

A Centrifugal Separation Procedure Detects Moderate Affinity cGMP Binding Sites in Membrane-Associated Proteins and Permeabilized Cells

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A centrifugal separation method involving centrifugation of cells or membrane preparations through a layer of silicone oil was developed for studying nucleotide binding in retinal photoreceptors. By adjusting the density and volume of the water-immiscible silicone oil, quantitative sedimentation of electropermeabilized photoreceptors or photoreceptor membrane preparations can be rapidly accomplished using a conventional microcentrifuge. The centrifugal separation method is superior to the filter binding assay for studies of cGMP binding sites in photoreceptors. In addition to high affinity cGMP binding sites which can be detected equally well by both separation methods, the centrifugal separation method also resolves moderate affinity binding sites that are not observed by filter binding.

Many protocols have been developed for separating bound solutes from solutes free in the external medium. Nonequilibrium methods include membrane filtration, rapid gel filtration, and ligand adsorption assays. However, these methods are accurate only if the dissociation of bound ligand is slow compared to the time required to separate bound from free ligand (1). Equilibrium meth-

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ods such as simple centrifugation binding assays and equilibrium dialysis measurements can detect low affinity ligand binding reactions, but often result in high nonspecific binding of the ligand as well as being more laborious than nonequilibrium binding assays [for review, see (2)]. We report here the use of centrifugal separation through silicone oil, in which the aqueous phase is stripped away from permeabilized cells or membrane preparations by passage through the oil layer. This method minimizes disruption of the equilibrium between bound and free cGMP, while also greatly reducing nonspecific entrapment of unbound ligand in the pellet.

Osmotically intact rod outer segments (ROS)² from frog retinas were prepared as described previously (3) and either gently permeabilized by electroporation³ or completely disrupted by homogenization. ROS suspensions were mixed with various concentrations of [³H]-cGMP, and the binding reaction was allowed to reach equilibrium at 22°C (3). The extent of [³H]cGMP binding determined by centrifugal separation was compared to that of a previously described filter binding assay (3). Binding data have been corrected for nonspecific binding in the presence of excess unlabeled cGMP and analyzed using the computer programs EBDA and LI-GAND (4,5).

The centrifugal separation procedure used in this study relies on centrifugation of photoreceptor cells or disrupted photoreceptor cell membranes through a water-immiscible silicone oil having a density greater than the aqueous medium but less than the cells or membranes. The silicone oils (William F. Nye, New Bedford, MA) were prepared by mixing Dow Corning 550 fluid ($\rho = 1.07$ g/ml) and Dow Corning 200 fluid (1.0 cS; $\rho = 0.818$ g/ml) in varying proportions to adjust the final density. A 0.4-ml polyethylene centrifuge tube was loaded with 5 μ l of 50% glycerol (to act as a cushion), and then the silicone oil layer was added (optimal volume range, 10–100 μ l). Finally, the photoreceptor suspension (20–25 μ l) was layered on top of the oil.

The 0.4-ml tubes were placed in 1-ml Fisher polystyrene centrifuge tubes and spun in a Fisher Model 59 microcentrifuge at 11,000g for the indicated times. All centrifugations were performed at room temperature. The 0.4-ml tube was subsequently sliced just above the interface between the oil layer and the bottom layer, and the contents of the bottom layer, along with residual oil, were removed for analysis.

The optimal conditions for centrifugal separation of ROS suspensions from their external medium were determined by measuring the extent of recovery of the integral membrane protein, rhodopsin (Rho), in the pel-

let after centrifugation. Homogenized ROS preparations were quantitatively sedimented (Rho recovery = $94 \pm 3\%$; $n = 3$) through silicone oils ranging in density from 1.017 to 1.028 g/ml. Lower densities cause the aqueous medium to sediment through the oil layer, while greater oil densities prevent complete sedimentation of Rho. Similar results have been obtained with electropermeabilized ROS preparations, except that an oil density of $\rho \geq 1.020$ g/ml is required to prevent inversion of the supernatant layer beneath the oil layer. The silicone oil layer also efficiently excluded aqueous layer solutes from entering either the oil layer or the bottom layer with the cell pellet. Addition of various radiolabeled compounds resulted in $<0.04\%$ of the total DPMs being recovered in the bottom layer of the tube after centrifugation. No detectable DPMs were observed in the oil layer itself. Over the range of 20–140 μ M Rho, the amount of cGMP binding was linearly proportional to the number of binding sites present; at lower Rho concentrations, the extent of binding of cGMP became more variable. Finally, the separation method is rapid; we observed 90% recovery of the total Rho in ROS suspensions when the centrifuge was turned on for 10 s at top speed (followed by an approximately 20-s deceleration time).

We first tested whether the high affinity class of cGMP binding sites previously reported in vertebrate photoreceptors [e.g., (3,6,7)] could be equally well determined with the centrifugal separation method as with the commonly employed filter binding assay. We maintained the total cGMP concentration in the submicromolar range in these experiments in order to minimize the contribution of moderate affinity binding sites (see below) to the analysis of the dissociation constant (K_D) and the maximum site density (B_{\max}). We found that both filter binding ($K_D = 58 \pm 10$ nM, $B_{\max} = 0.0045 \pm 0.0011$ mol cGMP/mol Rho; $n = 10$) and centrifugal separation ($K_D = 73 \pm 19$ nM, $B_{\max} = 0.0051 \pm 0.0010$ mol cGMP/mol Rho; $n = 11$) provide similar estimates for high affinity binding of cGMP to homogenized ROS preparations. Note that there appears to be greater precision in determining the K_D using the filter binding assay. The slightly higher values of K_D and B_{\max} for the centrifugal separation method may reflect a minor contribution from moderate affinity cGMP binding sites detected by this method.

The curvilinear Scatchard plot in Fig. 1 demonstrates that the centrifugal separation method permits the resolution of two distinct classes of cGMP binding sites when the ligand concentration ranges from 30 nM to 20 μ M cGMP. The two binding curves shown in Fig. 1 represent individual experiments of cGMP binding to electropermeabilized photoreceptors (circles) or to homogenized photoreceptor membrane preparations (triangles). The average binding parameters from four experiments for the high affinity cGMP binding sites (K_{D1}

² Abbreviations used: ROS, rod outer segment; Rho, rhodopsin; K_D , dissociation constant; B_{\max} , maximum site density.

³ Cote *et al.*, manuscript in preparation.

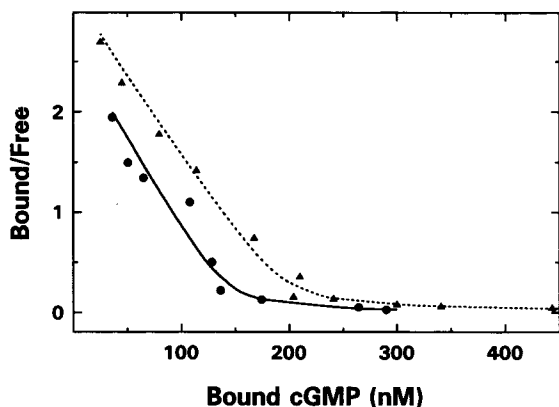


FIG. 1. Two classes of cGMP binding sites detected with the centrifugal separation method. Electropermeabilized (circles; 34 μM Rho) or homogenized ROS (triangles; 41 μM Rho) were incubated for 2–4 min with [^3H]cGMP (concentration range, 30 nM to 20 μM), and bound cGMP was separated from free cGMP by centrifugal separation through silicone oil ($\rho = 1.028$ g/ml for electropermeabilized ROS; $\rho = 1.024$ g/ml for homogenized ROS). The curves in the figure represent the fit of the data to a model assuming two classes of cGMP binding sites with the following binding parameters: for electropermeabilized ROS, $K_{D1} = 53$ nM, $B_{\text{max}1} = 0.004$ mol cGMP/mol Rho, $K_{D2} = 8$ μM , $B_{\text{max}2} = 0.008$ mol cGMP/mol Rho; for homogenized ROS, $K_{D1} = 62$ nM, $B_{\text{max}1} = 0.005$ mol cGMP/mol Rho, $K_{D2} = 20$ μM , $B_{\text{max}2} = 0.007$ mol cGMP/mol Rho.

$= 53 \pm 10$ nM, $B_{\text{max}1} = 0.0052 \pm 0.0011$ mol cGMP/mol Rho) are similar to the K_D and B_{max} values obtained when ligand binding is restricted to submicromolar concentrations of cGMP (see above). The second class of cGMP binding sites exhibits a binding affinity ($K_{D2} = 11.7 \pm 5.3$ μM) two orders of magnitude lower than the high affinity sites, and a site density ($B_{\text{max}2} = 0.0097 \pm 0.0032$ mol cGMP/mol Rho) twofold greater than the B_{max} for the high affinity class of sites. The relatively large error in estimating the K_D of the moderate affinity cGMP binding sites is due in part to the low specific activity of the [^3H]cGMP solutions at the highest cGMP concentrations used; this constraint is imposed by the requirement for a 1000-fold range of cGMP concentrations in order to quantitate both classes of sites [see also Ref. (4) for discussion of other sources of error]. We have also confirmed by ultrafiltration that rod photoreceptors contain two classes of cGMP binding sites with binding parameters in agreement with these results (3).

In contrast, filter binding assays were unable to estimate the binding parameters for the moderate affinity

class of cGMP binding sites. Occasional curvature in Scatchard analysis of filter binding data was detected at high ligand concentrations (not shown). In all such cases, however, analysis of the data with a two-site model using the program LIGAND did not reveal a statistically significant improvement in the fit compared to a one-site binding model. Similar observations to ours have been made by Macklin *et al.* (8) who compared the binding parameters for chemotactic peptide binding to neutrophil membranes by different techniques. We conclude that both electropermeabilized and homogenized ROS preparations contain two distinct classes of cGMP binding sites that filter binding assays cannot resolve, but which are amenable to study with the centrifugal separation method.

In conclusion, we have shown that the partitioning of electropermeabilized cells or homogenized cell extracts from their surrounding medium can be easily accomplished by centrifugation through a layer of silicone oil of the appropriate density. The rapid sedimentation of cells or membranes through the oil layer is advantageous in minimizing dissociation of bound ligand from the receptor–ligand complex, and probably accounts for the ability to detect moderate affinity classes of binding sites with the centrifugal separation method but not with the nonequilibrium filter binding assay.

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