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Immunocytochemical Localization of the $GABA_C$ Receptor ρ Subunits in the Mammalian Retina

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Polyclonal antibodies against the N terminus of the rat ρ1 subunit were generated to study the distribution of $GABA_C$ receptors in the mammalian retina. The specificity of the antibodies was tested in Western blots and transfected HEK-293 cells. No cross-reactivity with the $GABA_A$ receptor subunits α1–3, β1–3, γ2, δ or with the glycine receptor subunits α1 and β could be detected. In contrast, the ρ1, ρ2, and ρ3 subunits were all recognized by the antibodies. In vertical sections of rat, rabbit, cat, and macaque monkey retinae, strong punctate immunoreactivity was present in the inner plexiform layer. Weaker immunoreactivity was also present in the outer plexiform layer, and cell bodies of bipolar cells were faintly labeled. Double immunostaining of vertical sections and immunostaining of dissociated rat retinae showed the punctate immunofluorescence to colocalize with bipolar cell axon terminals. The puncta possibly represent clustering of the ρ subunits at postsynaptic sites.

Key words: $GABA_C$ receptor; ρ subunits; rat retina; rabbit retina; cat retina; macaque monkey retina; bipolar cell; immunocytochemistry; antibodies

GABA is the major inhibitory neurotransmitter in the vertebrate CNS (Sivilotti and Nistri, 1991). Three types of pharmacologically and physiologically distinct GABA receptors have been described. Activation of bicuculline-sensitive $GABA_A$ receptors causes the opening of integral membrane channels selectively permeable to chloride (Bormann et al., 1987; Macdonald and Olsen, 1994; Sieghart, 1995). By contrast, $GABA_B$ receptors couple to either K^+ or Ca^{2+} channels via G-proteins and second-messenger systems (Bormann, 1988; Bowery, 1989). They are activated by baclofen and are resistant to drugs that modulate the $GABA_A$ receptor. Early studies by Johnston and colleagues (1975) indicated that the partially folded GABA analog *cis*-4-aminocrotonic acid might activate selectively a third class of GABA receptor in the mammalian CNS. These receptors, which were tentatively designated $GABA_C$ (Drew et al., 1984), are insensitive to both bicuculline and baclofen (for review, see Bormann and Feigenspan, 1995).

$GABA_C$ receptors reportedly are present in various parts of the vertebrate brain, including spinal cord (Johnston et al., 1975), optic tectum (Nistri and Sivilotti, 1985; Sivilotti and Nistri, 1989), cerebellum (Drew et al., 1984; Drew and Johnston, 1992), and hippocampus (Strata and Cherubini, 1994; Martina et al., 1995). $GABA_C$ receptors first were observed in retina by Miledi and colleagues after expressing mRNA from bovine retina in *Xenopus* oocytes (Polenzani et al., 1991). Native $GABA_C$ receptors with similar properties were identified later on subpopulations of neurons in rat (Feigenspan et al., 1993; Feigenspan and Bormann, 1994b), fish (Qian and Dowling, 1993, 1994, 1995; Dong et al., 1994), and tiger salamander retinae (Lukasiewicz et al., 1994; Zhang and Slaughter, 1995).

Accumulating evidence indicates that $GABA_C$ receptors are composed of ρ subunits, which were originally cloned in man (Cutting et al., 1991, 1992). The human ρ1 and ρ2 subunits share 74% amino acid sequence identity, but only 30–38% when compared with $GABA_A$ receptor subunits (Cutting et al., 1991, 1992). When expressed in *Xenopus* oocytes, the ρ subunits form homooligomeric channels with the characteristic $GABA_C$ pharmacology (Cutting et al., 1991; Shimada et al., 1992; Kusama et al., 1993a,b; Wang et al., 1994).

The counterparts of the human ρ subunits now have been cloned in chick (ρ1–2; Albrecht and Darlison, 1995) and in rat (ρ1–3; Enz et al., 1995; Ogurusu et al., 1995; Zhang et al., 1995a; Ogurusu and Shingai, 1996), revealing a high degree of similarity at the protein level with the respective human sequences. In contrast to the receptors formed by the human ρ1 or ρ2 subunits (Shimada et al., 1992; Wang et al., 1994), $GABA_C$ receptors in the rat are almost insensitive to the Cl^- channel antagonist picrotoxinin (Feigenspan et al., 1993; Feigenspan and Bormann, 1994a; Pan and Lipton, 1995). This functional difference appears to reside in a single amino acid of the rat ρ2 subunit (Zhang et al., 1995a). The use of PCR and *in situ* hybridization has demonstrated a high expression of ρ1 and ρ2 subunits in rat bipolar cells (Enz et al., 1995), supporting the idea that ρ subunits are part of the $GABA_C$ receptor, because bipolar cells exhibit bicuculline-resistant $GABA_C$ responses. Although ρ1 appears to be restricted to the retina, the ρ2 subunit is expressed significantly in other parts of the CNS, most notably in the hippocampus and cortex (Enz et al., 1995).

The strong expression of ρ1/ρ2 subunits in the rat bipolar cell implies a possible role of $GABA_C$ receptors in mediating lateral inhibitory interaction in the mammalian retina. However, better spatial resolution than that of PCR and *in situ* hybridization is

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required to elucidate the synaptic mechanisms involving $GABA_C$ receptors. Therefore, we have raised polyclonal antibodies against the rat ρ1 subunit and studied the localization of $GABA_C$ receptors on mammalian retinal neurons.

MATERIALS AND METHODS

Preparation of antibodies. Total RNA was extracted from the retinae of an 8-week-old Wistar rat, and reverse transcription-polymerase chain reaction (RT-PCR) was performed as described (Chomczynski and Sacchi, 1987; Enz and Bormann, 1995). Specific oligonucleotide primers for the rat GABA receptor ρ1 subunit were synthesized (sense 5′-CG-**GGATCC**GCTGAGAGCACAGTGCACT-3′ pos. 46–64; antisense 5′-GA**AGATCT**GACGTTGTCTGTGGTGGTG-3′ pos. 513–495) using our own unpublished cDNA sequence data. Restriction sites for *Bam*HI and *Bgl*II were added to the primers (*bold letters*) to facilitate cloning of the PCR products. The PCR reaction (94°C, 5 min; 35 cycles at 94°C, 45 sec; 60°C, 40 sec; 72°C, 45 sec) was performed on a programmable thermocycler (Perkin-Elmer Cetus, Norwalk, CT) as described previously (Enz and Bormann, 1995). The PCR product was fused to an N-terminal histidine affinity tag in the *Bam*HI/*Bgl*II site of the bacterial expression vector pQE13 (Diagen, Hilden, Germany). After transformation in *E.coli* (strain M15[pREP4]), positive recombinants were analyzed by dideoxy sequencing (Sanger, 1977). Protein expression in one positive clone was induced by the addition of 2 mM isopropyl-β-D-thiogalactosid (Stratagene, La Jolla, CA), and after 5 hr incubation at 37°C, bacteria were lysed in 6 M guanidinium hydrochloride, pH 8. The fusion protein was purified using a Ni^{2+}–nitrilotriacetic acid resin column (Diagen) and dialysed against PBS, pH 7.4. The precipitated protein was lyophilized and used for antibody production in rabbits according to standard immunization procedures (Eurogentec, Seraing, Belgium). Briefly, rabbits were injected subcutaneously with 0.5 mg antigen emulsified in 0.5 ml of complete Freund's adjuvant, followed by injections in incomplete adjuvant after 2, 4, and 8 weeks. Obtained serum was affinity-purified using the diethyl aminoethylether (DEAE) column of the Econo-Pac IgG purification kit (Biorad, Richmond, CA). The eluate was further purified by an antigen-coupled column (Kem-En-Tec, Copenhagen, Denmark).

Other immunsera. The following polyclonal antisera against the $GABA_A$ receptor ($GABA_AR$) subunits (gift of H. Möhler) were used: α1, residues 1–16; α2, residues 1–9; α3, residues 1–15 (Benke et al., 1991a; Gao et al., 1993; Marksitzer et al., 1993); β1, residues 381–389 (Greferath et al., 1995); γ2, residues 1–15 (Stephenson et al., 1990); δ, residues 1–17 (Benke et al., 1991b). The mouse monoclonal antibodies bd17 ($GABA_AR$ β2,3; Boehringer Mannheim, Mannheim, Germany) (Schoch et al., 1985; Ewert et al., 1992) and mAb4a recognizing glycine receptor (GlyR) subunits (α1–3 and β; gift of J. Kirsch) (Pfeiffer et al., 1984; Schröder et al., 1991) also were used. Mouse monoclonal antibodies for the protein kinase C (PKC) α-isoenzyme (clone MC5; Amersham, Arlington Heights, IL) (Greferath et al., 1990; Walker et al., 1990) and calbindin-D (Sigma Aldrich, Deisenhofen, Germany) were used to label bipolar cells (Grünert et al., 1994).

Immunocytochemistry. Adult albino rats were deeply anesthetized with 4% halothane and decapitated. After enucleation, the eye was opened by an encircling cut and the eyecup was immersion-fixed in 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.4 for 15–60 min, depending on the primary antibodies used. The eyecup was washed in 0.1 M PB, and the retina was dissected out and cryoprotected by immersion in increasing sucrose concentrations (10–30%) in PB. Pieces of retina were sectioned vertically at 12 μm thickness on a cryostat, and the sections were collected on chrome-alum/gelatin-coated slides. Immunostaining was performed using the indirect fluorescence method. The primary antibodies and their dilutions used for receptor immunocytochemistry were: $GABA_AR$ α1 (1:10,000), α2 (1:2000), α3 (1:5000), β1 (1:50), β2,3 (1:50), γ2 (1:5000), GlyR α1–3, β (1:500), $GABA_CR$ ρ1 (1:100). The binding sites of the primary antibodies were revealed by secondary antibodies: goat anti-guinea pig conjugated to Cy3 (1:1000, Dianova, Hamburg, Germany), goat anti-mouse conjugated to Cy3 (1:1000; Dianova), goat anti-rabbit conjugated to Cy3 (1:1000; Dianova), goat anti-mouse conjugated to fluorescein isothiocyanate (FITC; 1:50; Dianova), and goat anti-rat conjugated to FITC (1:50; Dianova). In double-labeling experiments, sections were incubated in a mixture of the primary antibodies, and subsequently in a mixture of secondary antibodies. Controls were prepared by omitting the incubation with one of the two primary antibodies. In this case, only the immunoreactivity of the remaining primary antibody could be specifically detected.

Dissociated cells were prepared from adult rat retinae as described (Huba and Hofmann, 1988; Karschin and Wässle, 1990). Cells were fixed for 5 min in methanol/acetic acid (95:5/v:v) at −79°C, dryed for 15 min at room temperature, and preincubated for 20 min with 2.5% (w/v) goat serum in PB. Cells were incubated with the ρ1 antibodies diluted in preincubation buffer (1:100) for 5 hr, followed by the secondary antibody goat anti-rabbit conjugated to Cy3 (1:1000) for 30 min.

Human embryonic kidney cells (HEK-293 cells, ATCC CRL 1573) were transfected (Chen and Okayama, 1987) with cDNAs encoding $GABA_AR$ subunits α1–3, β1–3, and γ2 (gift of P.H. Seeburg), GlyR subunits α1 and β (gift of H. Betz), or ρ1 subunits (gift of G. R. Uhl). Proteins were expressed under control of the cytomegalovirus-promotor. Fixation of transfected HEK-293 cells and immunostaining was performed using the same procedure as described above for dissociated cells.

Immunofluorescence was examined and photographed with a Zeiss photomicroscope using the appropriate filter combinations (FITC: 450–490, FT 510, LP 520; CY3: BP 546, FT 580, LP 590). The fluorescence filters were wedge-corrected, so shifting from one filter to the other did not cause any displacement of the image. In some instances, very strong Cy3 fluorescence was also visible with the FITC filter. This could be blocked by an additional green interference filter (515–565) inserted into the microscope tube. Black-and-white photomicrographs were taken on Kodak TMY 400 film.

Gel electrophoreses and Western blot analysis. Membranes of HEK-293 cells expressing $GABA_AR$-subunits α1, α3, GlyR subunits α1 and β, or the ρ1 subunit were prepared as follows. Briefly, cells were homogenized in buffer (4 mM HEPES, 220 mM mannose, 70 mM sucrose, 0.1% benzamidin, 0.025% benzethonium chloride, 1.25% aprotinin, 0.005% α2-macroglobuline, pH 7.5) and sedimentated for 3 min at 1000 × *g*. To collect the membrane fraction, the supernatant was centrifuged at 20,000 × *g*.

Bacterial fusion proteins for the N termini of $GABA_AR$ subunits β3, γ2, δ, and ρ2,3 subunits were generated in *E.coli* strain M15[pREP4] using PCR cloning techniques as described. Oligonucleotide sequences were:

β3 sense 5′-GA**AGATCT**CAGAGCGTAAACGACCCCG-3′ pos. 75–93,
antisense 5′-GA**AGATCT**CCCAATGTTTCTCTTCAACCG-3′ pos. 732–711 (Ymer et al., 1989);
γ2 sense 5′-GA**AGATCT**CAAAAGTCAGATGATGACTATG-3′ pos. 114–135,
antisense 5′-GA**AGATCT**CCCCATTCTTCTGCTCAGAT-3′ pos. 816–796 (Shivers et al., 1989);
δ sense 5′-GA**AGATCT**CACCATGGCGCCAGAGCA-3′ pos. 54–71,
antisense 5′-GA**AGATCT**ACCCCGGTTCCTCCGAAG-3′ pos. 744–726 (Shivers et al., 1989);
ρ2 sense 5′-GC**GGATCC**AGAAAACCCAGGAGGAAGAG-3′ pos. 60–79,
antisense 5′-GC**GGATCC**GTGACGTCGCAGAGTGAAGT-3′ pos. 779–760 (Ogurusu et al., 1995);
ρ3 sense 5′-GC**GGATCC**TGGATCACACTGATGCTGGA-3′ pos. 40–59,
antisense 5′-GA**AGATCT**GTGCCTCCGTAGCACAAAG-3′ pos. 789–771 (Ogurusu and Shingai, 1996).

Restriction sites for *Bam*HI and *Bgl*II were added to the primers (*bold letters*) to facilitate cloning of the PCR products. Cloning of DNA, and expression and purification of fusion-proteins were performed as described.

Membrane proteins of HEK-293 cells and bacterial fusion proteins were subjected to SDS-PAGE gel electrophoresis (Laemmli, 1970), transferred to nitrocellulose sheets (Schleicher and Schüll, Dassel, Germany), and detected using the enhanced chemiluminescence system (Amersham). The following dilutions of antisera were used: $GABA_AR$ α1 (1:20,000), α3 (1:20,000), β2,3 (1:100), γ2 (1:10,000), δ (1:500); GlyR α1–3, β (1:1000); $GABA_CR$ ρ1 (1:300 for HEK cell membranes; 1:1000 for *E.coli* fusion proteins). As secondary antisera, goat anti-guinea pig (Dianova), goat anti-mouse (Dianova), and goat anti-rabbit (Dianova), conjugated to horseradish peroxidase, were used in a dilution of 1:5000 each.

RESULTS

Characterization of the ρ1 antibodies

We raised polyclonal antibodies against the $GABA_C$ receptor ρ1 subunit by synthesizing a fusion protein corresponding to the

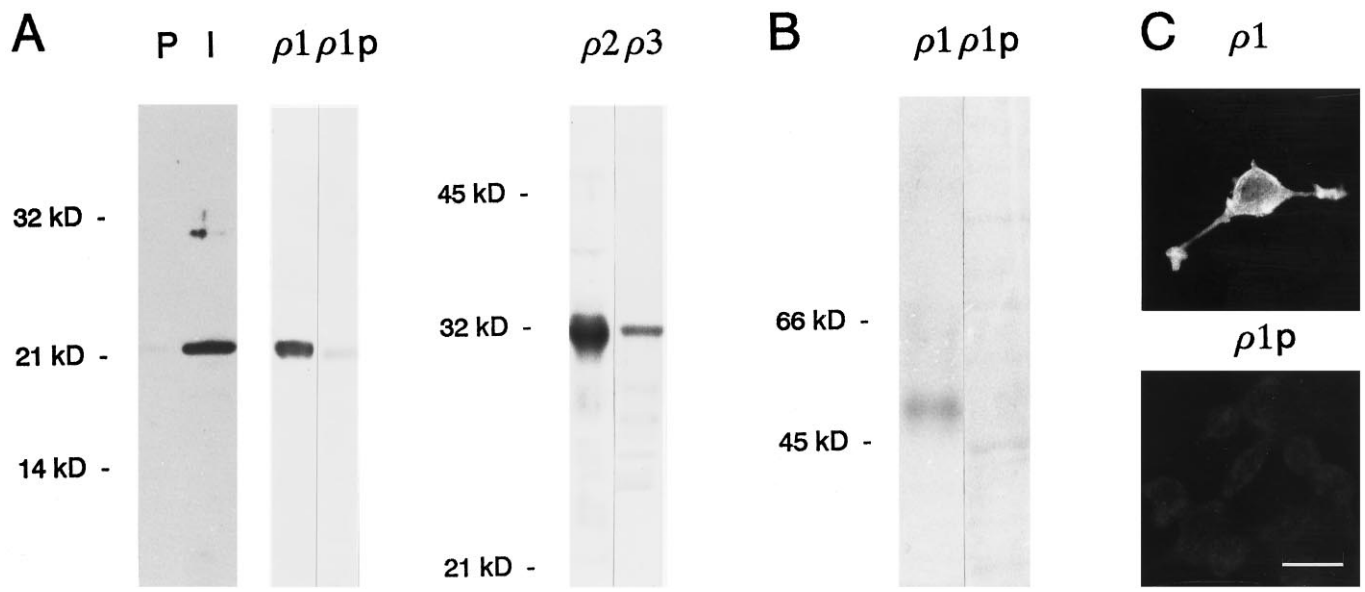

Figure 1. Characterization of ρ1 polyclonal antibodies. *A*, Detection of 20 ng antigen with DEAE-purified (*lane I*) and antigen affinity-purified (*lane ρ1*) immunsera. Preincubation with the antigen (1 mg antigen per ml serum) inhibits the signal (*lane ρ1p*). Incubation with the DEAE-purified preimmunserum does not show any specific signal (*lane P*). The N termini of the rat ρ2 and ρ3 subunits were also detected by the ρ1 antibodies (*lanes ρ2* and *ρ3*). The low molecular weight marker (Biorad) is indicated on the *left*. *B*, Crude membrane fractions with human ρ1 subunit transfected HEK-293 cells showed a specific signal of 50 kDa after incubation with the affinity-purified polyclonal antibodies (*lane ρ1*) that can be inhibited by preincubation with the antigen (*lane ρ1p*). Low molecular weight marker as in *A*. *C*, HEK-293 cells expressing the human ρ1 subunit labeled with ρ1 polyclonal antibodies (*ρ1*). Staining is prevented by preincubation with the antigen (*ρ1p*). Scale bar, 10 μm.

N-terminal region (position 16–171) of the rat ρ1 subunit. The specificity of the polyclonal antibodies obtained in rabbits after injection of the fusion protein was tested on Western blots and with transfected HEK-293 cells. Figure 1*A* shows specific signals of the expected size for the ρ1 antigen incubated with the DEAE- and antigen-affinity-purified antibodies (*lanes I* and *ρ1*), which are inhibited by preincubation with the antigen (*lane ρ1p*). No signal is present after incubation of the antigen with DEAE-purified preimmunserum (*lane P*). The N termini of the ρ2 and ρ3 subunits were also recognized by the antibodies (*lanes ρ2* and *ρ3*). In crude membrane fractions of HEK-293 cells transfected with the human ρ1 cDNA, a specific signal of ~50 kDa could be detected (Fig. 1*B*, *lane ρ1*). This signal is prevented by preincubating the immunserum with the antigen (Fig. 1*B*, *lane ρ1p*). Membrane fractions of untransfected cells showed no signal (not illustrated). Immunostaining of HEK-293 cells expressing ρ1 with our polyclonal antibodies revealed specific labeling (Fig. 1*C*, *ρ1*) that could be blocked by the antigen (Fig. 1*C*, *ρ1p*), whereas untransfected cells were not stained (not illustrated).

Because the primary sequence of the antigen has several regions identical to other $GABA_AR$ and GlyR subunits, we tested the cross-reactivity of the ρ1 polyclonal antibodies for some of those subunits. Western blot analysis of HEK cell membranes containing $GABA_AR$ α1 and α3 or GlyR α1 and β subunits showed no detectable signal after incubation with the polyclonal ρ1 antibodies (Fig. 2*A*, − *lanes*). The same result was obtained with fusion proteins for the N terminus of $GABA_AR$ subunits β3, γ2, and δ (Fig. 2*B*, − *lanes*), whereas the ρ1 antigen could easily be detected under the applied conditions (Fig. 2*B*, *lane A*). In both experiments, however, specific signals were obtained with the antibodies against $GABA_AR$ and GlyR subunits (Fig. 2, + *lanes*). Cross-reactivity was absent also in intact HEK-293 cells transfected with $GABA_AR$ subunits α1–3, β1–3, γ2, and GlyR subunits α1 and β, whereas clear labeling was seen after staining the cells with the specific $GABA_AR$ or GlyR antibodies (not shown). Finally, when antibodies against $GABA_AR$ or GlyR subunits were preincubated with ρ1 antigen, labeling of HEK-293 cells transfected with those subunits was not prevented (not shown), indicating specific inhibition of our ρ1 antibodies by the antigen. In conclusion, our polyclonal antibodies recognize the three ρ subunits known to date, and staining can be blocked by preincubation with the antigen. Cross-reactivity to $GABA_AR$ or GlyR subunits could not be detected under various conditions.

Localization of the ρ subunits in mammalian retinae

Vertical sections of rat, rabbit, cat, and monkey retinae were immunostained with the antibodies against the ρ1 subunit. Figure 3 shows fluorescence micrographs of rat (Fig. 3*A*), macaque monkey (Fig. 3*B*), and rabbit (Fig. 3*C*) retinae. The general labeling pattern is similar, and strong punctate immunoreactivity is found throughout the inner plexiform layer (IPL). Weaker, but specific, immunoreactivity is also present in the outer plexiform layer (OPL). The labeling pattern of the cat retina (not shown) is in close agreement with the results from rat, rabbit, and monkey retinae. The punctate immunofluorescence is not homogeneously distributed within the IPL of the rat retina (Fig. 3*A*). Rather, several distinct bands can be recognized.

Our previous light microscopic studies of the localization of glycine and GABA receptors also revealed a punctate immunofluorescence that was shown by electron microscopy to correspond to a clustering of the receptors at postsynaptic sites (Grünert and Wässle, 1993; Sassoè-Pognetto et al., 1994; Greferath et al., 1995). The punctate labeling of the IPL in Figure 3 is closely similar, and we interpret the puncta as an aggregation of ρ subunits at postsynaptic sites. However, only electron microscopy will be able to show this convincingly. The question is at which processes the immunoreactive puncta might be localized. The previous finding by *in situ* hybridization that rod bipolar (RB)

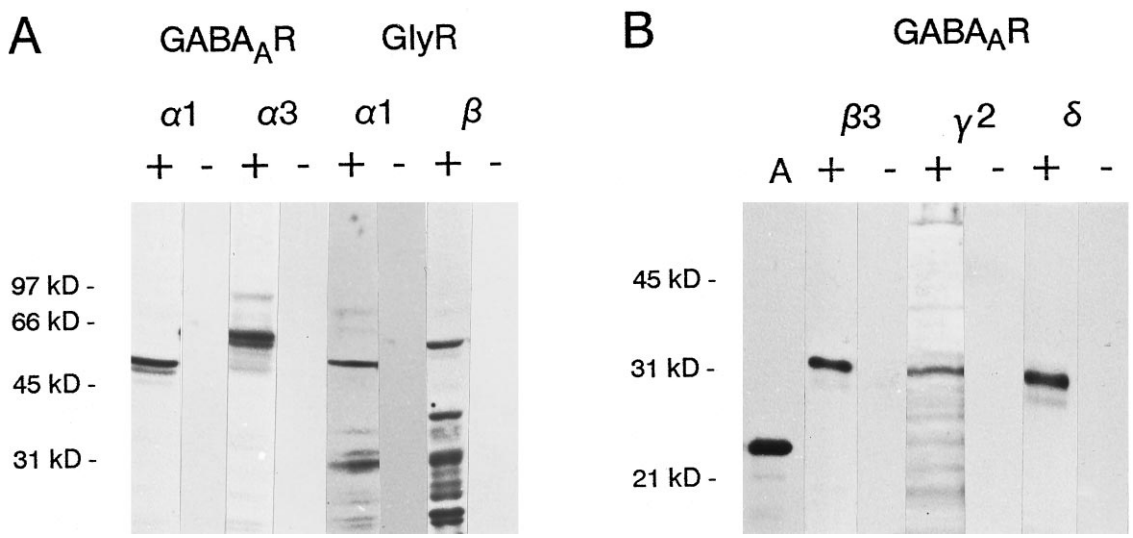

Figure 2. Cross-reactivity of ρ1 polyclonal antibodies with GABA$_A$R and GlyR subunits. *A*, Western blots of HEK-293 cell membranes containing GABA$_A$R α1, α3, or GlyR α1, β subunits. Expression of these proteins is detected after incubation with subunit-specific antibodies (+ *lanes*), whereas incubation with the ρ1 polyclonal antibodies shows no signal (− *lanes*). The low molecular weight marker (Biorad) is indicated on the *left*. *B*, Western blots of bacterial fusion proteins of GABA$_A$R β3, γ2, and δ subunit N termini showing specific labeling with subunit-specific antibodies (+), but no signal for the ρ1 polyclonal antibodies (−). Under these conditions, 20 ng of the ρ1 antigen can be detected (*lane A*). Signals lower than the expected molecular weight of a subunit are probably attributable to cleavage of the protein by proteases. Low molecular weight marker as in *A*.

cells express ρ1 and ρ2 messages (Enz et al., 1995) indicates that the immunoreactive puncta might be localized on RB cells. We investigate this possibility in the following section.

RB cells express punctate immunoreactivity for the ρ subunits

Double-immunofluorescence experiments were performed on vertical sections of the rat retina (Fig. 4). Labeling for the ρ subunits (Fig. 4*A*) was revealed with secondary antibodies coupled to Cy3. RB cells (Fig. 4*B*) were labeled with an antibody against the α-isoform of PKC and visualized with secondary antibodies coupled to FITC. Their cell bodies are in the outer part of the inner nuclear layer (INL), and their axons descend to the inner margin of the IPL where they terminate in a broad band of varicose swellings. The RB cell dendrites form the outer margin of the OPL. Comparison of the staining pattern in the OPL of Figure 4, *A* and *B*, suggests that the RB cell dendrites express weak immunoreactivity for the ρ subunits (*arrowheads*). Closer inspection with high-power objectives suggests an aggregation of the ρ subunits on the dendritic tips of RB cells. Enlarged micrographs of the inner half of the IPL of Figure 4, *A* and *B*, are shown, respectively, in Figure 4, *C* and *D*. The punctate immunofluorescence becomes apparent in Figure 4*C*. A point-by-point comparison between *C* and *D* of Figure 4 (*arrows*) shows that the immunoreactive puncta form aggregates that correspond to the varicose swellings of RB cell axon terminals. Inspection with high-power objectives and changing the focus of the microscope shows convincingly that the puncta coincide with the axon terminal membrane of RB cells. However, only electron microscopy can prove this definitely.

Dissociated rat retinae were investigated to corroborate the localization of the immunoreactive puncta on the RB axons. It has been shown previously that all cells in the dissociates that have the typical appearance of bipolar cells are RB cells (Karschin und Wässle, 1990). Nomarski micrographs of typical dissociated RB cells are shown in Figure 5, *A* and *C*. These cells have small cell bodies and long axons that terminate in varicose swellings. Most of the dendritic processes have been lost during dissociation. The culture dish was immunostained for the ρ subunits, and the corresponding fluorescence micrographs (Fig. 5*B,D*) show a concentration of the label at the axon terminal. Punctate immunofluorescence is present in Figure 5*D* along the descending axon (*arrows*) and on the varicose axon terminal system.

Do cone bipolar cells express punctate immunoreactivity for the ρ subunits?

Punctate immunofluorescence within the IPL (Figs. 3, 4) is not restricted to the inner part where RB cells terminate, but exists across the entire IPL. This suggests localization of the puncta not only on RB cell axon terminals, but also on cone bipolar (CB) cell axon terminals. Most of the markers, such as recoverin, that specifically label CB cells of the rat retina (Milam et al., 1993; Euler and Wässle, 1995) could not be applied here because they were raised in the same species (rabbit) as the ρ1 antibodies. Therefore, we addressed this question for the macaque monkey retina, where PKC immunoreactivity is found in RB cells and in the type DB4 cone bipolar cell (Grünert et al., 1994). A vertical section through parafoveal monkey retina is shown in Figure 6*A*. Strong labeling of RB cell dendrites in the OPL, of RB cell perikarya in the INL, and of RB cell axon terminals at the inner margin of the IPL is apparent. However, as indicated by the two horizontal arrows in Figure 6*A*, there is also a band of small varicosities more toward the center of the IPL. These have been shown previously to correspond to the axon terminals of DB4 cone bipolar cells (Grünert et al., 1994). Figure 6, *B* and *C*, shows, respectively, a section of a macaque monkey retina that was double-labeled for PKC immunoreactivity (Fig. 6*B*) and for ρ subunit immunoreactivity (Fig. 6*C*). Comparison of the labeling of the OPL in Figure 6, *B* and *C*, reveals differences. Only the flat tops of putative CB cells are significantly labeled in Figure 6*C* (*arrowheads*); no labeling of RB cell dendrites can be observed. It is possible that the density of GABA$_C$ receptors at RB cell dendrites in the monkey retina is low. The IPL of Figure 6, *B* and *C*, is shown at higher magnification in Figure 6, *D* and *E*, respec-

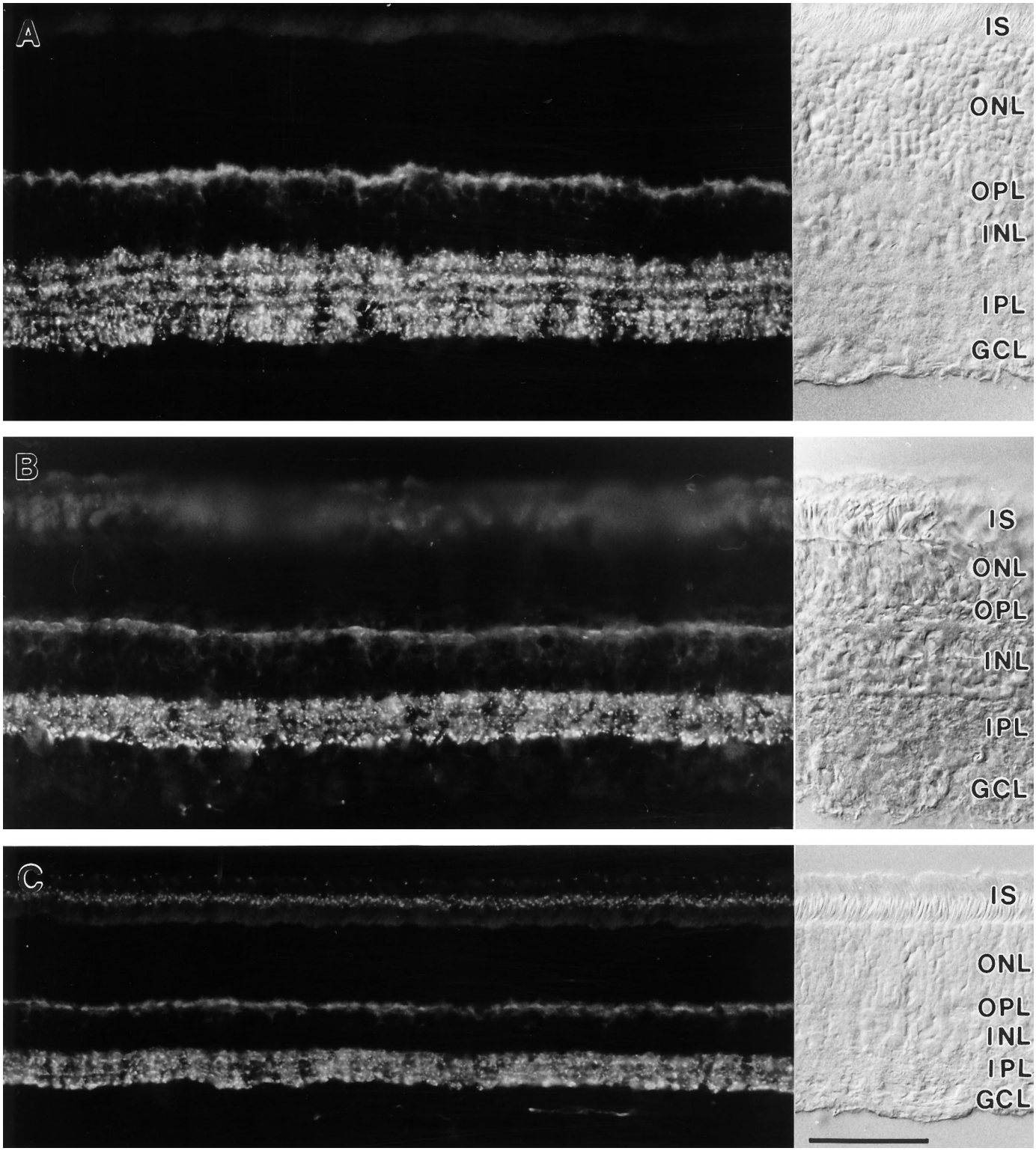

Figure 3. Fluorescence micrographs of vertical cryostat sections through rat, monkey, and rabbit retinae that were immunolabeled for the ρ subunits (Cy3-coupled secondary antibodies). The Nomarski micrographs to the *right* show the retinal layering. *IS*, Inner segments; *ONL*, outer nuclear layer; *OPL*, outer plexiform layer; *INL*, inner nuclear layer; *IPL*, inner plexiform layer; *GCL*, ganglion cell layer. Scale bar, 50 μm. *A*, In the rat retina, strong punctate immunolabeling is found in discrete bands in the IPL. There is also distinct fluorescence in the OPL, and some cell bodies in the outer part of the INL are faintly labeled. *B*, In the macaque monkey retina, strong punctate immunoreactivity is found throughout the IPL. Close to the GCL, a band of more brightly labeled, larger puncta becomes apparent. In the OPL, faint dashed labeling can be observed; single dashes probably represent the dendritic tops of cone bipolar cells at the position of individual cone pedicles. Some bipolar cell bodies are faintly labeled. *C*, In the rabbit retina, strong punctate immunofluorescence can be observed in the IPL, and there is a slight indication of lamination. In the OPL, dashed labeling can be observed. The punctate label of inner/outer segments is nonspecific and has been observed with many rabbit antisera.

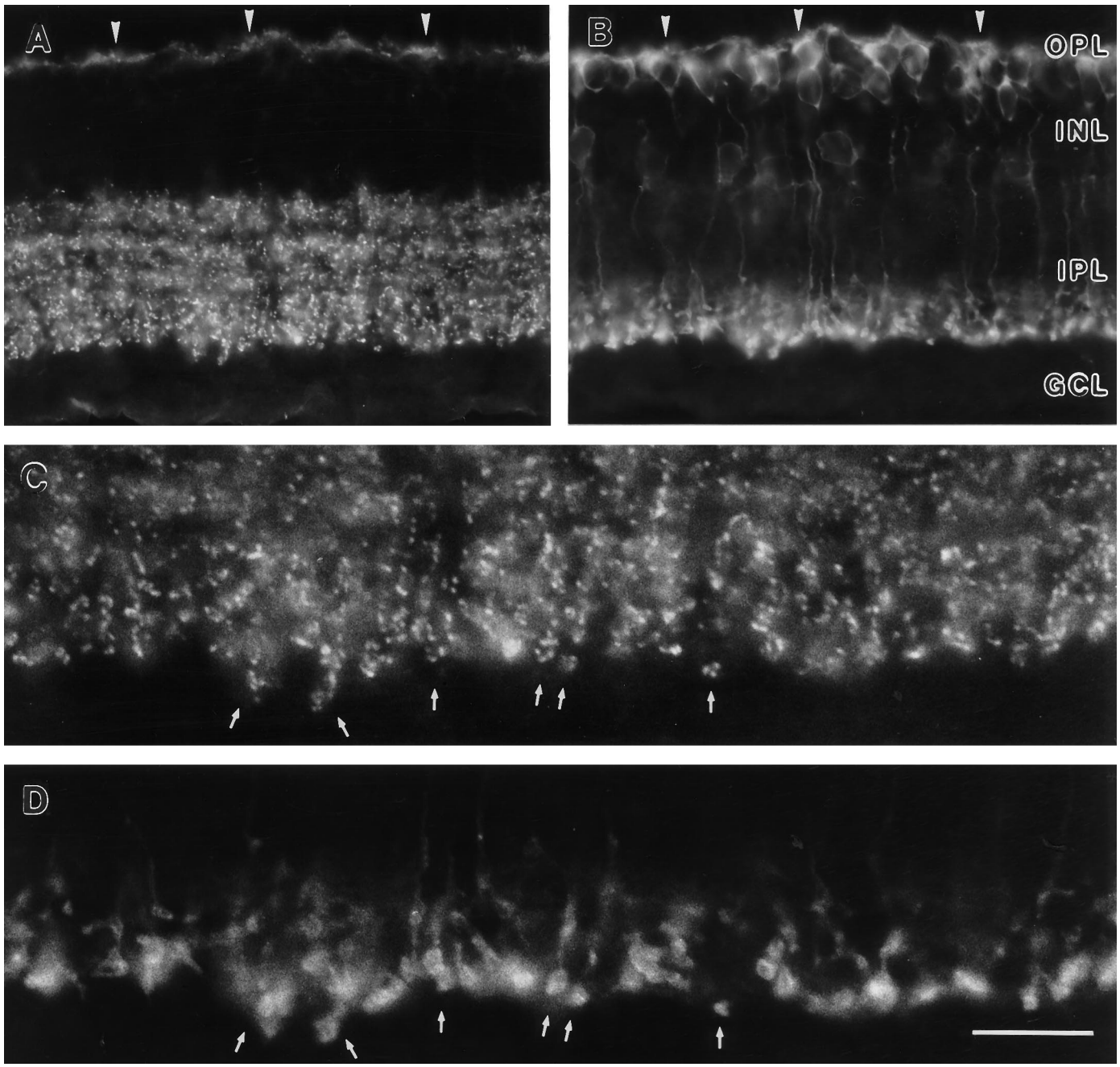

Figure 4. Fluorescence micrographs of a vertical cryostat section through a rat retina that was double-immunolabeled for the ρ subunits and for PKC. *A*, The strong punctate fluorescence specific for the ρ subunits becomes apparent in the IPL. Note also the weaker and partly punctate label in the OPL (*arrowheads*). *B*, PKC immunoreactive RB cells and some few amacrine cells can be seen (retinal layers as in Fig. 3). *C*, Part of the IPL in *A* shown at higher magnification. *D*, Part of the IPL in *B* shown at higher magnification. Many puncta at the lower half of *C* colocalize with RB axon terminals in *D*. Scale bar, 10 μm in *C* and *D*.

tively. The axons of RB cells terminate in large varicosities at the inner margin of the IPL (Fig. 6*E*, *bottom*). Comparison with Figure 6*D* shows that many of the immunoreactive puncta coincide with such RB cell axon terminals. Toward the center of the IPL in Figure 6*E*, several axonal varicosities of putative DB4 cells are shown (*small arrows*). Immunoreactive puncta that would be colocalized with these varicosities are depicted in Figure 6*D* (*small arrows*). This suggests that ρ immunoreactive puncta are present on RB as well as on DB4 cell axon terminals. However, punctate immunoflurescence is also present in the outer part of the IPL, suggesting that other bipolar cells express clusters of ρ subunits.

This possibility was investigated more directly by double labeling monkey retinae with an antibody against calbindin ($CaBP\text{-}D_{28K}$) and with the antisera against the ρ1 subunit. It was shown previously that antibodies against calbindin label the diffuse bipolar cell type DB3 (Grünert et al., 1994). Figure 7*A* shows the calbindin immunofluorescence. Cones and DB3 cells are strongly labeled; horizontal cells and some amacrine cells are weakly labeled. The axons of the DB3 cells terminate in a narrow

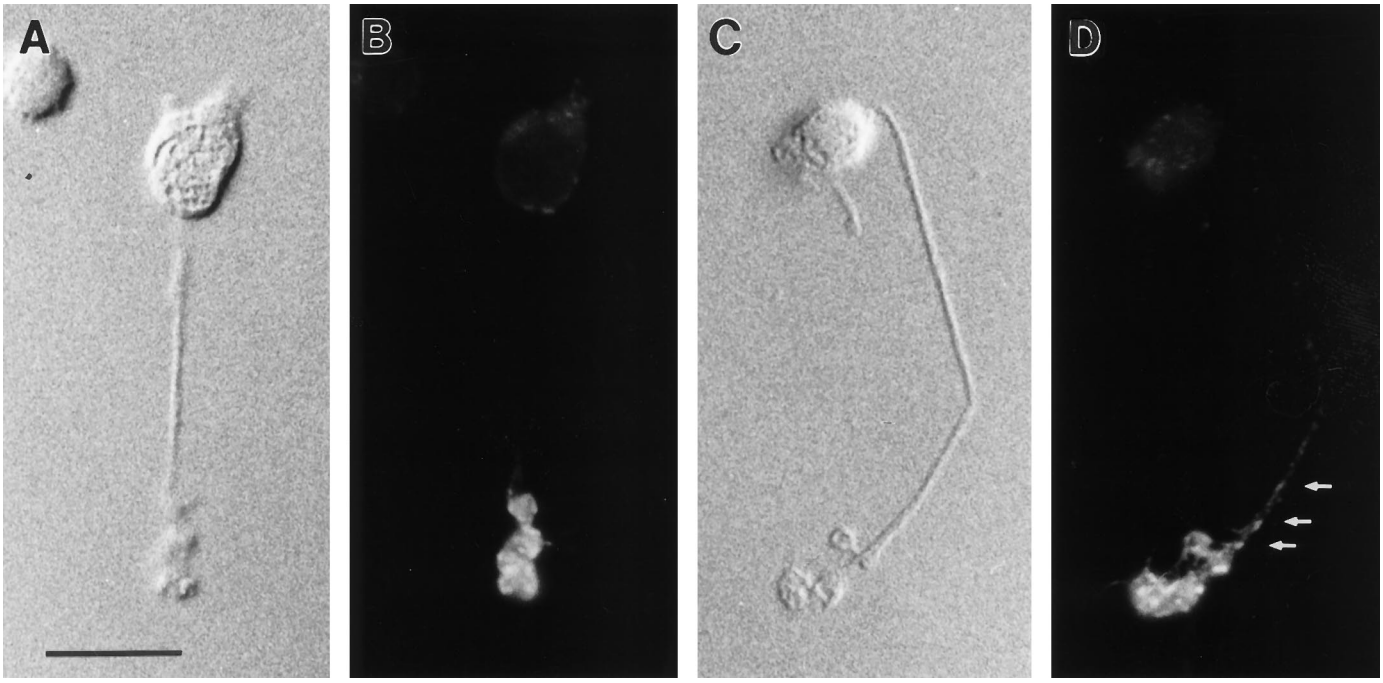

Figure 5. Micrographs of dissociated RB cells of the rat retina. *A* and *C* are Nomarski micrographs of two RB cells. *B* and *D* are the corresponding fluorescence micrographs showing immunoreactivity for the ρ subunits. Labeling is strongest in the axon terminals. Puncta of stronger immunofluorescence possibly represent clustering of the subunits at postsynaptic sites. Scale bar, 10 μm.

stratum in the outer half of the IPL, where they form a sparse band of varicosities (*arrowheads* in Fig. 7*A*). Figure 7*B* shows the ρ immunofluorescence. Strong punctate label is present throughout the IPL, and (as indicated by the *arrowheads*) punctate label is also present in the stratum where DB3 axons terminate. However, higher resolution is needed to find immunoreactive puncta on the DB3 axon terminals. Figure 7, *D* and *E*, shows the IPL of a double-labeled section at higher magnification. Several DB3 axonal varicosities can be seen in Figure 7*D* (three of them are marked by *small white arrows*). The distribution of ρ immunoreactive puncta in the IPL is shown in Figure 7*E*, where there are several examples of colocalizations of immunoreactive puncta and DB3 axonal varicosities (*arrows*).

This result was further quantified. High-power prints of 13 sections double-labeled for calbindin and the ρ subunits were made. The axon terminals of DB3 cells were traced onto acetate foil, and the number of ρ immunoreactive puncta coinciding with the axonal varicosities were counted. Of the 157 varicosities encountered, 97 did not coincide with a ρ punctum; 47 coincided with one ρ punctum, and only 13 coincided with two or more puncta. The average number of puncta per varicosity was 0.5. The same analysis was also performed for PKC/ρ double-labeling experiments (Fig. 6). Only axonal varicosities of RB cells terminating close to the ganglion cell layer were considered. RB cell axonal varicosities are comparable in size to those of DB3 cells. Of the 189 RB cell varicosities, 9 did not coincide with a ρ punctum; 59 coincided with one punctum and 121 coincided with two or more puncta. The average number of puncta per varicosity was 2.2. Thus, there are approximately four times as many ρ puncta on RB axonal varicosities as on DB3 cells. The bright immunofluorescence at the inner margin of the IPL in Figures 3*B* and 7*B* is in accordance with the strong expression of the ρ subunits by RB cells in the primate retina.

DISCUSSION

Specificity of the antibodies

We have raised polyclonal antibodies against the ρ1 subunit to study the localization of $GABA_C$ receptors in the mammalian retina. The antibodies were directed against the N-terminal region (position 16–171) of the rat ρ1 subunit. This region is different from that of the well known $GABA_A$ receptor or glycine receptor subunits: <60% sequence similarity is present. The specificity of the antibodies was confirmed on Western blots and with HEK-293 cells transfected against $GABA_A$ R subunits α1–3, β1–3, γ2, and δ, or GlyR α1 and β subunits. Most of these subunits previously had been localized to different cell types of the retina (Hughes et al., 1991; Brecha, 1992; Vardi et al., 1992; Greferath et al., 1993, 1995; Grigorenko and Yeh, 1994; Enz and Bormann, 1995). Both of the experiments with transfected cells and with Western blots (Figs. 1, 2), as well as the staining pattern of the retina (Figs. 3–7), show that the antibodies against the ρ1 subunit do not recognize $GABA_A$R or GlyR subunits. However, the N-terminal region (position 16–171) of the ρ1 subunit is highly similar to the ρ2 (82%) and to the ρ3 (78%) subunits. From position 78–171, this similarity reaches 94% (ρ2) and 93% (ρ3), respectively. Therefore, it is not surprising that our antibodies recognize the ρ1, ρ2, and ρ3 subunits (Fig. 1). We also tried to raise antibodies against shorter parts of the N-terminal as well as the cytoplasmic region, where ρ1, ρ2, and ρ3 show less similarities. However, these attempts were unsuccessful.

Localization of the ρ subunit to rod bipolar cells

The double-labeling experiments using an antibody against PKC and the antibodies against the ρ1 subunit showed convincingly the localization of ρ subunit immunoreactive puncta on the axon terminals of RB cells in the inner part of the IPL. Previous electron microscopic studies of the RB axon terminals in the cat

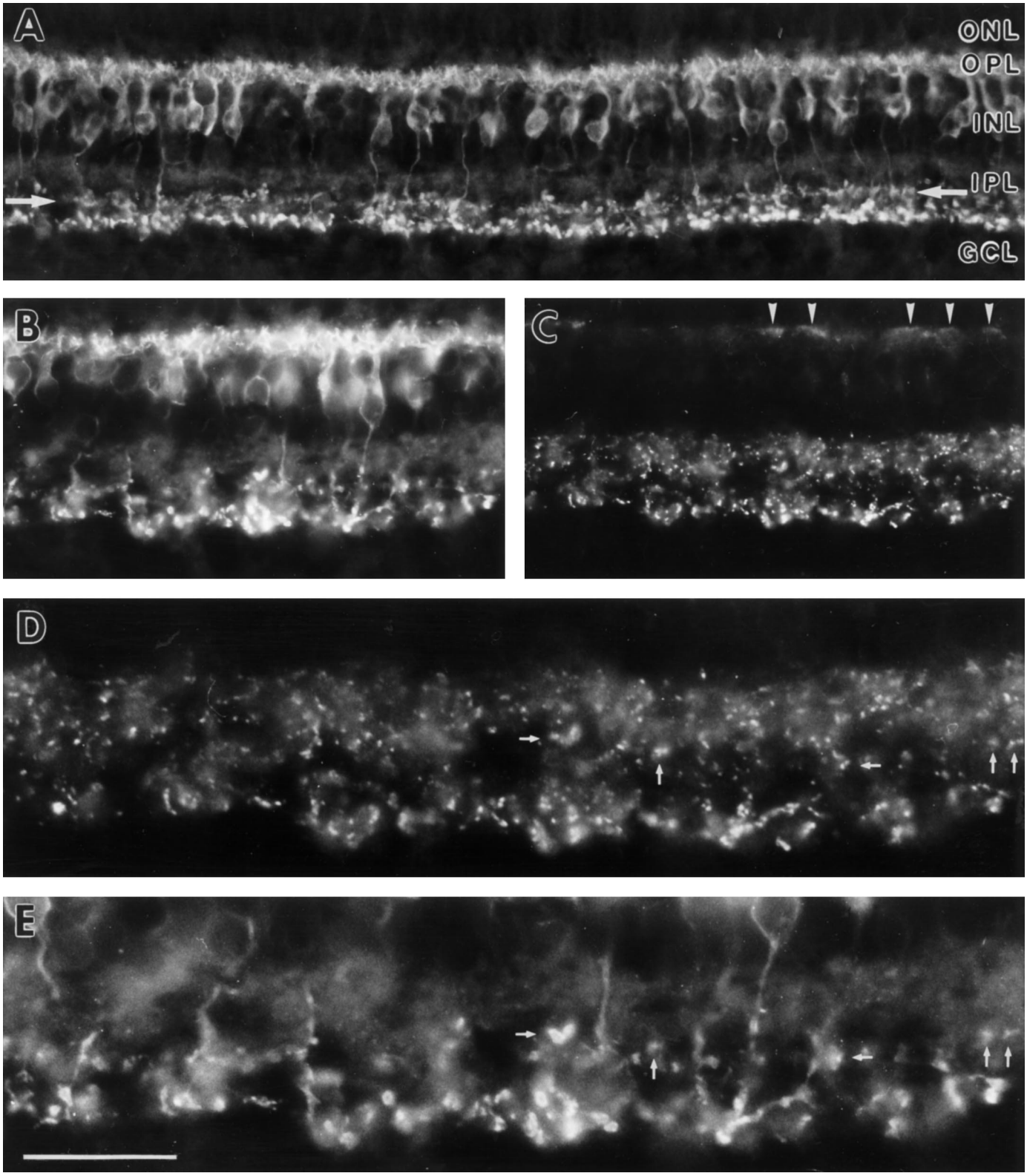

Figure 6. Fluorescence micrographs of vertical cryostat sections through the parafoveal retina of a macaque monkey. *A*, PKC immunoreactivity is found in RB cells. As indicated by the two *horizontal arrows*, there is a second band of smaller varicosities slightly below the center of the IPL. This has been shown previously (Grünert et al., 1994) to represent the axon terminals of cone bipolar cell DB4. *B* and *C* show a section that was double-labeled for PKC and the ρ subunits, respectively. Note that the RB cells that are prominently labeled in *B* have many dendritic terminals in the OPL. These terminals are not labeled in *C*. Instead, as indicated by the *arrowheads*, flat tops of putative CB cells are labeled. *D* is a magnified print of *C*, and *E* is a magnified print of *B*. Comparison of the lower parts of *D* and *E* shows that many ρ immunoreactive puncta colocalize with RB axon terminals. Colocalizations can also be observed (*small arrows*) for the smaller axonal varicosities of DB4 cells, which are found more toward the center of the IPL. Scale bar, 10 μm in *D* and *E*.

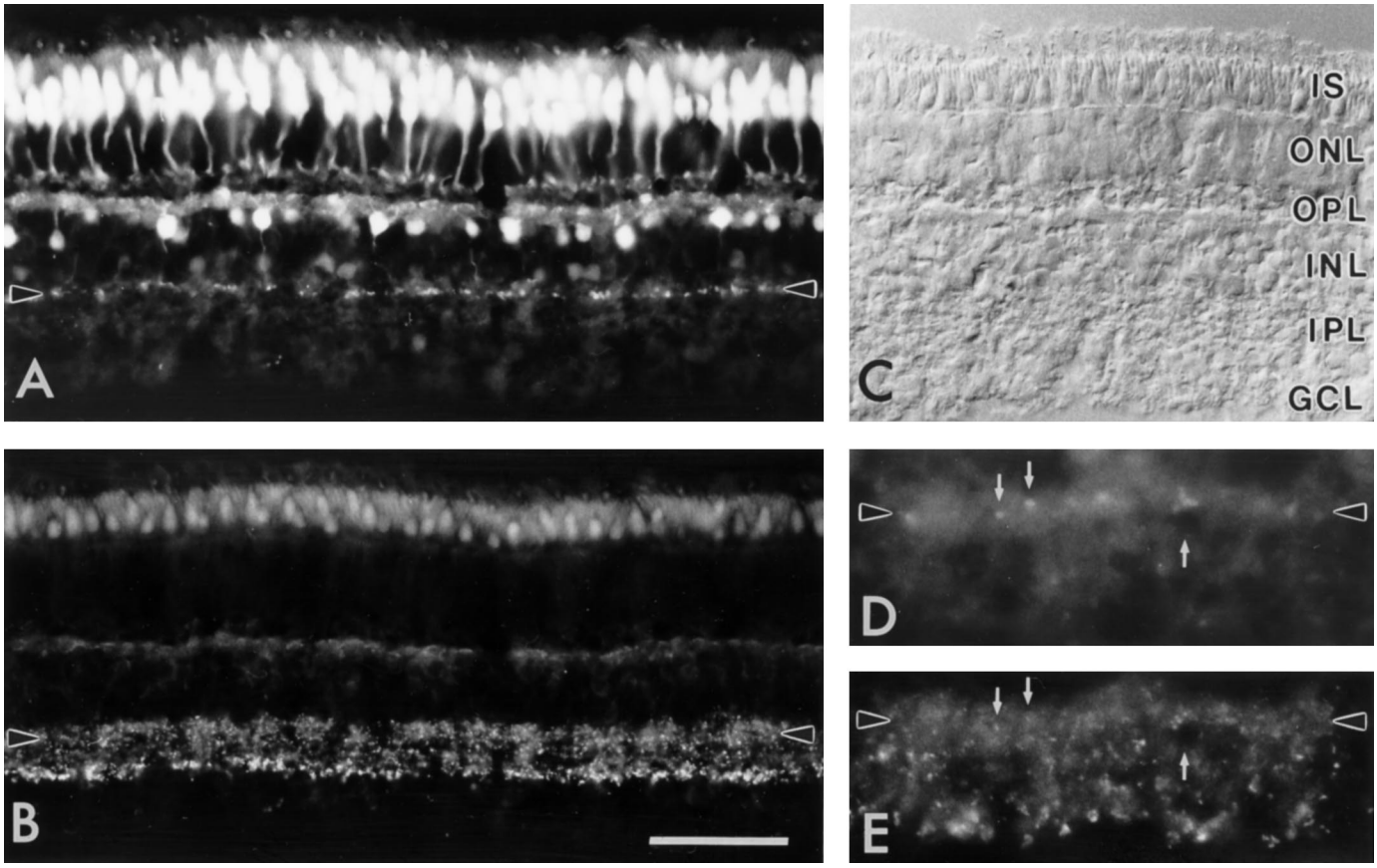

Figure 7. Fluorescence micrographs of vertical cryostat sections through the parafoveal retina of a macaque monkey. The sections were double-immunolabeled for the ρ subunits and for calbindin. *A*, Cones and some DB3 cone bipolars are strongly immunoreactive for calbinden. The axon terminals of DB3 cells form a narrow band of varicosities in the outer part of the IPL (*arrowheads*). The retinal layers are indicated in the Nomarski micrograph (*C*). Abbreviations as in Figure 3. *B*, Same section as in *A*, but revealing the localization of the ρ subunits. Strong punctate fluorescence is found throughout the IPL, including the stratum where DB3 cells have their axon terminals (*arrowheads*). *D* and *E* show the IPL of a double-immunolabeled section at higher magnification. Some calbindin-immunoreactive DB3 axonal varicosities are in focus in *D* (*small arrows*). The *arrows* in *E* show ρ-immunoreactive puncta that would be colocalized with these varicosities. Scale bar, 50 μm in *A–C* and 20 μm in *D* and *E*.

(Sterling and Lampson, 1986), macaque monkey (Grünert and Martin, 1991), rabbit (Strettoi et al., 1990), and rat retina (Chun et al., 1993) showed that they receive their major input from amacrine cells in the inner third of the IPL. Most of this input actually is localized on the axonal varicosities. The great number of ρ-immunoreactive puncta on the varicosities is in accordance with these ultrastructural results. There was also a weak but distinct labeling of the extrasynaptic membranes of RB cells. Their cell bodies and dendrites seem to express low amounts of ρ subunits. In well stained sections, dendritic tips of rod bipolar cells that are inserted into the rod spherules could be observed. This suggests that GABA released from horizontal cells might influence RB cells in the OPL through ρ subunits. However, the strong punctate immunofluorescence in the IPL suggests that the density of ρ subunits in these "hot spots" is substantially higher. Electrophysiological results are in accordance with this differential distribution of the ρ subunits. When GABA was applied to dissociated RB cells (Karschin and Wässle, 1990; Suzuki et al., 1990; Gillette and Dacheux, 1995), responses from the axon terminals were considerably larger than somatic or dendritic responses.

There is evidence from electrophysiology (Feigenspan et al., 1993; Feigenspan and Bormann, 1994a) and from histology (for review, see Greferath et al., 1995) that RB cells also express conventional bicuculline-sensitive $GABA_A$ receptors in addition to $GABA_C$ receptors. The evidence is strong for the expression of the α1 and γ2 subunits and less so for the β2/3 subunits. Immunoreactivity for the α1 and γ2 subunits is punctate, suggesting that these subunits are concentrated at postsynaptic sites comparable to the ρ subunits described here. This raises the interesting question of whether the ρ subunits and the α1 or γ2 subunits occur in the same puncta, or whether they are expressed at different synapses. One also would like to know whether the ρ subunits form homomeric receptors or whether they coassemble with specific subunits of the $GABA_A$ receptor. It has been shown that $GABA_A$ receptor subunits α1, β1, and γ2 do not coassemble with the ρ1 subunit (Shimada et al., 1992).

Localization of the ρ subunit to cone bipolar cells

There is no evidence that amacrine or ganglion cells express the ρ subunits. Extrasynaptic, diffuse labeling of amacrine or ganglion cell bodies was not observed in the present immunocytochemical study, and immunoreactive puncta did not decorate their cell body outlines. Such labeling has been observed with antibodies against certain subunits of the $GABA_A$ receptor (Greferath et al., 1995). In a previous *in situ* hybridization experiment, message for the ρ1 or ρ2 subunits was largely restricted to bipolar cell bodies (Enz et al., 1995). Finally, GABA-induced currents of amacrine and ganglion cells could be reliably blocked by the application of bicucul-

line (Ishida, 1992; Boos et al., 1993; Feigenspan et al., 1993) and thus are mediated by $GABA_A$ receptors. We therefore interpret the punctate label found throughout the IPL as expression of the ρ subunits by CB cell axons and not as labeling of amacrine or ganglion cell processes. There are approximately 10 types of CB cells in the mammalian retina (cat: Famiglietti, 1981; Kolb et al., 1981; McGuire et al., 1984; Pourcho and Goebel, 1987; Cohen and Sterling, 1990a,b; rabbit: Famiglietti, 1981; Mills and Massey, 1992; Jeon and Masland, 1995; monkey: Boycott and Wässle, 1991; rat: Euler and Wässle, 1995). Their axons terminate in different strata of the IPL: those of OFF-CB cells in the outer part, and those of ON-CB cells in the inner part. Because ρ immunoreactivity is present throughout the IPL, both ON- and OFF-CB cells must be labeled. This was shown here directly for two types of CB cells of the macaque monkey retina. Puncta of ρ immunoreactivity were found both on DB3 cells, which are putative OFF-bipolars, and on DB4 cells, which are putative ON-bipolars. Patch-clamp recordings from different types of CB cells in a rat retina slice preparation (Euler et al., 1996) have revealed bicuculline-resistant $GABA_C$ responses in both RB and in different types of CB cells.

As in the case of RB cells, there was also a weak diffuse labeling of the extrasynaptic membranes of CB cells. This also holds for their dendrites in the OPL. The dashed labeling, found in the OPL of rabbit and monkey retinae (Figs. 3*B,C*), suggests that more labeling might be present on dendritic terminals contacted by cone pedicles. Hence, GABA released from horizontal cells would have access to $GABA_C$ receptor there.

The question of whether all CB cells express the ρ subunits cannot be answered at present. Punctate labeling of the IPL of the monkey retina is not particularly strong in the strata where midget bipolar cells terminate, and it is possible that midget bipolar cells express only low amounts of the ρ subunit.

Physiological role of $GABA_C$ receptors

$GABA_C$ receptors are 10 times more sensitive to GABA than $GABA_A$ receptors (Polenzani et al., 1991; Feigenspan and Bormann, 1994a), and they show a weaker steady-state desensitization than the $GABA_A$ subtype (Polenzani et al., 1991; Qian and Dowling, 1993). Thus, gating of $GABA_C$ receptors on dendrites and axon terminals of bipolar cells is expected to occur at very low GABA concentrations and to be more efficacious than that of $GABA_A$ receptors (Bormann and Feigenspan, 1995). $GABA_A$ receptors are upregulated by substances such as dopamine, adenosine, histamine, enkephalin, somatostatin (Feigenspan and Bormann, 1994c), or vasoactive intestinal peptide (Veruki and Yeh, 1992, 1994; Feigenspan and Bormann, 1994c). The signal chain involves protein kinase A. $GABA_C$ receptors are downregulated by serotonin or glutamate via activation of PKC (Feigenspan and Bormann, 1994b; Kusama et al., 1995). The preferential labeling by PKC immunoreactivity of RB cells might be important in this context.

GABAergic amacrine cells are presynaptic to GABA receptor clusters in the IPL. They comprise many different morphological types (Vaney, 1990) and colocalize with many other neuroactive substances, which they perhaps corelease with GABA (Brecha et al., 1984, 1988; Massey and Redburn, 1987; Kosaka et al., 1988; Vaney and Young, 1988; Vaney, 1990; Wässle and Boycott, 1991). It is possible that there is a close match between the type of amacrine cell that provides the presynaptic terminal and the type of GABA receptor that is clustered at the postsynaptic site. More ultrastructural information is needed to determine, for instance, whether ρ subunits are clustered at the postsynaptic sites of certain types of amacrine cells. Further questions concern the distribution of ρ1, ρ2, and ρ3 subunits. RT-PCR studies with single RB cells in the rat retina indicated that the ρ2 subunit is expressed at a much higher level than the ρ1 subunit (Enz et al., 1995). This result was confirmed by Zhang et al. (1995b) using Northern hybridization and primer extension techniques. Preferential expression of the ρ2 subunit over the ρ1 subunit in rat bipolar cells raises the question of how oligomeric $GABA_C$ receptors are composed of these two proteins. Clearly, subunit-specific antibodies for all three ρ subunits are needed to understand the synaptic details of $GABA_C$ receptor localization.

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