Functional coupling of a Ca^{2+} /calmodulin-dependent nitric oxide synthase and a soluble guanylyl cyclase in vertebrate photoreceptor cells

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Electrophysiological recordings on retinal rod cells, horizontal cells and on-bipolar cells indicate that exogenous nitric oxide (NO) has neuromodulatory effects in the vertebrate retina. We report here endogenous NO formation in mammalian photoreceptor cells. Photoreceptor NO synthase resembled the neuronal NOS type I from mammalian brain. NOS activity utilized the substrate L-arginine ($K_m = 4 \mu\text{M}$) and the cofactors NADPH, FAD, FMN and tetrahydrobiopterine. The activity showed a complete dependence on the free calcium concentration ($[\text{Ca}^{2+}]$) and was mediated by calmodulin. NO synthase activity was sufficient to activate an endogenous soluble guanylyl cyclase that copurified in photoreceptor preparations. This functional coupling was strictly controlled by the free $[\text{Ca}^{2+}]$ ($\text{EC}_{50} = 0.84 \mu\text{M}$). Activation of the soluble guanylyl cyclase by endogenous NO was up to 100% of the maximal activation of this enzyme observed with the exogenous NO donor compound sodium nitroprusside. This NO/cGMP pathway was predominantly localized in inner and not in outer segments of photoreceptors. Immunocytochemically, we localized NO synthase type I mainly in the ellipsoid region of the inner segments and a soluble guanylyl cyclase in cell bodies of cone photoreceptor cells. We conclude that in photoreceptors endogenous NO is functionally coupled to a soluble guanylyl cyclase and suggest that it has a neuromodulatory role in visual transduction and in synaptic transmission in the outer retina.

Key words: cGMP/nitric oxide/photoreceptors/soluble guanylyl cyclase

Introduction

Visual excitation in outer segments of vertebrate photoreceptor cells is mediated through the light-triggered hydrolysis of the intracellular messenger guanosine 3',5'-cyclic monophosphate (cGMP). The decrease in the cytoplasmic cGMP level leads to the closure of cGMP-gated cation channels in the plasma membrane (Stryer, 1991; Kaupp and Koch, 1992; Lagnado and Baylor, 1992; Pugh and Lamb, 1993). The resulting hyperpolarization of the

photoreceptor cell is the first step in converting the light signal into neural pathways of the retina (Dowling, 1987; Copenhagen, 1991; Wu, 1992; DeVries and Baylor, 1993). Concomitant with a photoreceptor's light response is a decrease in the cytoplasmic $[\text{Ca}^{2+}]$ of the outer segment (Yau and Nakatani, 1985; Ratto *et al.*, 1988; Korenbrot and Miller, 1989; Lagnado *et al.*, 1992). A change in the cytoplasmic $[\text{Ca}^{2+}]$ is part of a negative feedback mechanism that modulates the phototransduction process during recovery and light adaptation (Matthews *et al.*, 1988; Nakatani and Yau, 1988). Therefore, any Ca^{2+} -sensitive regulation of photoreceptor enzymes is of potential importance for light adaptation. Such a modulatory effect of Ca^{2+} was observed for several photoreceptor proteins, including a particulate guanylyl cyclase (pGC) (Koch and Stryer, 1988; Dizhoor *et al.*, 1991; Lambrecht and Koch, 1991), a rhodopsin kinase (Kawamura, 1993) and a cGMP-gated ion channel (Hsu and Molday, 1993).

Involvement of nitric oxide (NO) in rod outer segment physiology was postulated recently based on the finding that electrophysiological recordings on frog rods exhibited an NO sensitivity of the light response. When an exogenous source of NO, i.e. the NO donor compound sodium nitroprusside (SNP), is applied as NO donor, a rod cell shows a stable dark voltage with a slight depolarization and an acceleration of the recovery phase of the light response (K.F.Schmidt *et al.*, 1992). A possible target enzyme of NO, a soluble guanylyl cyclase (sGC), is biochemically detected in rod outer segment (ROS) preparations (Margulis *et al.*, 1992; Sitaramayya and Margulis, 1992). Similar effects on the light response are observed when L-arginine and NADPH, substrate and cofactor of a nitric oxide synthase (NOS), are intracellularly applied (Tsuyama *et al.*, 1993). However, *in situ* hybridization studies show the presence of an sGC mainly in the inner retina (Ahmad and Barnstable, 1993), and evidence for NOS and the function of NO in the retina is rather circumstantial and controversial so far. For example, NOS is present in the amacrine cell layer of the inner nuclear layer, but was shown to be absent in photoreceptor cells by immunocytochemistry and NADPH-diaphorase histochemical staining (Vaney and Young, 1988; Sagar, 1990; Dawson *et al.*, 1991). In contrast, reports claim a NOS activity to be present in ROS preparations (Venturini *et al.*, 1991).

NO could also serve as a neuromodulator of synaptic transmission in the retina. Thus, involvement of NO in retinal cell physiology has been reported for the hemi-gap-junction channels in horizontal cells (DeVries and Schwartz, 1989, 1992) and for on-bipolar cells where an NO-sensitive guanylyl cyclase was localized recently based on electrophysiological recordings (Shiells and Falk, 1992).

As a result of contradictory results in the literature, it

Table I. Distribution of protein content and enzyme activities in photoreceptor band I and II

Protein content or enzyme activity	Unit	Band I	Band II	Ratio II/I
Rhodopsin	mg.ml ⁻¹	9.71	1.91	0.2
pGC, basal	nmol.min ⁻¹ .ml ⁻¹	34.3	8.1	0.2
sGC, SNP-activated	nmol.min ⁻¹ .ml ⁻¹	0.09	3.46	38.4
NOS, Ca ²⁺ -activated	pmol.min ⁻¹ .ml ⁻¹	<0.05	108.5	>2170
NOS/sGC	nmol.min ⁻¹ .ml ⁻¹	<0.04	2.94	>73.5

Activities of pGC were determined with manganese as cofactor. sGC was stimulated by the addition of 100 μ M of the NO donor SNP. NOS activity was measured by the formation of citrulline (NOS) or in a coupled bioassay (NOS/sGC).

was the aim of our study (i) to determine the distribution of NOS and sGC activities in photoreceptor preparations and (ii) to investigate a possible functional coupling of both enzymes in a signal transduction pathway. We report here the existence of a Ca²⁺/calmodulin-regulated NOS in outer and inner segments of mammalian photoreceptor cells and show that endogenous NO regulates an sGC activity that copurified in photoreceptor preparations.

Results

Presence of NOS and sGC activities in photoreceptor cells

After a typical gradient centrifugation of crude outer segment suspensions, we obtained two rhodopsin-containing bands (band I and band II in Table I). Band I was a pure outer segment preparation, whereas band II contained outer segments attached to parts of the inner segments (i.e. the ellipsoid; for a detailed description of band I and II see Materials and methods). We measured NOS and sGC activities in these preparations of photoreceptor cells. NOS was located in photoreceptor band II only (Table I), whereas sGC was found predominantly in band II and also in band I in a ratio of 1:40. While rhodopsin and pGC represent typical constituents of band I, NOS and sGC were markers for the band II preparation (see ratios in Table I).

Characterization of photoreceptor NOS

Photoreceptor NOS activity was characterized further using L-citrulline formation as the assay system (Bredt and Snyder, 1990; Schmidt *et al.*, 1991). The activity was located in the supernatant fraction of bovine retinas and in fractions enriched in photoreceptor band II, whereas the 100 000 g particulate fraction was without activity in the presence of saturating concentrations of all known cofactors of NOS, including FAD, FMN, NADPH and tetrahydrobiopterine (H₄B).

We used fractions enriched with band II to analyze further the molecular properties of photoreceptor NOS. The enzyme was clearly dependent on the substrate L-arginine, with a K_m of ~ 4 μ M, and was inhibited by N^ω-methyl-arginine and N^ω-nitro-arginine. Additional cofactors that induced a pronounced increase in specific NOS activity were the flavins FAD and FMN and H₄B (Figure

Fig. 1. Molecular properties of photoreceptor NOS. (A) Dependence of NOS activity on H₄B. NOS activity increased >3-fold by the addition of H₄B and was half maximal at 100 nM. Activity was measured by the citrulline assay. (B) Calcium-dependent NOS activity is mediated by calmodulin, but not recoverin. NOS activity is inhibited by 10 μ M of the calmodulin antagonist calmidazolium. Inhibition is reversed by increasing concentrations of calmodulin. Addition of recoverin had no effect. NOS activity without calmidazolium is 100%. Data show the average values from duplicates or triplicates. (C) Western blot analysis of NOS type I in the crude supernatant fraction of bovine brain and retina. Lane 1, bovine brain cytosol crude supernatant fraction (25 μ g); lane 2, ADP-Sepharose pool of bovine retina crude supernatant fraction (1 μ g). Numbers on the left side indicate molecular weight standards in kDa.

1A). NOS activity was >3-fold stimulated by exogenous H₄B ($EC_{50} \approx 100$ nM).

NOS was completely inhibited by complexing [Ca²⁺]

Fig. 2. Distribution of particulate and soluble GC activities in photoreceptor preparation. Rhodopsin content ($\blacktriangle$), particulate GC activities ($\square$) and soluble GC activities ($\star$) on a linear sucrose density gradient. Outer segments (OS) purified by sucrose gradient density centrifugation were applied to a second identical sucrose gradient and analyzed for the indicated parameter. The activity of sGC was stimulated by the addition of 100 μ M SNP. Activity of pGC was basal, i.e. non-stimulated at high free $[Ca^{2+}]$.

with EGTA below 10^{-9} M and by different calmodulin antagonists like trifluoperazine (TFP), W-7 and calmidazolium. NOS activity in the supernatant fraction of band II was inhibited by 10 μ M of the competitive calmodulin inhibitor calmidazolium to ~26% of control activity, which effect was almost completely reversed by increasing concentrations of calmodulin but not of recoverin (Figure 1B). Recoverin had no effect on NOS either at high (1 μ M) or low (<1 nM) free $[Ca^{2+}]$, and in the absence or presence of calmodulin. Specific activity of NOS was 9.8 pmol.min⁻¹.mg⁻¹ in band II preparations. Illumination of retinas or photoreceptor preparations had no effect on the subcellular distribution, total activity or regulation of NOS.

These properties of photoreceptor NOS strongly resemble those of the neuronal NOS type I from mammalian brain (Bredt and Snyder, 1990; Schmidt *et al.*, 1991) and are also consistent with the immunological results using a type I NOS-specific antibody (Figure 1C; H.H.H.W.Schmidt *et al.*, 1992). The serum recognized a single 160 kDa protein band in the supernatant fraction of both bovine brain and bovine retina (Figure 1C). However, retinal NOS had to be enriched by 2',5'-ADP Sepharose chromatography to detect NOS-I by Western blot.

Characterization of photoreceptor soluble guanylyl cyclase

A possible target of endogenously produced NO, an sGC, copurified with NOS-I in band II (Table I). Earlier reports suggested that a soluble GC is present in outer segments (Margulis *et al.*, 1992) or that the particulate GC in outer segments is activated by NO (Horio and Murad, 1991). To test what kind of guanylyl cyclase in photoreceptor cells is stimulated by NO, we analyzed the soluble and particulate fractions of photoreceptors separated on a continuous sucrose gradient (Table I) and rechromatographed our photoreceptor preparation on a second sucrose gradient (Figure 2). To measure sGC activities in photoreceptors, preparations were frozen in a hyperosmotic buffer or sonicated to break off the plasma membrane. To some extent, soluble GC activities that were sensitive to

exogenous NO (SNP) comigrated with the rhodopsin peak, but the activities were always higher in fractions at a higher sucrose concentration and lower rhodopsin content (Figure 2). We observed consistently these small sGC activities in outer segments fractionated on a sucrose gradient, which exhibited <0.5% of the basal non-stimulated activity of the pGC (when activities were normalized to the rhodopsin concentration taking the data in Table I). The soluble GC activity was stimulated 17-fold in the presence of 100 μ M SNP. In contrast, the activity of the pGC always comigrated with rhodopsin, and purified pGC, a 112 kDa protein (Koch, 1991), was not sensitive to NO. A very similar distribution of rhodopsin content and GC activities was observed on the first sucrose gradient (not shown).

Functional coupling of NOS/sGC

Endogenous NO formation in band II was sufficient to stimulate sGC (see Table I). Functional coupling of NOS and sGC activities was monitored by measuring sGC activities under conditions that led to enzymatic NO synthesis (i.e. incubation with L-arginine and NADPH in a bioassay; see Materials and methods). Complexation of Ca^{2+} with EGTA resulted in an ~10-fold reduction of sGC activity (Figure 3A). The effect was half maximal at 0.84 μ M and was markedly reduced below 0.5 μ M free $[Ca^{2+}]$. The calmodulin antagonist TFP inhibited sGC via inhibition of NOS. Inhibition by TFP showed an EC₅₀ value of 10 μ M (Figure 3B). This inhibitory action of TFP was competitively overcome by the addition of 1–2 μ M mammalian brain calmodulin shifting the EC₅₀ value for TFP to 56 μ M, indicating that the Ca^{2+} sensitivity was mediated by calmodulin.

About 50–100% of the SNP-sensitive GC activity (Figure 3C) was observed after incubation with 100 μ M L-arginine and 100 μ M NADPH, when endogenous NO synthesis stimulated sGC in the assay. Both stimulatory effects were strongly inhibited by 1 μ M hemoglobin. The L-arginine-dependent stimulation of sGC activity was stereospecific. D-Arginine was not a substitute for the L-isomer. Addition of N^ω-methyl-L-arginine (NMMA), known as a potent inhibitor of the enzymatic conversion

Fig. 3. Functional coupling of NOS and sGC. (A) Calcium-dependent photoreceptor NOS activity as monitored by endogenous soluble GC activity. The assay buffer contained 100 μ M of L-arginine and NADPH and 12.5 μ g of cytosolic proteins. Data were corrected for 20–30% of cGMP hydrolysis. (B) Inhibition of cGMP synthesis by the calmodulin antagonist TFP. GC activity reflects a Ca²⁺/calmodulin-regulated NOS activity. The IC₅₀ for TFP is shifted from 10 μ M (●) to 56 μ M in the presence of 1.6 μ M mammalian brain calmodulin (○). Assay mixture contained 13.5 μ g cytosolic proteins per incubation. Comparison of the NO-sensitive activities of the soluble (C) and the particulate (D) GC from bovine photoreceptor. Either a soluble extract of cytosolic photoreceptor proteins (11.7 μ g protein) or a suspension of washed OS membranes (103 μ g rhodopsin) was assayed for GC activity. Incubations were carried out in the presence of 100 μ M SNP, 100 μ M SNP plus 1 μ M hemoglobin (SNP + Hb) or without any addition (control). The L-arginine-dependent cGMP formation was measured in the presence of 100 μ M L-arginine and 100 μ M NADPH (L-Arg). The concomitant presence of 100 μ M L-NMMA and 1 μ M hemoglobin resulted in suppression of cGMP synthesis by the soluble GC. No influence of nitric oxide on the membrane-bound GC was detected (d). Data represent results of three sets of experiments.

of L-arginine to NO, resulted in a 73% reduction of cGMP synthesis (Figure 3C). No change in activity was observed when the influence of SNP, L-arginine, D-arginine, NMMA and hemoglobin on the pGC in ROS membrane preparations was tested (Figure 3D).

The hydrodynamic and biochemical characterization of photoreceptor NOS and sGC suggests that photoreceptor cells contain an NOS and an sGC, which are functionally coupled and mainly located in cell parts attached to the outer segments (for example the ellipsoid part of the inner segment) but not in the outer segment itself.

Localization of NOS and sGC in photoreceptor cells

Having shown that photoreceptor NOS and sGC are functionally coupled, we determined the subcellular distribution of NOS-I immunoreactivity in mammalian photoreceptors using monospecific immunoprobe. Isolated whole rabbit retina and frozen sections of a macaque monkey retina were stained with anti-NOS-I antibody (Figure 4a, c and d). In rabbit retina, a broad band of staining was observed across the region of photoreceptor inner segments. Heaviest labeling was demonstrated in

Fig. 4. Immunohistochemical localization of NOS and sGC in photoreceptor cells. Transverse sections of isolated, whole retina show immunocytochemical labeling of (a) the source of nitric oxide, nitric oxide synthase (NOS), and (b) the intracellular target, soluble guanylyl cyclase (sGC); scale bar = 17 μ m. In (a), NOS immunoreactivity is observed in the inner nuclear (I) and ganglion cell layers (G), as well as in the inner and outer segments of the photoreceptor (P). In (b), staining of sGC immunoreactive neurons is demonstrated in many cell types in the ganglion cell and inner nuclear layers as well as in the cell bodies of cone photoreceptors in the outer nuclear layer (arrow). NOS immunoreactivity in mammalian photoreceptors: a broad band of staining across the inner segments (arrowhead) and discrete localization of cone photoreceptor outer segments (arrow) is seen in transverse sections of both rabbit (c) and macaque (d); scale bar = 11 (c) and 9 μ m (d).

the ellipsoid region, while the rest of the inner segment was lightly labeled. We observed light staining of cone photoreceptor outer segments but only variable staining of rod outer segments. In macaque retina, distinct staining was seen in cone photoreceptor inner and outer segments (Figure 4d). The specificity of photoreceptor labeling was verified by the absence of staining observed with antiserum which was preadsorbed with purified NOS-I.

Cell bodies of cone photoreceptors in rabbit retina were specifically immunoreactive with an mAb to sGC (Figure 4b). Labeling observed in cone outer segments was inconclusive since here we were unable to resolve questions about the specificity of the stain.

Discussion

NO has been found in a variety of tissues to serve a second messenger function (for reviews see Bredt and Snyder, 1992; Nathan, 1992; Schmidt *et al.*, 1993). The presence of the NO-forming enzyme NOS has been demonstrated in many parts of the brain using immunocytochemistry and NADPH diaphorase histochemistry. The presence of NOS in the retina is only well documented

for amacrine and ganglion cells (Vaney and Young, 1988; Sagar, 1990; Dawson *et al.*, 1991), but several electrophysiological observations point to a neuromodulatory role of NO in different retinal cell layers. (i) NO influences the dark voltage and the light response of isolated frog rods (K.F.Schmidt *et al.*, 1992; Tsuyama *et al.*, 1993). (ii) Gap-junction channels in horizontal cells are closed by a pathway, which includes an NO-sensitive guanylyl cyclase. Horizontal cells contain different second messenger pathways that modulate junctional conductances in these cells. Another pathway probably operates through dopamine and a cAMP-dependent protein kinase (DeVries and Schwartz, 1989, 1992). As the release of dopamine in the retina is thought to participate in the modulation of synaptic circuitry and to control the shift from rod to cone vision during light adaptation [for a review see Copenhagen (1991), Wu (1992) and DeVries and Baylor (1993)], NO might have similar functions in the outer retina (for example it could be part of the feedback loop between horizontal cells and cones to stabilize photoreceptor output). (iii) The concentration of cGMP in retinal on-bipolar cells is expected to rise by the activity of an NO-sensitive GC. This would then open a cGMP-gated cation channel

Fig. 5. Model of two pathways of cGMP synthesis in vertebrate photoreceptor cells and possible intra- and intercellular signaling of NO in the outer retina. In outer segments (OS) a particulate GC (pGC) is activated when the cytoplasmic $[Ca^{2+}]$ decreases after light stimulation. Activation of pGC is mediated by a calcium-binding protein (CaBP) and contributes to the recovery of the photoresponse. In the inner segments (IS) a nitric oxide synthase (NOS) is activated at high cytoplasmic $[Ca^{2+}]$ via calmodulin (CaM). NO can have several effects: it can stimulate a soluble GC in inner segments and could modulate synaptic transmission. NO could also diffuse to neighboring targets and could effect outer segment proteins that undergo ADP ribosylation. Activation of a soluble GC in on-bipolar cells or horizontal cells is also conceivable and would control conductances in these cells.

and depolarize the bipolar cell (Nawy and Jahr, 1990, 1991; Shiells and Falk, 1990). Retinal on-bipolar cells are excited by the photoreceptor synaptic transmitter glutamate that is released during dark periods. Glutamate binds to a specific 2-amino-4-phosphonobutyrate (APB)-type receptor resulting in the hydrolysis of cGMP by a G protein-coupled phosphodiesterase [for a review see Copenhagen (1991)]. Hyperpolarization of photoreceptors by light stimuli decreases the synaptic glutamate release and weakens the glutamate-induced cGMP hydrolysis.

However, in all these cases the cellular source of locally formed NO in the outer retina is unclear and biochemical information on a retinal NOS and its functional coupling to an sGC is very limited so far. We show here, by immunocytochemical, hydrodynamic and biochemical methods, that an NOS type I is present in mammalian photoreceptor cells. Retinal NOS was indistinguishable from brain NOS-I both biochemically and on protein immunoblots. The presence of significant amounts of NOS-III (particulate) and NOS-II (Ca^{2+} -independent) can be excluded based on the subcellular distribution and lack of Ca^{2+} -independent NOS activity, respectively. The dependence of retinal NOS on exogenous H_4B in homogenates and crude supernatant fractions suggests that the concentration of this cofactor/activator of NOS may become rate limiting under certain conditions, for example in retinal disease states.

In the outer retina, the capacity to generate NO was immunocytochemically localized to the portion of the photoreceptor distal to the cell body. Although labeling was observed across the entire span of the inner segment, the ellipsoid region of the inner segment was most heavily labeled. This evidence is corroborated by the finding of heavy NADPH diaphorase labeling across inner segments (not shown). Light, but discrete, NOS immunoreactivity

was also found in the outer segments of cone photoreceptors. A similar staining pattern was also observed using NADPH diaphorase suggesting that, although the highest level of NOS activity was in the inner segments, some NOS activity is present in the cone outer segments. The immunohistochemical results match exactly with the hydrodynamic and biochemical data (Table I; Figures 2 and 3) localizing NOS activity to a fraction of ROS containing inner segment components such as the ellipsoid region, but not to pure ROS.

Localization of NOS in inner segments, in particular in the ellipsoid region, is an important further step to unravel the physiological role of NO/cGMP in visual transduction of the outer retina. Presence of the NO target enzyme, a soluble GC, in the retina, has been described mainly for the inner retina by *in situ* hybridization, only weaker hybridization signals were observed in the outer retina (Ahmad and Barnstable, 1993). In our preparation, sGC copurified with the NOS activity, i.e. in band II. Immunocytochemically, sGC was localized in the cell bodies of photoreceptors. Similar to the distribution of NOS activities, soluble GC activities were very low in pure outer segments, consisting of <0.5% of the basal non-stimulated activity of the particulate GC (see Table I). For our study, it was crucial to exclude that the sGC activity measured originated from a contamination of other retinal cells (i.e. bipolar cells). Such a contamination was unlikely, because isolation of bipolar cells from the retina requires proteolytic digestion with collagenase and papain (Karschin and Wässle, 1990). Furthermore, our preparations were not labeled by a specific marker for on-bipolar cells, i.e. an mAb against PKC. Nevertheless, it is conceivable that a soluble GC was released from the cytoplasm of these cells when the retinas were shaken in the first dissection step of the photoreceptor preparation. Crude retinal extracts

contain, in fact, a high amount of soluble GC activity; however, this activity is lost during the first flotation and sedimentation steps of the photoreceptor preparation as described in Materials and methods.

Thus, we conclude that NO has no significant effect on the cGMP synthesis in outer segments as suggested elsewhere (Venturini *et al.*, 1991). It may, however, influence the ADP ribosylation of outer segment proteins and therefore modulate phototransduction. In fact, rod outer segments contain an ADP ribosyltransferase activity that is influenced by the NO donor compound SNP. For example, transducin known to be ADP-ribosylated by bacterial toxins is also ADP-ribosylated by stimulation through either SNP or endogenously produced NO (Ehret-Hilberer *et al.*, 1992; Pozdnyakov *et al.*, 1993).

Photoreceptor cells are likely to contain two pathways of cGMP synthesis in separated compartments of the cell (Figure 5). In the outer segment, cGMP levels are low in the dark and rise during illumination (i.e. at low free $[Ca^{2+}]$) by a negative feedback control of the pGC (Koch and Stryer, 1988). In inner segments at high free $[Ca^{2+}] > 0.5 \mu M$, the Ca^{2+} /calmodulin-regulated NOS produces NO which stimulates sGC. One possible intracellular target of cGMP could be the cGMP-gated conductance in the inner segments (Matthews and Watanabe, 1988). At present it is unknown how ion conductances like the cGMP-gated channel and the calcium currents in the inner segment control the cytoplasmic free $[Ca^{2+}]$ (Bader *et al.*, 1982; Corey *et al.*, 1984; Maricq and Korenbrot, 1988; Barnes and Hille, 1989; Barnes and Deschenes, 1992). There is evidence that some ion conductances of the inner segment participate in shaping and modulating the synaptically transmitted photoresponse (Bader *et al.*, 1982). This function would be in agreement with the general concept of the neuromodulatory function of NO, e.g. in synaptic plasticity in hippocampus, cerebellum and spinal cord.

Besides controlling an intracellular cGMP pathway in inner segments, NO could diffuse out of the cell and thus influence neighboring cells within a diffusion distance of $\sim 100 \mu m$ (extrapolated from vascular tissue; Malinski *et al.*, 1993). NO released from photoreceptor cells could diffuse to the on-bipolar cells and activate this cyclase as long as the cytoplasmic $[Ca^{2+}]$ in inner segments is high enough to induce the calmodulin-dependent NOS ($> 0.5 \mu M$; see Figure 3A). To our knowledge, no exact data exist about the $[Ca^{2+}]$ in inner segments and how these concentrations change during a light stimulus. This will be important to determine in future studies, since cGMP levels in both photoreceptor segments are strikingly different in their dependence on free $[Ca^{2+}]$.

Materials and methods

Preparation of ROS and sucrose fractionation

Bovine photoreceptor ROS were purified under dim red light according to Schnetkamp and Daemen (1982) with some modifications. Retinas from freshly obtained bovine eyes were gently shaken in 115 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 10 mM HEPES/KOH pH 7.5 and 1 mM DTT (ROS buffer) containing 1.5 M sucrose (0.8–1.0 ml/retina). The suspension was centrifuged for 7 min at 7000 r.p.m. (Beckman JA-14) to sediment chunk pieces of retinas. The supernatant was filtered through a nylon cloth, the filtrate was diluted with ROS buffer 1:1 and centrifuged again. The pellet (crude ROS) was resuspended in the ROS buffer

containing 0.6 M sucrose (5 ml per retina) and layered on a linear continuous sucrose gradient (0.6–1.5 M sucrose in ROS buffer). After centrifugation (90 min; 24 000 r.p.m. in an SW28 Beckman rotor), ROS bands were collected, diluted with a low salt buffer and pelleted by centrifugation. ROS were stored in darkness at $-80^\circ C$. Analysis of ROS fractions on a second sucrose gradient was performed as in the following. ROS were layered on a sucrose gradient (0.6–1.5 M sucrose in ROS buffer; see above) and centrifuged for 90 min at 24 000 r.p.m. in an SW 28 rotor (Beckman). After centrifugation, fractions were collected from the top to the bottom and analyzed for guanylyl cyclase activities, rhodopsin and protein content. Fractions from a sucrose gradient that were not frozen or pelleted in a low salt buffer need a sonication step to release cytoplasmic proteins. Intactness of the plasma membrane was controlled according to Schnetkamp and Daemen (1982) using the NADPH recycling system.

Properties of band I and band II of ROS preparations

Two rhodopsin-containing bands were obtained after sucrose gradient centrifugation of crude outer segments (bands I and II in Table I). Band II appeared at a slightly higher sucrose density. Occasionally, an upper very faint band appeared above the other two. Band I was rich in rhodopsin and particulate GC activities, but very low in soluble GC and NOS activities. Band II contained less rhodopsin and pGC activities, but had a higher sGC and NOS activity. Both ROS bands contained an intact plasma membrane and showed an almost identical pattern of soluble proteins typically found in ROS preparations, for example transducin, PDE, arrestin, recoverin and others. On Coomassie Blue-stained gels, three additional proteins were visible in band II. These proteins were only faintly visible in band I after silver staining. Cytoplasmic fractions were analyzed for the presence of different marker enzymes as α -glucosidase (endoplasmic reticulum), β -galactosidase (lysosomes) and succinate cytochrome *c* reductase (mitochondria) using standard protocols (Tisdale, 1967; Zimmerman *et al.*, 1976; Rauscher, 1984). Marker enzyme activities for lysosomes and the endoplasmic reticulum were not detectable (α -glucosidase, β -galactosidase). Succinate cytochrome *c* reductase activity in both fractions was $< 0.1\%$ of the value typically found in mitochondria. Gentle shaking of retinas to break off rod outer segments at the cilium connecting to the inner segment can yield a preparation of leaky ROS, intact sealed ROS and ROS attached to the ellipsoid part of the inner segment (Birnbaum and Bownds, 1985). However, to obtain whole photoreceptor cells with outer segments, inner segments, cell bodies and synaptic pedicles (Townes-Anderson *et al.*, 1988), or to isolate other retina cells, for example bipolar cells, it would be necessary to digest the retina with proteases (Karschin and Wässle, 1990). We conclude from our data that band I was a pure outer segment preparation, band II contained outer segments attached to parts of the inner segments (i.e. parts of the ellipsoid) and intact inner segments were absent from our preparation. In particular, band II did not contain complete inner segments attached to outer segments, since marker enzyme activities for subcellular organelles were absent and the composition of soluble proteins was almost identical to the composition of pure outer segments. In addition, a possible contamination of our preparation by on-bipolar cells was tested with an mAb against protein kinase C (PKC) that specifically labeled on-bipolar cells in mammalian retinas (Wässle *et al.*, 1991). The antibody recognized PKC (80 kDa) in extracts of a whole retina, but not in supernatants obtained from photoreceptor bands I and II.

Tissue homogenization and preparation of NOS

Frozen bovine retinas or photoreceptor preparations were homogenized under dim red light using a procedure identical to one described for the purification of NOS from rat cerebellum (Schmidt *et al.*, 1991). Retinal NOS was enriched further by affinity chromatography on 2',5'-ADP Sepharose, as described for rat cerebellar NOS (Schmidt *et al.*, 1991).

Protein purification

Recoverin was purified from bovine rod outer segments using a cytoplasmic extract of an illuminated outer segment suspension, which was subsequently chromatographed on a Superdex 75 HiLoad gel filtration column (Pharmacia) and a Mono Q HR5/5 (Pharmacia) anion-exchange column (Lambrecht and Koch, 1992). NOS type I was purified from rat brain according to Schmidt *et al.* (1991).

Western blotting

For Western blotting, crude supernatant fractions of bovine retinas and brain were separated by electrophoresis on 7.5% polyacrylamide gels,

electrotransferred to nitrocellulose and developed for anti-NOS-I immunoreactivity (H.H.H.W. Schmidt *et al.*, 1992) by the indirect immunoperoxidase method and enhanced chemiluminescence detection (Amersham).

Immunohistochemistry

The animals used in this project were handled in accordance with the guidelines set by the Association for Research in Vision and Ophthalmology (USA). Adult New Zealand White rabbits were sacrificed and enucleated. For whole mount preparation, the eye ball was hemisected and flattened by making radial cuts in the eye cup. While submerged in PBS, the retina was gently separated from the choroid using a sable hair brush. The flat, isolated retina was then transferred onto a piece of filter paper and fixed in 4% paraformaldehyde overnight. Retinas from macaque (*Macaca fascicularis*) were obtained from the Regional Primate Research Center (supported by NIH grant RR00-166) at the University of Washington. Retinas were fixed by perfusion of the monkey with 10% formalin and placed in 2% formaldehyde, 0.1 M lysine, 0.01 M sodium periodate in 0.1 M sodium phosphate buffer. Fixed retinas were frozen and cut in 30 µm sections.

The tissue was washed and incubated in PBS containing a rabbit polyclonal antibody to rat NOS-I diluted 1:1000, 10% goat serum and 0.4% Triton X-100. After the retina was incubated with biotinylated anti-rabbit IgG (Vector), antibody labeling was visualized with an avidin-biotin complex (ABC kit from Vector). Tissues were preincubated with 3,3'-diaminobenzidine HCl (DAB) in a Tris-HCl buffer with 0.1 M imidazole and 0.8% NaCl₂. The whole mount was then incubated in a 1:1 solution of DAB and H₂O₂.

For soluble guanylyl cyclase immunoreactivity, the whole mount procedure above was followed with the following changes. Monoclonal anti-sGC directed against the α_1 -subunit of rat lung sGC (Nakane *et al.*, 1983) was diluted 1:2000 and incubated with 0.4% Triton X-100 and 10% horse serum in PBS. Anti-mouse IgG was used as a secondary antibody diluted in 1:200.

Frozen sections of monkey retina were preincubated in PBS containing 10% goat serum and 0.4% Triton X-100 and treated with 0.3% H₂O₂. Frozen sections were incubated in PBS containing a polyclonal antibody to NOS diluted 1:2000 and 10% goat serum for 3 days. The tissues were washed overnight in PBS and subsequently placed in biotinylated anti-rabbit IgG. Following a wash, staining was visualized as described above for whole mounts.

Enzymatic assays

Dark-kept purified photoreceptor bands I and II were centrifuged for 15 min at 100 000 g. The supernatant (i.e. the cytosolic fraction) was carefully removed. The cytosolic and the membrane fractions were used for assaying different enzymatic activities (see below).

GC activity with Mn-GTP as substrate was determined with an HPLC assay in a buffer described previously (incubation volume 50 µl) (Koch, 1991). The incubation time was 30–60 min (soluble GC) or 10 min (particulate GC). No hydrolysis of cGMP was detected using Mn²⁺ as cofactor, as determined with [³H]cGMP (Lambrecht and Koch, 1991). sGC was activated by saturating concentrations of SNP (100 µM). Determination of GC activity at different free [Ca²⁺] was performed with Mg²⁺ instead of Mn²⁺ as cofactor. The free [Ca²⁺] was adjusted by Ca²⁺/EGTA buffers and calculated with a calcium buffer program, as described previously (Lambrecht and Koch, 1991). The NOS catalyzed conversion of L-arginine to NO, and citrulline was assayed in supernatant fractions of photoreceptor preparations via the formation of [³H]-citrulline from [³H]-L-arginine, measured by liquid scintillation counting (Bredt and Snyder, 1990; Schmidt *et al.*, 1991). Functional coupling of NOS and sGC was measured in a bioassay (Mayer and Böhme, 1989) using an endogenously present soluble GC as target of the produced NO. Reactions were performed in the presence of 100 µM L-arginine and 100 µM NADPH.

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