Visualization of Cyclic Nucleotide Binding Sites in the Vertebrate Retina by Fluorescence Microscopy

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Abstract. Cyclic nucleotides play a major role in cell signaling, especially in the nervous system. They act as cytoplasmic messengers in a wide range of physiological responses, but the spatial distribution of their sites of action within cells and tissues is not well-known. In the vertebrate retina, there is a class of well-characterized cGMP binding sites which control the permeability of cation channels in the rod outer segments (ROS), while cAMP is involved in several other systems in the inner retina. Biochemical studies of the cGMP-activated permeability in ROS have not distinguished between the subcellular compartments of disk and plasma membrane. By a new method using fluorescein-conjugated cyclic nucleotides, we have found strong cyclic GMP binding to the plasma membrane of the ROS, both on frozen sections of retina and in freshly isolated, leaky ROS. We also found a high density of cGMP binding sites on structures resembling the inner segment calycal processes. Little specific binding could be detected on the disk membranes or on any other retinal layer. In contrast, fluorescent cAMP did not label ROS, but gave a striking pattern of labeling on several deeper layers of the retina. These results suggest that the ROS plasma membrane has a much higher density of cGMP-controlled cation channels than the disk membranes, and point to other retinal layers where cAMP is likely to shape cellular responses. This method opens up novel morphological approaches to the study of cyclic nucleotide regulation.

CYCLIC nucleotides regulate a wide variety of cellular functions by binding to regulatory sites such as protein kinases or ion channels in sensory receptors. In the vertebrate retina, which is stratified into photoreceptor and neural layers, cyclic nucleotides have distinct distributions and roles. Ferrendelli et al. (1980) examined their distributions in freeze-dried sections of retinal layers. They found very high cGMP levels in the photoreceptors, and fairly uniform cAMP levels throughout the retina, with perhaps a higher level in the photoreceptor inner segments, and high adenylate cyclase activity in the inner plexiform layer. The role of cGMP in visual transduction is now well-understood. This cyclic nucleotide acts as the cytoplasmic messenger whose concentration is modulated by a cascade of signal transduction enzymes, triggered by excitation of the light receptor protein, rhodopsin (reviewed by Stryer, 1986). Electrophysiological recordings have clearly demonstrated that the target of this biochemical cascade is a cGMP-activated cation channel (Fesenko et al., 1985; Yau and Nakatani, 1985; Cobbs and Pugh, 1985; Zimmerman and Baylor, 1986; Nakatani and Yau, 1988). Studies describing a class of cGMP regulatory sites in rod outer segment (ROS)[1] membranes which activate cation fluxes (Caretta et al., 1979, 1985; Koch and Kaupp, 1985; Cook et al., 1987) were interpreted as showing the presence of the channels in both ROS plasma membrane and in the cytoplasmic disk membranes, since cGMP activated fluxes were observed in ~30% of total ROS membrane vesicles, and also in leaky rods.

The protein compositions of ROS disk and plasma membranes are distinct (Kamps et al., 1982; Molday and Molday, 1987). The plasma membrane contains about half as much rhodopsin as the disk membrane in addition to other components not present in disks. However, disk membranes are continually formed by protrusions of the ciliary plasma membrane at the base of the outer segments (Steinberg et al., 1980). At this point the sorting of membrane proteins bound for disks and ROS plasma membrane must occur. Chaitin et al. (1984) found a high concentration of actin in this region, and they proposed it to be involved in protein sorting and membrane expansion.

A characteristic of cyclic nucleotide regulatory sites is that they avidly bind analogues with substitutions on the C8 position. So far the presence of cyclic nucleotide binding sites has been demonstrated only in vitro with methods that do not provide information on their spatial distribution. To visualize this distribution in the vertebrate retina, we used analogues of the cyclic nucleotides cAMP and cGMP with a fluorescein group substituted on C8, and observed binding of the fluorescent cyclic nucleotides to retinal sections and isolated ROS by fluorescence microscopy.

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1. *Abbreviations used in this paper*: IBMX, isobutylmethylxanthine; ROS, rod outer segments; SAF, 8-thioacetamido-fluorescein.

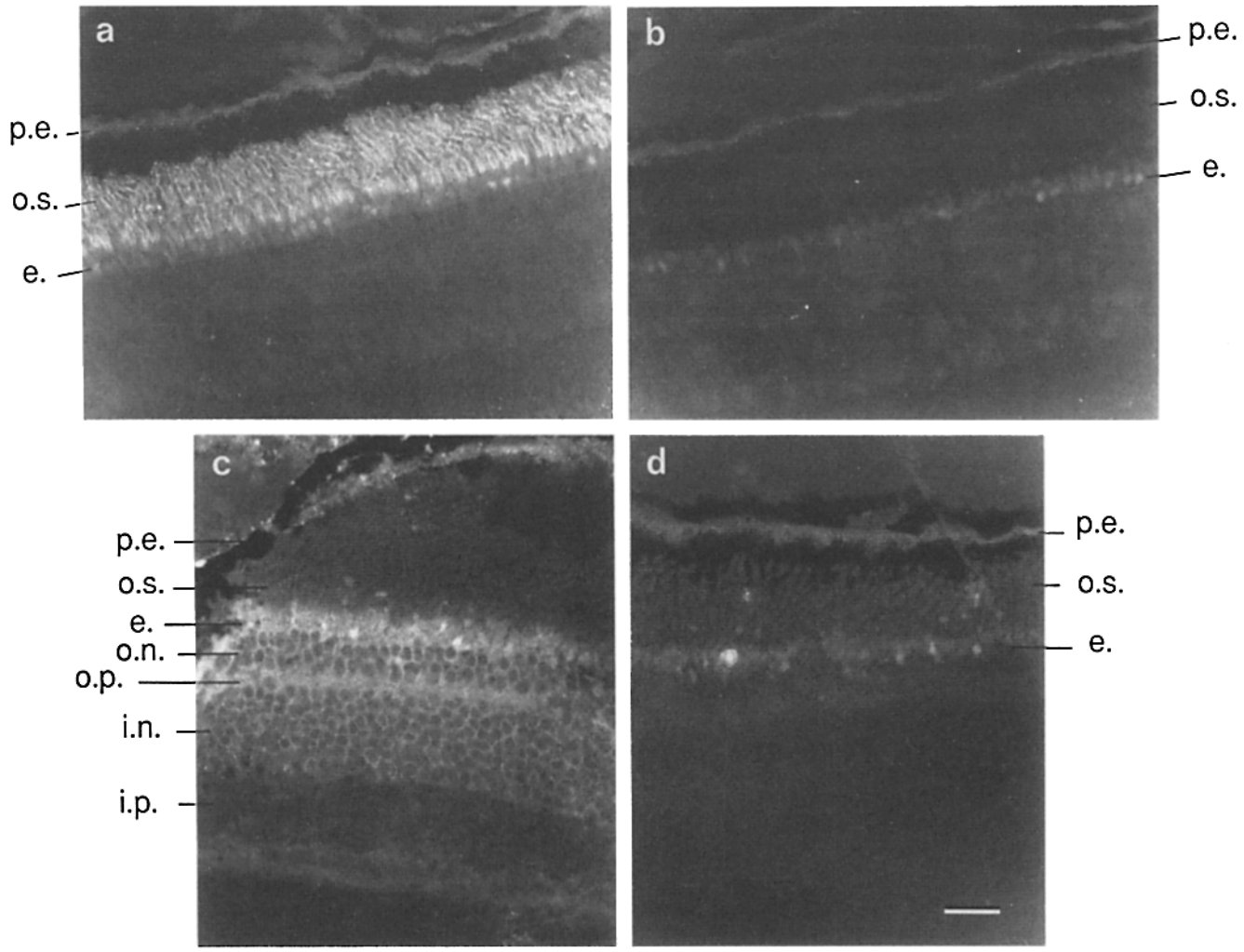

Figure 1. Fluorescent cyclic nucleotide binding to frozen sections of unfixed retina. (*a*) SAF-cGMP (1 μM) staining of the outer segment layer. The labeling did not change with time after application of fluorescent nucleotide to the section. (*b*) Control section, with 1 μM SAF-cGMP and 100 μM 8-Br-cGMP added together. Autofluorescent ellipsoids can be seen on both sections. (*c*) SAF-cAMP (1 μM) staining of the retina. The ROS are unlabeled, but the inner segments, outer nuclear, outer plexiform, and inner nuclear layers are progressively labeled with time after application of the nucleotide. This section was incubated for 30 min. (*d*) Control section, incubated for 30 min with 1 μM SAF-cAMP and 1 mM cAMP. Only the ellipsoid autofluorescence remains. *p.e.*, pigment epithelium; *o.s.*, outer segments; *e.*, ellipsoids; *o.n.*, outer nuclear layer; *o.p.*, outer plexiform layer; *i.n.*, inner nuclear layer; *i.p.*, inner plexiform layer. Bar, 50 μM.

Materials and Methods

Synthesis of 8-Thioacetamido-fluorescein Cyclic Nucleotides

The fluorescent substitution on C8 of cAMP and cGMP was done by the method previously described for cGMP (Caretta et al., 1985), yielding 8-thioacetamido-fluorescein (SAF)-cAMP and SAF-cGMP (Italian Patent Application 42506A/85). Briefly, cyclic nucleotides were transformed into the 8-Br derivatives by reaction with bromine (Muneyama et al., 1971; Miller et al., 1973). The 8-Br derivatives were reacted with thiourea to form the corresponding isothiouronium salts from which the 8-thioderivatives were obtained. Reaction of the 8-thioderivatives with iodacetamide fluorescein (Molecular Probes Inc., Junction City, OR) gave the SAF derivatives. The products were purified by TLC on Silica gel (Merck 7774) with butanol/acetone/acetic acid/5% ammonia/water (35:25:15:14:16 [vol/vol]). Chemical characterization: SAF-cAMP: Rf = 0.7, λ_{max} (alkaline methanol) = 492 nm; NMR shifts in DMSO (ppm): 10.81 (1), 10.20 (2), 8.29 (1), 8.19 (1), 7.63 (1d), 7.56 (2), 7.28 (1d), 6.62 (6d), 5.68 (1); ϵ_{492} (alkaline methanol) = 72,000 $M^{-1}cm^{-1}$ (extrapolated to infinite dilution); solubility: alkali, DMSO. SAF-cGMP was previously described (Caretta et al., 1985).

Fluorescence Microscopy of Frozen Retinal Sections

Toad eyes (Bufo bufo) were rapidly frozen by plunging into liquid nitrogen-cooled pentane. 10-μM sections were cut on a cryostat. The sections were air dried and stained with the SAF-cyclic nucleotides in 130 mM KCl, 20 mM Tris-HCl, 0.5 mM EDTA, pH 7.6. They were used within 2 h of sectioning as they became increasingly autofluorescent. Mild paraformaldehyde fixation did not abolish the specific staining but caused additional non-specific binding that was not inhibited by competing nucleotides and was therefore avoided. To look at the spatial distribution of cyclic nucleotide binding sites, the tissue was placed in a dilute solution of the fluorescent nucleotide (1 μM was found to be convenient for viewing and photography) and equilibrium binding was observed with a Zeiss epifluorescence microscope. Photographs were taken with Ilford HP5 film, ×25 objective, 15–30-s exposure.

Isolated ROS

ROS were isolated from dark adapted toad retinas by vortexing them in a small volume of the same buffer as above. The ROS suspension was mixed with SAF-cGMP and immediately observed in a Zeiss epifluorescence microscope. Again, a SAF-cGMP concentration of 1 μM was used, but values

Table I. Effect of Control Nucleotides on SAF-cGMP Labeling and on Permeability Activation of ROS

Nucleotide	Concentration/mM	Inhibition of labeling	Permeability activation
SAF-cGMP	0.001		+
cGMP	1*	±*	±*
cGMP + IBMX	1.0 + 0.5	+	+
IBMX	0.5	–	–
8-Br-cGMP	0.03	+	+
8-thiobenzyl-cGMP	0.03	+	+
2′,3′-cGMP + IBMX	1.0 + 0.5	–	–
deoxy cGMP + IBMX	1.0 + 0.5	–	–
GMP	1.0	–	–
GTP	1.0	–	–
cAMP	1.0	–	–

Results were identical for isolated ROS and retinal sections. Fluorescein alone did not cause any labeling. The permeability measurements were made as in Caretta et al. (1988).
* True concentration not known because of hydrolysis. Intermediate levels of labeling and permeability activation were observed.

between 0.3 and 15 μM gave similar results. The leaky ROS retained their structural integrity for up to 30 min at room temperature. Photographs were taken with Ilford HP5 film, ×100 objective, 45–60-s exposure.

Results

Cyclic Nucleotide Binding Sites in Sections of Retina

When cryostat sections of unfixed, rapidly frozen mouse or toad retina were placed in a solution with 1 μM SAF-cGMP, the photoreceptor outer segments were promptly and strongly labeled. The outlines of the sectioned ROS were clearly visible, despite the disruption due to sectioning of unfixed tissue (Fig. 1 *a*). This distribution was not due to impeded diffusion of the fluorescent nucleotide, because antibodies diffuse freely into the ROS in cryostat sections, giving uniform labeling of disk membrane antigens (see, for example, Lerea et al., 1986). There was an area of brighter labeling at the base of the ROS, in the region where the inner segment membrane extends calycal processes around the outer segment, just above the ellipsoids. 8-Br-cGMP abolished the ROS staining (Fig. 1 *b*) indicating competition for a cGMP binding site. Only a bright autofluorescence in the ellipsoids remained in this case. Other retinal layers were not labeled by SAF-cGMP. When the toad retina sections were stained with SAF-cAMP (1 μM), strong labeling appeared in the photoreceptor inner segments, outer nuclear and outer plexiform layers, but not in the outer segments of the photoreceptors nor in the inner plexiform or ganglion cell layers (Fig. 1 *c*). This labeling was reduced by simultaneous addition of 1 mM cAMP (but not by other nucleotides, the adenine nucleotide equivalents of Table I), indicating that specific cAMP binding sites were visualized (Fig. 1 *d*). Unlike the staining with SAF-cGMP, this labeling increased over a 30-min incubation. A similar labeling pattern was observed in mouse retinal sections (not shown). Neither SAF-cGMP nor SAF-cAMP labeling was reduced by isobutylmethylxanthine (IBMX), a ligand for the catalytic site of phosphodiesterase, ruling out binding of fluorescent nucleotides to these sites.

cGMP Binding Sites in ROS

To obtain better resolution of the SAF-cGMP labeling, a suspension of isolated toad, mouse or bovine ROS was observed in the presence of 1 μM SAF-cGMP. It is known that when isolated ROS are treated with a membrane impermeant fluorescent dye, e.g., N,N′-didansylcystine, some are found to be leaky and allow penetration of the dye into the cytoplasmic space, which appears uniformly fluorescent, whereas others are sealed and exclude the dye (Yoshikami et al., 1974). Only some of the ROS were labeled with SAF-cGMP, and the fluorescence labeling was not uniform. When the edges of the cylindrical ROS were in focus, the label was in a bright line defining their surfaces and there was weak fluorescence in the interior of the ROS (Fig. 2, *a* and *b*). This peripheral labeling could not be attributed to refraction effects because it could be observed in a 35% sucrose solution, nor could it be ascribed to restricted diffusion of the fluorescent nucleotide within the ROS, since the interior of labeled ROS at high SAF-cGMP concentrations (e.g., 15 μM) was almost as bright as the background fluorescence of free labeled nucleotide. By comparison, immunofluorescent labeling of rhodopsin or transducin gives uniformly fluorescent ROS (Witt et al., 1984). In deteriorating ROS (i.e., >1 h old) the separating disk stacks appeared dark, while the fluorescent surface layer could be seen to aggregate and detach from the disk stacks. Addition of 100 μM 8-Br-cGMP with 1 μM SAF-cGMP completely abolished the peripheral labeling (Fig. 2 *c*), but leaky ROS could still be discerned since they were slightly brighter than the background, probably because of nonspecific binding (Fig. 2 *c*). In bright field they could not be distinguished from sealed ROS (Fig. 2 *d*). By focussing on the top and bottom surfaces of the toad ROS so that the plasma membrane was viewed face on instead of edge on, areas of brightly stained lines and rings were often seen at one end of the ROS (Fig. 2 *e*). When the focus was on the outline of the ROS, this region appeared as a bright, diffuse band (Fig. 2 *b*, *left*). This may correspond to the band of brighter staining at the bases of the ROS in the section (Fig. 1 *a*). These observations are consistent with a high density of cGMP binding sites in the calycal processes.

Nucleotide Specificity

A series of control experiments was done to establish the nucleotide specificity of the binding sites for SAF-cGMP, both in retinal sections and in isolated ROS (Table I). Provided that IBMX was present to slow down its hydrolysis by phosphodiesterase, cGMP or the 8-substituted analogues, added simultaneously with SAF-cGMP, blocked the fluorescent labeling. Other analogues were ineffective at competing for the SAF-cGMP binding sites. This series is compared to the nucleotide specificity for activating the membrane permeability (Table I). Labeling of ROS plasma membranes is abolished by nucleotides which activate the cation channels. In vitro these compete with SAF-cGMP for binding sites on photoreceptor membranes (Caretta et al., 1985). The larger and more hydrophobic the substituent on C8, the higher the affinity. Analogue concentrations at half-maximal activation of the cation conductance are given by Caretta et al. (1985). The cGMP binding sites and the cGMP activated permeability also show the same sensitivity to detergent and urea treatment and resistance to trypsin (Capovilla et al., 1983). Labeling of noncatalytic sites on the phosphodiesterase is excluded by in vitro binding studies in which removal or read-

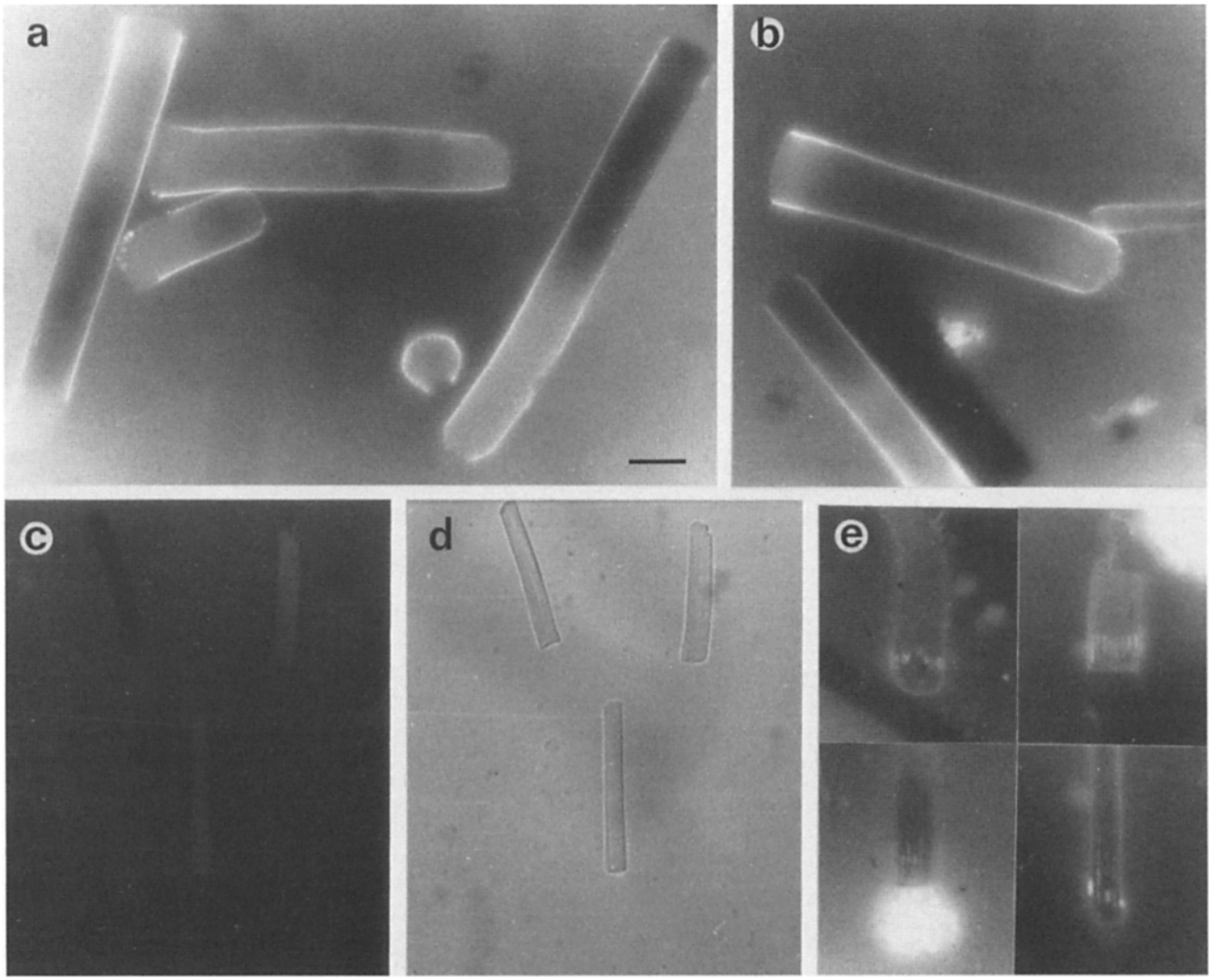

Figure 2. Fluorescent cGMP binding sites in isolated toad ROS. (*a* and *b*) ROS in 1 μM SAF-cGMP. An end-on view can be seen in the lower part of *a*, where a short fragment is oriented vertically, giving a broken, circular profile. The scalloped edges of its plasma membrane can be seen to follow the pattern of disk incisures. In the lower part of *b*, a sealed ROS is seen as a dark area. Gradations of fluorescent intensity are apparent within the labeled ROS. However, the darker areas may be due to depletion of label by photobleaching during the photographic exposure in regions of the ROS far from open ends or breaks in the plasma membrane. (*c*) *Control*, 100 μM 8-Br-cGMP was added with the 1 μM SAF-cGMP. No staining is observed, and the leaky rods can be barely detected as slightly lighter than the 1 μM SAF-cGMP background, while the sealed rod is slightly darker. (*d*) Bright field image of the area in *c*. (*e*) Four views of the end structure, in which cGMP binding sites are seen in a series of stripes when the plane of focus is at the top or bottom surface of the rod instead of at the midplane. The very bright area at the bottom left is a photoreceptor ellipsoid, containing mitochondria. These showed bright autofluorescence, which was independent of all added nucleotides. Bars, (*a*, *b*, and *e*) 10 μM; (*c* and *d*) 40 μm.

dition of the phosphodiesterase has no effect on SAF-cGMP binding (Caretta et al., 1988).

Discussion

The most likely identity of the SAF-cGMP binding sites is the cGMP receptor of the cation channel that mediates the electrical response of photoreceptors to light (see Stryer, 1986). Only cGMP analogues that activate the cation conductance gave competitive inhibition of labeling. The proteins specific to ROS plasma membrane observed by Kamps et al. (1982) and Molday and Molday (1987) probably include the putative cGMP activated channels isolated by Cook et al. (1987) and/or Matesic and Liebman (1987). Our labeling provides the first evidence for a dramatic difference in the density of cGMP binding sites in plasma and disk membranes of ROS. Sorting of membrane proteins into disk surface, disk rim, and plasma membrane is thought to occur as new membrane is formed at the inner face of the connecting cilium (Steinberg et al., 1980). During this process, the cGMP binding protein may interact with the ciliary cytoskeleton to effect its partitioning to the plasma membrane. We were able to detect plasma membrane labeling above the background at free SAF-cGMP concentrations from 0.3 to 15 μM. Assuming our lower limit of detection is 0.3 μM, this sets an upper limit of 0.3 μM to the cGMP binding site con-

centration in disk membranes. Above 15 μM the fluorescence started to show quenching. Thus, the cGMP binding site concentration in the plasma membranes is at least 15 μM, as it was visible against a background fluorescence concentration of 15 μM. In binding studies, it was determined that $\sim 10^6$ SAF-cGMP binding sites are present per bovine ROS (Caretta et al., 1985). Localization within ~ 100 μm^2 of plasma membrane implies that their density in bovine ROS plasma membrane is $\sim 1{,}000$ μm^{-2}, even if the disk membranes are assumed to contain 0.3 μM binding sites. This predicts a plasma membrane concentration of binding sites much higher than 15 μM, the maximum we can measure by fluorescence.

A significant proportion of the binding sites is present in the linear structures at the bases of the ROS (Fig. 2 *e*), which are likely to be identical with the inner segment calycal processes. These processes, whose function is unknown, consist of extensions of the inner segment plasma membrane enclosing bundles of actin filaments (Burnside, 1978; Steinberg et al., 1980). They can remain partially intact in dissociated toad ROS (Spencer et al., 1988). With fluorescent actin staining, they appear as slightly tapered structures (Del Priore et al., 1987). It is interesting to note that the calycal processes of dark-adapted toad rods contain 48k protein, which shifts to the outer segments in the light (Mangini and Pepperberg, 1988). 48k protein is a soluble component of the photoreceptors, also known as retinal S antigen or arrestin, because of its involvement in autoimmune uveoretinitis, and its role in quenching light activation of the cGMP phosphodiesterase, respectively (for references see Mangini and Pepperberg, 1988). Taken together, these observations suggest a possible participation of the inner segment calycal processes in visual transduction.

The observation of cAMP binding to noncatalytic sites in central layers of the retina is novel, and provides a morphological basis for further studies of cAMP regulation in the retina. The high concentration of cAMP binding sites found in the photoreceptor inner segments (Fig. 1 *c*) may be related to the mechanisms of photomechanical movement in lower vertebrate rods and cones analyzed by Burnside and her colleagues (see Burnside and Ackland, 1987). Their studies on the motility mechanism, mainly in teleost cones, have shown that cAMP-dependent protein kinase inhibits myosin light chain kinase, preventing myoid contraction in cones. A similar system operates in rods, although cAMP stimulates myoid contraction. Our labeling is consistent with a major regulatory role for cAMP in toad photoreceptor inner segments and the most likely identity of the binding site is cAMP-dependent protein kinase. However, we have also observed labeling in the inner segment layer of mouse retina, which does not exhibit photomechanical movements of its photoreceptors. cAMP binding sites in the outer plexiform/inner nuclear layers may be related to the role of cAMP in regulating gap junction coupling of horizontal cells (Piccolino et al., 1984). A dopamine-activated adenylate cyclase reduces receptive field size by reduction of gap junction permeability, perhaps via a cAMP-dependent kinase.

In summary, this work demonstrates a simple method for localizing regulatory cyclic nucleotide binding sites in cells and tissues, and allows the correlation of physiological, morphological and biochemical observations.

We thank Andrea Cavaggioni for discussions; A. Cavaggioni and Virna Conti for providing the fluorescent cyclic nucleotides; and Hugh Perry for comments on the manuscript.

Received for publication 25 August 1988 and in revised form 23 November 1988.

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