molecular weight cutoff 6000–8000) against 10 volumes of Ringer’s solution. The Ringer’s solution is then replaced and dialysis repeated overnight at 4°. Dialysis adjusts the osmolality and the pH to that of the Ringer’s solution, and removes free polyvinylpyrrolidone in the original Percoll suspension. On addition of 5 mM glucose, this isosmotic Percoll solution (defined as 100% Percoll) is used for subsequent dilutions.

Volumetric dilutions of 100% isosmotic Percoll are made with Ringer’s to prepare 5, 30, 42, and 60% Percoll solutions. The density of the diluted Percoll solutions is easily checked by measuring the refractive index (Ringer’s $\eta = 1.3340$, 100% isosmotic Percoll $\eta = 1.3510$).

Isolation of Retinas

Bullfrogs (\textit{Rana catesbeiana}) 10–15 cm in length are obtained from Niles Biologicals (Sacramento, CA) at least 2 weeks prior to use in experiments. The animals are housed in stainless steel tanks with continuously flowing 20° water. Inside the light-tight tank is a light controlled by a timer

\textsuperscript{16a} A. E. Granovsky, K. G. Musakov, and N. O. Artemyev, \textit{Methods Enzymol.} 315 [40], 1999 (this volume).

\textsuperscript{16b} T. A. Cook and J. A. Beavo, \textit{Methods Enzymol.} 315 [40], 1999 (this volume).
to permit a 12-hr light–12-hr dark daily cycle. This serves to entrain the circadian oscillations known to occur in the retina and also to define the diurnal disk membrane renewal process.\textsuperscript{17,18} During the “daytime,” the illumination is controlled in 15-min periods: 5 min of dark, 5 min of continuous light, and 5 min of 1-Hz flashing light. The animals are fed twice weekly 3 g of pureed commercial dog food supplemented with 1\% (w/w) AIN-76 Vitamin Mixture (Purina Test Diets, St. Louis, MO) from which the watersoluble vitamins are omitted.

Dissection of frog retina is performed 1–6 hr before the onset of “daytime.” All operations are performed in a darkroom using infrared illumination (Kodak, Rochester, NY, \#11 filters) and a Model 6100M infrared-imaging converter (Electrophysics, Nutley, NJ). The enucleated eye is opened by a shallow incision around the circumference of the eyeball, just anterior to the sclera. The cornea, lens, and attached retina are lifted out of the eyecup with forceps, and the retina is then teased from its points of attachment and placed in \(\sim0.7\) ml 5\% Percoll–Ringer’s solution in a glass depression slide. This approach minimizes contamination of the retina with the pigment epithelium layer.

Purification of Frog Rod Outer Segments

The two isolated retinas are held by their edges with a forceps and gently shaken together in 0.8 ml of 5\% Percoll in a 1.5-ml siliconized microcentrifuge tube. As the solution becomes cloudy with dissociated photoreceptors, the retinas are transferred to another tube with fresh 5\% Percoll, and the agitation of the retinas is gradually increased. This process is repeated three or four times until the yield of dissociated cells declines and the retinas begin to fall apart. Finally, the resulting pieces of retina are vigorously drawn up and expelled into 0.8 ml of 5\% Percoll using a wide orifice 1000-\(\mu\)l micropipette tip to further disrupt the retinal pieces.

Once the visible particles in each of the tubes have settled, the suspension of ROS is applied to the centrifuge tube containing the discontinuous Percoll gradient. The particles and retinal pieces from each tube are pooled and resuspended in 5\% Percoll. The retinal particles are further disrupted by gentle vortexing or pipetting, and the supernatant suspension is added to the gradient tube. This procedure is repeated until the supernatant solution does not appear cloudy.

Discontinuous Percoll density gradient centrifugation\textsuperscript{19,20} permits the separation of ROS from other constituents of the crude suspensions ob-

\textsuperscript{17} S. Bisinger, R. Hoffman, and M. Matthes, \textit{Science} \textbf{194}, 1074 (1976).
tained from shaking and disrupting the retinas. As a density medium for amphibian ROS purification, Percoll offers several advantages over sucrose\textsuperscript{21}: the isosmotic Percoll medium permits purification of osmotically intact cells; lower levels of contamination by other retinal neurons are seen; and an ~10-fold faster separation time is possible when preformed gradients are used.

Discontinuous gradients are typically prepared by carefully layering 60\% Percoll, then 30\% Percoll, and finally 0.5 ml of 5\% Percoll in a 12- to 15-ml polycarbonate tube so that a sharp interface exists between each layer. The distance between the 5/30\% and 30/60\% Percoll interfaces should be ~1 cm for optimal separation and recovery of the purified ROS band. The ROS suspensions are layered on top of the 5\% Percoll cushion, and centrifuged (8000–12,000 g for 10 min at 4°) in a fixed-angle rotor. Following centrifugation, the band at the 30/60\% Percoll interface contains a mixture of osmotically intact and "leaky" ROS. Because frog PDE is a peripheral membrane protein and does not dissociate from the disk membrane in Ringer's solution, the yield of enzyme is enhanced by including leaky ROS. In instances where osmotically intact ROS are required, an additional 42\% Percoll layer ($\eta$ = 1.3413) can be added to the discontinuous Percoll gradient to separate leaky ROS (30/42\% Percoll interface) from intact ROS (42/60\% Percoll interface). In either instance, microscopic examination of the bands collected with a 15-gauge syringe needle generally show <1\% contamination of the purified ROS suspension with spherical cells, cone photoreceptors, or visible cellular debris. There is significant day-to-day variation, however, in the fraction of ROS that retain the ellipsoid portion of the inner segment; typically $\leq$30\% of the ROS are attached to the ellipsoid region. (Methods are available to enrich for frog ROS still attached to the inner segment.\textsuperscript{22})

The Percoll present in the ROS suspension is removed by diluting the Percoll concentration to $\leq$20\% with Ringer's, gently sedimenting the ROS, and discarding the Percoll-containing supernatant. The ROS pellet is typically resuspended in a pseudo-intracellular medium or in a Tris buffer for subsequent use.

Gaining Access to the Cytosol

Several methods can be used to disrupt the plasma membrane of Percoll-purified ROS to permit biochemical assays, including sonication, freeze-thawing, forceful passage through a narrow-gauge syringe needle, electro-


permeabilization, and homogenization. The most consistent and reliable results in our laboratory have been obtained using homogenization with a Potter–Elvehjem tissue grinder closely following the procedure of Dumke et al. The resuspended ROS are placed in an ice-cold, 2-ml glass tissue grinder, and a custom-made nylon pestle [attached to a Talboy Model 134-1 stirrer (VWR Scientific Products, West Chester, PA)] is used to homogenize the ROS; the stirrer is operated at 50% maximum speed. The number of strokes needed to completely disrupt cellular morphology at the light microscopic level must be empirically determined for each mortar and pestle, and the suspension is kept in an ice water bath throughout to minimize heating. The commercially available Teflon pestles do not fit tightly enough to completely homogenize ROS, thus necessitating use of a custom-made nylon pestle. The complications that result if stacks of disk membranes remain in the homogenate require careful attention to the complete disruption of the ROS membrane system.

Following homogenization, the rhodopsin concentration is determined by difference spectroscopy to provide an initial estimate of the PDE concentration. As much as 1 mg of rhodopsin can be recovered from one frog when "leaky" and intact ROS are combined. The homogenate is incubated in the dark at 22°C for 30 min to degrade endogenous cGMP nucleotides that would interfere with measurements of [3H]cGMP binding, as well as GTP that could otherwise activate transducin on light exposure.

**Determination of PDE Holoenzyme and Py Concentration in Frog ROS**

**Use of ROS Homogenates as Source of Frog PDE**

Because frog photoreceptor PDE is the only protein in amphibian ROS that binds to cGMP with high affinity ($K_D < 1 \mu M$), we are able to carry out many of our experiments with ROS homogenates. Likewise, because no other PDE isoforms have been detected in ROS, hydrolytic activity determinations can be made using ROS homogenates without concern for contaminating activities from other phosphodiesterases. Using unfractionated ROS homogenates allows PDE regulation to be stud-

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ied under relatively physiologic conditions with the full complement of phototransduction proteins and under conditions where light activation of nucleotide-supplemented ROS homogenates approached the light sensitivity of the excitation pathway in vivo.

Purification to homogeneity of the amphibian photoreceptor PDE holoenzyme is possible but not routinely undertaken in our laboratory, primarily because of the instability of the purified enzyme once removed from the membrane. This contrasts with bovine rod PDE, which has been successfully purified and stored for extended periods.

Determination of PDE Content of ROS Homogenates

An initial estimate of the PDE concentration that is accurate to within ±20% can be obtained by measuring the rhodopsin concentration of the ROS homogenate, along with a knowledge of the molar ratio of rhodopsin to PDE (270:1). Frog-to-frog variations in the rhodopsin:PDE molar ratio are likely to be the major factor limiting the accuracy of this estimate. The PDE concentration in ROS homogenates can also be estimated by measuring the maximum amount of \(^{3}H\)cGMP bound \((B_{\text{max}})\) when the cGMP concentration is sufficient to saturate all of the high-affinity binding sites, and using a binding stoichiometry of 2 mol cGMP bound per mole of PDE holoenzyme. A third approach is to measure the rate of cGMP hydrolysis of transducin-activated PDE in a ROS homogenate (>4 \(\mu M\) rhodopsin, with 10 mM cGMP), and to calculate the enzyme concentration using the published turnover number, \(k_{\text{cat}} = 4400\) cGMP per PDE holoenzyme per second. Finally, the PDE concentration in ROS can be estimated by first proteolytically cleaving the Py subunits to activate the enzyme, and then titrating the amount of purified, recombinant Py needed to stoichiometrically restore full inhibition of the enzyme (see later section). The latter two methods that rely on activity measurements correlate very well (<10% variation) with estimates of PDE concentration from \(^{3}H\)cGMP \(B_{\text{max}}\) binding value.

Determination of Py Concentration in ROS Homogenates

Because the Py subunit is central to the regulation of PDE activity and cGMP binding, it is important to estimate its concentration accurately. The expression and purification of the recombinant bovine rod Py subunit is described in detail elsewhere in this volume. Following purification of

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Fig. 1. Titration of trypsinized frog PDE with Py. Trypsinized frog PDE (Pαβ concentration, 5 nM) was incubated with the indicated concentrations of recombinant bovine Py for 6 min at room temperature. To determine the extent of inhibition of PDE activity, 10 mM cGMP (final concentration) was added, and portions quenched at three time points. The extent of cGMP hydrolysis was determined with the colorimetric phosphate assay. The curve is the fit to a logistic equation where the IC50 value is 0.96 Py/Pαβ (r² = 0.972). The dotted line is the regression line (r² = 0.997) for data points up to only 2.1 Py/Pαβ and intersects the x axis at 2.18 Py/Pαβ. The results demonstrate that the Py is 90–95% active.

>95% pure recombinant Py by reversed-phase high-performance liquid chromatography (HPLC), spectroscopic measurements in 45% acetonitrile, 0.1% trifluoroacetic acid in conjunction with amino acid analyses of identical samples provided an experimental determination of the extinction coefficient for Py of ε277 = 7550 OD·M⁻¹ under these conditions. We have also determined the biological activity of purified Py by testing its ability to inhibit activated frog PDE under conditions where there is a linear relationship between Py added and inhibition (i.e., [PDE] ≫ Kp for Py binding).31 We find that the addition of 2.0 moles of Py per mole of PDE will titrate >90% of the hydrolytic activity of activated frog PDE (Fig. 1), indicating that the purified Py is close to 100% active.

We have also used purified, recombinant Py as a standard for quantitative immunoblot analysis of the total Py content in frog ROS homogenates.

Frog ROS homogenates prepared as described in the previous section were centrifuged in a Beckman Airfuge (5 min at 130,000g at room temperature) to sediment ROS membranes from cytosolic proteins. Equivalent portions of the original ROS homogenate, membrane, and supernatant fractions, as well as known amounts of Py were subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE). After electrophoretic transfer to a nitrocellulose membrane, the blot is probed with a rabbit polyclonal antipeptide antibody, UNH9710 (amino acids 63–87 of bovine Py). Detection of anti-Py antibody is performed using a horseradish peroxidase-linked secondary antibody directed to the primary antibody, followed by luminescent visualization of the antibody complex.\(^{32}\) As shown in Fig. 2, all of the Py originally present in ROS homogenates cosediments with PDE in the ROS membrane fraction, with no detectable Py immunoreactivity present in the supernatant fraction. The molar stoichiometry of Py to Pαβ has been determined to be 1.8 ± 0.2 mol Py per mole of Pαβ (n = 14). These results demonstrate that frog PDE has the same Py-subunit stoichiometry as had been determined for bovine rod PDE and that no excess Py is present in the cytoplasm of frog ROS.

Preparing Various Forms of Activated Frog PDE

Solutions

Pseudo-intracellular medium is a buffer that partially mimics the composition of the ROS cytosol and is routinely used for resuspending ROS pellets for homogenization. It consists of (in mM): 77 KCl, 35 NaCl, 2.0 MgCl\(_2\), 1.0 CaCl\(_2\), 1.18 EGTA ([Ca\(^{2+}\)\(_{\text{free}}\)] = 240 nM), 10 HEPES, pH 7.5. Just before use, the solution is supplemented with final concentrations of 1.0 mM dithiothreitol (DTT), 0.5 μg/ml leupeptin, 0.2 mM Pefabloc, and 0.7 μg/ml pepstatin.

As an alternative, we have also used a Tris-based buffer consisting of 100 mM Tris, 10 mM MgCl\(_2\), 0.5 mM EDTA. This solution is supplemented on the day of the experiment with 0.5 mg/ml bovine serum albumin (BSA), as well as DTT and protease inhibitors (as used earlier).

Nonactivated PDE

Following homogenization and nucleotide depletion of the ROS homogenate, the preparation can be exposed to room light for the subsequent

Fig. 2. Determination of the Py content in frog ROS. Samples of frog ROS homogenate (H, ○), as well as an equivalent amount of fractionated membrane (M, □) and supernatant (S) components, were loaded on a 15% SDS–PAGE gel, along with known concentrations of Py (numbers below each standard lane indicate the nanograms of Py loaded.). The membrane and homogenate samples each contained 3.0 pmol nonactivated PDE holoenzyme, whereas the supernatant sample did not contain any detectable PDE (as judged by an activity assay). The Py content of unknown samples was determined by comparing to the quadratic curve fit \( r = 0.997 \) of the known Py samples (●). In this experiment, the homogenate and membrane samples contained 5.9 and 6.0 ng Py, respectively, whereas the amount in the supernatant was below the detection limits of the assay \( (\leq 2 \text{ ng Py}) \). The data are representative of one of four similar experiments.

Experiment without concern for activation of PDE. When adjusted to rhodopsin concentration of 4 μM, this “nonactivated” PDE preparation binds 1.8–2 mol cGMP per mole of PDE holoenzyme, and has an enzyme activity that is \(~2\%\) of the fully transducin-activated rate. When stored at 4°, the cGMP binding stoichiometry gradually declines over several days, presumably due to PDE denaturation and/or loss of Py by proteolysis.

Transducin-Activated PDE

To prepare transducin-activated PDE, the poorly hydrolyzable analog of GTP, GTPγS, is added to the ROS homogenate in a two- to fivefold
molar excess over the transducin concentration (assuming a molar ratio of 1:10 transducin : rhodopsin) in order to activate all transducin. The rhodopsin concentration must be kept $\geq 4 \mu M$ in order to prevent dissociation of membrane-bound transducin and a loss of maximal PDE activation.\(^{25}\)

**Extraction of Py from PDE to Prepare Membrane-Associated \(\alpha\beta\) Dimers**

ROS membranes containing PDE depleted of most (~70%) of its bound Py can be prepared by extracting Py as a complex with the activated $\alpha_1$-GTP subunit of transducin.\(^{15,33}\) Nucleotide-depleted ROS homogenates (20 $\mu M$ rhodopsin) in pseudo-intracellular medium are first exposed to light at 4°C, and then incubated with 10 $\mu M$ guanosine 5'-triphosphate (GTP) for 0.5 min. On diluting the preparation 10-fold with GTP-containing buffer, the homogenate is immediately centrifuged at 40,000g at 4°C for 30 min. The membrane pellet is washed to remove residual GTP, and the ROS membranes are resuspended and homogenized in pseudo-intracellular medium (lacking GTP). PDE activity measurements and quantitative immunoblot analysis of the Py content of these Py-depleted membranes indicate that 50–80% of the total Py is removed by this treatment in any given experiment. Repeating the extraction procedure with additional GTP has been found to be mostly ineffective, although changing the ionic strength during Py extraction has been reported to affect Py release from ROS membranes.\(^{34}\) The presence of a significant fraction of $\alpha\beta\gamma$ and/or $\alpha\beta\gamma_2$ complicates the interpretation of experiments using this preparation for studies of PDE regulation by Py and its noncatalytic sites.

**Limited Proteolysis of PDE with Trypsin to Prepare $\alpha\beta$ Catalytic Dimers**

Brief exposure of PDE holoenzyme to trypsin is known to release the inhibitory constraint of Py on frog\(^{35}\) and bovine\(^{29}\) PDE. To prepare trypsinized PDE, we use ROS homogenates (adjusted to 40 $\mu M$ rhodopsin) that are prepared in the Tris homogenization buffer from which protease inhibitors are omitted. TPCK-treated trypsin (100 $\mu g/ml$ final concentration) is added for 10 min at 4°C, then the reaction halted with a sixfold excess

of soybean trypsin inhibitor. As shown in Fig. 1, inhibition of trypsinized frog PDE is nearly complete when 2 mol Pγ per mole of Pαβ is added back to this Pαβ preparation; this suggests that the primary locus of action of trypsin under these conditions is on Pγ, not on the catalytic subunits. Unlike the case of bovine rod PDE where trypsin first releases PDE from its membrane-associated state prior to full degradation of the Pγ subunits, the frog enzyme shows a similar time course for cleavage of the C-terminal site of membrane attachment and hydrolysis of the Pγ subunit. Although trypsin proteolysis is much more efficient in removing bound Pγ than the Pγ extraction from membrane-associated PDE described in the previous section, there remains the concern that some aspects of the structure and/or function of the Pαβ dimer may be altered by this treatment.

Assays for PDE Hydrolytic Activity

The high catalytic constant for activated photoreceptor PDE combined with a relatively large value of the $K_m$ for substrate permits the use of less sensitive assays for cyclic nucleotide hydrolysis in addition to the standard radiotracer assay. One method relies on the measurement of protons generated as a product of the PDE catalytic mechanism; in a minimally pH-buffered solution, changes in pH recorded with a pH microelectrode provide a continuous assay of the rate of cyclic nucleotide hydrolysis. This method has been described in a previous volume of this series, and critical factors for use of this assay have been discussed thoroughly. One little appreciated advantage of continuous monitoring of PDE activity is that the kinetic parameters can be determined from both initial velocities and from the complete reaction progress curve. In our laboratory we routinely use both the radiotracer assay and a colorimetric assay to quantify PDE activity.

PDE Activity Assay Using Radiolabeled Substrates

Solutions and Materials

Buffer A: 20 mM Tris, pH 6.8, at room temperature.
Buffer B: buffer A + 0.5 M NaCl.
0.1 M HCl.

0.1 M Trizma base will result in a final pH of 7.5 ± 0.5 when mixed with an equal volume of acid.

2.5 mg/ml *Crotalus atrox* snake venom (Sigma, St. Louis, MO) in water. Addition of 100 μM isobutylmethyl xanthine (IBMX) is sometimes warranted with cAMP as substrate if the venom preparation has cAMP PDE activity.

Polystyrene chromatography columns (Evergreen Scientific, Los Angeles, CA).

DEAE-Sephadex A-25 resin is initially swelled overnight in a 10-fold excess (w/v) of buffer B. Decant the liquid to remove fines, and add sufficient buffer B to give 6 parts settled resin to 10 parts resin plus buffer. Add 1 ml of resuspended resin to the column to yield 0.6 ml settled resin. Equilibrate columns with 8 ml of buffer B, then 8 ml buffer A before use.

**Activity Assay**

The reaction is started by mixing 9 volumes of the PDE sample with a 10× cyclic nucleotide reaction mixture containing ~10^5 dpm of [3H]cGMP or [3H]cAMP per sample plus the appropriate amount of unlabeled nucleotide to give the desired concentration. In addition to the experimental samples, control samples should also be run (i.e., blanks lacking enzyme and “total hydrolysis” samples in which all substrate is converted to product). Portions (≤50 μl) are withdrawn and quenched in 100 μl of 0.1 M HCl.

The samples are neutralized with 0.1 M Trizma, then the 5'-nucleoside monophosphate product is quantitatively converted to adenosine/guanosine by addition of 25 μg of snake venom, and incubation at 37° for 5–15 min (empirically determined for each batch of venom). The nucleoside is separated from unreacted substrate by passage through the DEAE-Sephadex A-25 column equilibrated in buffer A; cyclic nucleotide is retained, while the nucleoside elutes on washing the column with four 0.5-ml washes of buffer A. The eluate is mixed with 4 ml Ultima Gold XR (Packard, Meriden, CT) and counted in a Packard TR2300 scintillation counter. The columns are regenerated by washing in 8 ml buffer B, followed by storage in buffer A containing 0.1% sodium azide.

The radiotracer assay has unparalleled sensitivity (<0.1 pmol of product can be detected reliably), and is the method of choice for samples containing low enzyme activities or at low substrate concentrations. However, the need for radioactivity and the labor-intensive chromatography step are drawbacks to this method.

**Colorimetric Determination of PDE Activity Based on Phosphate Production**

This method relies on the stoichiometric production of inorganic phosphate in a coupled enzyme assay of photoreceptor PDE and snake venom
5'-nucleotidase: cyclic nucleotide → 5'-nucleoside monophosphate → nucleoside + inorganic phosphate.\textsuperscript{41} Colorimetric quantitation of the inorganic phosphate produced using a 96-well microplate reader permits rapid assay of large numbers of samples. In the following protocol, it is assumed that the PDE reaction is carried out in a separate tube, and portions removed and quenched in acid in the 96-well plate.

\textbf{Solutions and Materials}

0.1 \textit{M} HCl.

0.5 \textit{M} Trizma base (tested with HCl to ensure that the neutralized solution has a pH of 7.5 ± 0.5).

2.5 mg/ml snake venom (see earlier section). If the snake venom has significant contamination of phosphate, it can be removed by gel filtration of an initial preparation of 3.75 mg/ml snake venom using a PD-10 column (Pharmacia).

Phosphate standard solution: 1.0 mM KH$_2$PO$_4$.

Molybdate solution: 0.4 \textit{N} H$_2$SO$_4$, 0.2\% (w/v) ammonium molybdate, 2.0\% (w/v) sodium dodecyl sulfate (SDS), and 2\% (w/v) ascorbic acid (added just before assay).

96-Well plates, untreated (Corning, New York).

Repetitive dispensing pipettes and 8-channel pipettors.

Microplate reader with dual-wavelength option, reading at 700–750 nm with a 450-nm reference wavelength (to reduce light scattering artifacts).

\textbf{Protocol}

Substrate is added to a PDE-containing sample, and ≤20-\textmu{l} portions are removed at various times and quenched with 50 \textmu{l} of 0.1 \textit{M} HCl in a microplate well. The quenched sample should contain between 0.5 and 60 nmol hydrolyzed nucleotide in order to be accurately determined. The acidified samples are neutralized with 0.2 volume of 0.5 \textit{M} Trizma. (The volumes of sample, acid, and base can be varied, as long as it is verified that the acid quenching is instantaneous and that the addition of base restores the pH to neutrality.) The samples are then treated with 10 \textmu{l} snake venom per earlier section. Standards are prepared by addition of 1–70 nmol of the 1 mM KH$_2$PO$_4$ solution in water to the same final volume as the experimental samples.

Add 150 \textmu{l} of the molybdate solution and incubate at 37° for 20 min. The results of the standard phosphate samples are fit to a second-order

polynomial equation, and used to interpolate the concentration of phosphate in the unknowns.

**Variations**

If the PDE activity is substantially less than the 5'‐nucleotidase activity in the snake venom preparation, simultaneous incubation of PDE and snake venom is possible.¹⁴ In this instance, the reaction can be quenched with 2% SDS instead of acid, and the neutralization step avoided. The SDS should be omitted from the molybdate solution. Also, verify that the snake venom preparation has undetectable levels of PDE activity, especially if using cAMP as the substrate.

To determine the PDE concentration in a large number of samples (e.g., column chromatography fractions), 10 μl of PDE can be added to wells containing 10 μl of a 5× PDE assay buffer (1× concentrations: 20 mM Tris, pH 7.5, 10 mM MgCl₂, 0.5 mg/ml BSA). The samples can be activated by addition of 10 μl of 100 μg/ml TCPK‐treated trypsin for a sufficient time to optimally activate the PDE, then treated with 10 μl of 0.6 mg/ml soybean trypsin inhibitor. Then 10 μl of cGMP (10 mM final concentration is typical) is added to all wells, and the reaction quenched with acid. The remaining steps follow the standard protocol.

**Comments**

The phosphate assay agrees to within 10% with the radiotracer assay described in the previous section. Although the colorimetric assay is more than a thousandfold less sensitive than the radiotracer assay, often the assay conditions and/or amount of enzyme can be adjusted to permit use of this economic, rapid, and nonradioactive procedure.

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Membrane Filtration Assays for Measurements of cGMP Binding to Noncatalytic cGMP Binding Sites on PDE

**Principles**

Membrane filtration techniques for quantitating the binding of cyclic nucleotides to their receptor proteins⁴² are widely used for characterizing the regulation of cyclic nucleotide‐dependent protein kinases and cGMP‐binding phosphodiesterases (i.e., PDE2, PDE5, and PDE6). It is often the method of choice because of its high sensitivity, excellent partitioning of

free from bound ligand, and its low extent of nonspecific binding. The major drawbacks to membrane filtration are the nonequilibrium nature of the separation process and its restriction to studying relatively high-affinity ligand–receptor interactions.

To accurately use membrane filtration to estimate the equilibrium binding parameters \((K_D, B_{\text{max}})\) of a ligand for its receptor, several criteria need to be satisfied: (1) the binding reaction must closely approach equilibrium prior to separating free from bound ligand; (2) the separation of bound from free ligand must not perturb the equilibrium during filtration; (3) the extent of nonspecific binding of ligand to the membrane must be determined precisely; and (4) the appropriate binding model must be selected to interpret the binding data.

**Determining Approach to Equilibrium**

Accurate calculation of the binding parameters demands that the binding reaction be essentially (e.g., >97%) at equilibrium. This can be empirically determined by monitoring the time course of increase in binding. However, because the approach to equilibrium slows down as ligand concentration decreases, the time course should be performed at the lowest ligand concentration to be used. Alternatively the time to attain 97% of equilibrium \((T_e)\) can be shown\(^{43}\) to depend on the association \((k_{+1})\) and dissociation \((k_{-1})\) rate constants:

\[
T_e = \frac{3.5}{k_{+1}[L] + k_{-1}}
\]  
(1)

where \([L]\) is the free ligand concentration. Note that at low ligand concentrations \((k_{+1}[L] \ll k_{-1})\), the approach to equilibrium can be estimated if \(k_{-1}\) is known.

**Estimating Extent of Dissociation of Bound Ligand during Separation of Free From Bound Ligand**

The major constraint limiting the usefulness of membrane filtration is the rate of dissociation of the ligand–receptor complex during the separation procedure. It is the dissociation rate constant that determines how much ligand will dissociate during the time it takes to filter and rinse the receptor–ligand complex.\(^{44}\) The amount of time needed to complete filtration and


rinsing of a sample with the loss of \( \leq 10\% \) of the bound ligand \( T_{10\%} \) can be calculated from \( T_{10\%} = 0.14/k_1 \). Later in this section an alternative filtration protocol using ammonium sulfate is presented to specifically address this issue. This concern can also be addressed by using another method (see later section) that does not perturb the equilibrium of receptor–ligand binding.

**Quantitating Contribution of Nonspecific Ligand Binding to the Filter Membrane**

Usually, membrane filtration techniques offer a high ratio of specific to nonspecific ligand binding. Nonspecific binding is a general term comprised of several components: ligand binding to the filter, incomplete washing of unbound ligand during filtration, and true nonspecific binding of ligand to the sample containing the receptor. The first component can be examined by performing filtration and rinsing of the radiolabeled ligand solution in the absence of the receptor. The efficacy of removing unbound ligand can be assessed and optimized by varying the number of rinses and the volume of the rinse solution. The last component to total nonspecific binding can be judged by incubating the receptor with the labeled ligand to which a large excess of unlabeled ligand (typically, a concentration \( \geq 100 \) times the \( K_D \)) has been added. In our experience, nonspecific binding of radiolabeled cGMP in the presence of a large excess of cold cGMP is identical in the presence or absence of ROS membranes, and never exceeds 1% of the bound cGMP.

**Analysis of Ligand Binding Data**

Although graphical presentations of transformed data (e.g., Scatchard plot) remain helpful for visual inspection of ligand binding results, graphic approaches to determining binding parameters have major shortcomings and should be avoided.\(^{45}\) Instead, nonlinear curve-fitting techniques should be employed with exact models of the predicted binding reaction(s). The development of specialized computer programs using iteratively weighted, nonlinear regression analysis to evaluate equilibrium and kinetic radioligand binding data (e.g., LIGAND,\(^ {46}\) KELL\(^ {47}\)) provides several important advantages: no transformation of the raw data (or their associated errors) is introduced; the exact binding model is used; statistical comparison of different models (e.g., one versus two sites, cooperativity, etc.) can be


carried out to determine the most likely model; the weighting model that is most appropriate for the errors associated with different y-axis values can be selected; and the free ligand concentration is calculated by an iterative procedure that takes into account potential ligand depletion. The collection of programs marketed as KELL (Biosoft, Ferguson, MO) are derived from the original LIGAND program and are routinely used in our laboratory for kinetic and equilibrium binding studies of the noncatalytic sites of PDE.

Filter Binding Assay to Detect High-Affinity Noncatalytic cGMP Binding Sites on PDE

The following filter binding protocol is useful for measuring cGMP binding to high-affinity (i.e., \(K_D < \mu M\)) sites on amphibian PDE to determine the \(K_D\) and the maximum binding stoichiometry \((B_{\text{max}})\) under equilibrium conditions, as well as the dynamics of cGMP association and dissociation.

The first consideration in performing a radiolabeled cGMP binding experiment with PDE is to ensure that all endogenous cGMP has been destroyed and all binding sites are unoccupied. Although 30-min incubation of frog ROS homogenates is sufficient to deplete the cGMP, the considerably slower cGMP dissociation rate for bovine rod PDE has until recently hampered studies of the noncatalytic sites on the mammalian rod photoreceptor enzyme.

A second consideration arises from the fact that the ligand for the noncatalytic sites is also the substrate at the active site of PDE, necessitating that enzyme activity be abolished to prevent destruction of cGMP. PDE5-selective inhibitors such as E4021 and zaprinast are far more effective than EDTA in this regard, with \(K_i\) values for the activated enzyme of 1.7 and 32 nM for E4021 and zaprinast, respectively. The efficacy of inhibition is confirmed by determining the extent of \(^\text{3H}\) cGMP hydrolysis under the actual binding assay conditions. Because of the high discrimination of the noncatalytic sites for binding cGMP compared with cGMP analogs or PDE inhibitors, competition between cGMP and these compounds for binding to the noncatalytic sites is extremely weak.

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Solutions and Materials

Pseudo-intracellular medium (see previous section).
Wash buffer is identical to the pseudo-intracellular medium, except it lacks DTT and protease inhibitors.
100 mM zaprinast (Sigma) stock solution is dissolved in 1-methyl-2-pyrrolidinone.
The exact composition of the radiolabeled ligand solution depends on the nature of the experiment. In general, the concentration of cGMP is varied by adding a fixed amount of [3H]cGMP (NEN, Dupont, Boston, MA; \(\sim 10^4-10^5\) dpm per sample to be filtered) to various amounts of unlabeled cGMP (Sigma). The concentration of unlabeled cGMP is verified by its UV absorption spectrum (\(e_{254} = 1.29 \times 10^4\) OD \(\cdot M^{-1}\) at pH 7). Zaprinast is typically added to a final concentration of 100 \(\mu M\).
Filter disks: 25-mm diameter, 0.45-\(\mu\)m pore size, mixed cellulose esters MF-Millipore (Bedford, MA) membrane filters.
The vacuum filtration device (Hoefer 10-place manifold; Amersham/Pharmacia, Piscataway, NJ) is attached to a vacuum source that permits 1 ml of standard wash buffer to be filtered in \(\sim 1\) sec.
Ultima Gold (Packard) scintillation fluid is used to measure radioactivity eluted from the filters.

General Procedure for Filter Binding Assay

Following nucleotide depletion of the PDE-containing ROS homogenates (4 \(\mu M\) rhodopsin) for 30 min at room temperature in pseudo-intracellular medium, the cGMP binding reaction is initiated by addition of 10-fold concentrated [3H]cGMP solution containing zaprinast. Immediately before filtration of the ROS sample, the membrane filter is prewet with 1 ml ice-cold wash buffer. ROS (25-\(\mu\)l portions) are directly pipetted to the filter, and immediately rinsed with three 1-ml portions of wash buffer. This process should be completed within \(\leq 4\) sec.
It is not advised to add the PDE sample to a tube containing wash buffer and then filter the diluted sample. Diluting the PDE will alter the equilibrium binding of \(\gamma\) to \(\alpha\beta\) and thereby lower the cGMP binding affinity to those enzyme molecules lacking bound \(\gamma\).
Nonspecific binding is measured by supplementing the [3H]cGMP solution with a 1000-fold excess of unlabeled cGMP prior to adding to the PDE sample. Aliquots of the original [3H]cGMP ligand solution and the final incubation mixture are pipetted into scintillation vials to determine the actual specific activity of the cGMP solution and the total DPMs applied to the filter.
The filters are placed in scintillation vials, mixed with 4 ml of scintillation fluid, shaken for 15 min, and then analyzed in a scintillation counter.

Validation of Method

Values for the $K_D$ and $B_{\text{max}}$ for the high-affinity noncatalytic sites on nonactivated frog PDE using the standard membrane filtration assay agree well with two equilibrium methods described later, namely, ultrafiltration\textsuperscript{23} and a centrifugal assay.\textsuperscript{52} For general use, the membrane filtration method is preferred for several reasons: greater reproducibility, lower experimental error, and ability to process large numbers of samples. The major limitations of the filter binding method result from the loss of cGMP bound to lower affinity sites during the filtration process. This drawback prevents the filtration method from being able to detect a second class of cGMP binding sites present in amphibian ROS,\textsuperscript{23,52} as well as obscuring the transition from high- to low-affinity cGMP binding that results following P$_{\alpha\beta}$ release from P$_{\alpha\beta}$.\textsuperscript{15}

Alternative Filter Binding Protocol Using 96-Well Multiscreen Assay Plate

For high-throughput screening of cGMP binding to PDE,\textsuperscript{51} we have modified the preceding filter binding protocol to use Multiscreen (Millipore, MAHA N45) filtration plates containing the same mixed cellulose ester membrane as earlier. Each well is prewet with wash buffer in order to obtain a good seal of the plate with the Multiscreen vacuum manifold. A 20-$\mu$l portion is added, then immediately rinsed with three 100-$\mu$l washes. A disadvantage to this method is the slower time for filtration of the sample and wash solutions ($\sim$10 sec). The filters are punched from the base of the plate into 7-ml scintillation vials. Water is added, the vial vortexed, then 3 ml of Ultima Gold scintillation fluid is added and the vials treated as earlier.

Use of Ammonium Sulfate to Stabilize Bound cGMP during Membrane Filtration

In an attempt to reduce the likelihood of dissociation of bound cGMP from PDE during the membrane filtration procedure, we have recently utilized an ammonium sulfate solution to stabilize bound cGMP. This approach has been used before to precipitate soluble binding proteins as well

as for stabilizing protein-bound nucleotide. This method is particularly well suited for extending the operating range of the filtration assay to lower affinity binding sites where ligand exchange is more rapid and nucleotide dissociation will otherwise occur during filtration and washing. In addition, the ammonium sulfate stop solution can be used to more precisely define the exact duration of the association or dissociation reaction in kinetic studies.

Solutions

Saturated (100%) ammonium sulfate solution is prepared by adding 353.3 g ammonium sulfate to 500 ml distilled water. After dissolving completely, cool to 4° to achieve complete saturation.

200 mM Tris, pH 7.5.

Buffered ammonium sulfate (BAS) is made by adding 1 volume of 200 mM Tris to 19 volumes of saturated ammonium sulfate (final 95%).

A 2% SDS solution is used to solubilize the radioactivity trapped on the filter.

The scintillation fluid used is Ultima Gold XR (Packard), which has a high sample load capacity.

Procedure

Incubate the PDE-containing samples with [3H]cGMP as earlier. Meanwhile, chill tubes containing 200 μl of BAS to 4° in an ice water bath. When ready to stop the binding reaction, add a 20-μl portion of PDE to the BAS solution and vortex immediately. Once stabilized with BAS, the samples can remain on ice for several minutes prior to filtering.

Immediately prior to filtration, prewet the 25-mm filter disks with 2 ml BAS (no vacuum), and apply vacuum after filter is thoroughly wetted. Then apply the entire sample to the filter, and wash with three 1-ml portions of BAS. The filters are then placed in vials with 2 ml 2% SDS, and shaken for 10 min. Add 3.5 ml Ultima Gold XR, mix well, and count.

Comments

There is no evidence that addition of BAS affects the dynamics of [3H]cGMP exchange with the noncatalytic sites of PDE except to prevent already bound nucleotide from dissociating. Addition of a 1000-fold excess of unlabeled cGMP to the BAS to prevent additional [3H]cGMP association shows no detectable change in the amount of cGMP bound over a 10-min

period. Over a longer period of time, bound \(^{[3]}\text{H}\)cGMP stabilized with BAS dissociates its bound cGMP at a linear rate of 0.1% per min. Inclusion of cold cGMP has a minor effect on the stability of the cGMP–PDE complex (0.2% dissociation per minute).

The use of BAS results in several-fold higher levels of nonspecific binding of radiolabel to the filters compared with the standard filtration procedure. The absolute levels of nonspecific binding remain well below 1% of the total disintegrations per minute applied to the filter.

Ammonium sulfate concentrations as low as 50% are effective in stabilizing cGMP bound to PDE for short periods of time. If samples are to be filtered shortly after adding the PDE sample to BAS, then lower concentrations of ammonium sulfate can be used.

Dissociation Kinetics of cGMP Release from Noncatalytic Sites

Although equilibrium binding parameters provide important information about the extent of occupancy of the noncatalytic cGMP binding sites on PDE in the resting state, an understanding of the role of these binding sites in shaping the photoresponse also requires knowledge of the rate of cGMP exchange to and from the noncatalytic sites. In addition, small (<10-fold) differences between the \(K_D\) values for the two high-affinity sites on PDE not easily detected using equilibrium methods can be more readily resolved using kinetic approaches.

Release of bound cGMP from the high-affinity noncatalytic sites on PDE can be evaluated by either a rapid drop in the free cGMP concentration ("concentration jump") or by isotopic dilution ("cold chase"). Concentration jump experiments are classically performed by volumetric dilution of the solution containing an equilibrium mixture of free ligand and the receptor–ligand complex, resulting in net dissociation of bound ligand as the new equilibrium state is approached. Volumetric dilution of cGMP–PDE complexes is not feasible, since the Py–Pαβ binding equilibrium is also affected, which then affects the cGMP binding affinity to the noncatalytic sites. Calvert et al.\(^{55}\) have circumvented this problem in a novel manner by inducing cGMP dissociation following addition of the PDE1 (calmodulin-dependent) isoform to rapidly destroy all free cGMP and to induce net dissociation (and destruction) of bound cGMP. The advantage of this "enzymatic concentration jump" method is that it more closely mimics the conditions likely to pertain during the light activation phase of the photoresponse. However, establishing the proper conditions

and controls to perform these experiments is more difficult than for the cold chase protocol.

**Cold Chase Protocol for Measuring \[^3H\]cGMP Release from Noncatalytic Sites on PDE**

This approach relies on greatly reducing the specific activity of the free \[^3H\]cGMP by adding a large (>100-fold) molar excess of unlabeled cGMP. When a bound cGMP molecule dissociates from PDE, an unlabeled cGMP will reassocitate, leading to progressive decline in bound radiolabeled ligand over time.

PDE is prepared as described earlier, and the binding of 1 \(\mu M\) \[^3H\]cGMP (sufficient to saturate the high-affinity sites) allowed to approach equilibrium. Just prior to initiating dissociation of the bound \[^3H\]cGMP, portions of the incubated PDE sample are assayed to determine the maximum extent of binding. Then 1 mM unlabeled cGMP (final concentration) is added at time zero, and samples filtered at various times thereafter. Nonspecific binding controls are included in which the 1 mM cGMP is premixed with the radiolabeled cGMP before incubation with PDE. The filters are processed as described earlier for the general filtration protocol.

For the case of nonactivated PDE in which the dissociation kinetics typically follow a single exponential decay, <10 time points covering 10–90% dissociation can provide sufficient data for accurate estimation of \(k_{-1}\). The biphasic kinetics seen with transducin-activated PDE,\(^{15}\) for example, require twice as many data points to adequately define the fast and slowly dissociating components. The cold chase method described here has been shown to give equivalent results to the enzymatic concentration jump method for determining the dissociation rate constant of cGMP from nonactivated frog PDE.\(^{55}\)

**Equilibrium Methods to Determine cGMP Binding to Lower Affinity cGMP Binding Sites**

Various methods are available to detect binding of ligands to receptors without significantly perturbing the equilibrium of the binding reaction, including equilibrium dialysis, gel filtration, centrifugation, and ultrafiltration.\(^{56}\) These equilibrium methods are required for relatively weak ligand–receptor interactions where dissociation of bound ligand will occur during the process of separating free from bound ligand. They are also useful as

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an adjunct to nonequilibrium methods to confirm the validity of the binding parameters. Disadvantages of the equilibrium methods described later include relatively high levels of nonspecific binding, lower sensitivity, and difficulty in performing kinetic studies.

Centrifugal Ultrafiltration Method for Measuring cGMP Binding to ROS Homogenates

In this method, cGMP bound to specific binding sites is partitioned from free cGMP using a permeable membrane that permits the ligand to pass through while retaining the receptor.\(^{57,58}\)

**Procedure**

Nucleotide-depleted ROS homogenates are incubated with 0.04–20 \(\mu M\) \([\text{T}^3\text{H}]\text{cGMP}\) as described earlier for the membrane filtration assay. In addition, \([\text{[^{14}C]}\text{sorbitol}\) is added as an internal volume marker. A 140-\(\mu l\) portion is added to the top chamber of an Ultrafree-MC ultrafiltration unit (30,000 nominal molecular weight cutoff, polysulfone membrane; Millipore). The unit is centrifuged (5 min at 6000 \(g\) at 4\(^\circ\)) in a fixed-angle centrifuge rotor. Defined volumes of the filtrate, retentate, and initial reaction mixture are analyzed by dual-label scintillation counting to determine \(^3\text{H}\) and \(^{14}\text{C}\) disintegrations per minute in each fraction. Nonspecific binding controls are treated identically, except that a >100-fold excess of unlabeled cGMP is added to the \([\text{T}^3\text{H}]\text{cGMP}\) solution before mixing with the ROS sample.

**Comments**

When this method was used to measure cGMP binding affinity to PDE in the submicromolar range, the \(K_D\) and \(B_{\text{max}}\) values obtained agreed reasonably well with those from membrane filtration experiments.\(^{23}\) This equilibrium method has an operating range greater than that of membrane filtration; experiments carried out at cGMP concentrations up to 20 \(\mu M\) revealed a second class of cGMP binding sites (\(K_D = 8 \mu M\)) in ROS homogenates that was not detected by filtration.\(^{23}\)

Centrifugal Separation Assay Using Silicone Oil

Simple sedimentation assays have been employed with membrane-associated receptors to separate free from bound ligand by centrifugation.

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The resulting pellet is often washed with buffer, in which case low-affinity ligands will dissociate. The alternative, to assay the pellet without washing, results in substantial nonspecific binding due to free ligand present in the pellet volume.

We developed the use of centrifugal separation through silicone oil\(^\text{52}\), in which the aqueous phase containing unbound ligand is stripped away from the membrane-associated receptors by passage through a layer of silicone oil. This method relies on the fact that ROS membranes have a greater density than the silicone oil through which they pass; in contrast, the aqueous solution (containing unbound ligand) remains above the oil layer following centrifugation. The advantage of using the silicone oil layer is that nonspecific entrapment of unbound radiolabeled ligand is greatly reduced compared with a simple centrifugation of membranes from supernatant. Also, passage of the ligand–receptor complex through the oil does not appear to perturb the binding equilibrium. One limitation of this method is that it is applicable only to membrane-associated receptors; soluble receptor proteins will remain in the upper aqueous layer with free ligand and not be detected.

**Solutions**

Dow-Corning silicone oils are obtained from William F. Nye, Inc. (New Bedford, MA). To obtain the proper density, Dow Corning 550 fluid (density 1.07 g/ml) and Dow Corning 220 fluid (1.0 cS, density 0.818 g/ml) were mixed to achieve a final density of 1.02 g/ml.

**Verifying Quantitative Partitioning of Membranes and Free Ligand**

It is important to ascertain that the sedimentation conditions (oil density, centrifugal force) result in complete sedimentation of the membrane-associated receptor through the silicone oil layer and into the pellet. We assay the recovery of rhodopsin in the pellet as a measure of quantitative sedimentation of ROS membranes. We have observed that the manner in which the ROS membranes are prepared (see earlier section) can affect the membrane density.

It is also important to verify that the radiolabeled ligand is effectively excluded from entering the oil layer. \(^{14}\text{C}-\text{Labeled compounds that do not bind to ROS membranes (e.g., sorbitol) can be added both as volume markers and to quantify the ability of small molecules to enter into the oil layer or to cosediment with the membranes in the pellet.**

**Centrifugal Separation Protocol for ROS Membranes**

Silicone oil (150 \(\mu\text{l}, 1.020 \text{ g/ml}) is added to a 175-\(\mu\text{l}\) polyethylene Beckman Airfuge tube, and briefly spun to sediment the oil from the side walls
of the tube. (A 5-μl cushion of 50% glycerol can be added before the oil to enhance the ease of recovery of the pellet.) ROS homogenates are incubated with [3H]cGMP (0.04–20 μM) as earlier. Portions (25 μl) are then layered on top of the silicone oil layer. Centrifugation is carried out at room temperature for 1 min at 130,000g in the Beckman Airfuge. After removing the samples from the centrifuge, the tubes are immersed in a dry-ice–ethanol bath to freeze the aqueous layer. The tubes are cut on a diagonal through the oil layer, and the frozen top layer is discarded. The contents of the bottom half of the tube (including residual oil) are transferred to scintillation vials. The pellets at the bottom of the tubes are resuspended with 50–100 μl of detergent (2% SDS or 50 mM hexadecyltrimethylammonium chloride) and added to the vials. Scintillation fluid is added and the radioactivity sedimenting through the oil layer quantified. Nonspecific entrapment of unbound [3H]cGMP can be assessed by incubating an identical sample with a large excess of unlabeled ligand.

Comments

This centrifugal separation assay has been used with submicromolar levels of [3H]cGMP to determine the $K_D$ (73 ± 19 nM) and $B_{\text{max}}$ (0.005 ± 0.001 mol cGMP/mol rhodopsin) for the high-affinity class of noncatalytic sites on frog PDE. These values are in excellent agreement with membrane filtration measurements, although it is noted that the error associated with the centrifugal separation procedure is greater than that observed with filter binding measurements. When the ligand concentration is extended up to 20 μM, the centrifugal separation procedure is able to detect a second class of lower affinity binding sites, in general agreement with the ultrafiltration method discussed earlier. Depending on the type of ROS preparation the $K_D$ falls in the range of 5–12 μM. The relatively large error in estimating the binding parameters for this lower affinity class of sites is primarily due to the low specific radioactivity of the [3H]cGMP solutions at the highest cGMP concentrations. Use of [32P]cGMP in future work would overcome this limitation.

Conclusions

The experimental approaches described in this chapter have advanced our understanding of the roles that the noncatalytic cGMP binding sites play in the visual transduction pathway of frog rod outer segments. Under conditions where PDE is not activated and Pγ is associated with the Pαβ dimer, the high-affinity class of cGMP binding sites will be completely occupied. Furthermore, the slow rate of cGMP dissociation from the high-
affinity sites rules out any dynamic changes in binding site occupancy on the timescale of visual excitation. Thus, the high-affinity sites on nonactivated PDE effectively sequester much of the total cGMP content in the rod outer segment and effectively reduce the cytoplasmic free cGMP concentration. On activation of PDE by transducin, the high-affinity cGMP binding sites on PDE undergo changes in binding affinity and dissociation kinetics, but the regulatory significance of these changes is not fully understood at present. One likely scenario is that allosteric interactions between Py and the noncatalytic binding sites may regulate the ability of Py to act in concert with RGS9 to control the lifetime of activated PDE during the photoresponse (see Introduction section).

Much less is known about the locus and physiologic significance of the moderate-affinity binding sites in ROS, which can only be detected using equilibrium binding assays. This second class of sites may play a role in dynamically buffering the free cGMP concentration in the outer segment. The moderate-affinity cGMP binding sites may also represent a second noncatalytic cGMP binding domain on PDE, based on the identification in the primary sequence of two conserved putative cGMP binding domains per PDE catalytic subunit. It remains for future investigations to determine precisely how cGMP binding to distinct classes of binding sites in the photoreceptor encode information that is used to regulate the amplitude, duration, and/or state of adaptation of the phototransduction cascade.

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