Identification and localization of an immunoreactive AMPA-type glutamate receptor subunit (GluR4) with respect to identified photoreceptor synapses in the outer plexiform layer of goldfish retina

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Summary

L-glutamate, the main excitatory synaptic transmitter in the retina, is released from photoreceptors and evokes responses in second-order retinal neurons (horizontal, bipolar cells) which utilize both ionotropic and metabotropic types of glutamate receptors. In the present study, to elucidate the functional roles of glutamate receptors in synaptic transmission, we have identified a specific ionotropic receptor subunit (GluR4) and determined its localization with respect to photoreceptor cells in the outer plexiform layer of the goldfish retina by light and pre-embedding electron-microscopical immunocytochemistry. We screened antisera to mammalian AMPA (alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate)-preferring ionotropic glutamate receptors (GluR 1–4) of goldfish retina by light- and electron-microscopical immunocytochemistry. Only immunoreactive (IR) GluR4 was found in discrete clusters in the outer plexiform layer. The cones contacted in this manner were identified as long-wavelength ("red") and intermediate-wavelength ("green") cones, which were strongly immunoreactive to monoclonal antibody FRet 43 and antisera to goldfish red and green-cone opsins; and short-wavelength ("blue") cones, which were weakly immunoreactive to FRet 43 but strongly immunoreactive with antiserum to blue-cone opsin. Immunoblots of goldfish retinal homogenate with anti-GluR4 revealed a single protein at $M_r = 110$ kDa. Preadsorption of GluR4 antiserum with either the immunizing rat peptide, or its goldfish homolog, reduced or abolished staining in retinal sections and blots. Therefore, we have detected and localized genuine goldfish GluR4 in the outer plexiform layer of the goldfish retina. We characterized contacts between photoreceptor cells and GluR4-IR second-order neurons in the electron microscope. IR-GluR4 was localized to invaginating central dendrites of triads in ribbon synapses of red cones, semi-invaginating dendrites in other cones and rods, and dendrites making wide-cleft basal junctions in rods and cones; the GluR4-IR structures are best identified as dendrites of OFF-bipolar cells. The results of our studies indicate that in goldfish retina GluR4-expressing neurons are postsynaptic to all types of photoreceptors and that transmission from photoreceptors to OFF-bipolars is mediated at least in part by AMPA-sensitive receptors containing GluR4 subunits.

Introduction

L-glutamate is the main excitatory synaptic transmitter in the retina. In the outer plexiform layer (OPL) it is released tonically from photoreceptors, at a relatively high rate in the dark and a lower rate in the light (Copenhagen & Jahr, 1988; reviewed in Dowling, 1987 and Wu, 1994). Responses of second-order retinal neurons (horizontal, bipolar cells) to this chemical signal are mediated by glutamate receptors, or GluRs

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(reviews: Miller & Slaughter, 1986; Massey, 1990). To understand the functional properties and roles of specific glutamatergic synapses in the OPL, it is important to know the molecular structure and subunit composition of GluRs on the second-order neurons. Furthermore, by correlating the GluR composition and physiology of well-characterized retinal synapses, one might better understand the roles of native receptors containing specific GluR subunits.

The glutamate receptor family comprises two main groups, ionotropic and metabotropic (reviews: Nakanishi, 1992; Seeburg, 1993; Nakanishi & Masu, 1994). At least five different pharmacological types of glutamate receptors, distinguished by their selectivity for different glutamate agonists, have been identified. Ionotropic receptors fall into three groups, selective for alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionate (AMPA), kainate (KA), or N-methyl-D-aspartate (NMDA), whereas metabotropic receptors are selective for agonists such as 1-amino-cyclopentyl-1,3-dicarboxylate (ACPD) and L-2-amino-4-phosphonobutyrate (L-AP4 or APB). Ionotropic receptors are thought to be gated transmembrane channels formed as hetero-oligomers (probably pentamers) of closely-related subunits: GluR 1–4 (AMPA-type), GluR 5–7 and KA 1–2 (KA-type), or NR 1–2 (NMDA-type); whereas metabotropic receptors are single protein units (mGluR 1–8) having seven membrane-spanning domains, coupled to second-messenger systems through G-proteins (reviews: Monagan *et al.*, 1989; Gasic & Heinemann, 1991; Nakanishi & Masu, 1994). It is supposed that the expression of genes that encode different receptor subunits can result in the synthesis and assembly of receptors with a variety of pharmacological properties and kinetics. For example, while functional receptors formed by assembly of identical GluR subunits into homomers are generally permeable for Ca^{++}, the presence of an edited form of one subunit (the 'R' form of GluR2) confers very low Ca^{++} permeability to AMPA receptors, whatever other subunits they may contain (Burnashev *et al.*, 1992; Hollmann *et al.*, 1994).

Because photoreceptors are glutamatergic, they generally show a high glutamate content and transporter activity (Marc & Lam, 1981; Marc *et al.*, 1990; Rauen *et al.*, 1996), and the second-order bipolar and horizontal cells are strongly responsive to glutamate. Both ionotropic and metabotropic receptors are present in the OPL, where they mediate respectively sign-conserving (light-hyperpolarizing or OFF) and sign-inverting (light-depolarizing or ON) synaptic transmission (reviews: Miller & Slaughter, 1986; Massey, 1990; Nakanishi, 1995). It is not certain, however, exactly which GluR subunits mediate synaptic transmission to any specific second-order neuron. Horizontal cells in cyprinid fish are known to receive sign-conserving synaptic input via KA- and AMPA-sensitive receptors

(Murase *et al.*, 1987; Yang & Wu, 1991), and pharmacological studies have demonstrated the involvement of an AMPA-receptor in the retraction of carp horizontal cell spinules in the dark (Weiler & Schultz, 1993). OFF-centre bipolar cells in the rat are depolarized rapidly by glutamate and are sensitive to both AMPA and KA (Hartveit, 1995). In contrast, ON-centre bipolar cells are hyperpolarized by glutamate and its analog L-AP4, acting through a metabotropic receptor (Murakami *et al.*, 1975; Miller & Slaughter, 1986; Nawy & Jahr, 1990; Shiells & Falk, 1990; Wu & Yang, 1991). There is evidence, however, that ON-centre bipolars may express ionotropic as well as metabotropic receptors (Hughes *et al.*, 1992; Müller *et al.*, 1992; Brandstätter *et al.*, 1994; Hughes, 1997). This uncertainty, as to which second-order neurons express which ionotropic GluRs, underlines the importance of identifying and localizing GluR subunits in a retina in which synaptic transmission in the OPL has been well characterized.

In the retinas of various mammalian species, the expression of GluR genes has been studied by *in situ* hybridization histochemistry (Hughes *et al.*, 1992; Müller *et al.*, 1992; Shigemoto *et al.*, 1992; Hamassaki-Britto *et al.*, 1993; Akazawa *et al.*, 1994; Brandstätter *et al.*, 1994; Hartveit *et al.*, 1994, 1995), and in the goldfish retina, expression of GluR4 gene (clone GFGR52) was localized to the ganglion cell and inner nuclear layers (Ueda & Hieber, 1995). GluR-like proteins have been localized to various retinal layers or classes of neurons by immunocytochemistry (Morigawa *et al.*, 1995; Peng *et al.*, 1995; Qin & Pourcho, 1996). However, except for the presumed APB-receptor, mGluR6 (Nakajima *et al.*, 1993; Nomura *et al.*, 1994; Morigawa *et al.*, 1995), specific GluR types or subunits have not been identified at specific retinal synaptic sites.

The goldfish retina is very favourable for the purposes of this study. Goldfish photoreceptors (Marc & Sperling, 1976; Stell & Hàrosi, 1976), horizontal cells (Stell & Lightfoot, 1975) and bipolar cells (Ishida *et al.*, 1980) have been well characterized, and much is known of their synaptic connections and physiology (see above). Homologs of glutamate receptor subunits GluR3 and GluR4 (Ueda & Goldman, 1992; Ueda & Hieber, 1995) and NMDA-RI (Hieber & Goldman, 1995) are expressed in the goldfish retina and are >90% homologous to their rat counterparts. Probes were expected to be available, therefore, to identify not only the cells participating in the OPL but also many of the GluR subunits in goldfish. As a result, we were able to identify genuine GluR4 subunit in the goldfish retina and to localize it to synapses of identified photoreceptors. Some of our findings have been summarized previously in preliminary form (Schultz *et al.*, 1995).

Materials and methods

Animals

Common goldfish (*Carassius auratus*), 10–15 cm in standard length, were obtained from a local fish dealer. They were kept in aerated water at about 20°C, under ordinary fluorescent room lights on a 12:12 h light:dark cycle, and sampled during hours 3–6 of the light phase. Animals were enucleated immediately after administration of a lethal overdose of MS-222 (tricaine methanesulphonate; Sigma) followed by cervical transection.

Antisera

Subunit-specific polyclonal rabbit antibodies raised to oligopeptides (13–14 amino acids) of rat AMPA-receptor subunits GluR1 (Ab #9), GluR2/3 (common epitope; Ab #25), and GluR4 (Ab #22), and affinity-purified against the immunizing peptide (Wenthold *et al.*, 1992), were obtained from either Dr R. Wenthold, National Institutes of Health, Bethesda, MD, USA, or Chemicon International Inc., California, USA. Monoclonal antibody FRet43, reported to label double cones in the retina of the zebrafish (Larison & BreMiller, 1990), was obtained from Ms R. BreMiller, University of Oregon, Eugene, OR, USA. Polyclonal antibodies (pAb) against synthesized goldfish cone opsin peptides were raised in rabbits. These peptides represent a partial sequence of the N-terminal region of cone opsins in the goldfish decoded by Johnson and colleagues (1993). pAb to red-cone opsin (GFred) residues 2–16, green-cone opsin (GFgr-2) residues 6–19, and blue-cone opsin (GFblu) residues 2–16, were used in this study. All these pAbs showed immunolabelled bands at 36 kDa, and sometimes at 76 kDa (probable opsin dimers), in Western blots of goldfish retinal membranes (Ohtsuka *et al.*, in preparation).

Peptides

The immunizing peptide for GluR4 antiserum, i.e. the 14 carboxyl-terminal amino-acid sequence of rat GluR4, was obtained from Dr R. Wenthold, National Institutes of Health, Bethesda, MD, USA. A peptide for the corresponding C-terminal tetradecapeptide in goldfish GluR4 (H-RHGPALAVVSSNLP-OH; molecular mass 1417.63) was custom-synthesized by Chiron Mimotopes US, Emeryville, CA. This peptide, supplied at 49% purity, was purified to better than 98% by reverse-phase HPLC, dried in a vacuum centrifuge, reconstituted in distilled water, and subjected to amino acid analysis (Edman degradation) to verify its composition and determine its weight fraction. The amino acid sequences of the two proteins were strongly homologous even at their less well-conserved carboxyl-termini, which contain the region to which the GluR4 antibodies were directed (Fig. 1). Stock solutions of both peptides were made at 10^{-3} M in distilled water and frozen in small aliquots at $-80°$C until use.

Light-microscopical immunocytochemistry

Enucleated eyes were hemisected through the equator, fixed overnight at 4°C in 4% buffered paraformaldehyde with picric acid (Zamboni & DeMartino, 1967), cryoprotected in 30% sucrose, embedded in O.C.T. cryomatrix, and frozen rapidly in liquid N₂. All remaining steps except sectioning were done at ambient temperature, about 21°C. Sections 6–12 μm thick were thaw-mounted onto gelatin-coated slides, air-dried, soaked in phosphate-buffered saline at pH 7.4 (PBS), and non-specific binding of secondary antibodies blocked in PBS containing 1% normal goat serum. Slides were then incubated overnight in primary antibody in PBS + 1% Triton X-100 and washed in PBS. Binding sites were visualized by biotinylated secondary antibodies followed by ABC reagent (Avidin DH and biotinylated horseradish peroxidase) and visualized using hydrogen peroxide and diaminobenzidine tetrahydrochloride. Alternatively, FITC-conjugated anti-mouse (or anti-rabbit) goat IgG (Jackson ImmunoResearch Labs., PA) was used as a secondary fluorescent antibody. For immunofluorescent double-labelling, the latter procedure was repeated with the primary antibody raised in different species, and Texas Red-conjugated anti-mouse (or anti-rabbit) goat IgG (Jackson ImmunoResearch Labs., PA) was used as a secondary antibody.

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679a EPTVFTKTTNEGVARVRKSKGKYAFLLESTMNEYTEQRKPCDTMKVGGNLDSKGYGVATPKGSQLGNAVN
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700b EPSVFTRTTAEGVARVRKSKGKFAFLLESTMNEYTEQRKPCDTMKVGGNLDSKGYGVATPKGSSLGNAVN

767a LAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKDKSSQALSLSNVAGVFYILVGGLGLAMLVALVEFCY
     |||||||||||||||||||||||||||||||||||||·| |||||||||||||||||||||||||:||||
770b LAVLKLNEQGLLDKLKNKWWYDKGECGSGGGDSKDKTS.ALSLSNVAGVFYILVGGLGLAMLVALIEFCY
                                                                 TM4
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837a KSRAEAKKLKLTFSEAMRNKARLSITGSLGENGRVLTPDCPKAVHSGPSFRHGPALAVVSSNLP
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839b KSRAEAKRMKLTFSEATRNKARLSITGSVGENGRVLTPDCPKAVHTGTATRQSSGLAVIASDLP
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Fig. 1. Comparison of deduced amino acid sequences for goldfish GFGR-52 (a) and rat GluR4 (b) in the carboxyl-terminal region included by clone GFGR-52. Identical amino acids are joined by vertical lines, amino acids resulting from conservative nucleotide substitutions by double dots, and amino acids having similar physico-chemical properties by single dots. TM, putative transmembrane domain. The sequence used for production of antibodies to rat GluR4, which was the basis for rat and goldfish peptides for preabsorption, is double underlined.

The working dilution of the fluorophore-conjugated secondary antibodies was 1:100.

Electron-microscopical immunocytochemistry

For ultrastructural immunocytochemistry we followed a procedure described by Eldred and colleagues (1983), modified by eliminating glutaraldehyde fixation as suggested by Dr S. Yazulla (personal communication). Eye cups were fixed in 4% paraformaldehyde and 3% sucrose in 0.1 M phosphate buffer (pH 7.4) for 30 min at 20°C and transferred into 4% paraformaldehyde and 3% sucrose in 0.1 M bicarbonate buffer (pH 10.4) at 4°C overnight. Retinas were removed from the eye cups, washed in PBS for 1 h, cryoprotected in 30% sucrose, frozen and thawed three times at $-20°C$, and sliced into 0.5–1 mm sections with a razor blade. After incubation for 3 h in 5% normal goat serum in phosphate-buffered saline, pH 7.4 (PBS), sections were transferred into GluR4 antiserum (1:50 in PBS) and incubated for several days at 20°C. Bound GluR4 antibodies were detected with a VectaStain Elite ABC-Kit (Vector Laboratories, California). After a wash in PBS, sections were placed into biotinylated anti-rabbit IgG overnight at 20°C, washed in PBS, incubated in solution A overnight, washed in PBS, incubated in solution B overnight, washed in PBS, and incubated with H_2O_2 and 3,3′-diaminobenzidine (Sigma) in PBS. Small pieces of retina were postfixed in buffered 2% OsO_4 for 1 h, dehydrated in increasing concentrations of methanol, and embedded in Epon/Araldite. Ultrathin sections at 60–100 nm (silver–gold) were prepared on an Ultracut E ultramicrotome (Reichert-Jung, Germany), collected on Formvar-coated copper grids, stained with uranyl acetate and lead citrate, and carbon-coated. Sections were viewed and photographed on a Hitachi 7000 electron microscope operated at 80 kV.

Western blotting

The ability of the GluR4 antibodies to recognize the goldfish homolog of GluR4 was evaluated by immunoblotting of whole homogenates of goldfish retina. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE) was performed according to Laemmli (1970). Freshly isolated goldfish retinas were homogenized in 20 mM Tris-HCl, pH 6.8, containing 10 mM $MgCl_2$ and 1 mM EDTA without protease inhibitors, using a Tissue-Tearor (Biospec Products, Bartlesville OK) at 30 000 rpm. Homogenate was then diluted in sample buffer (0.06 M Tris-HCl, pH 6.8; 0.1% v/v glycerol; 2% SDS; 5% beta-mercaptoethanol; 0.1% bromphenol blue) and boiled for 5 min. Aliquots of homogenate (30 µg protein per lane) and molecular mass standards (Bio-Rad) were fractionated on a 7.5% separating 4% stacking SDS-polyacrylamide gel, and the separated proteins were transferred electrophoretically onto nitrocellulose membranes (Towbin *et al.*, 1979). The blot membranes were pre-blocked with 5% non-fat dry milk in PBS for 1 h and incubated with the primary antibody overnight. After rinsing in PBS for 30 min, the blots were incubated for 60 min with an alkaline phosphatase-conjugated secondary antibody (1:1500; Bio-Rad) in 25 mM Tris-HCl, pH 7.4, in 140 mM NaCl. The alkaline phosphatase was visualized using 5-bromo-4-chloro-3-indolyl phosphate as substrate and nitro-blue tetrazolium as chromogen (Bio-Rad). All procedures were done at 20°C.

Controls

As controls for specificity of staining in tissue sections and blots, the primary antibody to GluR4 was replaced by antibodies preabsorbed overnight at 4°C with 10^{-6} to 10^{-4} M rat or goldfish GluR4 peptide (above), rabbit antisera to unrelated antigens, or vehicle alone. Preabsorption controls for antibodies to goldfish red, green and blue-cone opsins were done in the same manner with the appropriate cone-opsin peptides.

Results

Immunoreactive glutamate receptors in goldfish retina: light-microscopical observation

GluR1-, GluR2/3-, and GluR4-immunoreactive (IR) structures were all seen in vertical sections of the goldfish retina. GluR1-IR and GluR2/3-IR cell bodies were seen in the proximal levels of the inner nuclear layer (INL) and ganglion cell layer, and GluR1- and GluR2/3-IR processes were present in the inner plexiform layer (IPL) (results not illustrated). As we were interested primarily in synaptic pathways in the outer plexiform layer (OPL), we did not characterize these structures further.

GluR4 immunoreactivity, in contrast, was seen only faintly if at all in neuronal structures in the INL and IPL, but was evident in the OPL and proximal parts of Müller's cells (Fig. 2). The Müller's cells were labelled most intensely from the ganglion cell layer (GC) to the INL, with stout GluR4-IR processes going directly across the IPL as described by Peng *et al.* (1995); they also wrapped around cell bodies in the INL and appeared to be responsible for faint staining of the external limiting membrane (Fig. 2A). The most strikingly GluR4-IR structures, however, were hemispherical or ovoidal clusters in the OPL (Fig. 2A). The dimensions (3–5 µm horizontally, 1–2 µm vertically) and intermittent placement of these clusters were reminiscent of those of cone pedicles, or more specifically, the postsynaptic invaginations into cone pedicles. Utilizing the greater sensitivity and resolution afforded by peroxidase-coupled antibodies, we could see clearly that the large GluR4-IR clusters were located in cone invaginations, where the dendritic terminations of subclasses of horizontal and bipolar cells are located. We also observed punctate GluR4-IR structures <0.5 µm in diameter scattered between the cone pedicles, i.e. at the level of the rod synaptic endings (Fig. 3). Therefore, both cone and rod photoreceptors appeared to be presynaptic to GluR4-immunoreactive second-order neurons. The identity of the cones making these contacts is the subject of the next section of this report. All staining with the
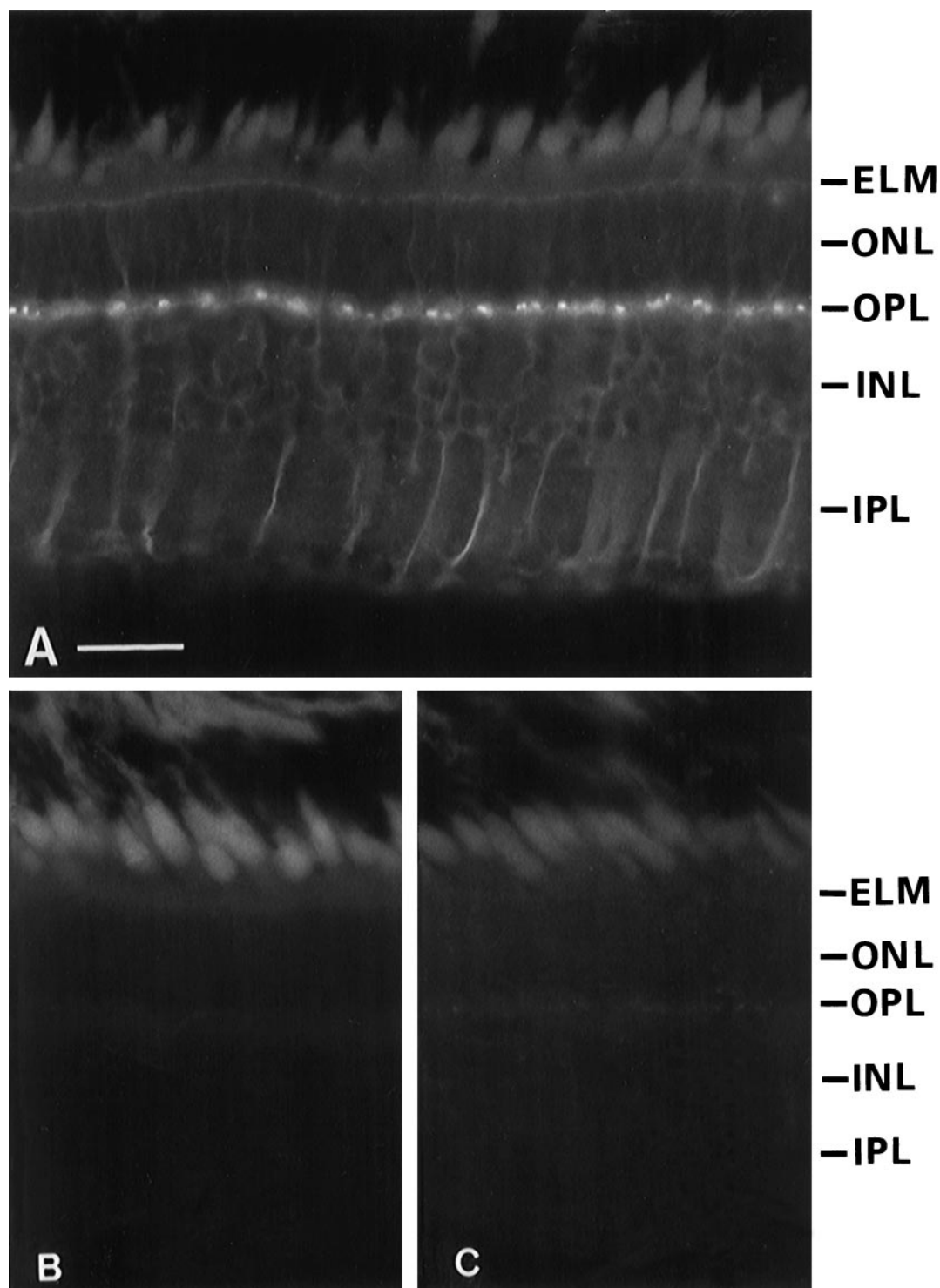
Fig. 2. Immunofluorescence localization of AMPA-receptor subunit GluR4 in vertically-sectioned goldfish retina. (A) Intense staining of GluR4-immunoreactive aggregates in the outer plexiform layer (OPL), and moderate staining of Müller's glial cells in all other layers (ELM, external limiting membrane; ONL, outer nuclear layer; INL, inner nuclear layer; IPL, inner plexiform layer). (B) Same as in A, but after preabsorption of primary antibodies with 10^{-4} M GluR4 peptide; (C) same, but after preabsorption with 10^{-4} M goldfish GluR4 peptide: abbreviations as in A. Scale marker = 50 μm.

affinity-purified antibodies to rat GluR4 was blocked completely by preabsorption with the immunizing (rat) GluR4 oligopeptide, at concentrations of 10^{-6} to 10^{-4} M (Fig. 2C); with the homologous goldfish GluR4 oligopeptide, blocking was complete at 10^{-4} M but only partial at 10^{-6} M (Fig. 2B).

Since GluR4 immunoreactivity was confined to the OPL, and not strongly expressed on cell bodies in the
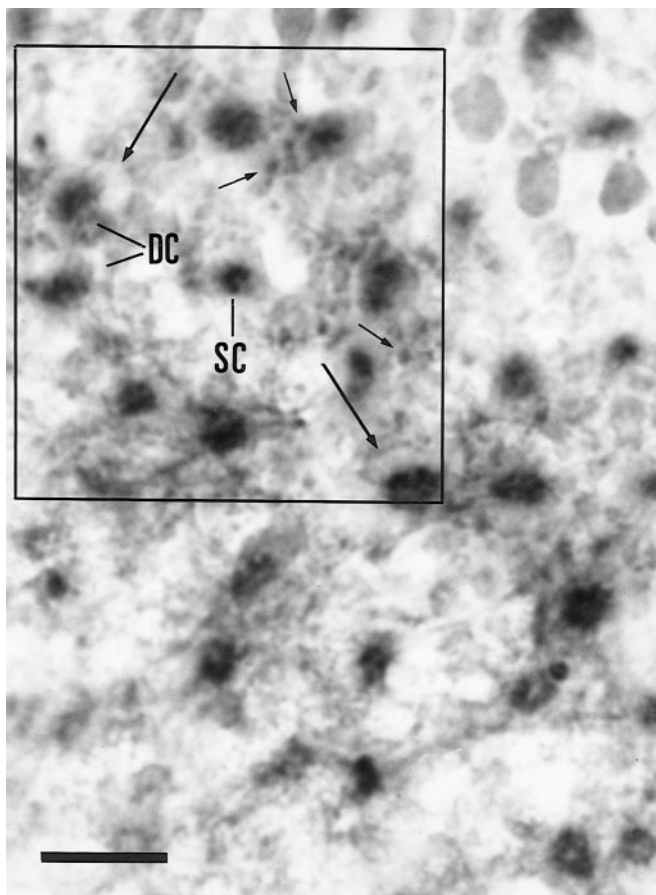
Fig. 3. Immunoperoxidase localization of GluR4 in the outer plexiform layer in horizontally-sectioned goldfish retina. The mosaic pattern of four pairs of double cones (DC) around a short single cone (SC) enclosed in a rectangle is clearly evident. GluR4-immunoreactive aggregates are surrounded by the cytoplasm of cone pedicles, into which they are invaginated (large arrows), and are also present as small punctate densities in the region occupied by rod synaptic endings, between the cone pedicles (small arrows). Scale marker = 20 μm.

Fig. 4. Immunoblots of goldfish retinal homogenate with Ab #22 to rat GluR4 C-terminal oligopeptide. Lane 1, molecular mass standards (M_r indicated at left); lane 2, unpreabsorbed antibodies; lane 3, antibodies preabsorbed with 10^{-4} M goldfish GluR4-peptide; lane 4, antibodies preabsorbed with 10^{-4} M rat GluR4 peptide; lane 5, primary antibodies omitted.

INL, it was not possible to identify the neurons responsible for GluR4 immunoreactivity in the OPL. Careful examination of horizontal sections at high resolution (Fig. 3) suggested that the GluR4-IR dendrites were located near, or proximal to, the apices of the synaptic ridges, and therefore that they might be dendrites of bipolar rather than horizontal cells. This issue could be addressed definitively only by electron-microscopical immunocytochemistry (see below).

Western blots of whole goldfish retinal homogenate, probed with the antibodies to rat GluR4, showed a single immunoreactive band with an estimated molecular mass of 110 kDa (Fig. 4). Preabsorption of the GluR4 antibodies with either rat or goldfish GluR4 peptide to 10^{-4} M abolished the immunoreactivity of this band completely, and nothing was labelled in the

blot membrane when the primary antibody had been replaced by vehicle alone (Fig. 4).

GluR4 clusters are postsynaptic to all photoreceptors

In horizontal sections through the OPL (Fig. 3), the GluR4-IR structures appeared as approximately circular aggregates having a higher staining intensity at the circumference than in the centre. The distribution of these circular aggregates within the OPL resembled the cone mosaic pattern, in which scattered single cones are enclosed within square to rhombic arrays of double cones (Marc & Sperling, 1976; Stell & Hàrosi, 1976). Individual cones, particularly the single cones, could not be identified securely solely on the basis of position. It was important to do so, however, because of uncertainty as to the transmitter utilized by the short-wavelength (blue) cones. For this reason we utilized double-immunofluorescent labelling for GluR4 along with specific cone types.

Monoclonal antibody FRet43, at a dilution of 1:5 to 1:10, was found to stain double cones, and some kind of interneuron in the INL having processes in the IPL (Fig. 5A), as reported previously in zebrafish retina (Larison & BreMiller, 1990). The staining of double cones was very intense, involving all parts of these

photoreceptors from the outer segments to the synaptic terminals and their telodendrons (Fig. 5B). At higher antibody concentrations (dilution 1:1 to 1:4), shorter and broader single cones also were labelled (not shown). On morphological criteria these were identified as short single, or blue cones. This identification was confirmed by double-labelling with FRet43 and antiserum to blue-cone opsin, in which the outer segments, inner segments (Golgi apparatus), axons and axon terminals of the weakly FRet43-IR short single cones were specifically blue-opsin-IR (Fig. 5B). Antibodies to red and green-cone opsins, in contrast, labelled only the outer segments of FRet43-IR double and long single cones – never their inner segments or axons, and never the weakly FRet43-IR short single cones. Any individual cone, therefore, could be identified unambiguously by its pattern of immunoreactivity to FRet43 and antisera to the three cone opsins (Table 1).

In vertical sections of retina labelled simultaneously with FRet43 and GluR4 antibodies, the synaptic pedicles of all strongly FRet43-IR cones appeared to enclose GluR4 aggregates (Fig. 5A). In horizontal sections labelled similarly, GluR4 aggregates could be seen clearly in the subsynaptic invaginations of both strongly FRet43-IR double-cone pairs, as well as in the invaginations of both strongly and weakly FRet43-IR single cones within the rhombic double-cone mosaic units (Fig. 5C). Double-labelling with antibodies to GluR4 and blue-cone opsin confirmed dramatically that GluR4-IR dendrites were postsynaptic to blue as well as to red and green cones (Fig. 5D). As a general rule, the GluR4 aggregates opposite blue cones were smaller and less symmetrical (or, more irregular) than those opposite red and green cones.

In view of these observations, it appears that all of the red, green, and blue cones (as well as rods) in goldfish retina make synaptic inputs to second-order neurons via ionotropic glutamate receptors that contain the GluR4 subunit.

Ultrastructural immunocytochemical of GluR4 in the outer plexiform layer

We confirmed that intensely labelled GluR4-IR processes invaginated the synaptic endings of rods as well as cones. Electron-dense HRP-DAB reaction product was seen to fill the cytoplasm of these dendritic processes. GluR4 labelling was never observed in the dendrites of rod or cone horizontal cells, which were recognized readily without special staining. Therefore we identified the GluR4-IR processes as bipolar cell dendrites, although we could not exclude the possibility that some might be telodendrons from neighboring photoreceptors (as in the turtle; Kolb & Jones, 1985) (see also Stell & Kock, 1982).

All cone synaptic endings (pedicles) that were sectioned through the subsynaptic invagination were found to make contact with GluR4-IR dendrites. The number of labelled processes differed between different cones, however; some showed a high density of processes, mostly located centrally in the clusters of processes entering the cone cavity, whereas in the invaginations of other cone pedicles only a few labelled processes were found. These differences might be due to differences in plane of sectioning, i.e., apex *vs* equator of the hemispherical subsynaptic cavity, or they might reflect real differences in the numbers of BC dendrites contacted by cones of different types. For example, red-sensitive cones and rods are contacted by dendrites of all (⩾6) known types of mixed bipolar cells, whereas green-sensitive cones are contacted by dendrites from only some of them and blue-sensitive cones are not contacted by mixed bipolar cells at all (Ishida *et al.*, 1980).

Several types of contact were seen between cones and GluR4-IR dendrites. In red-sensitive cones, in which ribbon synapses frequently include dyads of HC dendrites and triads formed by two HC dendrites and one BC dendrite (Stell, 1976; Stell *et al.*, 1977, 1982; Stell & Kock, 1982; and unpublished data), the long, fine invaginating central BC dendrites were always GluR4-IR (Fig. 6A). Therefore these GluR4-IR dendrites in goldfish cone synapses participate in ribbon synapses in the same way as those of the type I OFF-centre bipolars of Saito and colleagues (1985) (*a3* mixed bipolars of Stell & Kock, 1982). In contrast, the near-triadic dendritic terminals of the type II ON-centre bipolars of Saito and colleagues (1985) (*b2* or *b3* mixed bipolars of Stell *et al.*, 1977, and Ishida *et al.*, 1980; Stell & Kock, 1982) were never GluR4-IR. In the all-HC triads of other cones, GluR4-IR dendrites ended instead as near-invaginating elements (not illustrated). In cones of all kinds, many GluR4-IR dendrites were seen to make wide-cleft junctions with lobular projections from the cone pedicle into the subsynaptic cavity (Fig. 6A). Most of the invaginating GluR4-IR processes, however, were not involved in any kind of specialized junction or contact with the cone pedicle. Such GluR4-IR structures may represent the proximal or post-terminal, unspecialized portions of dendrites that do make wide-cleft or ribbon junctions.

All rod spherules were invaginated by one or more fine GluR4-IR processes. Sometimes these processes penetrated all the way to the synaptic ridge, forming triads with horizontal cell dendrites in the ribbon synapse as described for type I OFF-centre bipolars by Saito and colleagues (1985). More often they penetrated only partially, ending blindly among immunonegative processes, as described for type II OFF-centre BCs by Saito and colleagues (1985). In a few cases we were able to show that such partially-penetrating GluR4-IR dendrites made punctate or hemispherical wide-cleft junctions with rod spherules,
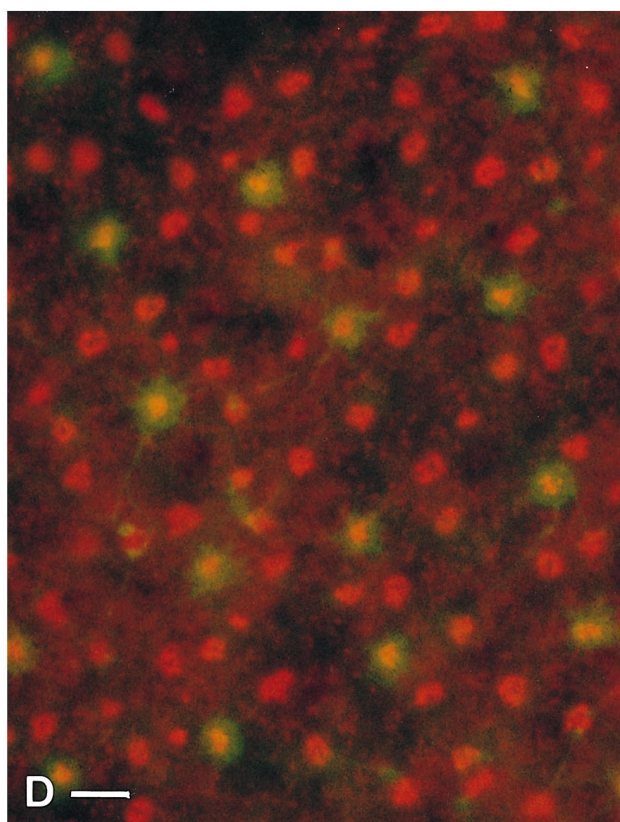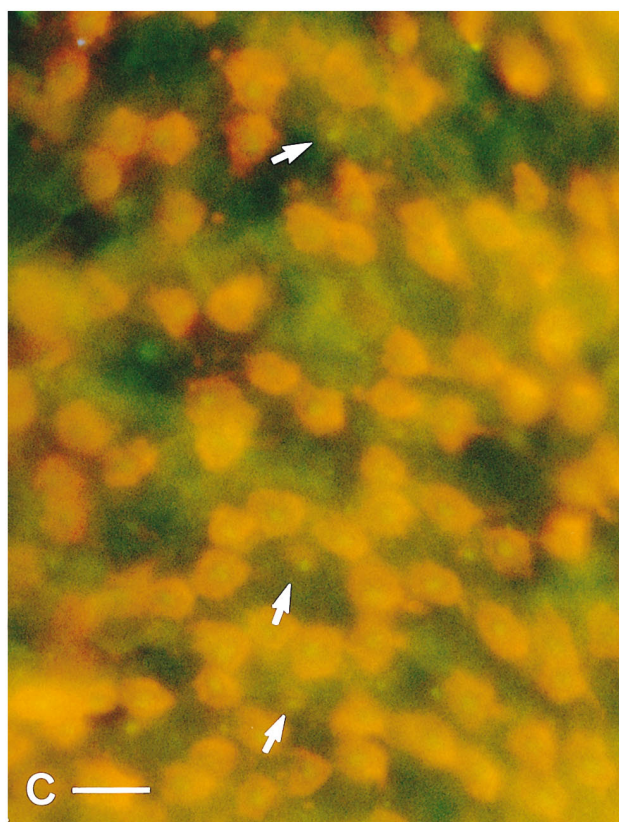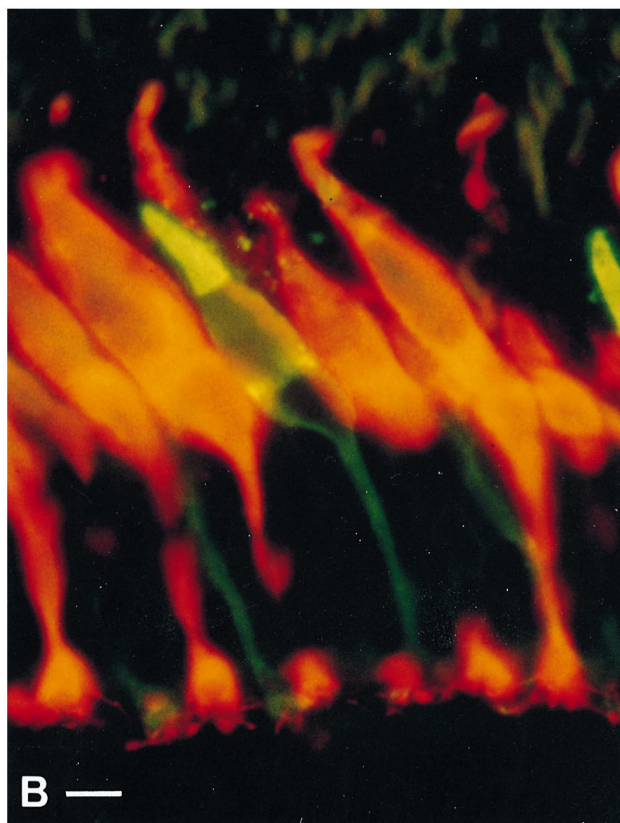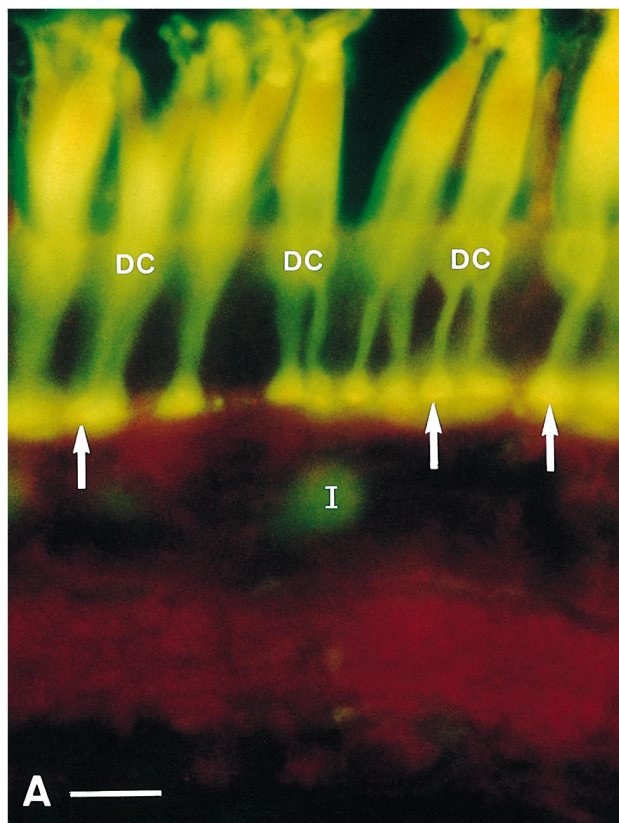
Table 1. Immunocytochemical signatures of goldfish cones

	Cone type		
Antiserum	Red (Long double, long single)	Green (Short double, long single)	Blue (Short single)
FRet43	Strong (entire cell)	Strong (entire cell)	Weak (entire cell)
R opsin	Strong (outer segments only)	–	–
G opsin	–	Strong (Outer segments only)	–
B opsin	–	–	Strong (outer segments, Golgi app.) moderate (soma, axon)

accompanied by a specialized close apposition to a rod horizontal cell dendrite (Fig. 6B), as reported previously for Golgi-impregnated type *a1* and *a2* mixed rod-cone OFF-centre BCs (Stell *et al*., 1977; Stell & Kock, 1982). GluR4 labelling was never observed in dendrites of horizontal cells or ON-centre bipolar cells, which are easily recognizable in rod synapses (Stell, 1976).

Discussion and conclusions

Immunoreactive GluR4 in goldfish retina is genuine GluR4

It is always potentially hazardous to study complex substances in one species by means of probes designed for another, unrelated one. For example, Peng and colleagues (1995) obtained completely disparate results in goldfish retina with two antibodies to rat GluR2, one subtype-specific and one to a shared GluR2/3 epitope. Peng and colleagues (1995; Fig. 4C) also reported a localization of immunoreactive GluR4

in goldfish retina similar to that reported here, using antibodies to a slightly different C-terminal oligopeptide of rat GluR4 (Blackstone *et al*., 1992), but were unable to detect it in Western blots.

In the present case, by means of immunochemical methods we were able to identify a glutamate receptor subunit in goldfish retina that is strongly homologous to rat GluR4. Most of the homology, however, lies in the pre-TM4 loop and TM4 itself, rather than the C-terminal region to which Ab 22 is directed (C-terminal sequences of 14 amino acids are 50% homologous; Fig. 1). Therefore it was necessary to take special pains to assure ourselves that the antibodies to rat GluR4 recognized a homolog to GluR4, and nothing else, in the goldfish retina. Ab 22 was affinity-purified against an immobilized C-terminal rat GluR4 nonapeptide (LAVIASDLP, 67% homologous to goldfish *LAV*VS*S*NLP), not the immunizing peptide or its glutaraldehyde-protein conjugate (Wenthold *et al*., 1992), and therefore is likely to be more specific for the C-terminus of goldfish GluR4 than if purified against the complete tetradecapeptide. This specificity was

Fig. 5. Immunocytochemical identification of cone types contacting GluR4-IR dendritic terminals in outer plexiform layer of goldfish retina (double immunofluorescence). (A) Vertical section; FRet43 (FITC) and GluR4 (TRITC). Double cones (DC) are strongly FRet43-IR (yellow-green), unidentified interneuron in inner nuclear layer (I) is weakly FRet-IR, and cone pedicles are invaginated by TRITC-labelled GluR4-IR aggregates (arrows) which appear yellow because of superposition with FITC-labelled cone pedicles. Scale marker = 25 μm. (B) Vertical section; FRet43-IR red- and green-sensitive cones (TRITC, orange) and blue-cone opsin-IR short single cones (FITC, yellow-green). Scale marker = 10 μm. (C) Horizontal section through OPL; strongly FRet-IR red- and green-sensitive cones and weakly Fret43-IR blue-sensitive cones (TRITC, yellow-orange) are invaginated by GluR4-IR dendritic clusters (FITC, yellow green). Scale marker = 10 μm. (D) Horizontal section through OPL; blue-cone opsin-IR blue-sensitive cones (FITC, yellow-green) and red- and green-sensitive cones (unstained) are invaginated by GluR4-IR dendritic clusters (TRITC, red-orange). GluRr aggregates in blue cones appear yellow because of superposition with FITC-labelled cone pedicles. Scale marker = 10 μm. Abbreviations: FITC = fluorescein isothiocyanate-coupled secondary antibody; TRITC = Texas Red isothiocyanate-coupled secondary antibody; OPL = outer plexiform layer; INL = inner nuclear layer, IR = immunoreactive.
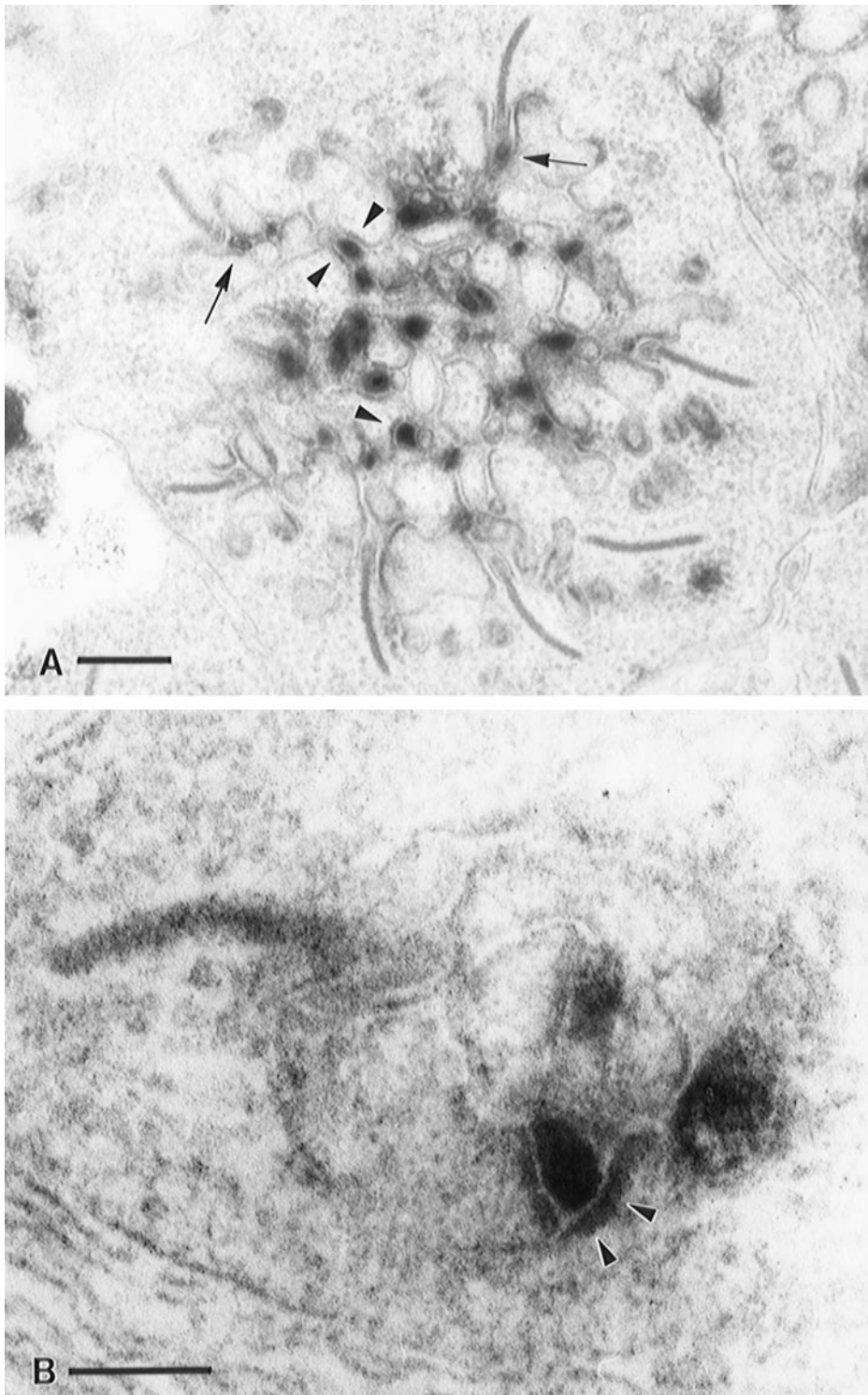
Fig. 6. Electron micrographs illustrating the labelling pattern of GluR4-IR OFF-bipolar cell dendritic terminals in cone pedicle and rod spherule. (A) GluR4-ir BC terminals form central elements with HC-BC triads in a red pedicle (arrows). The arrowheads indicate wide-cleft junctions between the photoreceptor and an OFF-BC. Scale marker = 0.5 μm. (B) GluR4-IR OFF-BC terminal entering a rod spherule and making a wide-cleft junction with the photoreceptor membrane. Scale marker = 0.25 μm.

further supported by the presence of a single GluR4-IR protein in Western blots of goldfish retinal homogenates, having an apparent molecular mass (110 kDa) close to the 108 kDa reported in rat brain (Blackstone

et al, 1992; Wenthold *et al.*, 1992). The staining of this protein in blots and tissue sections was blocked by preabsorption with rat or goldfish GluR4 peptide, confirming that the antibodies responsible for staining

have high affinity for the C-terminus of goldfish as well as rat GluR4. The lower affinity of Ab 22 for the goldfish GluR4 peptide (i.e. the requirement of higher concentration to blocks staining completely) is not unexpected, given the homology of only 67% between the rat and goldfish peptides.

We conclude that the goldfish homolog of rat GluR4 is expressed in the goldfish retina, and that it is detectable by Ab 22 under the very different conditions of immunocytochemistry and immunoblotting. Within the limitations of these methods, therefore, this report describes the localization of genuine GluR4 homolog in the goldfish retina.

AMPA receptors in the goldfish retina

Homologs to rat GluR3 and GluR4 mRNA were cloned previously from a goldfish retinal cDNA library (Ueda & Goldman, 1992), but the cellular location of GluR proteins was previously unknown. In the present study, using antibodies to GluR oligopeptides (Wenthold et al., 1992), we observed GluR1 and GluR2/3 immunoreactivity in the IPL of goldfish retina, a plausible location. In contrast, Peng et al. (1995), using antibodies to almost identical (though N-terminally extended) rat GluR peptides (Blackstone et al., 1992), found no immunoreactivity for GluR1 and completely disparate localizations for GluR2 (specific epitope) and GluR2/3 (shared epitope) in the goldfish retina. Our own preliminary tests with Ab 7 and Ab 25 did not include Western blotting or preabsorption controls. Therefore, it is uncertain at present where genuine GluRs 1–3 are located in the goldfish retina.

It is noteworthy that while Ab 7 and Ab 25 gave plausible localizations of AMPA-receptor subunits in the goldfish IPL, and therefore probably cross-reacted with goldfish homologues of GluR 1–3, they did not replicate the staining of GluR4 aggregates in the OPL. While it is possible that goldfish GluR 1, 2, and/or 3 may be present in the OPL only in an edited form not detected by these antibodies, C-terminally truncated forms of these subunits (which would escape detection by these antibodies) are not known. Novel AMPA-receptor subunits, other than GluR 1–4, have not been detected in rat brain (Wenthold et al., 1992) but in principle might exist in goldfish. Therefore GluRs 1–3 are likely to be absent from dendrites in the postsynaptic invaginations of goldfish photoreceptors. Since native AMPA-receptors are thought to be pentamers of two or more homologous AMPA-receptor subunits, and not mixtures of AMPA and kainate- or NMDA-preferring subunits (Brose et al., 1994; Puchalski et al., 1994), it is unlikely that GluR4 and non-AMPA-receptor subunits are co-assembled into functional receptors in the goldfish retina. However, most ionotropic GluR subunits can assemble into functional homomeric receptors in expression systems (see, for example, Herb et al., 1992), and evidence has been presented for the existence of native homomeric GluR1 receptors in the rat hippocampus (Wenthold et al., 1996) and NR1 receptors in the rat retina (Brandstätter et al., 1994). Therefore, on the basis of evidence available at present, the AMPA receptors described here in the goldfish OPL and Müller's cells could be homomeric assemblies of GluR4 subunits alone.

Glutamate as neurotransmitter of goldfish rods and cones

Under certain conditions, goldfish red and green cones readily accumulate exogenous glutamate (Marc & Lam, 1981) and contain high concentrations of endogenous glutamate (Marc et al., 1990), whereas blue cones and rods do not (Marc & Lam, 1981; Marc et al., 1990). For some time, therefore, it was thought that red and green cones were glutamatergic, whereas blue cones and rods might not be. Recently, however, evidence has been presented that these discrepancies are due to differences in adaptation mechanisms and that all goldfish photoreceptors are glutamatergic (Marc et al., 1995). Our finding that GluR4-containing ionotropic glutamatergic receptors are post-synaptic to all kinds of goldfish photoreceptors, coupled with the lack of plausible alternative candidates for the blue-cone and rod transmitters, is consistent with this interpretation.

GluR4 in goldfish OPL is located in bipolar cell dendrites

We found that GluR4 was localized to the interior of small neurites in the goldfish outer plexiform layer (OPL). Similar observations have been made in mammalian brain and spinal cord, using the same antibodies (Petralia & Wenthold, 1992; Jaarsma et al., 1993; Spreafico et al., 1994). Since these antibodies are directed towards the carboxyl terminus of GluR4 (Wenthold et al., 1992), such a localization would suggest that the C-terminus of GluR4 is located intracellularly, i.e. on the cytoplasmic side of the membrane. While the original topological model for ionotropic glutamate receptor subunits, based on similarity to the nicotinic acetylcholine receptor, placed the C-terminus on the extracellular side of the membrane (Nakanishi, 1992), further analysis of ionotropic glutamate receptors suggests that they are quite different from nAChR (Wo & Oswald, 1995). More recent studies on AMPA, kainate, and NMDA receptor subunits suggest that the original topological model was incorrect, and that the C-terminus is indeed intracellular (Tingley et al., 1993; Hollmann et al., 1994; Taverna et al., 1994; Bennett & Dingledine, 1995; Wo & Oswald, 1995). Diffusion of DAB reaction product away from the site of antibody binding on the inner surface of the postsynaptic membrane might produce the apparently uniform localization that we observed throughout the cytoplasm of fine dendritic tips. Alternatively, it has been proposed

that the intracellular staining may indicate that the immunoreactive protein localized in these studies is located in the cytoplasm, because of transport, assembly, and degradation of GluR subunits (Spreafico *et al.*, 1994).

The GluR4-bearing second-order neurons were identified by electron-microscopical immunocytochemistry. The immunocytochemical staining of the goldfish retina revealed GluR4-IR processes in contact with all cone pedicles and rod spherules, varying in number with the plane and level of sectioning and the type of photoreceptor. The dendritic terminals of rod and cone horizontal cells, which are easily recognized in virtually every section in the electron microscope, were never labelled for GluR4. On the contrary, the much finer dendrites that were found to be GluR4-IR frequently made contacts known to be typical of bipolar cell dendrites, such as basal junctions and invaginations into ribbon synapses. It cannot be excluded that GluR4 could sometimes be confined to the shafts of horizontal cell dendrites, since we did not trace HC dendrites all the way out of the subsynaptic enclosures of rod and cone synaptic endings to prove that they were completely unlabelled. In principle this is an unlikely scenario, as the functional consequences would be puzzling at best; and in practice, no ir-GluR4 was seen proximal to the level of cone synaptic endings in sections examined with the light microscope.

Therefore GluR4 expression in the OPL of goldfish retina seems to be the exclusive property of bipolar cells. This conclusion is consistent with the *in situ* hybridization results of Ueda and Hieber (1995), which showed that GluR4 message is present in cells in the INL, but the limited resolution of autoradiography in their study did not permit identification of the labelled cells. Some of the cells in the INL that express GluR4 message are likely to be Müller's glial cells, which have been shown previously to be GluR4-immunoreactive (Peng *et al.*, 1995). Strong expression of GluR4 in bipolar cells in the goldfish is rather surprising, because comparable observations have not been reported in other species (Hughes *et al.*, 1992; Müller *et al.*, 1992; Hamassaki-Britto *et al.*, 1993; Brandstätter *et al.*, 1994; Hartveit *et al.*, 1994; but see Morigawa *et al.*, 1995, and Qin & Pourcho, 1996).

GluR4-containing bipolar cells in goldfish OPL are OFF-bipolars

ON and OFF-centre bipolar cells in goldfish were first described by Kaneko (1970). Subsequently it was shown that the neurites of many cells in the fish retina, including bipolars, were stratified in the inner plexiform layer according to their function; the axons of OFF bipolars, for example, terminated in the outer or distal part of the IPL, called sublamina *a*, whereas those of ON bipolars terminated in the inner or proximal

part of the IPL, called sublamina *b* (Famiglietti *et al.*, 1977). This discovery made it possible to identify morphological features of synaptic inputs to the dendrites of bipolar cells that might be responsible for generating ON or OFF responses. Ultrastructurally different photoreceptor-bipolar contacts, the wide-cleft and narrow-cleft basal junctions, were described in the turtle retina by Lasansky (1971). Stell and colleagues (1977) reported that in goldfish retina the dendrites of ON-centre (mixed) bipolars made narrow-cleft junctions in photoreceptor synapses, while dendrites of OFF-centre bipolars made wide-cleft junctions in them.

Dendrites of mixed rod-cone bipolars go to the synaptic endings of both rods and cones (Stell *et al.*, 1977; Ishida *et al.*, 1980). We found that ir-GluR4 and wide-cleft junctions were absent from the rod-contacting dendrites of putative ON-centre bipolars (this study). This was not unexpected, since the inhibitory or sign-inverting inputs from rods to ON-centre BCs are thought to be mediated exclusively by APB-sensitive metabotropic glutamate receptors (Shiells, 1995).

Since cone pedicles are invaginated by dendrites from both mixed rod-cone bipolars (Ishida *et al.*, 1980) and pure cone bipolars (Sherry & Yazulla, 1993), it is likely that the GluR4-IR dendrites represent OFF-bipolars of both classes. Furthermore, since dendrites making wide-cleft junctions or central elements of red-cone ribbon triads were only very rarely immunonegative, it is apparent that as a rule wide-cleft junctions are concerned with synaptic transmission to GluR4-IR bipolar cell dendrites.

Role of GluR4 subunits in bipolar cell function

The ionic mechanisms of OFF-centre bipolar cells have been studied most thoroughly in urodele amphibians, such as the mudpuppy and tiger salamander. In these animals, at least some bipolars contact both rod and cone photoreceptors by a combination of invaginating, narrow-cleft, and wide-cleft junctions (Lasansky, 1978). Centre-OFF-responses of urodele bipolars are clearly mediated by AMPA-kainate receptors (Slaughter & Miller, 1983; Gilbertson *et al.*, 1991; Hensley *et al.*, 1993), probably of more than one pharmacological type (Gilbertson *et al.*, 1991; Hensley *et al.*, 1993; Kim & Miller, 1993), probably of more than one pharmacological type (Kim & Miller, 1993; Taylor & Copenhagen, 1993). In these cells, glutamate and non-NMDA agonists gate a desensitizing channel having high Na^+ and K^+ conductances, a reversal potential near zero, and an unusually high conductance to Ca^{++} (Gilbertson *et al.*, 1991). In carp, the response of OFF-centre bipolars was found to be mediated by a receptor that gates a Na^+ conductance, with an $E_{rev} > 50$ mV and a doubly-rectifying I-V relationship (Toyoda, 1973; Kaneko & Saito, 1983; Saito & Kaneko, 1983). These characteristics were found to hold for both rod and

cone-dominated inputs (Saito et al., 1984). The pharmacology of the glutamate receptors on OFF-centre bipolar cells has not been studied in fish. To the extent that comparable studies have been done, the receptors of OFF-centre bipolars in urodele and fish are similar in being due to glutamate-gated cationic channel which may be doubly-rectifying, but dissimilar in the ionic selectivity of the channel and perhaps the characteristics of receptors directly postsynaptic to rods and cones.

The GluR4 subunit appears to be expressed in a minority of bipolar cells (perhaps OFF-bipolars), and the GluR2 subunit not expressed, in a number of mammalian species (Hughes et al., 1992; Müller et al., 1992; Hamassaki-Britto et al., 1993; Brandstätter et al., 1994; Hartveit et al., 1994; but see Morigawa et al., 1995, and Qin & Pourcho, 1996). Perhaps in salamander, too, the glutamate receptors of OFF-centre bipolars are GluR4-rich (at least at the inputs from one class of photoreceptors) and GluR2-poor. We did not detect GluR4 in larval tiger salamander with the antibodies employed in this study (unpublished studies). However, the C-terminal amino acid sequence of GluR4 (detected by this antibody) could be substantially different in salamander from that in rat and goldfish.

The properties of GluR4-rich, GluR2-lacking receptors can be inferred from the properties of homomeric AMPA receptors assembled in expression systems (Seeburg, 1993). Receptors deficient in GluR2 are characterized by low Ca^{++} permeability and a linear V-I relationship (Burnashev et al., 1992). Consequently, homomers of GluR4 have high permeability to Ca^{++} (Hollmann et al., 1991) and a doubly-rectifying I-V relationship (Verdoorn et al., 1991; Dingledine et al., 1992). Native AMPA-type receptors composed mainly or exclusively of GluR4 may be assembled in the Bergmann glial cells of the cerebellum (Monyer et al., 1991; Martin et al., 1992; Petralia & Wenthold, 1992). Glutamate-activated channels in Bergmann glia have high Ca^{++}-permeability (Burnashev et al., 1992). This supports the notion that the gating of highly Ca^{++}-permeable channels by glutamate may be due largely to GluR4-dominated receptors. Therefore it is possible that the responses of OFF-centre bipolar cells in cyprinid fishes are indeed mediated by homomeric GluR4-containing AMPA receptors.

Acknowledgements

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